Current Topics in Microbiology and Immunology

Sabra L. Klein
Craig W. Roberts *Editors*

Sex and Gender Differences in Infection and Treatments for Infectious Diseases

Second Edition

Springer

Current Topics in Microbiology and Immunology

Volume 441

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ISSN 0070-217X ISSN 2196-9965 (electronic)
Current Topics in Microbiology and Immunology
ISBN 978-3-031-35138-9 ISBN 978-3-031-35139-6 (eBook)
https://doi.org/10.1007/978-3-031-35139-6

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

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Genetics of Sex Differences in Immunity

Shani T. Gal-Oz and Tal Shay

Abstract Women have a stronger immune response and a higher frequency of most autoimmune diseases than men. While much of the difference between men and women is due to the effect of gonadal hormones, genetic differences play a major role in the difference between the immune response and disease frequencies in women and men. Here, we focus on the immune differences between the sexes that are not downstream of the gonadal hormones. These differences include the gene content of the sex chromosomes, the inactivation of chromosome X in women, the consequences of non-random X inactivation and escape from inactivation, and the states that are uniquely met by the immune system of women—pregnancy, birth, and breast feeding. While these female-specific states are temporary and involve gonadal hormonal changes, they may leave a long-lasting footprint on the health of women, for example, by fetal cells that remain in the mother's body for decades. We also briefly discuss the immune phenotype of congenital sex chromosomal aberrations and experimental models that enable hormonal and the non-hormonal effects of the sex chromosomes to be disentangled. The increasing human life expectancy lengthens the period during which gonadal hormones levels are reduced in both sexes. A better understanding of the non-hormonal effects of sex chromosomes thus becomes more important for improving the life quality during that period.

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S. L. Klein and C. W. Roberts (eds.), *Sex and Gender Differences in Infection and Treatments for Infectious Diseases*, Current Topics in Microbiology and Immunology 441, https://doi.org/10.1007/978-3-031-35139-6_1

1 Evolutionary Perspective

Females mount a stronger immune response than males and hence have a better prognosis in most infectious diseases and higher rates of most autoimmune diseases, as discussed later in this book. This difference in the immune systems of females and males is consistent not only across mammalian species but also across other animal species that may have different sex determination mechanisms [reviewed in (Klein and Flanagan 2016)]. These differences give rise to two questions. First, what were the selective forces that shaped these differences in immune responses between females and males? And, second, if females have indeed developed a better defense mechanism that provides a selective advantage, why have males not developed similarly?

Both questions may be answered, at least in part, by examining the evolution of cost and benefit trade-offs in immune defenses and reproduction in females and males. One explanation lies in the evolutionary fitness of an individual, which can be defined in terms of the number of descendants that reach sexual maturation. For females, the number of descendants is limited by the length of the sexual maturity period, whereas for males, it is limited by the number of mates fertilized. Thus, females need to invest resources in longevity, which implies a stronger immune function to increase their survival probability. In males, allocation of resources from immune defense to mating may bring higher fitness gains than longevity (Zuk 2009; Zuk and Stoehr 2002; Rolff 2002). However, if the males also contribute to offspring rearing or if a stronger immune response improves both survival and mating success, e.g., by making individuals with fewer parasites more attractive, then such a trade-off may not be a strong selective force for a stronger immune response in females (Stoehr and Kokko 2006).

A complementary explanation is that sex-specific adaptations may involve genes that confer some immune advantages or disadvantages, either directly through pleiotropic effects or indirectly by inducing a positive selective sweep of their immediate genetic region. It would seem logical that such advantageous or disadvantageous immune functions that had evolved in one sex should have been selected for or against, respectively, in the other sex as well. However, this selection may be countered by the disadvantage of incorporating the sex-specific variant into the other sex or of removing the sex-specific variant, a phenomenon known as sexual antagonism. In sexual antagonism, a mutation that is beneficial to one sex may be harmful to the other, as demonstrated experimentally in non-immune systems (Rice 1992). A possible example of sexual antagonism in the immune system is the action of the male hormone, testosterone, which promotes the male phenotype features but decreases the immune response (Gubbels Bupp and Jorgensen 2018).

Notably, the female immune system faces challenges that the male immune system does not, namely, pregnancy, birth, and breast feeding, as described later in this chapter. Although the selective forces posed by these challenges acted only on females, they may also have contributed to the differences in immunity between sexes. Interestingly, it has been argued that fetal cells that are retained in the mother's

body for decades after birth—a phenomenon termed fetal microchimerism—constitute cellular representatives of cooperation and conflict of interest between males and females and between mother and fetus (Cómitre-Mariano et al. 2021; Kinder et al. 2017).

Finally, the immune sexual dimorphism of the host contributes to host–pathogen coevolution (Gipson and Hall 2016). For example, females can transmit pathogens via several routes that are not available to males, namely pregnancy, birth, and breast feeding. As a result, certain pathogens might have adapted for lower virulence in women, which translates into worse observable outcomes in men (Úbeda and Jansen 2016). Thus, when examining differences in pathogenicity between the female and male immune systems, one should bear in mind that the pathogens themselves might display different phenotypes in female and male hosts.

2 Sex Chromosomes

The genetic codes of mammalian males and females are identical, except for the sex chromosomes, for which females are homozygous (XX) and males are heterozygous (XY). The sex chromosomes are derived from an ordinary pair of recombining autosomes. During evolution, recombination between that pair of chromosomes has been progressively suppressed, and X and Y chromosomes have become highly diverged from each other and have ceased to recombine throughout their entire length (Lahn and Page 1999). However, two pseudoautosomal regions (PARs) on the sex chromosomes, termed PAR1 and PAR2, still recombine between the X and Y chromosomes during meiosis in males. Over time, most of the mammalian Y chromosome has been lost, and the current human Y chromosome is 62 Mbp long and encodes for only ~ 100 protein coding genes as well as repetitive and non-functional DNAs. The X chromosome is much longer (~ 155 Mbp) and encodes for ~ 800 protein coding genes. The human X and Y chromosomes have only recently been sequenced from telomere to telomere (Miga et al. 2020; Rhie et al. 2022). Both sex chromosomes are enriched for complex repetitive sequences, and the Y more than the X (Rhie et al. 2022).

In the text that follows this section, we focus on the ***human*** sex chromosomes and immune systems, except where explicitly stated.

2.1 X Chromosome Inactivation

In females, one of the two copies of the X chromosome is randomly silenced early in the development to compensate for the double dosage of the X chromosome compared to males. However, this X chromosome inactivation (XCI) is more dynamic than initially thought, especially during embryonic development and in the immune system.

The long non-coding RNA, X-inactive specific transcript (*Xist*), is expressed only from the inactive X chromosome and induces the XCI. During embryonic development in the mouse, starting from the two-cell stage, Xist is expressed only from the paternal copy of the X chromosome in female embryos. As a result, the paternal X is silenced during early cleavage stages. Though this paternal XCI is replaced with random XCI in the embryo, the extraembryonic tissues retain paternal XCI (Okamoto et al. 2004; Mak et al. 2004), which reduces the exposure of the maternal immune cells to non-self-proteins. Surprisingly, though XIST is transcribed from both the maternal and paternal X chromosomes in in vitro fertilized human embryos, none of the X chromosomes is inactivated (Petropoulos et al. 2016). In accordance, either the maternal or the paternal copies of the X chromosomes can go through XCI in human placenta cells, though the paternal copy of the X chromosome is more likely to be inactivated (Hamada et al. 2016). Thus, the exposure of the human mother to paternal antigens on the X chromosome may be greater than that of the murine mother.

However, even when XCI is in place, it is incomplete, with some genes escaping XCI and being expressed from both the active and inactive copies of the X chromosome. The mechanisms that determine which genes will escape from XCI are not fully understood. Although the escape from XCI leads to expression of a gene from both copies, the expression from the inactive X is usually lower than that from the active X and varies between chromosomal regions, tissue and individuals (Tukiainen et al. 2017; Carrel and Willard 2005). Therefore, expression of X escapee genes located outside the PAR is higher in females, which express them from both the active and the inactive X chromosomes, vs. males, which express those genes only from a single X chromosome. In contrast, expression of genes encoded on PAR1 is higher in males, which express them from the X and Y chromosomes, vs. females, which express them from the active and inactive (relatively low levels) X chromosomes (Tukiainen et al. 2017). Since XCI escapee genes are transcribed from two alleles in females, the two potentially slightly different translated proteins in females may confer wider activity in addition to higher expression, thus conferring functional advantages on females, compared to males which can only express one allele of genes in the X chromosome (Migeon 2007). For example, women who carry the T allele (TA or TT genotype) of a frequent single-nucleotide polymorphism (SNP) in toll-like receptor 7 (*TLR7*), rs179008, have lower levels of TLR7 protein production in their plasmacytoid dendritic cells, higher proportions of type I interferon-producing plasmacytoid dendritic cells, and a lower viral burden during acute HIV-1 infection compared to women homozygous in the A allele (AA genotype), whereas no differences were found between T0 and A0 males (Azar et al. 2020). In contrast, this more diverse repertoire of cellular proteins of X-linked genes in females compared to males may also increase the likelihood of developing an autoimmune disease (Youness et al. 2021).

It has recently been shown that XCI in the female immune system is dynamic and incomplete. Lymphocyte progenitors lack XCI. XIST localization to the X chromosome occurs in more differentiated progenitors, and from there XIST is dispersed in mature naïve B and T cells, only to be relocalized to one of the X chromosomes

when lymphocytes are activated (Syrett et al. 2019a, b). Natural killer cells, myeloid and lymphoid dendritic cells, and bone marrow-derived macrophages also display dynamic and variable patterns of Xist RNA localization (Syrett, Sindhava et al. 2019a, b). The functional implications of the relaxed form of XCI maintenance in the female mammalian immune system are unclear. It has been speculated that the female immune cells benefit from increased dosage of certain X-linked genes and that each type of immune cell has a specific set of X-linked genes exhibiting sex-biased expression (Syrett and Anguera 2019). This dynamic XCI maintenance and immune cell-specific XCI escape may contribute to immune cell sexual dimorphism and the female disposition toward autoimmunity (Syrett and Anguera 2019). Indeed, XIST is less localized in T cells from systemic lupus erythematosus (SLE) patients compared to healthy women, and X-linked genes are up-regulated in those patients (Syrett et al. 2019a, b).

As the female body is composed of two distinct cellular populations, one expressing the paternal X chromosome and the other expressing the maternal X chromosome, females present cellular mosaicism. This cellular mosaicism inevitably leads to interactions between the two different cell populations, which may be positive (cellular cooperation) or negative (cellular interference). In cellular cooperation, cells expressing the wild-type allele share the wild-type product with cells expressing the mutant allele, thereby reducing or eliminating the phenotype caused by the mutation. In cellular interference, cells expressing the mutant allele compromise the function of cells expressing the wild-type allele (Migeon 2020, 2007). Competition between the cell populations may lead to skewing of X inactivation in females, where the cell population that has a relative advantage will be present in more than half of the cells and may eventually lead to elimination of the other population (Migeon 2020, 2007). Although cellular cooperativity, interference and competition in female mosaicism cell populations have been rarely reported, given the large number of immune-related genes on the X chromosome, it is likely that such effects do exist and contribute to immune sexual dimorphism. Indeed, it is known that skewed X inactivation occurs in healthy women and that its frequency increases with age (Busque et al. 2009). Skewing is more frequent in women with certain female-predominant autoimmune diseases, such as scleroderma (Ozbalkan et al. 2005), autoimmune thyroid diseases (Ozcelik et al. 2006), juvenile idiopathic arthritis (Uz et al. 2009), rheumatoid arthritis, and systemic sclerosis (Kanaan et al. 2016). Notably, it is suspected that the skewed X inactivation is a factor contributing to the development of an autoimmune disease, and not the consequence of the disease; the basis for this premise is the thinking that the immune system has not been not trained to recognize a rare allele as self (due to its low expression) and is thus predisposed to develop antigens against it (Ozbalkan et al. 2005; Lambert 2009).

Notably, as XCI is maintained by chromatin silencing modifications, any condition that affects or interferes with chromatin silencing modifications could also interfere with the inactivation of genes on the silenced X chromosome, thus causing a female-specific phenotype even though the particular condition is sex independent (Migeon 2007). On the other hand, as the XCI in female cells requires many molecular components to silence an entire chromosome, XCI may compete with chromatin silencing

at autosomal loci, thereby indirectly affecting autosomal gene expression in females (Burgoyne and Arnold 2016).

2.2 *Immune-Related Genes on the Sex Chromosomes*

The X chromosome contains about 800 protein coding genes as well as microRNAs and long non-coding RNAs. The biology of the sex chromosomes causes several unique facets in the expression on their genes. First, as males have only one copy of the X chromosome, any mutation in a non-PAR gene on the X chromosome will be dominant, and the resulting disorder is expected to be male biased or male specific. Indeed, many X-linked diseases have a milder phenotype or no phenotype in women [reviewed in (Migeon 2020)]. An excellent example is the X-linked *FOXP3* gene, the master regulator of regulatory T cells. Mutations in FOXP3 cause the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), and an X-linked recessive disorder in men, caused by lack of regulatory T cells. Certain FOXP3 mutations in the male fetus may even lead to miscarriage, as the fetal immune system is not properly regulated (Rae et al. 2015).

Second, in females, several immune regulators are known to escape XCI in different cell types and conditions, including TLR7, CD40L, CD99, LAMP-2, IRAK-1, USP27X, DDX3X, and XIAP [reviewed in (Mousavi et al. 2020)]. This escape may lead to higher expression levels in females, which might contribute to sex differences in immunity and the sex bias in the frequencies of autoimmune diseases.

As mentioned before, the X chromosome also codes for long non-coding RNAs. Two long non-coding RNAs on the X chromosome, *XIST* and *FIRRE*, which are involved in the XCI mechanism also have an immune function. In addition to its role in XCI, *XIST* also suppresses acute inflammation by interacting with NF-κB to delay the migration of NF-κB into the nucleus (Shenoda et al. 2021). *FIRRE* is regulated by NF-κB and, in turn, regulated inflammatory genes on autosomal chromosomes (Lewandowski et al. 2019; Lu et al. 2017). Unlike *XIST*, *FIRRE* is expressed from both copies of the X chromosome in females (Hacisuleyman et al. 2014). In mice, the chromatin region of *Firre* is more accessible in female macrophages compared with males (Gal-Oz et al. 2019).

Compared to autosomes, the X chromosome is enriched for microRNAs, whereas the Y chromosome is depleted (Guo et al. 2009). Similar to the protein coding genes on the X chromosome, X-linked microRNAs are subject to XCI and escape from XCI. A few X-linked microRNAs have indeed been shown to be immune regulators (Pinheiro et al. 2011).

The Y chromosome contains very few genes, but it does contain a small number of genes that epigenetically regulate immune system gene expression (Case et al. 2013). Non-PAR homologous genes on the X and Y chromosomes might differ in their functional properties or expression patterns. For example, if the X- and Y-encoded histone demethylases are different in their substrate specificity or affinity,

it might lead to a genome-wide difference between males and females (Hughes and Page 2015).

2.3 Sex Chromosome Abnormalities and the Immune System

Sex chromosome abnormalities are conditions in which individuals have a karyotype other than XX females or XY males. The difference could lie in the addition or deletion of a complete copy of a sex chromosome or a part of a chromosome. The small size of the Y chromosome, the limited functions of its genes, and the X dosage compensation mechanism probably explain why chromosomal aberrations in the sex chromosomes are tolerated better than those on autosomes: In humans, there is only one viable monosomy, i.e., monosomy X, and there are also three viable autosomal trisomies, of which only trisomy 21 (Down syndrome) carriers can reach maturity, but several trisomies of sex chromosomes can reach maturity. The cells of individuals with sex chromosome abnormalities are often a mosaic in terms of sex chromosomes composition. Some sex chromosome abnormalities are lethal and prevent fetuses from developing correctly. Even the apparently non-lethal sex chromosome abnormalities might be subject to in utero selection. Sex chromosome abnormalities lead to a variety of phenotypes—some of which may be asymptomatic. These abnormalities may cause fertility and growth abnormalities and may also affect the immune system and the risk for various autoimmune diseases, as detailed below. In general, these effects are extreme manifestations of typical immune sexual dimorphism, whereas additional copies of the X chromosome make the immune phenotype more female like, and additional copies of the Y chromosome make the immune phenotype more male like.

Sex chromosome abnormalities provide valuable information regarding the effect of sex chromosomes on the immune system and the tendency to autoimmune diseases. However, there are inherent limitations in the study of sex chromosome abnormalities, namely these abnormalities are rare, as are most autoimmune diseases, and the estimation of an increased or decreased risk ratio is based on a very small number of individuals with co-morbidity, which are subject to biases caused by undiagnosed non-phenotypic carriers and strong in utero selection of viable newborns with sex chromosome aberrations. With the increase in non-invasive prenatal screening and the establishment of centralized digital medical registries in several countries, the estimates of risk ratio of autoimmune diseases in sex chromosomal aberrations will become increasingly more accurate and will be able to indicate clinical phenotypes in whom sex chromosomes make an important contribution to the phenotype.

Below we discuss some sex chromosome aberrations, focusing mainly on their immune-related phenotypes.

2.3.1 Turner Syndrome (45,X)

Turner syndrome, a complete or partial X chromosome monosomy, is the only viable human monosomy. Turner syndrome occurs in 1 in 2000–2500 females, and its most frequent karyotype is 45,X. Other karyotypes of Turner syndrome are 45,X/46,XX mosaicism or isochromosome X (Hjerrild et al. 2008). Turner syndrome manifests in atypical sexual development and skeletal, cardiac, endocrine, and metabolic characteristics. Patients also experience a variety of immune-related symptoms, such as chronic inflammation, a decrease in the levels of circulating lymphocytes, and changes in immunoglobulin ratios [reviewed in (Thrasher et al. 2016)], and are at a higher risk for several autoimmune diseases compared with 46,XX women (Jørgensen et al. 2010, De Sanctis and Khater 2019), i.e., women with Turner syndrome are at an almost fourfold increased overall risk for male-predominant autoimmune diseases and 1.7-fold increased overall risk for female-predominant autoimmune diseases (Jørgensen et al. 2010). Notably, the risk varies with the Turner syndrome karyotype, which is to be expected, as different karyotypes result in different numbers of copies of genes on different parts of the chromosome (e.g., patients with isochromosome Xq have three copies of all genes in the duplicated q arm and one copy of the genes on the p arm, as opposed to one copy of all X genes in patients with monosomy X).

Women with Turner syndrome share the male vulnerability to deleterious mutations in X-linked genes and exhibit haploinsufficiency of PAR genes, which, in males, has a Y chromosomal counterpart. Indeed, a few genes encoded from the PAR of the X chromosome that are involved in a variety of immune processes are differentially expressed between Turner syndrome and XX females (e.g., IL3RA) or XY males (i.e., P2RY8), possibly contributing to the increased autoimmunity in Turner syndrome individuals (Trolle et al. 2016). Another example of the possible effect of X-linked genes on immune activity is the ubiquitously transcribed tetratricopeptide repeat on the X chromosome (UTX). UTX is a histone H3 lysine 27 demethylase that escapes XCI and is thus down-regulated in Turner syndrome, leading to reduction in T helper cells (Thrasher et al. 2016).

2.3.2 Klinefelter Syndrome (47,XXY)

Klinefelter syndrome is a condition—affecting 1 in 500–1,000 men—in which individuals carry at least one copy of the Y chromosome and two or more copies of the X chromosome. The most common karyotype of Klinefelter syndrome is 47,XXY, and only 10–20% of cases have more copies—these are typically a mosaic of 46,XY/47,XXY/48,XXYY/48,XXXY/49,XXXXY (Giltay and Maiburg 2010). Due to the presence of the Y chromosome, individuals with Klinefelter syndrome have a male phenotype. However, as in women, in cells of individuals with Klinefelter syndrome, one of the X chromosomes is subject to XCI and escape from XCI [reviewed in (Navarro-Cobos et al. 2020)].

Men with Klinefelter syndrome also suffer from a higher risk for female-predominant autoimmune diseases, such as SLE, rheumatoid arthritis, Sjögren's syndrome, multiple sclerosis, and type 1 diabetes compared with XY men (Seminog et al. 2015), and their frequency of autoantibodies is also higher (Panimolle et al. 2021). Surprisingly, even though eosinophilic esophagitis is a male-predominant disease (Hruz and Nanan 2014), there is an increased risk for eosinophilic esophagitis in men with Klinefelter syndrome (Ghisa et al. 2022).

2.3.3 Triple X Syndrome (47,XXX)

One in every thousand females has an extra X chromosome (47 XXX), also known as triple X syndrome or trisomy X. Similar to XX females, in the triple X syndrome only one copy of the X chromosome is active, and the other two copies of the X chromosome are inactivated (Tartaglia et al. 2010). The syndrome is often asymptomatic, with an estimated 10% of carriers diagnosed on the basis of phenotype. The syndrome includes fertility issues and a wide range of behavioral and physical phenotypes, but no altered immune phenotypes have been reported (Tartaglia et al. 2010; Otter et al. 2010). Several anecdotal case reports of women with X trisomy (or a mosaic of more than three copies of X) have described a co-morbidity with a variety of immune-related conditions, including Sjögren's syndrome involving additional systemic organs (Miyakawa et al. 1997; Fujimoto et al. 2015), childhood-onset SLE (Yamazaki et al. 2021; Barbosa et al. 2020), and autoimmune thyroid disorder (Goswami et al. 2003). A systematic study of the frequency of X trisomy in female-predominant autoimmune disease cohorts found that the incidence of SLE and Sjögren's syndrome was more than double in women with trisomy X compared to XX women, but the frequency was unchanged in other diseases, such as primary biliary cirrhosis and rheumatoid arthritis (Liu et al. 2016). As the phenotypic abnormalities associated with trisomy X may result from overexpression of genes that escape the XCI, it is possible that the contribution of escapees to the increased risk for SLE and Sjögren's syndrome is higher than that for other female-predominant autoimmune disorders.

2.3.4 Jacob Syndrome (XYY)

Jacob syndrome patients carry one X and two Y chromosomes (47, XYY), with an incidence of 1 in 1000 males. Like other sex chromosome abnormalities, Jacob syndrome often remains undiagnosed. The symptoms of Jacob syndrome vary between men and can include tall stature, low muscle tone, and macrocephaly as well as developmental delays, behavioral issues, and fertility problems (Sood and Clemente Fuentes 2022). Men with Jacob syndrome are at increased risk for asthma (Bardsley et al. 2013), but no other immune-related phenotypes have been described as associated with Jacob syndrome. Therefore, it is generally assumed that the additional copy of the Y chromosome does not affect immune function.

2.3.5 Somatic Sex Chromosomes Aberrations

In addition to the congenital sex chromosome aberrations reported above, many individuals carry populations of cells with chromosome aberrations, including aberrations in sex chromosomes that can contribute to immune alterations. For example, the rate of monosomy X is higher in women with systemic sclerosis or autoimmune thyroid disease compared to healthy women (Invernizzi et al. 2005). The monosomy X rate increases with age and is more frequent in blood T cells and B cells compared to other blood cell types (Invernizzi et al. 2005). Hence, monosomy X might affect immune function.

3 Experimental Models for Studying the Sex Effect on Immunity

Disentangling the effect of the composition of sex chromosomes and gonadal hormonal effects on the immune system is challenging, as the hormonal profile is highly dependent on the sex chromosome composition and both factors affect the immune system. Whereas hormonal manipulations in experimental animal models have been used extensively, the study of the effect of sex chromosomes on the immune system is less common. Below we describe several experimental systems that have been developed to study the effect of the sex chromosomes independently of the hormonal effect and their application to the study of the immune system.

3.1 Four Core Genotypes Mouse Model

The four core genotypes (FCG) mouse model (De Vries et al. 2002) produces XX and XY gonadal males and XX and XY gonadal females, thereby enabling a comparison of the effects of XX and XY genotypes in same (gonadal) sex mice. In this model, XX females are crossed with $XY^{-}Sry^{+}$ males, where Y^{-} stands for a Y chromosome with deletion of the testis-determining gene Sry (Lovell-Badge and Robertson 1990), and Sry^{+} stands for insertion of an Sry transgene onto an autosome. With its classic two-way ANOVA design, the FCG model can test simultaneously for sex chromosome effects, hormonal effects, and the interaction of sex chromosome and hormonal effects (Arnold and Chen 2009). When same gonadal sex XX and XY mice differ, the difference is due to the sex chromosomes (mostly their composition), although it can also arise from different patterns of imprinting or X inactivation (Burgoyne and Arnold 2016). The FCG model has been used to study the effect of the sex chromosome complement on several types of immune responses, disease models, and viral infections, and the results reflect the complicated intricacy of the interplay between the sex chromosomes complement and gonadal hormones.

Immunization of FCG mice with an autoantigen showed that the Y chromosome, independently of sex hormones, was immunostimulatory, in contrast to testosterone, which was immunosuppressive, suggesting compensatory effect between the Y chromosome and testosterone (Palaszynski et al. 2005). The FCG model combined with mouse models of the female-predominant autoimmune diseases, experimental autoimmune encephalomyelitis (the most common mouse model for multiple sclerosis) and pristane-induced lupus (a mouse model of SLE), showed higher frequency of the diseases in XX animals compared to XY animals, independently of gonadal sex (Smith-Bouvier et al. 2008). In contrast, gonadectomy and hormone manipulation in the FCG model showed no effect of sex chromosomes composition on the numbers of total CD4+ and CD8+ cells in young mice, although gonadal hormones markedly affected those numbers (Ghosh et al. 2021).

In the FCG model with induced bladder cancer, both female gonadal sex and an XX genotype independently improved the overall survival. Mechanistically, the XX protection is conferred, at least in part, via KDM6A escape from XCI (Kaneko and Li 2018). The FCG model has also been used to demonstrate that sex chromosomes can impact susceptibility to some, but not all, viral infections; for example, the Y chromosome contributes to the severity of coxsackievirus B3 infection but not influenza A virus infection (Robinson et al. 2011).

3.2 Human Cell Lines with Sex Chromosome Aberrations

The natural occurrence of individuals with sex chromosome aberrations facilitates the study of the number of sex chromosome copies on gene expression, as was recently done on lymphoblastoid cell lines with one active X and 0–3 copies of inactive X (San Roman et al. 2022). The expression of more than third of the genes on the X chromosome was affected by X chromosome copy number. This finding was independent of the presence of a Y chromosome, indicating that the effect is genetic and not caused by a hormonal effect. Notably, although the change in expression of some of those genes can be explained by escape from X inactivation, the allelic ratio shows that for many of those genes, the inactive X copy number modulates the expression level of the gene copy that is on the active X chromosome, indicating *trans*-regulation (San Roman et al. 2022). Although such cell lines have not yet been used for the study of the immune response, they hold promise for the study of the sex chromosome effect on the human immune cell response.

3.3 Additional Mouse Models

Steroidogenic factor 1 (Sf1) knockout mice of both sexes are born without gonads or adrenals and die at birth unless they receive treatment. Although this model is useful to test whether XX and XY immune-related differences are retained when there is no

gonadal hormonal exposure (Luo et al. 1995), it is not used to study of sex differences in immunology, probably due to the structural and functional abnormalities in the spleen and in hematopoiesis of Sf1 knockout mice (Morohashi et al. 1999).

The mouse model of Turner syndrome (39X0) has a surprisingly mild phenotype and is mostly viable (Lopes et al. 2010; Ashworth et al. 1991). A possible explanation to the difference between the phenotype of human and murine X chromosome monosomy is the small number of genes on the murine PAR region and, in general, a lower number of escapees in mouse compared to human. This probably means that the mouse is a limited model for studying the effects of X copy number aberrations. Notably, due to the rapid evolution of the Y chromosome in terms of gene content, size, and structure, the mouse Y chromosome is far more different from the human Y chromosome vs. other chromosomes, which limits its use as a model (Hughes and Page 2015).

4 Female-Specific States and Their Immune Consequences

The female body is required to be tolerant to sperm and to the fetus, which contain foreign proteins, while protecting the developing fetus from pathogens. Also, after birth, the immunity of the newborn baby is supplemented via breast feeding [reviewed in (Camacho-Morales et al. 2021; Lokossou et al. 2022)]. Pregnancy-related and lactational-related states are specific to women and pose challenges to the immune system. Here, we focus on those aspects of these states that are most likely to contribute to the differences in immunity between women and men.

4.1 Pregnancy as a Graft

At the fetal–maternal interface, two immunologically distinct individuals meet. To preserve the pregnancy, the maternal immune system must ensure tolerance to the fetal material [reviewed in (Hsu and Nanan 2014)]. This tolerance can be achieved by a combination of anatomical separation (that minimizes the interface), reduced fetal antigenic potential, and maternal immune inertness (Billington 2003). Various models have been suggested to explain why the maternal immune system does not attack the fetus as it would be a graft [reviewed in (Chaouat 2016; Billington 2003)], and specific mechanisms have been identified [reviewed in (Erlebacher 2013)]. In parallel to maternal modifications, the fetal immunogenicity is reduced; for example, placental trophoblast cells do not express the strong histocompatibility antigens, HLA-A, -B, or -D, thereby reducing their immunogenicity to T cells (Wood 1994). However, trophoblasts do express paternal HLA-C antigens, which are recognized by maternal KIR receptors on uterine natural killer cells. The combination of a specific maternal KIR genotype with a fetal HLA-C2 increases the risk of preeclampsia (Hiby et al. 2004). The fetus may also actively reduce the maternal immune response,

for example, by releasing from the placenta exosomes that suppress maternal T cell signaling components (Sabapatha et al. 2006). However, in preeclampsia, the maternal response to the pregnancy endangers the fetus and the mother and involves an immune component. The risk of preeclampsia is higher in (1) oocyte-donation pregnancies compared with other methods of assisted reproductive technology or natural conception (Masoudian et al. 2016), (2) first pregnancy (Eskenazi et al. 1991), (3) subsequent pregnancy but with a different father (Robillard et al. 1993), and (4) shorter duration of sexual relationship with the father (Kho et al. 2009). These observations have given rise to the hypothesis that the maternal adaptive immune system is involved in preeclampsia and that prolonged exposure to a specific set of paternal antigens on sperm or fetus, by sexual relationships or pregnancies with the same partner, respectively, leads to tolerance to the paternal antigens [reviewed in (Robillard et al. 2011)].

4.2 Microchimerism in Fetal Cells

The mother and fetus exchange cell-free DNA, microRNAs, exosomes, and even cells. Such fetal cells can persist in the mother for decades (Bianchi et al. 1996). Rare cells found in one individual that originate from another individual and are genetically distinct from the host individual are termed microchimeric cells, and if the other individual is a fetus, the phenomenon is termed fetal microchimerism. Preeclampsia increases the frequency of fetal microchimerism in blood cells (Gammill et al. 2013) and, particularly, in immune cells (McCartney et al. 2022). There is contradictory evidence regarding the contribution of fetal microchimerism to maternal autoimmune diseases later in life (Gammill and Nelson 2010; Nelson 2012). Nonetheless, fetal microchimerism has contributed to the evolution of the female immune system and clearly remains a potential player in female immunity (that is missing in male immunity). It has even been suggested that fetal microchimerism has played an active part in the evolution of cooperation between sexes and between mother and fetus, as well as in the conflict of interest between those parties (Cómitre-Mariano et al. 2021). The immune effect of fetal microchimerism might depend on multiple factors, including the fetal sex and age and the (dis)similarity between the fetal and maternal HLA compositions (Nelson 2012). It has also been suggested that fetal microchimerism in immune cells may even trigger a graft-versus-host-like disease, activating and regulating the maternal immune responses in ways that increase the risk of autoimmune disease (Cómitre-Mariano et al. 2021). In contrast, fetal microchimerism may also improve wound healing in the mother and protect her from cancer [reviewed in (Fugazzola et al. 2011)].

5 Concluding Remarks

With the constant increase in human life expectancy, the period during which gonadal hormones levels are reduced in women and men will become increasingly longer. During this period, the non-hormonal differences between the sexes contribute more to the difference between sexes immunity than earlier in life, when hormonal changes are more prominent. A better understanding of the non-hormonal effects of sex chromosomes thus becomes more important for improving the life quality during that period.

Although the only genetic difference between women and men is seemingly the sex chromosome complement, extensive research has revealed this difference is multifaceted and is also influenced by additional genetic factors. Briefly, men and women with monosomy X are more vulnerable to mutations on X. The inactive X chromosome is a significant amount of silenced DNA, and as a result it is affected by global DNA silencing changes and may also affect such changes. All humans carry cells of their mothers, which may affect men and women differently, and women who have carried pregnancies and hence been exposed to fetal material may carry fetal cells for many years. Although the effect of the above differences on the functioning of the immune system and the likelihood of developing autoimmune diseases has been shown in some cases, their full consequences, and the mechanisms behind them are only just beginning to be understood.

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The Influence of Sex Hormones and X Chromosome in Immune Responses

Nina Anesi, Charles-Henry Miquel, Sophie Laffont, and Jean-Charles Guéry

Abstract Males and females differ in their susceptibility to develop autoimmunity and allergy but also in their capacity to cope with infections and cancers. Cellular targets and molecular pathways underlying sexual dimorphism in immunity have started to emerge and appeared multifactorial. It became increasingly clear that sex-linked biological factors have important impact on the development, tissue maintenance and effector function acquisition of distinct immune cell populations, thereby regulating multiple layers of innate or adaptive immunity through distinct mechanisms. This review discusses the recent development in our understanding of the cell-intrinsic actions of biological factors linked to sex, sex hormones and sex chromosome complement, on immune cells, which may account for the sex differences in susceptibility to autoimmune diseases and allergies, and the sex-biased responses in natural immunity and cancer.

1 Introduction

Autoimmune diseases (AIDs) differentially affect females and males. In developed countries, about 5% of the population suffers from autoimmune diseases, which is estimated to represent between 14 and 23 million people in the USA (Committe 2005), of which women are the most affected category (70 to 80%). Women also produced a greater antibody response than men, with higher range of protection and of better quality, to various types of pathogens and vaccines (Fink and Klein 2018).

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S. L. Klein and C. W. Roberts (eds.), *Sex and Gender Differences in Infection and Treatments for Infectious Diseases*, Current Topics in Microbiology and Immunology 441, https://doi.org/10.1007/978-3-031-35139-6_2

This sex bias in the anti-viral immune response has been clearly documented during the COVID-19 pandemic, whose infected female patients presented a lower risk of disease severity as compared to males at all classes of age above 50 (Scully et al. 2020; Takahashi and Iwasaki 2021).

This accumulated ability of women to induce a stronger immune response has been associated to a higher risk to develop AIDs, as scleroderma, rheumatoid arthritis (RA), multiple sclerosis (MS), Sjögren's syndrome or systemic lupus erythematosus (SLE), each of them with a specific high female/male sex ratio (Billi et al. 2019). This female predominance is commonly seen in SLE patients, of which, nine women for one man are affected (Whitacre 2001), female predominance is also observed in childhood before puberty or in postmenopausal women, when estrogen levels are low (Smith et al. 2019), suggesting that alternative mechanisms besides sex hormones may be at play.

The difference in antibody response is one of the best conserved sex differences in immunology within mammals. In accordance with it, studies have revealed that the differences are due to the composition of the immune system itself. In general, women have a higher number of T lymphocytes (LT) CD4/CD8, B lymphocytes (LB) and plasma cells (PC), while men have a higher proportion of Natural Killer cells (NK) and CD14 and CD16 monocytes (Klein and Flanagan 2016). This difference in the composition of the immune system has also been found in mice (Menees et al. 2021), suggesting that it represents an evolutionary advantage for females that have been conserved over time (Zuk 2009). The female bias in autoimmune diseases was initially mainly attributed to sex hormones. Indeed, estrogens in females and androgens in males can positively or negatively influence the maintenance, development and effector functions of various immune cell subsets from the innate and adaptive immune system (Laffont et al. 2017a; Laffont and Guery 2019; Wilkinson et al. 2022).

In addition to the action of sex steroid hormones, genetic mechanisms linked to sex chromosome effects have recently emerged as a potential contributor of sex differences in immune responses across a wide range of age (Youness et al. 2021; Jiwrajka and Anguera 2022), and men with Klinefelter syndrome (47,XXY) have an equivalent susceptibility to women (46, XX) to develop lupus (Liu et al. 2016), while women with turner syndrome (45 X0) appear protected (Bai et al. 2015). Thus, the dosage of the X chromosome seems to be involved in the development of lupus pathology, scleroderma and Sjögren's syndrome.

Understanding the X-linked genetic mechanisms by which biological sex influences the strength and magnitude of innate and adaptive immunity may therefore have implications not only for the treatment of these immunopathological disorders, but also for promoting optimal protective immunity in response to pathogens or for improving vaccine efficacy in both sexes.

2 Sex Hormone Signaling and X Chromosome Dosage Effects

2.1 Sex Steroid Hormones Signaling

2.1.1 Molecular Structure of ERs and AR

Sex steroid hormones, estrogen and androgen, essentially mediate their actions through estrogen receptor α and β and androgen receptor, respectively. ERs and AR belong to the large family of nuclear receptors whose members share similar structures and functions. Their molecular structures can be subdivided into six functional domains: the N-terminal transcriptional regulation domain (or A/B domain), the DNA binding domain (DBD or C domain), the hinge region (D domain), the ligand binding domain (LBD) and the C-terminal region (F domain) (Thomas and Gustafsson 2011). The highly variable A/B region, both in length and sequence, contains the AF-1 transactivation domain which can regulate transcription activity of targeted genes through the recruitment of co-activators or repressors. The A/B domain also contains target sites for phosphorylation by several kinases involved in signaling pathways downstream of growth factors or cytokines (Arnal et al. 2017). The C domain encompasses the DBD which is the most highly conserved domain among the different nuclear receptor members. The DBD consists of two zinc fingers that recognize specific hormone responsive element (HRE) within target genes to modulate their transcription allowing their specificity of action. The D domain is a flexible hinge region, poorly conserved, joining the DBD and the LBD. It contains, among other function domains, the nuclear localization domain (NLS) which is requires for the translocation of the receptor to the nucleus. The large C-term E/F region includes the LBD, the transcriptional activation function 2 (AF-2) and domain responsible for the binding with chaperons. Binding of the hormone to the LBD induces conformational changes allowing stabilization of the receptor dimers.

2.1.2 Mode of Action of ERα and AR.

Hormones and their receptors trigger cellular responses through different pathways that can be grouped into ligand-dependent and non-dependent pathways. In the classical ligand-dependent genomic pathway, binding of the hormone to its receptor induces the release from chaperones, which sequestered them in the nucleus, its homodimerization and its translocation to the nucleus. Liganded receptors can influence target gene transcription through direct DNA binding at HRE or indirect DNA binding via association with transcription factors such as AP-1 and SP-1 for ERα or ETS for AR. Co-activators or inhibitors of the transcription will further be recruited to AF-1 and AF-2 domains to regulate the transcription. In addition to this classical mode of action that is relatively slow, non-genomic signaling can rapidly be initiated, within couple of minutes, upon binding of the hormone to cell membrane-bound

receptors (Arnal et al. 2017). These membrane initiated steroid signaling effects (MISS) trigger cascades of kinases or phosphatases generating second messengers ultimately influencing transcription (Arnal et al. 2017; Dai et al. 2017). Cell surface-initiated androgen action can also occur via non-classical membrane AR, unrelated to nuclear steroid receptors (ZIP9, GPRC6A, OXER1), which engage G-protein coupled signaling mechanisms (Thomas 2019; Kalyvianaki et al. 2019). Besides these ligand-dependent pathways, ERα and AR can be reactivated by others signals such as cytokines like IL-6 or growth factors like epidermal growth factors (EGF) (Arnal et al. 2017; Bennett et al. 2010) that can trigger protein kinase cascade leading to receptor phosphorylation and subsequent activation in the absence of their ligands.

If the mode of actions of ER and AR is similar in many respects, the way the two receptors limit hormone responsiveness is very different (Lee and Chang 2003). In the presence of estrogen, ER is rapidly exported from the nucleus and degraded by the proteasome in the cytoplasm, limiting the duration of promotor occupancy by ER and allowing the constant binding of newly imported receptors (Reid et al. 2003). Conversely, level of AR protein is increased in the presence of androgen. This positive feedback loop could be due to a combination of nuclear localization promotion of the receptor by androgen (Astapova et al. 2021) together with the promotion of AR translation by the hormone (Siciliano et al. 2022). Recent study suggests that deprivation of androgen may lead to the ubiquitination and degradation of AR within the nucleus (Lv et al. 2021).

2.2 X Chromosome Gene Dosage and X Chromosome Inactivation Escape

2.2.1 General Principles of X Chromosome Inactivation

The process of X chromosome inactivation (XCI) begins during embryonic development and is inherited through somatic cell divisions over life. The initiation of XCI is genetically controlled by a regulatory region called the X inactivation center (XIC) located on the long arm of the X chromosome (Xq13.2) in humans (Loda et al. 2022). The inactivation center hosts many genes and transcriptional regulators. One of the best characterized locus is the gene coding for *Xist*, a long non-coding RNA (lncRNA) of approximately 19 Kb in Human and 18 kb in mice which covers in cis the X chromosome to be inactivated. *Xist* will induce the extinction of gene expression or "gene silencing". However, although required for the initiation of the process, *Xist* does not appear necessary in the maintenance of the inactive state in differentiated cells (Loda et al. 2022). During the initiation of XCI, the coating *in cis* of the inactive X chromosome (Xi) by the lncRNA *Xist* leads to a disruption of the structure of the chromosome as well as a complete reorganization of the chromatin. *Xist* then induces a cascade of epigenetic mechanisms leading to the transition from an open and transcriptionally active chromatin (euchromatin) to a condensed and inactive

chromatin (heterochromatin). Thus, locking of chromatin on the Xi is dependent on complex epigenetic mechanisms (Loda et al. 2022). During the initiation process, *Xist* will be recognized by SPEN, a transcriptional repressor which harbors RNA-binding domains. This interaction will lead to the activation of histone deacetylase 3 (HDAC3) targeting permissive epigenetic marks (euchromatin), such as acetylated histone H3 Lys27 (H3K27ac) thereby initiating chromatin locking. Concomitantly, the RNA polymerase II will be excluded from the future inactive compartment. Subsequently, the polycomb PRC1 and PRC2 protein complexes, via their enzymatic activity, will participate in the maintenance of gene extinction. All of these molecular modifications will eventually extend to the entire X chromosome to ensure inactivation, except in the pseudoautosomal regions PAR1 and PAR2, which are shared by both X and Y chromosomes and recombine during meiosis. In addition, the Xa, unlike Xi, is organized into topological associated domains (TADs) where transcriptionally active genes can interact with each others over long distances, forming loops. These loops or TADs, with an average size of 1 Mb (Nora et al. 2012), regulate gene expression by facilitating or preventing interactions between gene promoters and regulatory elements (enhancer) over long distances. From a structural point of view, these spatial conformations are made possible thanks to two architectural proteins: zinc finger protein 11 or CTCF (CCTC-binding factor) and cohesin which can modulate and separate the different TADs. In addition to being poor in TADs, the Xi presents a bipartite structure divided into two large domains called "mega-domains". These two mega-domains are separated by a microsatellite region DXZ4 on which the transcriptional repressor CTCF can also bind and modulate the three-dimensional conformation of the Xi (Rao et al. 2014; Giorgetti et al. 2016) (Fig. 1).

However, the process of XCI to repress X-linked gene expression is incomplete. Indeed, some genes will escape from the global gene extinction of Xi. These genes called "escapees" will then be expressed from the two chromosomes (Xa and Xi) at variable levels.

2.2.2 Escape from XCI: Facultative Versus Constitutive Escapees

As mentioned above, two categories of genes that escape XCI can be distinguished: those which escape constitutively and those which escape in a facultative manner (Fig. 2). Variable escapees can be expressed from the Xi in one or more given tissues but will be completely inactivated in others. At the population level, several studies have also described significant inter-individual variability in XCI escape (Tukiainen et al. 2017; Cotton et al. 2013). In a study investigating 51 variable escapees in monozygotic twins, only 3% of the 51 genes studied were different between twins, i.e., they escape in one twin and are subjected to XCI in the other twin (Cotton et al. 2015). These results therefore suggest the existence of genetic and hereditary factors explaining the great similarity in the "escapees" profile observed between twins. However, genetics alone does not explain all the inter-individual variability observed, given that 3% of variable escapee genes are found to be different between twins. Finally, this variability can also be traced over time. Indeed, it has been described

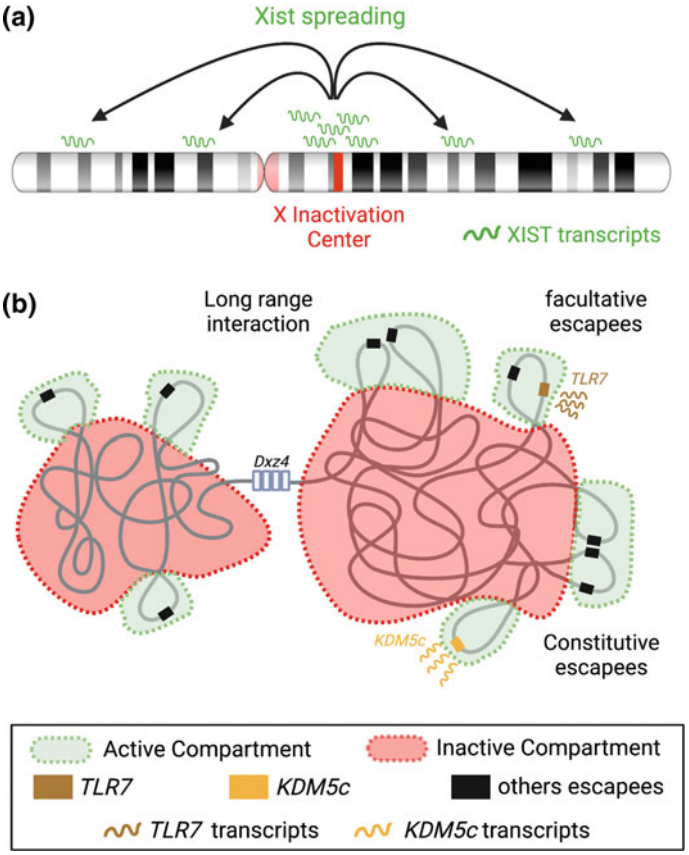

Fig. 1 3D organization of the Xi. The X inactivation process is genetically controlled by a regulatory region known as the X inactivation center (XIC) located on the long arm of the X chromosome (Xq13.2) in humans. During XCI initiation, Xist a long non-coding RNA (lncRNA), encoded on XIC, spreads throughout along the future X inactive chromosome and induces the extinction of gene expression or gene silencing on the Xi (**a**). The inactive chromosome (Xi) is organized into two repressive superdomains (red clouds) separated by DXZ4. As opposed to the active X chromosome (Xa) organized into topological associated domains (TADs) with a large euchromatin domain, the Xi is depleted in TAD but presents chromatin loops that are accessible to the transcriptional machinery (green clouds), allowing the constitutive or facultative escape of certain X genes. As example, *TLR7* is represented as a facultative escapee gene and *KDM5c* for a constitutive escapee (**b**). Created with BioRender.com

that certain genes are inactivated, notably during embryonic development, but are subject to reactivation later in life (Giorgetti et al. 2016). For instance, *Kdm5a* is partially inactivated during embryonic development but constitutively escapes from XCI later in development, while *Huwe1* (E3 ubiquitin-protein ligase) escapes early in development and is highly repressed after embryo implantation (Patrat et al. 2009; Yang et al. 2010). Several constitutive escapees, such as the histone demethylases,

KDM5c and *KDM6a*, are involved in the regulation of transcription by catalyzing the demethylation of di- and tri-methylated histone H3 Lys4 (H3K4me2 and H3K4me3) and of H3K27me3, respectively (Loda et al. 2022). This led to the hypothesis that constitutive escapees could also themselves directly contribute to the establishment/maintenance of XCI and/or to the regulation of others facultative escapees.

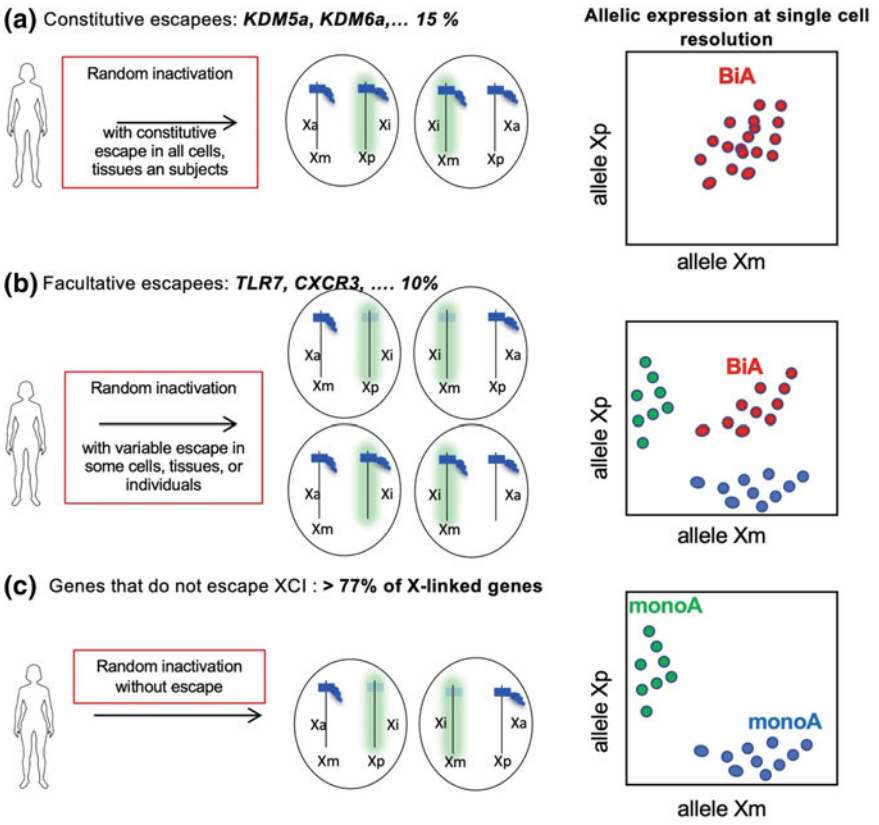

Fig. 2 X chromosome inactivation, facultative versus constitutive escapee genes. X chromosome inactivation occurs in all individuals whose chromosome conformation is XX; this mechanism is randomly carried out in one of the maternal or paternal X chromosome. XCI is initiated by the long non-coding RNA *XIST* (green), which becomes highly expressed on one allele and coats the future inactive X chromosome (Xi) *in cis*, leading to transcriptional repression. The result is a mosaic of cells where in theory half of the cells have an active chromosome (Xa) of maternal origin and the other half of paternal origin, and an inactivation of more than 77% of the X-linked genes in one of two X chromosomes (**a**). Nevertheless, some genes, as *KDM5a and KDM6a*, always escape from XCI in all cells, tissues and individuals, representing among the 15% of the X-linked genes, and are referred as constitutive escapee genes (**b**), whereas approximately another 10% of the X-linked genes, as *TLR7* and *CXCR3*, are tissue specific with dedicated functions and variably escape between cells, tissues and individuals. They are referred as facultative escapee genes (**c**). Dot plots on the right illustrate the putative allelic expression pattern of each alleles from the Xp and/or the Xm at single-cell resolution

2.2.3 Escapees of Interest in Immunity

Escape from XCI is a process found in many mammals, which underlines the importance and advantages of this mechanism in the female. As previously discussed, many immunity-related genes are carried by the X chromosome and some of them escape from XCI at varying levels (Youness et al. 2021). The facultative escapees generate much more cellular heterogeneity in the expression of X-linked genes, as compared to the constitutive escapees (Fig. 1). This diversity could be advantageous during the course of the development of adaptive immune responses, by selecting those cells endowed with enhanced functional properties in response to particular pathogens. However, this same mechanism could be deleterious and contribute to the development and exacerbation of AIDs and in particular of SLE (Youness et al. 2021). Indeed, a growing literature shows that some genes escaping from XCI such as *CD40L, CXCR3, BTK, IRAK-1, TLR7/TRL8 and CXorf21* (Table 1) are overexpressed in many autoimmune pathologies, including lupus, and are likely contributing factors to the development of AIDs (Youness et al. 2021). Using *CXCR3* bi-cistronic dual reporter mice, it was shown that biallelic *CXCR3*-expressing T cells were endowed with enhanced functional properties during *Leishmania Mexicana* infection (Oghumu et al. 2019). Because *CXCR3* is a T-bet-induced homing receptor highly expressed in Th1 and T-bet+ "age-associated B cells" (ABC), it is tempting to speculate that *CXCR3* biallelic cells will be more efficiently recruited to the site of inflammation, resulting in sex-associated predisposition to autoimmunity and resistance to infection.

3 Biological Role of Sex-Linked Factors in Autoimmunity

3.1 Sex Hormone and X Chromosome Regulation in Organ-Specific Autoimmunity: The Example of Multiple Sclerosis.

Multiple sclerosis (MS) is a T cell-mediated autoimmune disease with a female to male ratio of 3:1. Multiple sclerosis and its mouse model, experimental autoimmune encephalomyelitis (EAE), are characterized by the recruitment of inflammatory leukocytes, including autoreactive T cells, into the central nervous system (CNS), resulting in myelin damage (Dendrou et al. 2015). Reduced disease activity in women with multiple sclerosis is commonly observed during pregnancy suggesting that sex hormones, such as estrogens, could down-modulate the autoimmune response and associated neuro-inflammation (Abramsky 1994; Korn-Lubetzki et al. 1984; Confavreux et al. 1998). Because of the protective effect of pregnancy on EAE/MS, this disease is considered as a relevant model to study the mechanism by which sex hormones could control autoimmunity. Indeed, studies in EAE have clearly established protective effects of estrogen administration on disease activity (Bebo et al.

Table 1 Genes that escape from XCI in immune cells

Gene symbol	Gene nomenclature	XCI escapee type	Cell type	References
IRAK1	Interleukin 1 receptor associated kinase 1	Facultative	Variable escape in primary fibroblast cell lines	Carrel and Willard (2005)
CD40LG	CD40 ligand	Facultative	Escape in activated T cells and immortalized B cell lines generated from pediatric SLE patients or healthy females	Wang et al. (2016)
CXCR3	C-X-C motif chemokine receptor 3	Facultative		
IL13RA1	Interleukin 13 receptor subunit alpha 1	Facultative	Escape in pDC from healthy women	Hagen et al. 2020)
CYBB	cytochrome b-245 beta chain	Facultative		
TLR7	Toll like receptor 7	Facultative	Escape in monocyte, lymphocyte B and pDC from healthy women and Klinefelter syndrome males (XXY)	Souyris et al. (2018)
		Facultative	Escape in pDC from healthy women	Hagen et al. 2020)
KDM6a	Lysine demethylase 6A	Constitutive	Escape in hamster-human somatic cell hybrids and immune cells (unpublished)	Greenfield et al. 1998)
KDM5c (SMCX)	Lysine demethylase 5c	Constitutive	Escape in hamster-human somatic cell hybrids and mouse tissues (unpublished)	Greenfield et al. (1998; Agulnik et al. (1994)
BTK	Burton tyrosine kinase	Facultative	escape in pDC from healthy women	Hagen et al. (2020)
CXorf21/ TASL	Chromosome X open reading frame 21; also known as TASL (TLR adaptor interacting with SLC15A4 on the lysosome) (Heinz et al. 2020)	Facultative	Variable escape in primary fibroblast cell lines	Carrel and Willard (2005)

2001; Polanczyk et al. 2004a; Liu et al. 2003; Ito et al. 2001; Garidou et al. 2004; Wang et al. 2009; Lelu et al. 2011). Likewise, protective effects of estrogens have been reported in clinical trials using estriol (E3) (Sicotte et al. 2002; Soldan et al. 2003; Voskuhl et al. 2016) or estradiol (E2) (Pozzilli et al. 2015) in relapsing–remitting MS patients.

3.1.1 Direct Action of Sex Hormones in T Cells (Treg Th17)

As mentioned above, estrogens (E2, E3) emerged as a major pregnancy-associated sex hormones responsible for disease protection in MS/EAE (Spence and Voskuhl 2012; Laffont et al. 2015). It has been clearly established that E2 could inhibit CNS autoimmunity and inflammation through distinct non-overlapping mechanisms, involving anti-inflammatory and/or neuroprotective actions (Spence and Voskuhl 2012; Laffont et al. 2015). Multiple targets have been previously proposed to explain the anti-inflammatory actions of E2 on CNS autoimmunity (Wang et al. 2009, 2008; Polanczyk et al. 2004b; Papenfuss et al. 2011; Bodhankar et al. 2011). However, these conclusions were mostly based on correlative analysis of the effect of estrogen supplementation on various immune cell subsets in lymphoid tissues or in the CNS (Laffont et al. 2015). The use of conditional ER-mutant mice has clearly helped resolving contradictory results in the field (Laffont et al. 2015). Using such models to validate the cellular ER targets in vivo, estrogens or ER-selective ligands have been shown to exert potent neuroprotective effects by acting in CNS-resident cells, through either ERα expressed in astrocytes (Giraud et al. 2010; Spence et al. 2011, 2013) or ERß expressed in microglia (Saijo et al. 2011; Khalaj et al. 2013).

The immunosuppressive action of E2 supplementation on peripheral encephalitogenic CD4 T cell activation was certainly the most consensual effects particularly when using high doses of E2 or E3 (Laffont et al. 2015). This suppressive effect, characterized by inhibition of Th1/Th17 cell priming in inflammatory lymph nodes, was elicited with serum concentrations of E2 congruent with pregnancy values. Using such supplementation model, we previously reported a requirement for ERα expression in hematopoietic cells, and more specifically CD4+ T cells rather than dendritic or myeloid cells, or Foxp3+ Treg cells (Lelu et al. 2011; Garnier et al. 2018). Surprisingly, bystander rather than cognate CD4+ T cells were found to represent the primary target of E2 probably through the induction of trans-acting mechanism of suppression involving PD-1 expression on cognate $CD4^+$ T cells (Garnier et al. 2018). Because E2-mediated inhibition was reverted by in vivo immune check-point blockade using anti-PD-L1 antibody, it was suggested that E2 supplementation may act to promote PD-L1 expressing cells in vivo as PD-1/PD-L1 interactions have been shown to inhibit Th17 development (Hirahara et al. 2012; Zhang et al. 2017).

Few studies have examined the effect of natural pregnancy on EAE protection (Engler et al. 2017). Although such analyses are quite challenging, they have the advantage to study a physiological situation as compared to pharmacological supplementation. In such study, progesterone (P4) was reported to subvert signaling through the glucocorticoid receptor (GC) in Treg cells (Engler et al. 2017). P4 was found to

robustly induce Tregs during pregnancy and T cell-specific deletion of GR in pregnant animals undergoing EAE resulted in a reduced Treg increase and a selective loss of pregnancy-induced protection (Engler et al. 2017). Indeed, it has been reported that P4 can promote the generation of "induced" Tregs (iTregs) through inhibition of mTOR signaling in a mechanism partially involving progesterone receptor (PR) expression (Lee et al. 2012). Whether cross-talk or complementary/synergistic actions exist between ER, PR and GC signaling in distinct T cell subsets to coordinate suppression during pregnancy will certainly deserve further investigation.

In the EAE model, we clearly established that ERα signaling in Treg cells was dispensable for in vivo Treg cell development and for E2-mediated EAE protection (Garnier et al. 2018). However, evidence for ERα-mediated cell-intrinsic action of endogenous E2 on Treg cells has been recently provided at steady state. T cell-specific deletion of ERα in T cells promoted T cell activation-induced apoptosis and down-regulated Foxp3 expression. These effects were mostly observed in competition experiment with ERα-sufficient T cells upon adoptive transfer into Rag2-deficient mice (Mohammad et al. 2018). Ablation of ERα in T cells resulted in the development of mild-autoimmune phenotype with aging associated with anti-nuclear antibodies and increased proportion of T_{FH} cells, suggesting that ERα-signaling suppresses T_{FH} cell development (Kim et al. 2019).

Besides ERα in T cells and Treg cells, recent reports pointed to converging and reproducible actions of E2 or ER ligands on Treg cells through ERß activation. Although ERß-agonist ligand can protect from EAE development through microglia expression of ERß (Saijo et al. 2011), peripheral action of ERß ligand on CD4 T cells has been suggested to contribute to EAE protection by others (Aggelakopoulou et al. 2016). Whether this effect of ERß-ligand was mediated through ERß signaling in cognate $CD4^+$ T cells or Treg cells was not established. In experimental model of intestinal inflammation, ERß agonist ligands were found to be protective by promoting Treg cell expansion (Goodman et al. 2014). Indeed, using an in vitro model, the expression of ERβ, but not ERα, was found necessary in naïve CD4 T cells to generate TGF-ß-induced Foxp3 iTregs cells (Goodman et al. 2020). E2-mediated ERβ activation was required to maintain an effective immune suppression by limiting Treg-specific expression of the glucocorticoid transcription factor GILZ, not normally expressed in mature Tregs (Goodman et al. 2020). Thus, by inhibiting GLIZ which interferes with functional Treg suppression mechanism, E2 through ERß helps maintain peripheral tolerance in the gut (Goodman et al. 2020). A similar role for ERβ in Tregs has also been supported in a model of pneumococcal pneumonia characterized by accelerated resolution in female compared to male (Xiong et al. 2021). As the role of ERα and ERβ within the same cell type is complex and often antagonistic (Thomas and Gustafsson 2011), it will be critical to determine the respective contribution of each ER in the regulation of Treg cell functions and whether this involves liganded or unliganded ERs. Together, although ERβ appears to be required for in vitro iTreg development in vitro and for their in vivo maintenance in some experimental models, the role of ERα in driving Treg cells is less clear despite previous correlative studies pointing to increased proportion of Foxp3 Treg

in E2-treated mice (Polanczyk et al. 2004b; Garnier et al. 2018; Mohammad et al. 2018).

3.1.2 Indirect Effect on Central Mechanisms of Negative Selection of Autoreactive T Cells Through AIRE Crisscross Regulation in the Thymus

Evidence has been provided for sex hormones regulation of central T cell tolerance mechanisms in the thymus (Dragin et al. 2016; Zhu et al. 2016). The autoimmune regulator (*AIRE*) gene is a key transcription factor expressed in rare medullary epithelial cells (mTECs) in the thymus, which promotes expression of tissue-specific antigens (TSAs). Ag presentation of mTEC-derived TSAs by mTECs or local DCs deletes developing T cells that recognize self-antigens with high affinity, primarily through apoptosis. In addition, AIRE stimulates Treg induction and mTEC differentiation and maturation. Mutations in the *AIRE* gene sequence induce an autoimmune monogenic disease, the autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APCED) syndrome. In mice and humans, thymic AIRE expression is higher in males compared to females (Dragin et al. 2016; Zhu et al. 2016), and whereas the female sex hormones, estrogens, down-regulated AIRE expression in mTECs, androgens had the opposite effect by upregulating its expression (Dragin et al. 2016; Zhu et al. 2016). Of note, estrogen induced CpG hypermethylation at the *AIRE* promoter (Dragin et al. 2016), while the AR agonist ligand DHT activates the *AIRE* gene through androgen responsive element (Zhu et al. 2016). Male sex was associated with less severe EAE in an AIRE-dependent manner and upregulation of the MOG CNS autoantigen by mTECs, suggesting enhanced negative selection of self-reactive T cells as a potential mechanism of protection (Zhu et al. 2016). Hence, these data support the idea that males are less susceptible than females to autoimmune disorders due to the androgen-mediated reinforcement of central tolerance mechanisms, thereby limiting the output of autoimmune T cells in the periphery (Dragin et al. 2016; Kim et al. 2016).

3.1.3 X Chromosome Dosage Effects: FCG Mice and the Role of Kdm6a in EAE

X disomy or polysomy is clearly associated with a greater risk of developing autoimmunity (Scofield et al. 2008, 2022; Harris et al. 2016; Seminog et al. 2015). For SLE, the risk is ninefold higher in women (46, XX) than in men (46, XY) (Whitacre 2001). Relative to healthy men, the supernumerary X is associated with 15-fold greater susceptibility to SLE in men with Klinefelter syndrome (47, XXY) and 25-fold greater susceptibility in women with triple X syndrome (47, XXX) (Liu et al. 2016). Furthermore, although many autoimmune diseases have been reported in women with Turner syndrome (45,X0), these patients rarely develop SLE (Bai et al. 2015).

The effect of the sex chromosome complement on autoimmune disease susceptibility has been addressed specifically using a set of mouse strains known as the four core genotypes (FCG) (Smith-Bouvier et al. 2008). In this model, the testes-determining *Sry* gene was deleted from the Y chromosome (Y^-), and ovary-bearing animals of either XX or XY^- karyotype were generated. Backcrossing these females to males expressing a *Sry* transgene autosomally resulted in XX *Sry* and XY^- *Sry* testes-bearing animals. A comparison was thus possible between XX and XY^- mice against a shared female hormonal background and between XX *Sry* and XY^- *Sry* animals of a male hormonal background (Smith-Bouvier et al. 2008; Arnold and Burgoyne 2004). Relative to XY carriers, the XX sex chromosome complement was associated with enhanced susceptibility to pristane-induced (Smith-Bouvier et al. 2008) and spontaneous lupus (Sasidhar et al. 2012).

KDM6a, also known as *UTX*, has been recently proposed as a putative X-linked genes contributing to X chromosome effects on EAE/MS development (Itoh et al. 2019). KDM6a removes the repressive marks associated with the triple methylation of lysine 27 of histone H3 (H3K27me3) and then associates with the methyltransferase KMT2D which can add the activating marks on lysine 4 of histone H3 (H3K4me3) (Berletch et al. 2015). *KDM6a*/*Kdm6a* constitutively escapes from XCI both in human and mouse, which means that this gene can be expressed at similar level from both X chromosomes across many different tissues including immune cells (Berletch et al. 2015). Deletion of *Kdm6a* selectively in T cells promoted a naïve T cell phenotype and a shift toward Th2 polarized cytokine production, thereby diminishing neuro-inflammation and protecting mice from developing MS-like symptoms (Itoh et al. 2019). The observation that *Kdm6a* expression was associated with a collection of immunological processes having the potential to skew immunity toward inflammatory responses suggested that *Kdm6a* is a master switch for many genes involved in autoimmunity (Itoh et al. 2019). Indeed, the diabetes drug metformin, known to regulate *KDM6A* by blocking its demethylase activity, is thought to increase gene silencing across the genome (Cuyas et al. 2018). Since *Kdm6a/Utx* expression in $CD4^+$ T cells promotes EAE, escape from XCI and overexpression of *Kdm6a* in females is consistent with the increased susceptibility of women to MS (Itoh et al. 2019).

T cell-specific deletion of *Kdm6a* has been also examined in the context of chronic LCMV infection, where it was found to promote Tfh lymphocyte accumulation thereby promoting optimal GC reaction and protective humoral immunity (Cook et al. 2015). Mechanistically, *Kdm6a*/*Utx* was required in Tfh cells to promote upregulation of IL-6Rα and other Tfh related genes, while repressing Th1 signature genes (Cook et al. 2015). This indicates that the mechanisms of action of Kdm6a in T cells may be broadly divergent according to the cellular and environmental context and the type of immune responses examined. Although evidence was provided in the Cook et al. studies (Cook et al. 2015) for a dosage-dependent effect of Kdm6a, whether selective expression of *Kdm6a* from the Xi was important for EAE development or clearance of persistent virus infection was not established.

3.2 *Sex Hormones and X Chromosome Effects in Systemic Autoimmunity (SLE)*

3.2.1 Cell-Intrinsic Action of Sex Hormones and Sex Chromosomes in Immune Cells (e.g., pDCs and B Cells)

SLE is characterized by a strong sex bias, with females being affected about 9 times more often than males (Arnaud et al. 2014). In murine models of SLE, mainly affecting females, castration and E2 supplementation have been reported to influence disease onset and severity (Roubinian et al. 1978). B cells and pDCs are among the immune cell populations involved in SLE pathogenesis that have been shown to be directly regulated by estrogens. By enhancing the expression of CD22 and SHP-1 by B cells, estrogens decreased the signaling threshold of the B cell receptor and allowed autoreactive B cell survival via the positive up regulation of anti-apoptotic factor Blc-2 and by promoting the accumulation of marginal zone B cells (Grimaldi et al. 2002; Hill et al. 2011). Whether estrogens promoted the accumulation of autoreactive B cells in MZ compartment through positive selection mechanisms is unknown.

Estrogens may also promote somatic hypermutation by increasing the expression and function of the enzyme activation-induced cytidine deaminase (AID) in B cells (Pauklin et al. 2009). Whether this effect is particularly relevant during the GC reaction, where AID is up-regulated in germinal center B cells in response to T-dependent antigens will deserve further investigations. Although, constitutive ERα-deficiency or E2-blockade experiments in mouse SLE models has led to inconsistent or contradictory results, specific ERα inactivation in B cells delayed disease development in (NZBxNZW) F1 mice (Tabor and Gould 2017). This was associated with decreased B cell activation in young pre-autoimmune mice, suggesting that B cell-intrinsic ERα signaling may control B cell activation and autoantibody production (Tabor and Gould 2017). On the other hand, B cell-extrinsic actions of the male sex hormone testosterone have been reported to negatively control splenic B cell numbers (Wilhelmson et al. 2067) or antigen-specific B cell positioning during the GC reaction, thereby also contributing to the sexual dimorphism associated with lower humoral immunity in males (Zhao et al. 2020).

Besides B cells, pDCs are an important contributor of SLE pathogenesis through their capacity to produce type I IFN upon activation of their endosomal TLR, TLR7 or TLR9, by self-nucleic acids bound in complexes to autoantibodies (Theofilopoulos et al. 2017). Interestingly, a marked sex bias exists in the type 1 IFN production by human pDCs, with pDCs from females releasing larger amounts of type 1 IFN in response to stimulation by TLR7-specific ligands (Berghofer et al. 2006). Subsequent studies showed that this sex bias was due to an increased frequency of pDCs capable to secrete IFN-α, rather than a sex bias in IFN-α production on a per cell basis (Meier et al. 2009; Seillet et al. 2012; Azar et al. 2020). E2 supplementation of postmenopausal women was found to substantially enhance IFN-α production by blood pDCs not only in response to TLR7 stimulation, but also to TLR9 ligands. Importantly, enhanced cytokine production by pDCs was elicited not only by synthetic

ligands of TLR7 or TLR9, but also by natural ligands such as self-nucleic acid-containing immune complexes present in the sera of SLE patients (Seillet et al. 2012). Cell-intrinsic ERα activation by estrogens was required to up-regulate pDC innate functions in vivo, but also in vitro (Seillet et al. 2012, 2013; Griesbeck et al. 2015; Laffont et al. 2014). Estrogen signaling was subsequently found to up-regulate the expression of the transcription factor IRF5 in mouse and human pDCs, which may act by enhancing IFN-α production in synergy with IRF7 (Griesbeck et al. 2015) (Fig. 3).

However, besides estrogens, X-linked genetic factors independently contribute to the strong sex bias in the TLR7-driven response of pDCs (Seillet et al. 2012; Laffont et al. 2014). In a humanized mouse model, the frequency of IFN-α- and TNF-α-producing human pDCs in response to TLR7 ligands was greater in female than in male host mice. This confirmed that estrogens positively regulated the functions of human pDCs, but interestingly, functional differences were also observed depending on the sex of the human stem cell donors (Laffont et al. 2014). These data provided the first evidence for a critical role of X chromosome dosage on the functional competence of female pDCs and suggested that the X chromosome complement and sex hormone estrogens may act in concert to confer elevated production of type I IFN by pDCs in response to TLR7 ligands (Laffont et al. 2014). This genetic effect may explain the persistence of the female superiority in the TLR7-driven IFN-α response

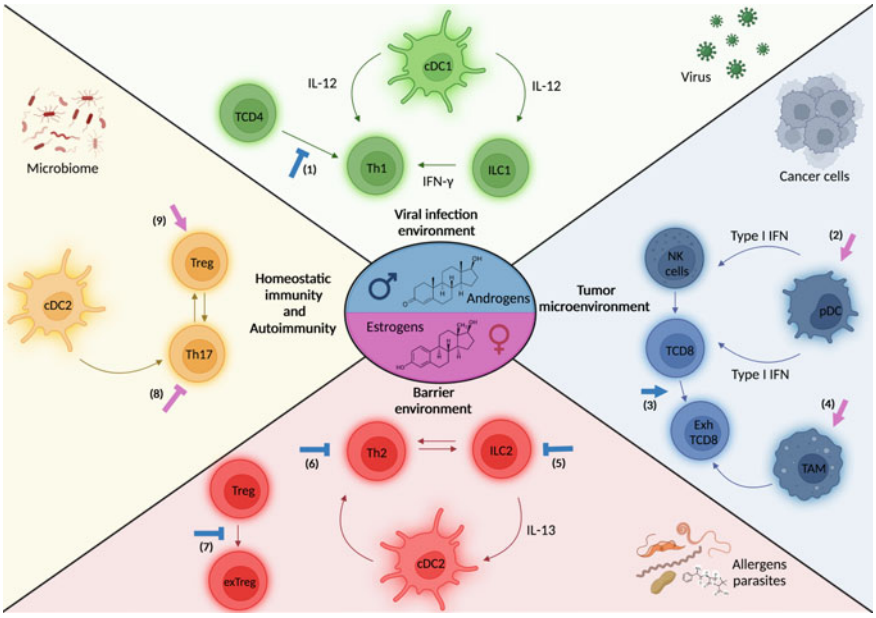

Fig. 3 Global mechanisms of action of sex hormones on the immune system. (1): ref 195; (2): 101 and 103–105; (3): ref 196–197; (4): ref 190; (5): ref: 159–163; (6): ref 176; (7): ref 175; (8): ref 66–67; (9): ref 73–75. Created with BioRender.com

of pDCs across ages, including postmenopausal and elderly women (Congy-Jolivet et al. 2022).

TLR7 gene is on the X chromosome and escapes from XCI in immune cells (Souyris et al. 2018, 2019; Hagen et al. 2020). *TLR7* belongs to the category of facultative "escapees" as expression from the Xi was not observed in all cells, but in a distinct subset ranging from 10 to 40% of immune cells, including B cells, pDCs and monocytes. So far, *TLR7* biallelic cells have been found in all female subjects tested (Souyris et al. 2018; Hagen et al. 2020), including HIV-1-infected women under anti-retroviral therapy (ART) (Abbas et al. 2022). Of note, the frequency of pDCs with biallelic expression of *TLR7* was similar between uninfected control and HIV/ART females, despite enhanced functional differences in the TLR7-driven pDC responses in HIV/ART women (Abbas et al. 2022). Consistent with the increased innate cytokine responses of pDC from HIV/ART females, a significant upregulation of *TLR7* gene expression was observed in pDCs including mono-allelic ones where *TLR7* was expressed from the Xa. These results provide evidence for an increased TLR7-driven pDC responsiveness associated with enhanced transcriptional activity of the *TLR7* locus on both X chromosomes, in pDCs from HIV/ART females. This suggested that enhanced TLR7 mRNA levels in pDCs at single-cell resolution could promote enhanced responsiveness, whatever the mechanism, enhanced transcriptional activity of the *TLR7* loci on both X chromosomes, *TLR7* biallelic expression or both (Souyris et al. 2018; Hagen et al. 2020; Abbas et al. 2022). Indeed, single-cell resolution analysis of *gene* expression in female pDCs at steady state revealed that *TLR7* biallelic pDCs not only expressed higher levels of *TLR7* mRNA but also more transcripts coding for all IFN-α subtypes and IFN-β (Hagen et al. 2020). This observation suggested that the expression of basal level of IFN-I mRNA in *TLR7* biallelic pDCs could be a consequence of a higher *TLR7* mRNA expression thereby functionally discriminating mono-allelic and biallelic pDCs (Hagen et al. 2020). Indeed, constitutive production of low levels of IFN-I in pDCs has been reported to act in an autocrine/paracrine manner to drive high levels production of IFN-I by pDCs (Kim et al. 2014). Thus, pDCs with high level expression of *TLR7* mRNA due to the cell-autonomous action of XCI escape of *TLR7* (Souyris et al. 2018; Hagen et al. 2020) could belong to a group of early responder pDCs with superior ability to produce IFN-I (Wimmers et al. 2018).

Concerning adaptive immune cells like B cells, being *TLR7* biallelic could be even more advantageous for B cells due to antigen-driven positive selection processes occurring at various checkpoints of B cell responses. This is based on the hypothesis that female B cells with biallelic expression of *TLR7* would exhibit enhanced functional responses to pathogen-like antigens or self-antigens containing TLR7 ligands compared to mono-allelic cells (Laffont and Guery 2019; Souyris et al. 2018, 2019). In support of causality, using an in vitro model of naïve B cell differentiation, *TLR7* biallelic B cells displayed over twofold greater propensity to class switch relative to mono-allelic B cells, in response to TLR7 stimulation, whereas no association was found when B cells were stimulated through TLR9 (Souyris et al. 2018). Likewise, B cells with biallelic *TLR7* expression were enriched within the $CD27^{hi}$ proliferating plasmablasts following treatment with TLR7-specific ligands, whereas

counter-selection of biallelic cells was instead observed in CD27hi PC that responded to a TLR9 ligand from the same donors (Souyris et al. 2018).

A strong indication that the number of X chromosomes, not just sex itself, is important in lupus susceptibility comes from Klinefelter syndrome males, who carry one or more extra X chromosomes and develop lupus and Sjögren's syndrome with a risk similar to females (Scofield et al. 2008, 2022; Harris et al. 2016; Seminog et al. 2015). *TLR7* escapes XCI in immune cells from 47, XXY Klinefelter syndrome males (Souyris et al. 2018). Because increased TLR7 signaling is a known risk factor in lupus in mice (Pisitkun et al. 2006; Deane et al. 2007; Brown et al. 2022), we propose that higher TLR7 dosage arising from X inactivation escape connects the presence of two X chromosomes in females and individuals with Klinefelter syndrome with the greater risk of developing lupus. In the FCG model, it has been shown that XX mice are more susceptible to SLE development than XY mice, independently of gonadal type, supporting the important contribution of the X chromosome complement also in mice (Sasidhar et al. 2012).

Regarding the causal relationship between XCI escape of *TLR7* alone or in combination with other genes and the sex differences in the development of spontaneous autoimmune diseases, important question still remains regarding the stability of XCI escape in immune cells and the underlying molecular mechanisms controlling XCI escape at single-cell resolution, particularly those related to facultative "escapees" like *TLR7*. In addition, the direct causal link between escape from XCI, namely the additional expression of genes from the Xi, and the greater susceptibility to develop autoimmune diseases remains to be demonstrated and will require the implementation of new genetic tools.

3.2.2 X Chromosome Inactivation Mechanisms in SLE

It has been suggested that *XIST* plays a central role in the sex bias observed especially in SLE and rheumatoid arthritis (RA) (Sierra and Anguera 2019). As discussed earlier, coating of the Xi by the lncRNA *XIST/Xist* induces gene expression silencing by recruiting polycomb complexes PRC1 and PRC2 that are responsible for deposition of repressive epigenetic marks and chromatin locking. The acquisition of these repressive epigenetic marks is involved in the stable maintenance of the inactive state (Fang et al. 2019). Although *XIST/Xist* is required for the initiation of the XCI process, it seems that it is dispensable to maintain the Xi status. Previous work reported that the absence of *Xist* in mature differentiated cells did not affect the maintenance of XCI (Brown and Willard 1994; Li et al. 1993). In general, in somatic cells such as fibroblasts, *XIST/Xist* is localized on the Xi as a "cloud" as visualized by RNA-FISH, suggesting that XCI is strongly maintained in this cell type. Dynamic regulation of *XIST/Xist* has been reported in lymphoid cells both in Human and mice (Sierra and Anguera 2019; Syrett et al. 2017), whereas common lymphoid progenitors (CLP) and common myeloid progenitors (CMP) present an inactive territory covered by *Xist* and H3K27me3 repressive marks, and this is not the case of mature

lymphoid cells and pDCs. Xi chromatin gradually changes during B cell development, initiated by the progressive loss of *Xist* together with heterochromatin marks, as cells differentiate into immature B cells (Syrett et al. 2017; Savarese et al. 2006). Thus, within mature follicular naive B cells, *Xist* is absent at the Xi level, which is associated with a depletion of repressive marks on chromatin. Although *Xist* is not visible on the Xi by RNA-FISH, it is however continuously transcribed and localized diffusely in the nucleus. Remarkably, following in vitro B cell activation by TLR ligands LPS (TLR4) or CpG (TLR9), *Xist*, as well as the repressive marks, relocate to the Xi according to a dynamic process involving the transcription factor YY1 (Syrett et al. 2017). YY1 can then recruit Xist either directly or indirectly via *Xist* binding proteins, allowing its relocation to the Xi. All these observations were also found in T cells (Syrett et al. 2019). Interestingly, a defect of *XIST* relocalization on the Xi was observed after in vitro activation of B cells, $CD4^+$ and $CD8^+$ T cells from lupus patients (Syrett and Anguera 2019; Pyfrom et al. 2021). Similar observations were made in NZB/NZW F1 mice predisposed to lupus (Coz et al. 2018). Nevertheless, the consequences of this defect in the repositioning of *XIST/Xist* on Xi in activated lymphocytes remain to be defined as combined RNA-FISH analysis of *XIST* clouds and escapees were not performed, and no difference was observed in the frequency of biallelic cells, in particular for *Tlr7*, between the B cells of mice prone to lupus compared with wild-type mice (Coz et al. 2018). Of note, in mouse, escapees always appear to evade coating of *Xist* and localize outside the *Xist* compartment in RNA-Pol II-rich area (Chaumeil et al. 2006; Chow et al. 2010). Thus, the role of *Xist* repositioning to the Xi in the negative regulation of the expression of facultative escapees following lymphocyte activation needs further investigations.

Conditional deletion of *Xist* in neurons showed no impact on XCI, although a lack of expression of the repressive marks (H2AK119ub1, H3K27me3) on the Xi was observed. This suggested that maintenance of transcriptional repression of Xi is largely independent of Xist in differentiated cells (Adrianse et al. 2018). However, evidence has been provided for a role of *Xist* in XCI maintenance using targeting deletion or knock-down of this *Xist/XIST* gene in murine hematopoietic cells (Yildirim et al. 2013) and in human female B cell lines (Yu et al. 2021). In both studies, the *Xist* deficiency led to overexpression of many genes, a large part of which corresponds to X-linked genes, such as *Tlr7/TLR7*, *Cxcr3*, *TLR8* or *TASL/Cxorf21*. In addition, *Xist* knock-down in B cell lines after co-stimulation of BCR and TLR7 led to the development of unconventional T-bet + CD11c + memory B cells (Yu et al. 2021). It should also be noted that in Yildrim's study, mice with *Xist* deficiency in the hematopoietic compartment develop fatal leukemia (Yildirim et al. 2013).

In conclusion, all of these data suggest that reactivation of facultative escapees from the Xi could be due to an alteration in the maintenance of the inactive state, possibly mediated by *XIST/Xist*. However, as mentioned earlier (Fig. 2), constitutive escapees always evade coating by *Xist*. Whether this also occurs in activated T and B cells for facultative escapees, such as *Tlr7, Tasl* or others candidate genes, remains to be investigated.

4 Sex Differences in Allergic Asthma: A Prominent Role of Sex Steroid Hormone Signaling

4.1 Sex Bias in Asthma Prevalence and Severity

Asthma is the most common chronic condition in children. WHO data show that in 2019, asthma affected 262 million people worldwide and was responsible for more than 450,000 deaths per year. Asthma is a complex upper airway disease with a heterogeneous pathophysiology that can be classified into two main endotypes: the more common type 2 inflammation related and the non-type 2, IL17 or IFNγ inflammation related (Hammad and Lambrecht 2021). As it is the case with many inflammatory diseases, there are marked sex differences in asthma incidence, prevalence and severity. While in childhood the incidence of the disease is increased in boys, this trend is reversed at puberty. In adults, asthma is not only more frequent but also more severe in women (Zein et al. 2016). Unsupervised clustering studies have identified asthma phenotypes that show the strongest sex-related differences: a sub-group of young women who reports early atopic asthma and another sub-group of obese older women who report severe non-eosinophilic late asthma (Haldar et al. 2008; Moore et al. 2010).

Changes in the prevalence of asthma around puberty, both exacerbation in women and amelioration in men, suggest a plausible involvement of sex hormones in sex-related differences in this disease. The role of sex steroid hormones, estrogen in women and androgen in men, and their mechanisms of action are certainly complex and numerous.

In women, hormone levels vary throughout life and many studies have investigated the effect of menstruation, pregnancy, contraceptive use or menopause with or without hormone replacement therapy (HRT). As recently summarized by two comprehensive reviews (Chowdhury et al. 2021; Miyasaka et al. 2022), all combined, and although sometimes contradictory, data point toward a promoting/aggravating role of estrogen in asthma. Periods related to increased concentration of estrogen, either during pregnancy (Schatz et al. 2003), or in pre- versus postmenopausal cohorts (Troisi et al. 1995; Scioscia et al. 2020) for example, are associated with increased asthma severity.

In comparison, data related to the effect of androgen on asthma are less controversial, and many studies in mice but also in Human are supportive of a protective role of androgen in the disease which is in agreement with the clear reduction of asthma around puberty (Ross et al. 2020). Studies have shown that increased levels of free testosterone are associated with amelioration of asthma both in men and women (Han et al. 2020). Another study further reports that elevated testosterone to estradiol ratio is associated with better lung function in women (Han et al. 2021). In agreement with these observations, two trials have shown that administration of DHEA (dehydroepiandrosterone), a precursor of androstenediol which can be further catabolized into testosterone by the 3β-hydroxysteroid dehydrogenase-1 (3β-HSD1) encoded by the HSD3B1 gene, improves asthma control in women with no major

adverse effect (Marozkina et al. 2019; Wenzel et al. 2010). In line with the beneficial effect of androgen in asthma, genetic studies have identified germline missense-encoding polymorphisms in the HSD3B1 gene that affect asthma. The HSD3B1 (1245A) variant limits DHEA conversion to androgens, whereas HSD3B1 (1245C) promotes DHEA conversion into potent androgens. Patients with severe asthma and homozygous for HSD3B1 1245C display better lung functions compared with AA or AC carrier patients, particularly in the context of glucocorticoid therapy (Zein et al. 2020).

Murine models of asthma recapitulate the greater susceptibility of female mice to develop severe asthma (Melgert et al. 2005; Blacquiere et al. 2010; Takeda et al. 2013; Laffont et al. 2017b; Fuseini et al. 2018). In line with this, the protective role of androgen on lung inflammation has been convincingly demonstrated in different studies as recently reviewed (Laffont and Guery 2019). Different non-exclusive mechanisms have been identified that could contribute to the protective effect of androgen on asthma, either through its action on the pulmonary tissue itself or by regulating the inflammatory response. Androgen has been shown to promote airway smooth muscle relaxation (Kouloumenta et al. 2006) and to reduce airway remodeling through epithelial-mesenchymal transition (EMT) (Xu et al. 2014).

More recently, focus has been made on the role of androgen on type 2 immunity. In this context, we will highlight in the next paragraph recent data from the literature that demonstrate the central role of androgens in immune cells: the regulation of group 2 innate lymphoid cells (ILC2) and Treg cells.

4.2 Impact of Sex Hormones in Type 2 Mediated Allergic Airway Inflammation

4.2.1 AR Signaling, A Master Regulator of Group 2 Innate Lymphoid Cells

The discovery of ILC2, more than 10 years ago now, as a potent innate source of type 2 cytokines within tissue adds early effectors in the cascade of events leading to the so-called type 2-high endotype of asthma (Hammad and Lambrecht 2021). ILC2s are closely related to Th2; they share the expression of the transcriptional factor GATA-3 and the production of IL-5, IL-13 and IL-9 upon activation. ILC2s are particularly enriched in mucosal tissues, such as the gastrointestinal and the respiratory tract, and are primarily activated by cytokines/alarmins (IL-33, IL-25 and TSLP) released by damaged epithelial cells following allergen exposure. A broad spectrum of environmental allergens (fungi, pollen, HDM) possesses a common intrinsic proteolytic activity leading to cleavage and fully activation of IL-33(Cayrol et al. 2018). Upon activation, ILC2s proliferate and secrete large amount of IL-5 and IL-13. IL-5 promotes differentiation and recruitment of eosinophils to the lung, whereas IL-13 plays pleiotropic role among which increasing mucus production and

bronchoconstriction. IL-9 favors mast cell proliferation and activation. Model of ILC2 deficiency has demonstrated, not only the key role of ILC2s in innate cell activation upon allergen encounter, but also their contribution to adaptive Th2 response (Halim et al. 2014). ILC2-derived IL-13 licenses lung DCs to migrate to the draining lymph node and to prime Th2 cells (Halim et al. 2014). ILC2s can also directly promote Th2 responses in the tissue through their capacity to express MHC class II molecules (Oliphant et al. 2014) or to deliver instructive signaling dependent on OX40L expression to promote IL-33-driven expansion of Th2 and Treg cells(Halim et al. 2018).

In Human, the role of ILC2s in the pathophysiology of asthma has also been documented. Numerous studies have demonstrated that the numbers and the activation status of ILC2 are increased in samples (sputum, blood) from pediatric and adult asthmatic patients compared with controls (Laffont et al. 2017b). In addition, GWAS studies identify some of the top susceptibility genes in asthma to be linked with ILC2 biology (IL-33, IL-33 receptor IL1RL1, RORA, IL2RB and IL-13) (Torgerson et al. 2011). Furthermore, a recent study has demonstrated that genetic polymorphisms associated with asthma are localized with gene regulatory elements in ILC2 not only in mouse models of asthma, but also in patients with allergic asthma (Stadhouders et al. 2018).

Given the recent identified role of ILC2s in asthma and to better understand the mechanisms behind sex bias in the disease, the potential regulation of ILC2 by sex-linked biologic factors has been suspected. ILC2s, both in terms of numbers and functional properties, are subjected to sex differences. Female tissues, either at steady state or upon inflammation, harbor increased numbers of ILC2 equipped with enhanced cytokine production capacity (Laffont et al. 2017c; Cephus et al. 2017; Warren et al. 2017; Kadel et al. 2018). The possible involvement of estrogen receptor signaling in ILC2 sex bias has been ruled out, as ovariectomy, estrogen supplementation or ER deficiency in the hematopoietic compartment had no impact on ILC2 frequency (Laffont et al. 2017c; Cephus et al. 2017; Warren et al. 2017; Kadel et al. 2018; Blanquart et al. 2022). The lack of a specific role of estrogen in ILC2 regulation is in agreement with the absence of estrogen gene receptor expression in tissue ILC2 (Robinette et al. 2015; Ricardo-Gonzalez et al. 2018; Jacquelot et al. 2019) or their progenitors (Laffont et al. 2017b), with the notable exception of ILC2 from the uterus which express ERα and can be modulated by estrogen (Bartemes et al. 2018).

ILC2s are however highly sensitive to regulation by androgen. ILC2s express high levels of *Ar* gene expression (gene encoding for androgen receptor), similar to those of Nr3C1 (gene encoding glucocorticoid receptor) (Robinette et al. 2015; Ricardo-Gonzalez et al. 2018; Jacquelot et al. 2019). *Ar* has even been characterized to be a prototypic ILC2 signature gene in tissue-resident ILC2s (Robinette et al. 2015), the expression of Ar being restricted to ILC2 and not to other members of the ILC family (Robinette et al. 2015).

Through intrinsic activation of Ar within ILC2, naturally produced androgen in male mice is responsible for the reduced number of ILC2 that populate pulmonary male tissues at steady state (Laffont et al. 2017c) and protect male mice from an

allergen-induced lung inflammation (Fuseini et al. 2018; Laffont et al. 2017c; Cephus et al. 2017). However, mechanisms behind these observations have not been identified. One explanation of the reduced representation of ILC2 in the lung would be that androgen acts early in ILC2 progenitors to limit their development and consequently their seeding in tissues in agreement with the expression of *Ar* in ILC2 progenitors (ILC2p) (Laffont et al. 2017c). Indeed, ILC2p frequency together with their capacity to proliferate is decreased in the bone marrow of male compared to female mice (Laffont et al. 2017c), while the frequency of BM PLZF^{+} ILC precursors (or ILCp), the upstream progenitor of ILC2p, is higher in males and increased by excess androgens (Kadel et al. 2018). Accordingly, in vitro, ILC2 development can be pharmacologically manipulated through the modulation of AR activation, using the full agonist ligand 5α-DHT, selective AR modulators (SARM) such as Cl-4AS-1 or flutamide, a pure AR antagonist (Laffont et al. 2017a; Blanquart et al. 2021). Studies are now required to demonstrate if AR signaling in ILC progenitors inhibits ILC2 development in vivo.

Together with its action on ILC2 development, it has been recently demonstrated that exogenous androgen can regulate pulmonary ILC2 through intrinsic AR activation (Blanquart et al. 2022). Lung resident ILC2s express a functionally active AR that can be manipulated by androgen not only in males but also in females. A short-term androgen therapy is sufficient to reduce pulmonary ILC2 both at steady-state and upon allergen challenge and the resulting inflammation in female mice (Blanquart et al. 2022). This rapid reduction most probably occurs independently of the androgen-mediated inhibition of ILC2 development in the bone marrow, but directly in lung as ILC2 maintenance and proliferation within tissue have been shown to be largely independent of distal source such as the bone marrow (Gasteiger 2015; Schneider et al. 2019; Huang et al. 2018) but likely through tissue differentiation of resident ILC progenitors (Zeis et al. 2020).

The inhibitory receptor KLRG1, that is tightly up-regulated in ILC2 upon androgen-mediated activation of AR (Blanquart et al. 2022), has been suspected to be responsible for the inhibitory action of testosterone. However, our recent study could demonstrate that although KLRG1 expression impairs in vitro ILC2 proliferation together with the competitive fitness of pulmonary ILC2 in a sex and androgen receptor expression dependent manner, its absence has no impact on ILC2 number and functions neither at steady state nor at inflammation (Blanquart et al. 2022). Increased level of alarmins in female tissue has also been put forward to contribute to sex bias in ILC2 number, and functions (Cephus et al. 2017; Matha et al. 2019) between sexes have been reported (Blanquart et al. 2022). However, these differences are unlikely to be responsible for the higher responsiveness of female ILC2s as IL-33 deficiency is not associated with changes in ILC2 number or function (Blanquart et al. 2022). The molecular mechanisms behind the androgen regulation of ILC2 number and function are yet to be identified. The effect of AR on ILC2 are summarized in Fig. 3.

4.2.2 AR Signaling Promotes T Regulatory Cells Function During Allergic Airway Inflammation

Recently, another mechanism highlighting the protective role of testosterone on allergic airway inflammation has been identified. Gandhi et al. (2022) have shown that Foxp3-induced Tregs from male mice are more potent than female once to suppress allergic airway inflammation. Furthermore, mice selectively lacking AR signaling in $Foxp3^+$ cells harbor higher lung inflammation. In the lungs, androgens were found to act by inhibiting the conversion of conventional $Foxp3^+$ Tregs into $ST2^+$ Tregs (ex-Treg) that have been reported to promote airway inflammation (Koh et al. 2020). Thus, androgen stabilizes $Foxp3^+$ Tregs through two concomitant actions, the limitation of IL-33 production by epithelial cells and the decrease of ST2 expression on Tregs by inhibiting the expression of the transcription factor GATA2 (Koh et al. 2020) (Fig. 3).

4.2.3 Indirect Effect of AR Signaling in ILC2 on Skin cDC and Th2 Instructive Signaling

ILC2-derived IL-13 has been shown to license lung DCs to migrate to the draining lymph nodes and to prime Th2 cells (Halim et al. 2014) (Fig. 3). In a recent study, Mayer et al. further highlight the key role played by IL-13 in the development of Th2 responses through the promotion of a subset of dermal type 2 conventional DCs (cDC2) (Mayer et al. 2021). In the healthy skin, they could describe a unique subset of $CD11b^{low}$ cDC2 that requires IL-13 for their development. They identified ILC2s as the main source of homeostatic production of IL-13 independently of any alarmin signaling. In the absence of IL-13 signaling in cDC2, the Th2 responses mounted following intra-dermal immunization with *Nippostrongylus brasiliensis* larvae are strongly impaired. Whether this mechanism is also at play within the course of allergy skin inflammation will require further investigation (Mayer et al. 2021). In agreement with androgen-mediated negative regulation of ILC2 in male tissues, Mayer et al. could show a selective decreased of $CD11b^{lo}$ cDC2 in male tissues, whereas their $CD11b^{hi}$ cDC2 counterparts were accordingly increased (Mayer et al. 2021). The indirect reduction of $CD11b^{lo}$ cDC2 by androgen could represent an additional mechanism that may explain why males are less likely to develop asthma (Fig. 3).

5 Biological Sex Influences Natural Immunity to Neonatal Pathogens and Cancer

In this chapter, we will discuss some recent and remarkable examples as to how biological sex and particularly sex hormones influence the natural protective immunity in response to environmental pathogens involved in bloodstream infection and in the control of solid tumor growth through hormonal regulation of immune cells from the innate and adaptive immune system.

5.1 Sex-Biased Dimorphism in Bloodstream Infections: The Role of Estrogens/Peritoneal Macrophages/B-1 Cells Axis

Gram-negative bacteria, more specifically *E. coli*, are the predominant organisms responsible for bloodstream infections and are often involved in fatal sepsis (Laupland 2013). A sex-biased dimorphism has been documented for bloodstream infections. Although women are at higher risk than men for exposure to *E. coli*, especially via the genitourinary tract, they experience significantly less disease severity and mortality from sepsis induced by Gram-negative bacteria (Laupland 2013). Evidence for a sex-biased immunological dimorphism in the clearance of bacteria entering the bloodstream was recently provided (Zeng et al. 2018). In this study, Zeng et al. made the unexpected observation that capture of blood born entero-pathogeneic *E. coli* (EPEC) by Kupffer cells (KCs) relies on complement C3 fragments in male but not female mice. In C3-deficient female, full protection was still observed due to the presence of preexisting natural antibodies (nAbs) to *E. coli* of IgM and IgG3 subclasses. These nAbs were absent in males. Exploring further the mechanisms, the authors discovered that the production of this innate anti-E. coli nAb was estrogen dependent and started after sexual maturity. Estrogen was found to target peritoneal macrophages to express cytokines like IL-10, BAFF and APRIL, acting on peritoneal B.1 cells to produce nAbs specific for LPS O-antigens. Production of these nAbs did not require prior exposure to the cognate antigen and can be maternally transferable, thereby protecting male and female offspring from lethal EPEC infection. Similar nAbs to EPEC were found in the serum of adult women suggesting that this sexual dimorphism has been evolutionary conserved to protect newborns against infection with EPEC at a time where they have not fully developed their humoral response and complement system (Zeng et al. 2018). This landmark study provided important insights into how sex hormones act to regulate the immune system in adult female to provide natural protection to their progeny from bloodstream infections.

5.2 *Sex Dimorphism in Solid Tumor Control: From Innate to Acquired Immunity*

5.2.1 Sex Bias in Cancer

Cumulative evidence supports a role for sex-based differences in the susceptibility to develop cancers affecting non-reproductive tissues such as the liver, colon, lung, and also in leukemia and melanoma. It is well established that lung, colon and liver cancers manifest differently in men as compared to women (Haupt et al. 2021). For instance, at all ages, women are less likely than men to develop colon cancer (Lawrence et al. 2007). Likewise, hepatocellular carcinoma (HCC), the most common primary liver cancer, is three to five times more frequent in men than women (Ghebranious and Sell 1998). Another prototypical example of a cancer with a sex bias in occurrence and survival is melanoma. A study based on 11,000 patients with melanoma showed that women are less prone to this type of cancer, and that once affected, they have a survival advantage increased by 38% compared to men, their cancer being less likely to metastasize (Joosse et al. 2012). A large-scale multidimensional study also demonstrated that cancers that showed a sex bias in susceptibility could be associated with sex-specific molecular signatures. Interestingly, more than 50% of genes differentially regulated by sex can potentially be pharmacologically targeted (Yuan et al. 2016).

The higher risk of the onset of cancers in men can be attributed to extrinsic factors such as increased exposure to carcinogenic environmental factors (chemical agents, tobacco, alcohol) but also to biological intrinsic factors of genetic or hormonal origin. Indeed, it has been shown that the sex hormones (androgens, estrogens) and the genes encoded on the sex chromosomes can act both on the tumor cells themselves, but also on the tumor environment, modifying, for example, the tumor growth (Clocchiatti et al. 2016). At the same time, estrogens and androgens can act systemically by influencing anti-tumor immune responses (Clocchiatti et al. 2016). The mechanisms responsible for this sexual dimorphism in cancers were until recently poorly documented, but several recent studies have highlighted the intrinsic actions of sex hormones via their nuclear receptor in cells of the innate and adaptive immune compartment, opening up new therapeutic perspectives in the management of cancer according to sex. The most significant results are summarized below.

5.2.2 A Role for Estrogens in the Sex Differences in the Immune Response to Cancer

Hepatocellular carcinoma (HCC), the most common liver cancer, occurs mainly in men and similar sex disparity is seen in mice given the chemical carcinogen diethylnitrosamine (DEN) (Naugler et al. 2007). DEN treatment in mice causes 100% HCC in males but only 10 to 30% in females. Relative female protection in this experimental model of HCC was attributed to estrogen-mediated regulation of IL-6 production

by Kupffer cells through Myd88-dependent signaling in vivo (Naugler et al. 2007; Rakoff-Nahoum and Medzhitov 2007). Estrogen supplementation in DEN-treated males inhibited IL-6 secretion from KCs exposed to necrotic hepatocytes, thereby reducing circulating IL-6 (Naugler et al. 2007). Of note, sex disparity was lost in IL-6-deficient mice (Naugler et al. 2007). These estrogen-related sex differences may well extend to other cancers, as hormone replacement therapy also reduces the incidence of colorectal cancer in postmenopausal women (Chlebowski et al. 2004; Rennert et al. 2009). Indeed, in a spontaneous model of intestinal tumorigenesis, Myd88 signaling was critical for spontaneous and carcinogen-induced tumor development (Rakoff-Nahoum and Medzhitov 2007). This suggested that Myd88 signaling pathway downstream of members of the TLR and IL-1R family has a critical role in intestinal tumorigenesis (Rakoff-Nahoum and Medzhitov 2007). Whether this involved Myd88 signaling in somatic cells only, immune cells or both, and was influenced by sex will deserve further investigations (Rakoff-Nahoum and Medzhitov 2007).

As estrogens could contribute to sex-dependent differences in cancer (Naugler et al. 2007), it is essential to identify the cellular targets of estrogens and the mechanisms by which ERα-signaling outside the malignant cells could contribute to regulate cancer chemotherapy and anti-tumor immunity. Such a mechanism has been recently provided in a mouse model of ovarian cancer (Svoronos et al. 2017) and melanoma (Chakraborty et al. 2021). E2 treatment was first reported to promote the mobilization of myeloid-derived suppressor cells (MDSC) of hematopoietic origin that exert immunosuppressive activity and were associated with tumor growth (Svoronos et al. 2017). However, whether this effect was mediated through ERα signaling in MDSCs or their immediate progenitors in vivo was unclear. In a recent study, MDSC gene signatures were not predictive of melanoma patient's response to immune check-point blockade, suggesting additional E2 targets in the tumor microenvironment (TME) (Chakraborty et al. 2021). Besides MDSCs, tumor associate macrophages (TAM) are the main immunosuppressive myeloid cell subsets in the TME. In melanoma models, E2 treatments promoted tumor growth by inducing the accumulation of M2-like TAM, thereby suppressing adaptive immunity (Chakraborty et al. 2021) (Fig. 3). Targeting ERα with Fulvestrant or selective inactivation of ERα in macrophages resulted in the upregulation of the M1/M2 ratio and exacerbated the cytotoxic CD8 + T cell anti-tumoral response upon check-point inhibitors blockage, thereby resulting in melanoma suppression (Chakraborty et al. 2021). Of note, tumor-derived factors were also necessary in conjunction with E2 signaling to promote the immune-suppressive phenotype in macrophages. This study highlighted the cell-intrinsic role of E2/ERα signaling together with uncharacterized tumor-associated factors in the regulation of TAM polarization (Chakraborty et al. 2021) (Fig. 3). Monocytes and macrophages are very well-established target of E2/ERα signaling, which have been previously reported to modulate macrophage polarization and macrophage-associated tissue repair functions (Calippe et al. 2008, 2010; Campbell et al. 2014; Keselman et al. 2017). Although the study by Chakraborty et al. (2021) is informative regarding the deleterious action of E2-ERα in TAM, which could be targeted in females to improve the anti-melanoma response to

immune check-point blockade therapy, this mechanism is unlikely to explain the lower incidence of melanoma and the better survival of females (Joosse et al. 2012). Of note, the studies identifying E2-mediated induction MDSCs and M2-like TAM were performed in ovariectomized mice using pharmacological supplementation with exogenous E2, whether this mechanism also occurs in the context of physiological production of E2 is unknown (Svoronos et al. 2017; Chakraborty et al. 2021). Thus, further works are warranted to identify the physiological mechanisms by which female sex may positively influence tumor immunity and better control of metastasis (Joosse et al. 2012).

5.2.3 Androgen Negatively Impacts Tumor-Specific CD8 T Cell Immunity

Despite earlier evidence that androgen inhibited Th1 cell differentiation by blunting IL-12-induced Stat4 phosphorylation through upregulation of the phosphatase *Ptpn1* in CD4 T cells (Kissick et al. 2014) (Fig. 4), little was known on the role of AR signaling in CD8 T cells. In prostate cancer patients undergoing androgen deprivation, a reduced expression of *Ptpn1* was observed in CD4 T cells although this was not associated with changes in IFN-γ or T-bet expression (Kissick et al. 2014), suggesting that this mechanism may have limited impact in vivo. The contribution of T cell immunity to the sex bias in cancer remained unclear until the recent discoveries that CD8 T cells, rather than CD4 T cells, may represent the primary targets of androgens (Kwon et al. 2022; Guan et al. 2022; Yang et al. 2022). In these three studies, a major role of AR signaling in CD8 T cells was established in the control of tumor growth and response to immune check-point blockade (Kwon et al. 2022; Guan et al. 2022; Yang et al. 2022). In the Kwon et al. study (Kwon et al. 2022), the authors observed in multiple cancer models a male-based tumor burden driven by gonadal androgen rather than sex chromosome effects using the FCG model. Using CD8-specific Ar knockout mice, they observed an enhanced intra-tumoral accumulation of a subset of antigen-experienced $CD8^+$ T cells called "progenitor exhausted T cells" in male mice as a consequence of cell-intrinsic AR signaling (Kwon et al. 2022). Likewise, Yang et al. (2022) also reported that AR signaling in $CD8^+$ T cells promoted the differentiation of stem cell-like CD8 T cells into terminally differentiated exhausted $CD8^+$ T cells in tumor-bearing male mice (Fig. 4). Interestingly, by extending the analysis to sc-RNA-seq data to tumor infiltrating $CD8^+$ T lymphocytes obtained from published research in human cancers, basal cell carcinoma and non-small cell lung cancer, Kwon and colleagues observed higher frequencies of terminally exhausted $CD8^+$ T cells in males compared to females (Kwon et al. 2022). Using multiple approaches, they further identified the transcription factor TCF1, encoded by *Tcf7*, as a critical target of Ar signaling. *Tcf7* expression in T cells was up-regulated by androgens resulting in the accumulation of $TCF1^+$ $CD8^+$ progenitor exhausted subsets associated with impaired tumor control (Kwon et al. 2022) (Fig. 4). The study by Guan et al. (2022) started from the analysis of patients with metastatic castration-resistant prostate cancer undergoing PD-1 targeted therapy. By comparing

the immune landscape of tumor infiltrating cells from responders and non-responder patients, they revealed distinct CD8 T cell states associated with response and resistance to PD-1 blockade. They found that favorable T cell signatures were associated with low AR-signaling activity suggesting a regulatory role for androgens (Guan et al. 2022). Indeed, using tumor mouse models, pharmacological inhibition of AR together with PD-L1 blockade was associated with enhanced tumor control associated with enhanced CD8 T cell function (Guan et al. 2022). Although conditional Ar^{KO} mice were not used in this latter study, the authors suggested that AR signaling inhibited IFN-γ expression in CD8 T cells, thereby limiting anti-tumor immunity and check-point blockade efficacy (Guan et al. 2022). Importantly, in all three studies, AR blockade ameliorated responsiveness to PD-1 targeted therapies (Kwon et al. 2022; Guan et al. 2022; Yang et al. 2022). This may appear counterintuitive as males without androgen deprivation therapy already benefit more from immune check-point blockade as compared to females (Conforti et al. 2019; Kwon et al. 2018), particularly for colorectal cancer (Haupt et al. 2021). A possible explanation could be that the CD8 T cell exhaustion program may vary depending on the tumor microenvironment. In some cancers, steady-state levels of androgens may promote the preferential accumulation of stem cell-like progenitor exhausted TCF1+ PD-1low CD8 T cells (T_{PEX}) with a memory/precursor-like phenotype prone for reactivation and long-lasting immunity in response to PD-1 blockade (Kallies et al. 2020). In other settings where men may experience worse outcome, AR blockade by inducing less T cell exhaustion could sensitize the host to more effective immune check-point therapy and effector-like cell development (Fig. 4).

Despite fundamental differences in approaches and proposed underlying mechanisms, these three studies complement each other and probably cracked a critical

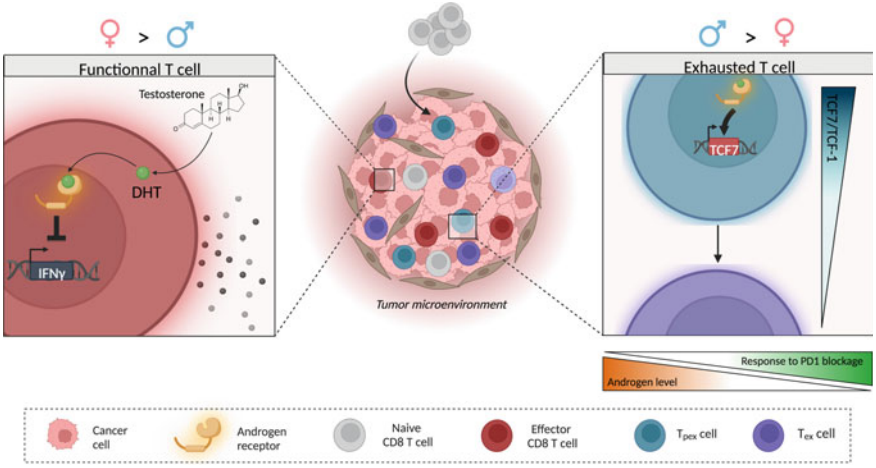

Fig. 4 Androgen negatively impacts tumor-specific CD8 T cell immunity. Androgen negatively regulates CD8 T cells by inducing an "exhausted precursor like" phenotype, characterized by the decreased production of IFNγ and increased expression of TCF1. Created with BioRender.com

mechanism contributing to the sex bias in cancer by identifying AR as a critical regulator of CD8 T cell immunity.

Acknowledgements The research carried out in the laboratory of JC Guéry benefits from the support of the Agence National de la Recherche (ANR-20-COV8-0004-01, ANR-20-CE15-0014-01, ANR-20-CE15-0027), the French National Agency for Research on AIDS and Viral Hepatitis (ANRS), SIDACTION, Fondation pour la Recherche Médicale (DEQ20180339187), the CSL Behring Research Funds, the Arthritis Foundation, the ARSEP Foundation, the FOREUM Foundation and the Inspire Program from the Région Occitanie/Pyrénées-Méditerranée (#1901175) and the European Régional Development Fund (MP0022856).

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Sex Differences in HIV Infection

Marcus Altfeld and Eileen P. Scully

Abstract Biological sex has wide-ranging impacts on HIV infection spanning differences in acquisition risk, the pathogenesis of untreated infection, impact of chronic treated disease and prospects for HIV eradication or functional cure. This chapter summarizes the scope of these differences and discusses several features of the immune response thought to contribute to the clinical outcomes.

1 Introduction

Since the initial recognition of the Acquired Immune Deficiency Syndrome (AIDS) and the identification of the causative agent HIV in the mid-1980s, the global HIV pandemic has been responsible for an estimated 40 million deaths (UNAIDS 2022). It has also demonstrated how intensive study of a single pathogen (HIV) can inform broader questions about the immune response, viral pathogenesis, in general, and the impact of host characteristics on the outcomes of infection. Aspects of both sex and gender impact HIV transmission and outcomes and highlight key features of pathogenesis, persistent challenges in the care for people living with HIV and potential barriers to an HIV cure (Addo and Altfeld 2014; Scully 2018) (Fig. 1).

Both sociobehavioral and biological factors are important determinants of HIV risk and disease pathogenesis. In this chapter, we are specifically discussing the role of biological sex, although this is at times difficult to disentangle from the influence of gender-based behavioral variables. In this chapter, we refer to women and men when the data referenced are derived from a clinical trial identifying participants as such, identifying cis and transgender participants when possible and use the terms

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S. L. Klein and C. W. Roberts (eds.), *Sex and Gender Differences in Infection and Treatments for Infectious Diseases*, Current Topics in Microbiology and Immunology 441, https://doi.org/10.1007/978-3-031-35139-6_3

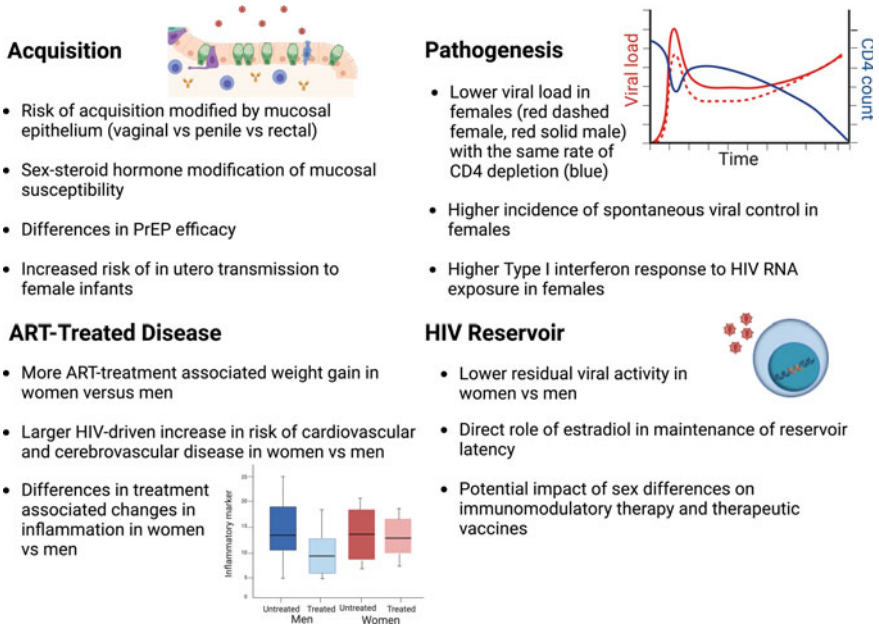

Fig. 1 Overview of selected sex differences in HIV acquisition, pathogenesis, treated disease and the HIV reservoir

female and male when referring to sex-based features such as the reproductive tract environments and to in vitro studies of biological factors.

2 Sex Differences in HIV Acquisition

Acquisition of HIV infection is the first level at which there is an impact of sex. Transmission risk varies by the type and body site of exposure, with bloodborne exposure linked to the highest rate of seroconversion (Patel et al. 2014). Sexual transmission risk varies substantially between receptive anal intercourse (138 per 10,000 exposures) and receptive penile-vaginal intercourse (8 per 10,000 exposures) (Patel et al. 2014). Both the anal and vaginal mucosal microbiome (Abdool Karim et al. 2019; Chen et al. 2021; Fulcher et al. 2022) and the respective mucosal immune environments are among the factors that modulate the risk of infection. The relative enrichment of immune cells in the gastrointestinal versus the vaginal mucosa, high frequency of infection-susceptible cells in the rectal environment, and characteristic of the epithelial layer (McElrath et al. 2013; Poles et al. 2001) are postulated to drive the specific risk of acquisition via anal sex. In the female reproductive tract, sex steroid hormone variation during the menstrual cycle impacts the phenotype of epithelial and immune cell complement (Wira et al. 2014). In a study of serodiscordant couples,

among those individuals with incident pregnancy, the per sex-act risk of HIV acquisition increased through pregnancy and peaked in the postpartum period, supporting that hypothesis that hormone exposure, which varies during pregnancy, may impact transmission risk (Thomson et al. 2018). Likewise, exogenous sex steroids in contraceptive therapy alter immune cell populations and microbiome composition, although it is difficult to parse these factors from sociobehavioral and gender features that also alter risk of HIV acquisition (reviewed in Griesbeck et al. (2016); Haddad et al. 2014)). In a large randomized trial comparing the risk of HIV infection among women receiving one of three methods of contraception including a non-hormonal option (depot medroxyprogesterone acetate, copper intrauterine device, and levonorgestrel implant) there was no significant difference in the risk of HIV acquisition (Evidence for Contraceptive O, Consortium HIVOT 2019), suggesting contraceptives are not a primary driver of risk. Taken together, the results from these studies demonstrate that in addition to the location and type of HIV-1 exposure, variations in sex steroid hormone can play a role in impacting the risk of HIV-1 acquisition, in particular in the female genital tract.

While there is no effective preventive vaccine for HIV, biomedical prevention with pre-exposure prophylaxis (PrEP) with antiretroviral agents does significantly reduce the risk of HIV acquisition. Initial studies establishing this therapy demonstrated substantial risk reduction in a population of men and transgender women who have sex with men (Grant et al. 2010). In subsequent studies, performance of oral PrEP in populations of women at risk has been less consistent, with some studies demonstrating significant protection (Baeten et al. 2012; Choopanya et al. 2013; Murnane et al. 2013) and others with low efficacy (Damme et al. 2012; Marrazzo et al. 2015). The weight of the data from these studies suggests that adherence is the driver of low efficacy and that this requirement may be more stringent for women than for men and transgender women, potentially related to the specific features of the anal versus vaginal mucosa (Sheth et al. 2016). For topical PrEP agents, specific components of the vaginal microbiome appear to modulate the local concentration and preventive efficacy of the PrEP drug tenofovir (Klatt et al. 2017), again highlighting the potential for sex-specific biology to directly impact efficacy.

Several studies have shown an increased female susceptibility to in utero HIV-1 transmission (Marinda et al. 2007; Taha et al. 2005; Thorne et al. 2004). In a recent cohort of over 170 in utero HIV-1-infected infants in South Africa, HIV-1-infected female infants significantly outnumber male infants by 1.7:1 (Adland et al. 2020). The mechanisms underlying these sex differences in in utero HIV-1 transmission are not fully understood, but comparative studies of cord blood from HIV-1-uninfected sex-discordant twins demonstrated that cord blood cells from newborn females were more activated and more susceptible to HIV-1 infection in vitro (Adland et al. 2020). These studies show that sex differences in HIV-1 acquisition are already manifested prior to birth.

3 Sex Differences in HIV Pathogenesis

Following HIV acquisition and in the absence of antiretroviral therapy (ART), there continue to be features of disease that are distinct between cisgender men and women. The most notable is the set point viral load, an average number of HIV copies/mL that is linked to risk of disease progression (Henrard et al. 1995; Mellors et al. 1996). Early studies enrolled predominantly cisgender men, but as data accumulated from both men and women, most studies demonstrated lower HIV viral loads in women as compared to men, in particular early during infection (Anastos et al. 2000; Bush et al. 1996; Evans et al. 1997; Farzadegan et al. 1998; Gandhi et al. 2002; Katzenstein et al. 1996; Lyles et al. 1999; Meditz et al. 2014; Moore et al. 1999; Napravnik et al. 2002; Sterling et al. 1999, 2001). However, the lower viral load in women did not stall pathogenesis, with similar rates of progression to AIDS. Guidelines for initiation of ART early during the HIV epidemic included consideration of the viral load, and the lower levels in women meant that in one cohort, only 37% of women who progressed to AIDS were eligible for treatment, as compared to 74% of men (Sterling et al. 2001). This is one clear example where consideration of biological sex is critical in setting clinical treatment guidance. The mechanisms that lead to lower viral load in women are still incompletely understood, but there is evidence for a role for the sex steroid hormone 17β-estradiol in suppressing HIV replication (Szotek et al. 2013) and data demonstrating lower per cell production of HIV RNA from $CD4^+$ T cells from lymph nodes (Meditz et al. 2014). It is also unclear why the lower viral loads in women are not associated with a slower kinetics in $CD4^+$ T cell decline. One hypothesis is that immune activation, a key determinant of AIDS progression (Giorgi et al. 1999), has a sex-specific threshold with higher responses to similar levels of viral exposure in women driving pathogenesis (discussed below).

Another notable sex difference in the natural history of HIV pathogenesis is in the frequency of spontaneous viral control. In rare individuals, HIV replication is controlled to low or undetectable levels in the absence of ART. Host genetic features including specific HLA genotypes and the delta 32 deletion in the *CCR5* gene are associated with the HIV control (Pereyra et al. 2010), but are neither necessary nor sufficient to predict the emergence of this phenotype. Although accounting for relatively few of the controllers who have been extensively studied, women are over-represented in population-based cohorts of spontaneous HIV control with associated odds ratios of control in women versus men ranging between 1.9 and > 5 across several studies (Crowell et al. 2015; Madec et al. 2005; Price et al. 2019; Yang et al. 2017). Of note, in a report of a small number of pediatric spontaneous controllers, there was again a female preponderance (10 of 11 cases) (Vieira et al. 2019), although the mechanisms of control appear to differ between children and adults. The biological basis for this enrichment of controller phenotypes among women is unknown but may provide insight into mechanisms of viral control across both men and women and pathways to a preventive vaccine.

4 Immune Mechanisms Underlying Sex Differences

While sex differences in the clinical manifestations of HIV-1 infection are well established, the underlying mechanisms are less well understood. Increasing evidence suggests that differences in antiviral immune responses of females and males might contribute to the observed sex differences in the clinical manifestations of HIV-1. Biological sex represents an important variable in immunity, and sex differences have been described for different components of the immune system, both in mouse models and in humans. The nature and strength of immune responses differ between females and males, resulting in differences in the prevalence, manifestations and outcome of autoimmune and infectious diseases (Klein and Flanagan 2016; Fish 2008). In general, females develop stronger immune responses against pathogens and vaccines, but also are at higher risk to develop autoimmune responses.

In the context of sex differences in the clinical manifestations of HIV-1 infection, increasing data indicate that these sex differences can be mediated by sex-specific differences in Type I interferon (IFN) responses. Type I IFNs are produced predominantly by innate immune cells and in particular by plasmacytoid dendritic cells (pDCs) that exhibit the strongest Type I IFN production on a per cell basis (Fitzgerald-Bocarsly et al. 2008). Multiple studies have demonstrated that Type I IFN responses of pDCs after toll-like receptor 7- (TLR7) stimulation are stronger in females compared to males (Berghofer et al. 2006; Griesbeck et al. 2015; Laffont et al. 2014; Meier et al. 2009; Seillet et al. 2012; Spiering and Vries 2021). Initial studies described that PBMCs derived from females secreted higher amount of IFNα in response to TLR7 stimulation and that these differences were due to sex differences in the production of IFNα by pDCs (Berghofer et al. 2006; Meier et al. 2009). HIV-1, as a single-stranded (ss) RNA virus, encodes for several GU-rich regions viral genome that can serve as TLR7 ligands and induce IFNα production (Beignon et al. 2005; Meier et al. 2007). In vitro studies demonstrated that pDCs derived from females produced more IFNα in response to stimulation with HIV-1-encoded TLR7 ligands than pDCs derived from males (Meier et al. 2009). In untreated HIV-1 infection, prior to initiation of ART, higher expression levels of interferon-stimulated genes (ISGs) were furthermore observed in immune cells of women living with HIV (WLWH) compared to men living with HIV (MLWH) after adjusting for the level of viral replication (viral load) (Chang et al. 2013), indicating stronger Type I IFN responses in WLWH in response to same amount of viral antigen compared to MLWH.

Studies be several groups have subsequently started to unravel the mechanisms underlying these differences in TLR7-induced responses between females and males (Laffont et al. 2014; Seillet et al. 2012; Douin-Echinard et al. 2008; Souyris et al. 2018; Souyris et al. 2019; Webb et al. 2018). The gene encoding for TLR7 is located on the X chromosome and can escape from inactivation of the second X chromosome, leading to higher TLR7 mRNA levels and also protein levels in B cells and pDCs derived from females with bi-allelic TLR7 expression (Souyris et al. 2018; Hagen et al. 2020). These higher TLR7 expression levels result in higher induction

of IFNα production following TLR7 stimulation in pDCs of females. Furthermore, sex hormones can regulate Type I IFN production of pDCs following TLR7 stimulation, probably by affecting signaling events downstream of TLR7 (Laffont et al. 2014; Seillet et al. 2012; Douin-Echinard et al. 2008). For example, IRF5, a critical signaling molecule in the TLR7 pathway, was expressed at higher levels in pDCs derived from females, and IRF5 expression levels were strongly associated with IFNα production after TLR7 stimulation (Griesbeck et al. 2015). Higher expression of IRF5 in female pDCs depended on estrogen, and pDCs from mice with an estrogen receptor (ERalpha) knockout expressed less IRF5 and exhibited lower IFNα production after TLR7 stimulation (Griesbeck et al. 2015; Laffont et al. 2014). Overall, these data suggest that stronger Type I IFN responses in WLWH compared to MLWH can be mediated by both the effects of sex hormones on innate immune responses to HIV-1 and by differences in expression levels of genes encoded by the X chromosome that escape inactivation (Fig. 2).

Type I IFNs play a critical role in HIV-1 pathogenesis (Sandler et al. 2014; Utay and Douek 2016) and differences in Type I IFN responses between the sexes can therefore affect different stages of HIV-1 infection (Addo and Altfeld 2014; Scully 2018). During primary viral infections, Type I IFNs can mediate strong antiviral effects through the activation of ISG that include viral restriction factors. Stronger Type I IFN responses in females compared to males in primary HIV-1 infection might therefore contribute to the better control of viral replication observed in WLWH than MLWH (Gandhi et al. 2002; Meditz et al. 2014) and might also result in the seeding of a smaller viral reservoir in WLWH (Gandhi et al. 2017; Prodger et al. 2020; Scully

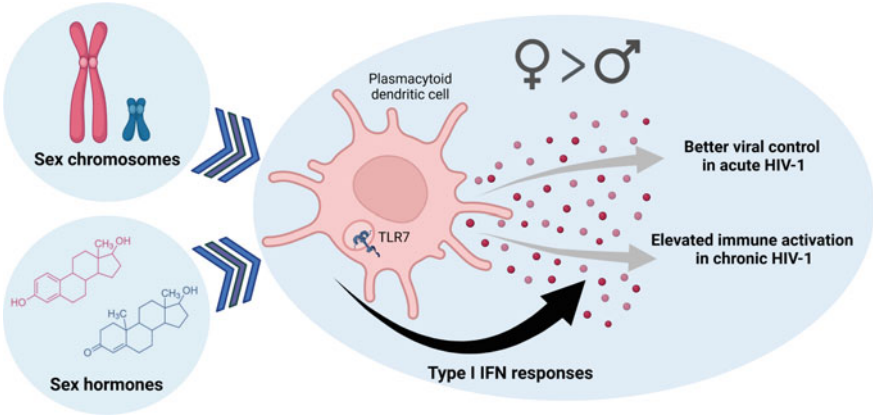

Fig. 2 Mechanisms underlying sex differences in Type I IFN responses by pDCs, and consequences for HIV-1 disease

Genes encoded by the X chromosome that escape inactivation of the second X chromosome and sex steroid hormones can both affect Type I IFN responses by pDCs following TLR7 stimulation, resulting in higher production of IFNα by pDCs from females. Higher levels of IFNA and ISGs in females can contribute to better control of HIV-1 replication in primary infections in WLWH, but also in higher levels of immune activation during chronic untreated HIV-1 infection in WLWH

et al. 2018). However, persistent induction of Type I IFNs in chronic untreated HIV-1 infection induces immune activation that contributes to the pathogenesis in chronic HIV-1 infection, including the loss of CD4+ T cells, and might therefore provide an underlying mechanism for the described faster CD4+ T cell loss and progression to AIDS in WLWH compared to MLWH after adjusting for the level of viral replication (Farzadegan et al. 1998; Gandhi et al. 2002; Meditz et al. 2014; Sterling et al. 2001). Stronger Type I IFN responses during acute viral infections in females compared to males might also have consequences for the characteristics of viral strains that establish an infection. Previous studies have described that primary HIV-1 infection is preferentially established by HIV-1 strains with a higher level of Type I IFN resistance (Gondim et al. 2021; Iyer et al. 2017), and additional studies are required to determine the impact of sex and sex-specific differences in Type I IFN responses during acute HIV-1 infection not only on the level of viral replication and on the size of the viral reservoir, but also on the quality to the HIV-1 strains that establish the HIV-1 reservoir.

The impact of sex on adaptive immune responses to HIV-1 is less well understood. Cytotoxic CD8+ T cells have been shown to play an important role in the control of HIV-1 replication. Some studies have suggested sex differences in CD8+ T cell function, including stronger antiviral activities against CMV (Abdullah et al. 2012; Poon et al. 2021), but sex differences in HIV-1-specific T cell responses have not been systematically studied to data. Strong HIV-1-specific CD8+ T cell responses have been reported in individuals that control HIV-1 replication in the absence of ART (HIV controllers), and several studies have described an overrepresentation of females in cohorts of HIV controllers, including post-treatment controllers (Crowell et al. 2015; Madec et al. 2005; Price et al. 2019; Yang et al. 2017). However, it remains unknown whether differences in the strength of antiviral T cell responses between the sexes might be linked to this overrepresentation of females in HIV controller cohorts. Furthermore, Type I IFN responses play a critical role in regulating the induction and function of antigen-specific CD8+ T cells, and potential differences in HIV-1-specific T cell responses between females and males might therefore not represent T cell-intrinsic aspects, but the consequence of extrinsic Type I IFN-mediated effects (Pujantell and Altfeld 2022). Overall, additional studies are required to better understand the potential effect of sex differences in adaptive immune responses, including antibody responses that have been described to be stronger in females compared to males (Klein et al. 2010), on sex differences in the clinical manifestations of HIV-1.

5 Sex Differences in ART-Treated Disease

Broadly considered, ART has similar efficacy in males and females, with modern treatments achieving high rates of viral suppression. However, the rate of adverse effects may differ for some agents. In a large, randomized trial of three treatment regimens identifying a significant excess of weight gain linked to specific ART, there was a disproportionate impact on women (Venter et al. 2019). In separate

studies, weight gain following ART initiation was linked to a smaller decrease in inflammatory markers (IL-6, TNF-RII, CXCL10, sCD163) specifically in women (Bares et al. 2021). Other studies have similarly demonstrated that the magnitude of the impact of ART on inflammatory markers is sex specific (Mathad et al. 2016; Ticona et al. 2015). Residual immune activation is linked to the risk of non-AIDS comorbid conditions (Raghavan et al. 2017), and HIV confers a greater increase in the relative risks of cerebrovascular and cardiovascular disease as compared to controls without HIV infection in women than in men (Chow et al. 2012, 2017; Triant et al. 2007). There are also sex and gender-specific features impacting the rates of bone and metabolic disease associated with HIV infection (Pond et al. 2021). Taken together, the data suggest that there are some off-target impacts of ART, including weight gain, that may be exacerbated in women as compared to men and that HIV has a greater impact on risk of comorbid conditions and residual inflammation in women as compared to men.

Despite the residual evidence of immune activation, markers of HIV reservoir activity are generally lower in women compared to men, with lower levels of residual HIV viremia by single copy assay and lower levels of cell-associated HIV RNA expression despite comparable levels of HIV proviral DNA (Gandhi et al. 2017; Scully et al. 2018). A lower frequency of HIV virus outgrowth was observed in females from a cohort from Uganda, but no significant difference was identified in a North American cohort (Prodger et al. 2020; Falcinelli et al. 2020). The apparently tighter control of residual virus activity could be linked to differences in establishment or maintenance of latency or in innate host restriction of viral replication. Similar to the observations in active replication, 17β-estradiol suppresses latency reversal in model cell lines and in ex vivo reactivation in cells from people living with HIV (Das et al. 2018). The potential role of estrogen is further supported by data indicating that there is an increase in HIV RNA expression during reproductive aging, while HIV DNA levels are stable to declining (Gianella et al. 2022).

These differences in HIV reservoir dynamics and potentially in the mechanisms of latency maintenance and threshold of immune response are relevant for efforts toward HIV eradication or functional cure. These interventions aim to either purge the reservoir or achieve a state of durable immune control of viremia. Host factors, including the transcriptional controls that either maintain latency or drive HIV transcription and the immune response itself, are the therapeutic targets for these efforts. As such, sources of host variation, including biological sex, become highly relevant (Gianella et al. 2016). A higher barrier to latency reversal may render curative approaches in women even more difficult to achieve, but conversely enhanced immune responses may enhance the efficacy of immunomodulatory treatments.

6 Summary

Differences in risks of acquisition, fundamental features of the antiviral response, and comorbid conditions associated with treated infection all show clear evidence of the impact of biological sex in HIV. These differences obligate tailored prevention and therapeutic approaches. They also provide leverage points where delineating the biological mechanisms underlying differences may inform the understanding of HIV pathogenesis more broadly.

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Effects of Biological Sex and Pregnancy on SARS-CoV-2 Pathogenesis and Vaccine Outcomes

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Abstract SARS-CoV-2 is the causative agent of COVID-19 in humans and has resulted in the death of millions of people worldwide. Similar numbers of infections have been documented in males and females; males, however, are more likely than females to be hospitalized, require intensive care unit, or die from COVID-19. The mechanisms that account for this are multi-factorial and are likely to include differential expression of ACE2 and TMPRSS2 molecules that are required for viral entry into hosts cells and sex differences in the immune response, which are due to modulation of cellular functions by sex hormones and differences in chromosomal gene expression. Furthermore, as comorbidities are also associated with poorer outcomes to SARS-CoV-2 infection and several comorbidities are overrepresented in males, these are also likely to contribute to the observed sex differences. Despite their relative better prognosis following infection with SARS-CoV-2, females do have poorer outcomes during pregnancy. This is likely to be due to pregnancy-induced changes in the immune system that adversely affect viral immunity and disruption of the renin-angiotensin system. Importantly, vaccination reduces the severity of disease in males and females, including pregnant females, and there is no evidence that vaccination has any adverse effects on the outcomes of pregnancy.

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S. L. Klein and C. W. Roberts (eds.), *Sex and Gender Differences in Infection and Treatments for Infectious Diseases*, Current Topics in Microbiology and Immunology 441, https://doi.org/10.1007/978-3-031-35139-6_4

1 Sex Differences in Viral Pathogenesis

Sex as a biological variable refers to the differences between males and females based on their sex chromosome complement, reproductive tissues, and concentrations of sex steroid hormones, all of which define most of us as being either male or female. For the past several decades, it has become increasingly apparent that the intensity, prevalence, and pathogenesis of infections caused not only by viruses, but by parasites, fungi, and bacteria can vary between the sexes (vom Steeg and Klein 2016). The mechanisms explaining how sex-based differences occur in immune responses include differences in genetics and epigenetics (X versus Y chromosome and differential epigenetic programming), concentrations of sex steroid hormones (i.e., progesterone, estrogens, and androgens), and environmental factors (Klein and Flanagan 2016). Although biological differences between the sexes can contribute significantly to differential immune responses and outcomes of infectious diseases, equally relevant are gender-associated factors that define the social or cultural norms that impact lifestyle choices, access to health care, and occupational exposures, and even vaccine hesitancy (Morgan and Klein 2019; Kini et al. 2022; Scully et al. 2021). Gender can intersect with sex to impact outcomes of many infectious diseases and responses to treatments (Mauvais-Jarvis et al. 2020; Shapiro et al. 2021).

The pathogenesis of infectious diseases as well as responses to vaccines that protect against infectious diseases can change throughout the life course (Klein and Flanagan 2016; Flanagan et al. 2017). For many viruses, disease severity is heightened in infants and young children as well as older adults (Troy and Bosco 2016; Hause et al. 2018). Among children (i.e., individuals < 18 years of age), males are more vulnerable to severe outcomes from respiratory viral infections, such as RSV, than females (Klein and Flanagan 2016). At the other end of the spectrum, among individuals 65 years and older, chronic inflammation combined with reduced antibody responses typically occurs at a faster rate among males compared with females (Marquez et al. 2020). The greater age-associated decline in immunity among males may partially explain how males are typically more susceptible to infections than females in later decades of life (Ursin and Klein 2021). During reproductive years (e.g., after puberty and prior to menopause in females), females often experience worse outcomes from respiratory viral infections than males (vom Steeg and Klein 2016; Klein and Flanagan 2016), which may be associated with immunological shifts associated with pregnancy as well as overall greater inflammatory immune responses and tissue damage following infection of non-pregnant, reproductive aged females as compared with age-matched males (Hause et al. 2018; Englund and Chu 2018). In this chapter, we will explore the specific effects of biological sex, gender, pregnancy, and aging on outcomes of coronavirus disease 2019 (COVID-19) and responses to COVID-19 vaccines.

2 Sex Differences in SARS-CoV-2 Pathogenesis

Around the world, males suffer worse outcomes from COVID-19, which was reported early before the pandemic was declared in China (Chen et al. 2020a; Guan et al. 2020) and was further supported by epidemiological evidence from countries in Europe and Asia as well as in the United States (Gebhard et al. 2020; Scully et al. 2020; Klein et al. 2020a). Throughout the pandemic, the Global Health 50/50 organization tracked sex-disaggregated data on severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) confirmed infections, hospitalizations, and deaths worldwide (The COVID-19 Sex-Disaggregated Data Tracker 2022). Despite similar number of confirmed SARS-CoV-2 cases (based on PCR or antigen tests) between males and females, hospitalizations, intensive care unit (ICU) admissions, and deaths from COVID-19 are greater for males than females. Based on a meta-analysis of over 10 million patients from 229 studies, male COVID-19 patients are 41% more likely to require ICU admission and are 35% more likely to die as compared with female patients (Pijls et al. 2022). Sex differences in the outcome of other beta coronavirus infections have been reported, including during the 2003 outbreak of severe acute respiratory syndrome coronavirus (SARS-CoV) and sporadic outbreaks of Middle East respiratory syndrome coronavirus (MERS-CoV), both of which cause greater mortality in male compared to female patients (Karlberg et al. 2004; Alghamdi et al. 2014). Whether these sex differences in COVID-19 outcomes reflect biological differences in cellular virus entry, virus replication, or transmission will be discussed. Further, how sex differences in innate and adaptive immune responses to SARS-CoV-2 result in differential outcomes from infection and vaccination will be explored. Finally, with reports of females being more likely to experience symptoms of post-acute sequalae of SARS-CoV-2 (PASC or long-COVID), greater consideration into the biology of these differences is required.

2.1 SARS-CoV-2 Entry, Replication, and Transmission

Angiotensin-converting enzyme 2 (ACE2) is the principal receptor for SARS-CoV-2 spike (S) binding and its entry into the host cell. This process is facilitated by a transmembrane serine protease 2 (TMPRSS2) enzyme which cleaves and activates viral S glycoprotein (Hoffmann et al. 2020). ACE2 and TMPRSS2 are expressed at varying levels in different tissues of the respiratory, cardiovascular, digestive, and urinary systems (Zou et al. 2020; Sungnak et al. 2020). ACE2 is an X chromosome-encoded gene, and a systematic transcriptomic analysis of X-chromosomal inactivation in 29 adult (20–70 years) human tissues revealed that the expression of ACE2 was greater in tissues derived from male than female patients, including in the lungs (Tukiainen et al. 2017). In mice, the presence of estradiol, which is found in higher concentrations among females than males, reduces the renal ACE2 activity in females (Liu

et al. 2010). Whether sex steroids affect ACE2 expression in the lungs has not been reported.

In the human nasal epithelium (age 4–60 years), age-dependent increases in ACE2 gene expression are observed (Bunyavanich et al. 2020). Similarly, in developing mouse lungs as well as in human lung tissues (collected between birth to 69 years of age), age-dependent increases in TMPRSS2 gene expression are reported (Schuler et al. 2021) indicating the potential association of low levels of receptor distribution in the respiratory tract and lower risk of COVID-19 in children. These studies, however, did not perform sex-specific analysis for receptor distribution. Okwan-Duodu et al. performed sex-specific analysis of ACE2 and TMPRRSS2 expression in the lungs of 13 adult male (mean age 51.8 ± 4 years) and 13 adult females (mean age 46.9 ± 6 years) (Okwan-Duodu et al. 2021). Though ACE2 expression was similar, TMPRSS2 expression was significantly greater in males compared to females. Moreover, ACE2 + TMPRSS2 + double-positive alveolar type 2 (AT2) epithelial cells were threefold enriched in male lungs (Okwan-Duodu et al. 2021). As these cells are important in mediating virus replication, the differences in receptor distribution may explain, to some extent, the risk of developing severe COVID-19 in adult males compared to adult females. Co-expression of ACE2 and TMPRSS2 also is observed in oral epithelial cells, with both expressed at greater levels in older individuals and with TMPRSS2 expressed at greater levels in males than females (Peng et al. 2021). Because SARS-CoV-2 infects the oral mucosa and salivary glands, with salivary viral burden correlating with COVID-19 symptoms (Huang et al. 2021), these data suggest that sex and age differences in TMPRSS2 expression might contribute to the age and sex distributions in COVID-19 outcomes.

Early data from Wuhan, China showed that male sex and older age were associated with prolonged virus shedding in symptomatic COVID-19 patients (Xu et al. 2020). Another retrospective study from the Zhejiang province in China also showed similar findings, where virus shedding was prolonged in male and older (> 60 years) patients with severe disease (Zheng et al. 2020). While Carrouel et al. reported neither age nor sex differences in salivary viral quantification in adults (18–85 years) with asymptomatic or mild COVID-19 (Carrouel et al. 2021), Kobayashi et al. reported 10 times lower viral load in the saliva obtained from symptomatic adult female compared to male patients (Kobayashi et al. 2021). A systematic review of SARS-CoV-2 shedding dynamics, however, indicated that disease severity rather than age or sex better predicts viral shedding kinetics (Chen et al. 2021). Thus, sex difference in SARS-CoV-2 replication and shedding might be dependent on other factors including age, disease severity, and comorbidities or an interaction of these factors.

Studies of sex differences in SARS-CoV-2 transmission are limited. One epidemiological survey among adult Brazilian couples living together without any protective measures during SARS-CoV-2 infection indicated that males are more likely to transmit virus to their spouse in the household setting (Silva et al. 2022). Another systematic review showed that the most typical super-spreader for SARS-CoV-2 is likely to be a male over 40 years of age and having a mild disease course (Brainard et al. 2023). Because males produce 34% more aerosol than females, it is plausible that males spread SARS-CoV-2 better than females (Good et al. 2021). Other

gender-associated factors that can contribute to spread (e.g., use or acceptance of non-pharmacological interventions [NPIs]) must also be considered as males across diverse countries were shown to be less accepting of NPIs than females (Galasso et al. 2020).

2.2 Development of Innate and Adaptive Immune Responses

SARS-CoV-2 infection of the respiratory system induces production of interferons (IFNs) and other cytokines and chemokines, though the interferon response to SARS-CoV-2 is less pronounced as compared to other human coronavirus infections (Zhou et al. 2020; Chu et al. 2020; Hadjadj et al. 2020). Increases in interleukin-6 (IL-6), IL-10, and C-reactive protein (CRP) are observed among severe COVID-19 patients at the time of inpatient admission (Tan et al. 2020). Similarly, reduced numbers of total lymphocytes, CD4+ and CD8+ T cells, CD19+ B cells, and NK cells but increases in monocytes, neutrophils, and neutrophil-to-lymphocyte ratio (NLR) occur in severe cases (Tan et al. 2020; Lucas et al. 2020). Longitudinal analyses of immune responses indicate a strong association of an early rise in the plasma cytokine level with severe COVID-19 outcome (Lucas et al. 2020). Moreover, patients with severe disease maintained the elevated levels of cytokines for a longer period compared to the patients with moderate disease (Lucas et al. 2020).

Among patients with moderate disease, Takahashi et al. observed significantly greater levels of IL-8 and IL-18 in male than female patients at baseline, whereas greater C–C motif chemokine ligand 5 (CCL5) responses are observed in male patients over the course of the disease (Takahashi et al. 2020). Females have greater numbers of intermediate monocytes (CD14+ CD16+), while males have higher frequencies of non-classical monocytes (CD14loCD16+), suggesting stronger activation of innate immune cells in males leading to the production of greater levels of inflammatory cytokines (Takahashi et al. 2020). Similar observations have been made in hamsters infected with the ancestral strain of SARS-CoV-2, in which infected males suffer worse outcomes than females and have greater frequencies of myeloid cells than lymphocytes (Dhakal et al. 2021; Ruiz-Bedoya et al. 2022). The NLR is positively correlated with negative COVID-19 outcomes, and male patients are more likely to have higher NLRs than females (Yan et al. 2020). Likewise, the initial and peak plasma levels of IL-6 and CRP are significantly greater in male than in female COVID-19 patients (Lau et al. 2021). Moreover, the effect of change in peak CRP level is more robust in males than females, where a one standard deviation higher peak in CRP level was associated with over 7- and ninefold increased odds of ICU admission and death in males compared to 2.7- and 2.8-fold increased odds in females, respectively (Lau et al. 2021). Auto-antibodies against at least one type of type I IFNs is reported in 10.2% of severe COVID-19 patients, and 94% of these patients bearing auto-antibodies were males (Bastard et al. 2020). Males with life-threatening COVID-19 have 5.22 times higher odds of having type I IFN

auto-antibodies (Bastard et al. 2020) highlighting their possible role in mediating sex difference in COVID-19 outcomes.

Male sex, older age, and hospitalization are associated with a greater antibody response in COVID-19 convalescent plasma donors (Klein et al. 2020b). Grzelak et al. reported that binding and virus neutralizing antibodies are significantly greater in males than in females at 1 month, while the decay of these antibodies is faster in male than female patients over a period of 6 months (Grzelak et al. 2021). Male-biased greater SARS-CoV-2 binding and neutralizing antibody titers also were reported in another study which further showed that the age and symptom grade are positively correlated with antibody levels only in males but not in females (Markmann et al. 2021). This study, however, observed no sex difference in antibody durability (Markmann et al. 2021). Another study showed significantly greater IgM antibody responses in male than female patients, but sex difference in IgG responses was inconsistent (Wu et al. 2021). In contrast, in hamsters infected with the ancestral strain of SARS-CoV-2, females develop greater IgG and neutralizing antibody responses against SARS-CoV-2 antigens and viruses, both peripherally in blood and locally in the lungs (Dhakal et al. 2021). Such disparities may be explained by differences in disease severity among patients in different studies as hospitalization with severe disease is the strongest predictor of antibody response than male sex and age (Klein et al. 2020b).

During COVID-19, female patients have greater frequencies of activated and terminally differentiated T cells compared to male patients (Takahashi et al. 2020). The inferior T cell response is associated with worse disease outcome in male but not female patients (Takahashi et al. 2020). Sex difference in mucosal-associated invariant T (MAIT) cells also is reported during COVID-19 where the extravasation and recruitment of these cells in the airway epithelium is greater in female than male patients (Yu et al. 2021). The qualitative and quantitative superiority of MAIT cells in females may play a role in providing better protection to females during COVID-19 (Yu et al. 2021).

2.3 Sex Differences in Post-Acute Sequelae SARS-CoV-2 Infection (PASC)

PASC or long-COVID is characterized by the occurrence of a wide range of physical and psychological symptoms beyond the acute recovery phase of COVID-19 (Aiyegbusi et al. 2021). The most common persistent symptoms during long-COVID include fatigue, shortness of breath, muscle pain, cough, chest pain, and altered smell or taste (Aiyegbusi et al. 2021). A recent meta-analysis and systematic review suggested that 43% of COVID-19 patients experience at least one symptom beyond 28 days of infection (Chen et al. 2022). The long-COVID prevalence is greater (54%) in hospitalized patients compared to the non-hospitalized (34%) patients (Chen et al. 2022).

Long-COVID is more prevalent in female (49%) compared to male (37%) patients, and females have 1.57 times greater odds of developing long-COVID (Chen et al. 2022). During long-COVID, ear, nose, and throat (ENT), gastrointestinal, psychiatric, and neurological disorders are reported more frequently by females, whereas males report more symptoms associated with endocrine and renal disorders (Sylvester et al. 2022). In the Mayo clinic, between January and April 2021, 75% of the patients admitted with long-COVID were females (Ganesh et al. 2022). While fatigue is more common in females, dyspnea is more prevalent in male patients. Increased IL-6 concentrations are observed in 61% of the patients, with most of them being females, suggesting the possible association of persistent inflammation with long-COVID (Ganesh et al. 2022). Long-COVID patients suffer from neurocognitive symptoms at least up to a year, and this is more common in female than male patients (Seessle et al. 2022). Antinuclear antibodies are detected more frequently in female long-COVID patients than in males, and a higher antinuclear antibody titer is associated with the greater number of symptoms in females but not males (Seessle et al. 2022). This suggests for the possible association of long-COVID with autoimmunity, as autoimmune diseases are more prevalent in females (Klein and Flanagan 2016).

2.4 Comorbidities, Sex Difference, and COVID-19

Comorbidities, including hypertension, obesity, diabetes, and malignancy, are known risk factors for COVID-19 (Guan et al. 1590; Richardson et al. 2020). COVID-19-associated ICU admission is 2–3 times greater in patients with hypertension, cardiovascular disease, and diabetes (Li et al. 2020a). The odds of death, compared to patients having no comorbidity, is 1.53 times higher for patients with one comorbidity and 3.82 times higher for patients with more than 10 comorbidities, indicating that risk increases with increasing number of comorbidities (Kompaniyets et al. 2021). Among COVID-19 patients, the incidence of comorbidities, including diabetes, hypertension, and cancer, is greater in males than in females (Chakravarty et al. 2020). However, sex-specific distribution of comorbidities is also reported where males have a higher prevalence of cardiovascular, pulmonary, and renal diseases during admission, while dementia and autoimmune diseases are more prevalent in females (Raparelli et al. 2020).

Males, in general, have a greater incidence of cardiovascular disease (Gao et al. 2019). A higher prevalence of heart disease during hospital admission with COVID-19 also is reported in males compared to females (Raparelli et al. 2020). Among heart failure patients, circulating plasma ACE2 level is significantly elevated in males than females suggesting a role in greater COVID-19 severity in males (Sama et al. 2020). Risk of developing severe or fatal COVID-19 disease is 2.3 times greater with type 1 diabetes and 1.3 times greater with type 2 diabetes compared with patients without diabetes (McGurnaghan et al. 2021). COVID-19 patients with diabetes are most predominantly males (McGurnaghan et al. 2021; Tramunt et al. 2021). Lymphocytopenia at hospital admission is the independent predictor of death at 28 days in

females, while increased plasma concentration of CRP is the predictor in both sexes (Tramunt et al. 2021). A population-based study from Italy showed that males with prostate cancer have 79% increased risk of getting SARS-CoV-2 infection compared to males without prostate cancer (Montopoli et al. 2020).

Obesity is an independent risk factor for severe COVID-19 (Simonnet et al. 2020). Visceral obesity, which is greater in males than in females, is reported to be positively associated with severe disease (Petersen et al. 2020; Foldi et al. 2021). With every ten square centimeter increase in visceral fat area, the risk of ICU admission is increased by 1.37-fold (Petersen et al. 2020). As visceral adipose tissue contains macrophages that promote inflammation and release of inflammatory cytokines, it is likely that greater visceral adiposity in males drives elevated cytokine response and severe disease (Foldi et al. 2021). A recent study in mice showed a sex-specific effect of diet-induced obesity (DIO), in which greater lung virus titers and shortened time to death due to obesity was observed in adult female, but not male, mice (Lee et al. 2021).

2.5 Role of Sex Steroids

In males, lower testosterone levels correlate with greater COVID-19 severity. Male COVID-19 patients have reduced levels of testosterone, elevated levels of luteinizing hormone, and reduced testosterone to luteinizing hormone ratio compared to healthy controls (Ma et al. 2021; Rastrelli et al. 2021). Likewise, male patients who require ICU admission, ventilator use, or who died from COVID-19, all have significantly lower concentrations of testosterone at hospital admission as compared to their respective non-severe counterparts (Dhindsa et al. 2021). The baseline plasma testosterone level in male patients is negatively associated with neutrophils, CRP, and IL-6, which are elevated in severe COVID-19 cases, and positively correlated with lymphocyte count, which is reduced with disease severity (Rastrelli et al. 2021; Dhindsa et al. 2021). Further, upregulation of androgen signaling pathways in classical (CD14+ CD16−) and non-classical (CD14−CD16+) monocytes obtained from the male patients that require ICU admission also is reported (Dhindsa et al. 2021). In females, a probable protective role of estradiol is reported. Menopausal females are more likely to be hospitalized for a longer duration compared to premenopausal female COVID-19 patients. Moreover, estradiol concentrations are negatively correlated with COVID-19 severity and concentrations of inflammatory cytokines, including IL-6 and IL-8 (Ding et al. 2021).

Testosterone and estradiol can influence the SARS-CoV-2 receptor expression and function. In male mice, androgen deprivation after castration results in reduced TMPRSS2 expression in type II alveolar cells, while ACE2 expression levels are reduced in lung parenchyma, type I and II alveolar cells, and bronchial epithelium (Deng et al. 2021). The expression of ACE2+ TMPRSS2+ double-positive cells also is lower in lungs of castrated compared with gonadally intact male mice. ACE2 and TMPRSS2 downregulation also is observed in human lung cancer cells

(i.e., H460) that are grown in androgen-deprived environment (Deng et al. 2021). Then, 17β-estradiol (i.e., the primary biologically active form of estrogen found in premenopausal females) treatment of differentiated normal human bronchial epithelial (NHBE) cells, derived from a female donor, results in significantly lower levels of ACE2, but not TMPRSS2 mRNA expression (Stelzig et al. 2020). In A549 human lung epithelial cells, however, 17β-estradiol treatment reduced expression of both ACE and TMPRSS2 mRNAs (Baristaite and Gurwitz 2022). These studies suggest that sex steroids can modulate SARS-CoV-2 receptor expression in the respiratory tract which may contribute to sex difference in SARS-CoV-2 pathogenesis and COVID-19 outcomes.

Sex steroid-related therapies also have been considered during the COVID-19 pandemic. Early studies suggested that androgen-deprivation therapy reduces the risk of severe COVID-19 in prostate cancer patients (Montopoli et al. 2020; Patel et al. 2020). A protective role for androgen deprivation, however, was not observed in subsequent studies (Gedeborg et al. 2021; Schmidt et al. 2021). In vitro studies suggested that anti-androgens, such as enzalutamide, can reduce TMPRSS2 expression and SARS-CoV-2 entry into human cells (Qiao et al. 2020; Leach et al. 2021). Anti-androgen treatment in humans, however, showed mixed results. In one study, male COVID-19 patients with mild disease were treated with dutasteride for 30 days after enrollment and experienced reduced virus shedding as well as inflammatory responses (Cadegiani et al. 2021). Another study showed that patients taking anti-androgens, dutasteride, finasteride, or spironolactone, for at least 6 months prior to hospitalization have significantly lower risk of ICU admission after SARS-CoV-2 infection (Wambier et al. 2020). Phase 2 clinical trial results, however, found that treatment with enzalutamide does not provide any therapeutic benefit in COVID-19 (Welén et al. 2022). One retrospective study observed that females who received hormone replacement therapy within 6 months of having COVID-19 have reduction in all-cause mortality (Dambha-Miller et al. 2022). Similarly, treatment with a commonly used contraceptive and hormone replacement therapy drug, Evra (norelgestromin and ethinylestradiol), resulted in significantly reduced hospital stay, mechanical ventilation, and intubation (Alfredo et al. 2022).

2.6 Role of Genetic Variations

Genetic associations with susceptibility to SARS-CoV and MERS-CoV infection have been reported (Made et al. 2022). For example, polymorphisms in mannose-binding lectin (MBL), chemokine (C–C motif] ligand 2 gene (CCL2], interferon gamma (IFNγ], and Regulated upon Activation, Normal T cell-Expressed and Secreted (RANTES] are associated with increased risk of SARS-CoV infection or mortality, while certain human leukocyte antigen class II alleles increase susceptibility to MERS-CoV infection (Tu et al. 2015; Chong et al. 2006; Ng et al. 2007; Hajeer et al. 2016). Studies with COVID-19 have also identified alteration in different genes that encode antiviral and pro-inflammatory proteins responsible for increased

severity (Made et al. 2022). For example, low expression of *IFNAR2* and high expression of *TYK2* and *CCR2* are associated with severe COVID-19 (Pairo-Castineira et al. 2021). Likewise, single nucleotide polymorphisms in genes encoding SARS-CoV-2 entry receptors ACE2 and TMPRSS2 also impact virus infectivity (Zhang et al. 2022). In a mouse model, mice with the apolipoprotein E2 (APOE2) or APOE4 had greater viral loads and suffered worse clinical outcomes after SARS-CoV-2 infection as compared to the mice bearing the most dominant APOE3 allele (Ostendorf et al. 2022). The clinical outcomes are more pronounced in male than female mice. Consistently, the association of APOE genotype with COVID-19 survival and male bias in adverse survival outcomes are also observed in human patients (Ostendorf et al. 2022).

Inborn errors of immunity (IEI) are observed more frequently in male patients admitted into the hospital with COVID-19, with the subsequent need of ICU admission also being greater in younger male compared to female patients (Meyts et al. 2021; Castano-Jaramillo et al. 2021). Severe COVID-19 patients have enrichment of genetic defects in the loci governing TLR3 and IRF7-mediated activation of type I IFN responses (Zhang et al. 2020). These inborn errors are observed in 3.5% (23/659) of the patients with severe COVID-19, and majority of them (15/23) were males (Zhang et al. 2020). IRF7 can be activated by viral nucleic acids through different signaling pathways including the TLR7-mediated pathway. TLR7 is encoded on the X chromosome and can escape X-inactivation process, resulting in its greater expression in female-derived immune cells (Souyris et al. 2018). TLR7 is highly expressed in plasmacytoid dendritic cells (pDCs), which are the predominant source of type I IFNs, and during viral infection, pDCs from females have greater type I IFN responses compared to males (Meier et al. 2009; Chang et al. 2013). Genetic loss-of-function variants of TLR7 are reported in four young adult (21–32 year) male COVID-19 patients admitted to ICU (Made et al. 2020). In these patients, the type I IFN response is downregulated. Association of genetic alteration on TLR7 and severe COVID-19 also have been reported in subsequent studies (Kosmicki et al. 2021; Solanich et al. 2021; Asano et al. 2021). Around 1.8% of the male patients under 60 years of age carry deleterious TLR7 variants (Asano et al. 2021). When peripheral blood mononuclear cells (PBMCs) or plasmacytoid dendritic cells (pDCs) derived from these patients are treated with TLR7 agonists and SARS-CoV-2, the expression of genes involved in type I IFN production, including IRF7, as well as interferon production is diminished (Solanich et al. 2021; Asano et al. 2021). These findings highlight the importance of TLR7-mediated type I IFN immunity in sex differences in SARS-CoV-2 pathogenesis.

3 Pregnancy and SARS-CoV-2 Pathogenesis

Pregnancy is associated with enhanced susceptibility to COVID-19 (Table 1). Pregnant women have worse outcomes from SARS-CoV-2 infection, including increased viral load, disease severity, and adverse effects on the developing fetus. Successful

pregnancy is dependent on physiological, endocrinological, and immunological adaptations from insemination to parturition. During pregnancy, the immune system is altered mostly in the local environment of the reproductive tract, but the immunological effects also are observed systemically. Local immune modulation could affect the outcome of pregnancy including the integrity of the fetoplacental unit, fetus infection, and survival. Pregnancy-induced systemic changes in the immune system are associated with modulation of both autoimmune, e.g., arthritis and systemic lupus erythematosus, and infectious diseases, including Influenza (Ostensen 1999; Abdullahi et al. 2021). There has been considerable concern regarding the potential detrimental effects of COVID-19 on fetal and maternal health.

Several studies highlight that adverse effects are likely to be increased in SARS-CoV-2-infected pregnant women compared to healthy non-pregnant control (Chen et al. 2020b; Akbar et al. 2022; Khan et al. 2020; Jering et al. 2021; Li et al. 2020b). In one study in Indonesia, the maternal mortality rate was 8.3% among females with COVID-19, compared to 1.3% in healthy females (Akbar et al. 2022). Infected females also are more likely to suffer increased rate of pregnancy-related complications, including hypertension, premature rupture of the membrane, and miscarriage (Akbar et al. 2022).

SARS-CoV-2 induces robust immune activation in pregnant females, including cytokine responses (Tanacan et al. 2021). SARS-CoV-2-infected pregnant females exhibit an overall increase in pregnancy complications, including miscarriage, gestational diabetes mellitus (GDM), gestational hypertension (GHT), intrahepatic cholestasis of pregnancy (ICHP), pre-eclampsia, and preterm delivery. Concentrations of CRP, IFNγ, and IL-6 are significantly greater in SARS-CoV-2-infected pregnant females, whereas uninfected pregnant females have greater numbers of peripheral blood lymphocytes and levels of IL-2, IL-10, and IL-17. In pregnant females, a significant positive association is observed between COVID-19 disease severity and increased concentrations of IFNγ and IL-6, whereas increased levels of IL-2 and IL-10 correlate with reduced severity (Tanacan et al. 2021). Another study also illustrates that IL-6 and IFNγ together with IL-1β, CXCL10, TNFα, and NF-kB transcript levels are increased in pregnant females infected with SARS-CoV-2 relative to uninfected pregnant females (Foo et al. 2021). In contrast, IL-13 levels are reduced in COVID-19 pregnant females relative to healthy pregnant females (Foo et al. 2021). Although it is difficult to assign cause and effect, these findings suggest that SARS-CoV-2-mediated adverse effects on pregnancy are associated with immunological modulation.

An alternative or even additional possibility that could explain the detrimental effects of SARS-CoV-2 infection on pregnancy is through direct interactions of the virus with the placenta. ACE2 is expressed on the placenta where its canonical role is to exert its protective effects over the actions of angiotensin II (AngII) in the renin–angiotensin–aldosterone system (RAAS), which is essential for maintaining heathy homeostasis during pregnancy (Tamanna et al. 2021). ACE2 expression is greater in placenta tissue during the first trimester. As ACE2 is used by SARS-CoV-2 to infect cells, this could facilitate viral infection of the placenta (and potentially the fetus). SARS-CoV-2 infection could be more severe in pregnant women because of

Table 1 Comprehensive analysis of COVID-19 outcomes in pregnant people

References	COVID-19 status	Pregnant person inclusion criteria	Cohort number	Comorbidities/ complications	COVID-19/ viral symptoms	Mode of delivery	Pregnancy outcome	PT delivery	PE	Key findings
Chen et al. (2020a, b)	+	COVID-19 detected by qRT-PCR with associated pneumonia admitted to Zhongnan Hospital, Wuhan (China), between January 20 and 31 2020	9	1 gestational hypertension (pre-infection) (11%) 1 influenza (11%)	8 (88%)	9 cesarean (100%)	No maternal or fetal death	4 (44%)	1* (11%)	5 lymphopenia (56%) 6 > CRP (66%) 2 PROM (22%)
Khan et al. (2020)	+	COVID-19 detected by qRT-PCR admitted to Renmin Hospital (China) during January 28–March 1, 2020	3	N/R	3 cough (100%) 2 fever (67%) 1 chest tightness (33%)	3 vaginal (100%)	No maternal or fetal death	1 (33%)	N/R	2 > CRP (67%)

(continued)

Table 1 (continued)

References	COVID-19 status	Pregnant person inclusion criteria	Cohort number	Comorbidities/ complications	COVID-19/ viral symptoms	Mode of delivery	Pregnancy outcome	PT delivery	PE	Key findings
Akbar et al. (2022)	+	Suspected COVID-19 infection displaying at least one symptom including abnormal blood count. Categorized by qRT-PCR, April–August 2020 (Indonesia)	62	37 pregnancy complications (60%)	15 (24%)	14 vaginal (23%) 43 C-section (71%) 3 conservative (5%)	6 maternal death (10%) **($p^a = 0.044$)** 5 fetal death (8%)	9 (15%)	1 (2%)	Increased hospital admission, hypertension, and PROM
	–		79	37 pregnancy complications (47%)	19 (24%) **	25 vaginal (32%) 50 C-section (63%) 4 conservative (5%)	1 maternal death (1%) 3 fetal death (4%)	7 (9%)	N/R	No significant findings

(continued)

Table 1 (continued)

References	COVID-19 status	Pregnant person inclusion criteria	Cohort number	Comorbidities/ complications	COVID-19/ viral symptoms	Mode of delivery	Pregnancy outcome	PT delivery	PE	Key findings
Li et al. (2020a, b)	+ (laboratory confirmed)	Patients displaying signs of COVID-19 pneumonia admitted to Hubei provincial Maternal and Child Centre (Wuhan), January 24–February 29, 2020. Categorized by PCR result and chest X-ray	16	3 diabetes (19%), 1 PROM (6%), 3 hypertension (19%), 2 hypothyroidism (13%), 1 PE (6%), 1 sinus tachycardia (6%)	4 fever (25%)	2 vaginal (12.5%) 14 C-section (87.5%)	No maternal or fetal death	3 (19%) 1 set of twins = 4 (24%) ***	1 (6%)	1 placental bleeding 2 lymphopenia (12.5%) 5 > CRP 1st postpartum blood test (31%) (**p^b < 0.001**)
	− (Suspected)		18	**2 chronic illness patients (13%)**: hypertension, PCOS, and Hepatitis B 13 gestational complication (72%) 1 chronic illness (6%): hypertension, PCOS, and Hepatitis B	1 fever (6%)	2 vaginal (11%) 16 C-section (89%)	No maternal or fetal death	3 (17%) 1 set of twins = 4 (21%)	N/R	2 gestational hypertension, 1 placental previa causing PT delivered 5 lymphopenia (28%) 11 > CRP 1st postpartum blood test (61%) (**p^c < 0.02**)

(continued)

Table 1 (continued)

References	COVID-19 status	Pregnant person inclusion criteria	Cohort number	Comorbidities/ complications	COVID-19/ viral symptoms	Mode of delivery	Pregnancy outcome	PT delivery	PE	Key findings
	− (2020)	Age matched women randomly selected from between 25 and 35 admitted during January 24–February 29, 2020	121	38 gestational complications (31%) 5 chronic illness (4%)	N/R	64 vaginal (53%) 57 C-section (47%) (**$p^{d} = 0.003$**)	No maternal or fetal death	7 (6%)	N/R	15 lymphopenia (13%) 68 > CRP (58%)
	− (2019)	Age matched women randomly selected admitted during January 24–February 11, 2019	121	32 gestational complications (33%) 0 chronic illness (0%)	N/R	77 vaginal (64%) 44 C-section (36%) (**$p^{e} < 0.001$**)	No maternal or fetal death	6 (5%)	N/R	14 lymphopenia (12%) 57 > CRP (47%)
Jering et al. (2021)	+	Women giving birth between April 1–November 23, 2020, within the premier healthcare database (Peng et al. 2021). Categorized by COVID-19 result	6380 ****	**Gestational complications**: 381 hypertension (6%) 667 diabetes (10.5%) ($p^{g} = 0.01$)	N/R	1847 C-section (29%) (**$p^{i} = 0.01$**)	34 stillbirth (0.5%) (**$p^{j} = 0.003$**) 9 maternal death (0.1%) ($p^{k} < 0.001$)	459 (7%) (**$p^{l} < 0.001$**)	564 (9%) (**$p^{m} < 0.001$**)	212 admitted to ICU (3%) $p < 0.001$ 22 thrombotic event (0.3%) $p < 0.001$

(continued)

Table 1 (continued)

References	COVID-19 status	Pregnant person inclusion criteria	Cohort number	Comorbidities/ complications	COVID-19/ viral symptoms	Mode of delivery	Pregnancy outcome	PT delivery	PE	Key findings
	–		400,066	**Chronic illness:** 19,117 hypertension (5%) 5716 diabetes (1%) **Gestational complications**: 29,584 hypertension (7%) (***p*** [h] **< 0.001**) 38,066 diabetes (9.5%)	N/R	109,865 C-section (28%)	1289 stillbirth (0.3%) 20 maternal death (0%)	23,234 (6%)	27,078 (7%)	1747 ICU (0.4%) 300 thrombotic event (0.1%)

Abbreviations: PROM, premature rupture of membrane; CRP, C-reactive protein; PT, preterm birth; PE, pre-eclampsia; PCOS, polycystic ovarian syndrome; N/R, not reported; ICU, intensive care unit. *Lowest birth weight seen in PE patient; **Pregnant women with suspected COVID-19 displaying at least 1 typical viral symptom including abnormal blood count; ***Significantly higher rate of premature birth defined as birth before 37 weeks of gestation compared to both 2019 and 2020 control ($p = 0.031$ and $p = 0.021$, respectively); ****Women with COVID-19 were significantly younger ($p < 0.001$) and more often part of an ethnic minority. [a]Maternal death significantly higher in positive COVID-19 cases; [b]CRP higher in the blood of COVID-19 patients postpartum compared to admission and following test; [c]CRP higher in the blood of suspected COVID-19 patients postpartum compared to admission and following test; [d]C-section delivery significantly higher in confirmed COVID-19 positive women compared to 2020 control; [e]C-section delivery significantly higher in confirmed COVID-19 positive women compared to 2019 control; [f]Diabetes higher in COVID-19 positive patients compared to control; [g]Gestational diabetes higher in COVID-19 positive patients compared to control; [h]Gestational hypertension higher in control than positive patients; [i]C-section higher in COVID-19 positive women compared to control; [j]Stillbirth higher in COVID-19 positive women compared to control; [k]Maternal death higher in COVID-19 positive women compared to control; [l]PT delivery higher in COVID-19 positive women compared to control; [m]PE higher in COVID-19 positive women compared to control

the disruption of the RAAS. Both COVID-19 and pregnancy are associated with increased risk of thrombosis due to their hypercoagulable state, meaning infection during pregnancy could exacerbate prothrombotic conditions, potentially due to increased AngII which is implicated in thrombus formation (Servante et al. 2021). Four studies have examined placentas for SARS-CoV-2, ACE2, and TMPRSS2 protein or transcript levels from control and SARS-CoV-2-infected pregnant females and used histopathology to gage the damage (Lu-Culligan et al. 2021; Taglauer et al. 2020; Mourad et al. 2021; Verma et al. 2021). Viral protein or RNA was detected in placenta of some of the COVID-19 positive pregnant females. One study observed that ACE2 protein levels were increased in placentas from SARS-CoV-2-infected females (Lu-Culligan et al. 2021), although none of the other studies could replicate this observation (Taglauer et al. 2020; Verma et al. 2021). One study, however, reported that ACE2 transcript levels were greater in the placentas of females with severe COVID-19 than in placenta tissue from females with mild disease or who were uninfected (Mourad et al. 2021). In this study, a significant increase in IFN-induced transmembrane proteins 1 and 3 (IFITM1 and IFITM3) in the placenta tissue of positive patients is observed compared to asymptomatic and negative patients (Mourad et al. 2021). IFITM3 plays a key part in innate immunity and has recently been linked to COVID-19, exerting both protective and pathogenic effects (Shi et al. 2021). There is no consistent alteration of placental TMPRSS2 levels in infected and non-infected pregnant females. In vitro studies using human trophoblast cells that were infected with SARS-CoV-2 expressing a GFP reporter (spike/VSV-eGFP-SARS-CoV-2-S) illustrate downregulation of ACE2 and angiotensin II receptor type 2 (AT_2R) after viral infection, whereas AT_1R and soluble fms-like tyrosine kinase-1 (sFlt1) are upregulated (Verma et al. 2021). A finding that is largely consistent in all studies is that placentas from pregnant females infected with SARS-CoV-2 have increased histopathology, including increased intervillous fibrin, chorionitis, or maternal vascular malperfusion (MVM). Together, these studies illustrate that SARS-CoV-2 is capable of infecting human placentas, but the contribution of direct infection to pregnancy-associated adverse outcomes is not clearly or consistently understood. More studies are needed to define the risks and mechanisms of RAAS disruption in pregnant females with COVID-19.

4 Sex Differences in COVID-19 Vaccine Outcomes

The COVID-19 vaccines saved an estimated 20 million lives from December 2020 to December 2021 (Watson et al. 2022). Despite the clear sex differences in disease pathogenesis described above, however, the large Phase III trials failed to consider sex differences in the a priori design or analyses of vaccine efficacy or safety (Vries et al. 2022; Jensen et al. 2022). This trend of failing to test for sex differences, or even to provide sex-disaggregated data, has continued in post-licensure research (Zhu et al. 2021). For example, of 75 COVID-19 vaccine studies included in one systematic review, only 24% provided sex-disaggregated data for their main outcome, and only

13% included a discussion of the implications of their findings for males and females (Heidari et al. 2021). Despite the lack of data, sex differences have been identified in the immunogenicity, effectiveness, and safety of the COVID-19 vaccines.

4.1 Immunogenicity

Most vaccine immunogenicity studies that have incorporated sex as a biological variable have focused on the BNT162b2 (Pfizer) vaccine. Among these studies, it has consistently been reported that females mount stronger IgG responses to vaccination than males in both the general adult population and in older adults (Lustig et al. 2021; Demonbreun et al. 2021; Visci et al. 2022; Kondo et al. 2022; Kageyama et al. 1861; Shapiro et al. 2022). This sex difference holds true when controlling for potential confounders, such as age and previous SARS-CoV-2 infection (Lustig et al. 2021; Visci et al. 2022), and in a meta-analysis combining data from 32 studies (Notarte et al. 2022). Furthermore, six months after receipt of the second vaccine dose, a large study from Israel revealed that neutralizing antibody titers were significantly lower in males than in females (Levin et al. 2021). One study of the AZD1222 (AstraZeneca) vaccine also found greater anti-spike IgG titers in females than males, but no sex differences are observed in pseudoneutralization titers, antibody isotypes, or antibody subclasses (Marchevsky et al. 2022).

In addition to females mounting stronger antibody responses to the COVID-19 vaccines than males, intersectional analyses have revealed several sex-specific effects of the humoral vaccine response. For example, in a sample of hospital-based healthcare workers, aging had a more pronounced negative association with antibody responses in males than females (Resta et al. 2021). Similarly, in a sample of older adults (above 75 years of age), the effect of aging on four different measures of humoral immunity (i.e., anti-spike IgG, anti-spike receptor binding domain IgG, ACE2-inhibiting antibodies, and neutralizing antibodies) is significant in males but not females, leading to sex-specific effects of aging (Shapiro et al. 2022). In the same study, sex-specific effects of frailty also are observed in older adults, whereby frailty is associated with impaired antibody responses in males but not females (Shapiro et al. 2022). The effects of both age and frailty are attenuated after reciept of the third vaccine dose, highlighing the importance of booster vaccines, particularly for older males. Turning toward the role of BMI in vaccine-induced antibody responses, once again effects are more pronounced in males than females, leading a signficant interaction between sex and BMI in anti-spike IgG titers (Yamamoto et al. 2022). Together, these data suggest that across diverse populations, males have lower antibody respones to vaccination and are also more vulnerable to intersecting factors, such as BMI and age, further decreasing antibody-mediated protection.

To our knowledge, a single study has investigated sex differences in vaccine-induced cell-mediated immunity after two doses of BNT162b2 (Kondo et al. 2022). In a small sub-sample (10 males and 10 females), Kondo et al. reported that post-vaccination, females have more SARS-CoV-2-specific $CD8^+$ T cells, memory

precursor effector cells ($CD8^+KLRG1^-CD127^+CD8^+$ T cells), and activated T cells ($CD8^+CD38^-HLA\text{-}DR^+$). Further, ex vivo stimulation of T cells from female participants leads to more antigen-specific $CD8^+$ T cells expressing CD107a (a marker cytotoxic potential), a greater intensity of CD107a staining, and greater secretion of IFNγ and TNFα than T cells isolated from male participants. The authors posit that this small study provides evidence that females mount stronger memory T cell responses after two doses of BNT162b2 than males. Given the importance of cell-mediated immunity in mRNA vaccine-mediated protection, more work is needed to better understand the implications of sex differences in this context.

4.2 Effectiveness

The large Phase III efficacy trials for the SARS-CoV-2 vaccines were not powered to detect sex differences (Polack et al. 2020; Baden et al. 2020; Sadoff et al. 2021; Falsey et al. 2021). With widespread use of the vaccines beginning in late 2020, however, several large studies have investigated whether there are sex differences in vaccine effectiveness. For example, in a cohort of over 2.5 million individuals in Scotland, male sex was associated with a greater risk of hospitalization or death from COVID-19 among vaccinated people (Agrawal et al. 2021). Similarly, in the United Kingdom, the reduction in the risk of infection associated with vaccination is greater in females than males (Menni et al. 2021). In a total population cohort study in Sweden, including individuals who received BNT162b2, mRNA-1273, AZD1222, or a combination of BNT162b2 and AZD1222, evidence emerged of higher vaccine effectiveness in females than males, particularly when time since vaccination was taken into account (Nordström et al. 2022). By 181 days post-vaccination, there is no detectable vaccine effectiveness in males, whereas vaccination remained efficacious in females (Nordström et al. 2022). In a follow-up study in July 2021–January 2022, when Delta and then Omicron were dominant variants, an interaction between sex, age, and comorbidity in the risk of severe COVID-19 among vaccinated individuals is reported (Kahn et al. 2022). Whereas for females aged 40–64 during the Delta-predominant period the risk of severe disease remains low regardless of the number of comorbid conditions, risk becomes elevated for males in the same age group who had ≥ 1 comorbidities. A similar trend is observed in the ≥ 65 age group, where the risk of severe disease in vaccinated males is approximately double that in females among those who had at least one comorbidity. Data also are consistent during the early Omicron-predominant period, albeit with a lower overall risk of severe disease. Together, like the immunogenicity data, vaccine effectiveness is found to be greater in females than males, and the factors that dampen effectiveness, such as the presence of comorbidities, have a larger effect in males than females.

4.3 Vaccine Reactogenicity

As with many other vaccines (Cook 2009), a stark sex difference in the rate of adverse events following SARS-CoV-2 immunization is reported. In the first month of vaccine use in the United States, 79% of the adverse events reported to the Vaccine Adverse Event Reporting System (VAERS) occurred in females. In a larger study in the United Kingdom, the proportion of people who experience either a local or a systemic adverse event is greater in females than males, regardless of vaccine type (BNT162b2 or the AZD1222) or dose number (Menni et al. 2021). A meta-analysis of four cross-sectional studies in Israel found that females report more local, systemic, and sensory adverse events than males across all age groups (Green et al. 2022). In addition, multiple studies report that both male sex and old age are associated with a lower rate of adverse events (Resta et al. 2021; Iguacel et al. 2021; Lee et al. 2021; Izumo et al. 2021). At the intersection of sex and age, among older adults, the rate of adverse events decreases with age in females only (Hoffmann et al. 2021). Sex differences in adverse events are often attributed to a gender bias, with women thought to be more likely to report adverse events than men (Cook 2009; Robb et al. 1996). When objective measures of adverse events, such as the presence of fever, are used following the SARS-CoV-2 vaccines, however, the female preponderance is maintained (Marchevsky et al. 2022). These data suggest that along with potential influences of gender, biological sex differences also contribute to the occurrence of adverse events.

In addition to local and systemic adverse events that were identified during clinical development (Polack et al. 2020; Baden et al. 2020; Sadoff et al. 2021; Falsey et al. 2021), several severe but extremely rare adverse events have received a tremendous amount of public attention. This includes anaphylaxis, myocarditis, and vaccine-induced immune thrombotic thrombocytopenia (VITT).

4.3.1 Anaphylaxis

The incidence of anaphylaxis after vaccination with an mRNA COVID-19 vaccine is greater in females than males. In the first month of vaccine administration in the United States (December 2021–January 2021), 94% of anaphylaxis cases associated with BNT162b2 and 100% of cases associated with mRNA-1273 were in females (Shimabukuro et al. 2021a). Similarly, of the first million vaccine doses administered in Japan, 70 of the 79 reported cases of anaphylaxis were in females (Somiya et al. 2021). While it was initially argued that the female bias may be explained by health-care workers, who are predominantly female, being prioritized for early vaccination, the trend continued throughout 2021 in Israel, where 78% of cases of anaphylaxis were in females (Anis et al. 2022). Convincingly, a systematic review of 26 articles found that female sex, along with a history of allergy, were the two most common risk factors for anaphylaxis (Alhumaid et al. 2021).

While the mechanism underlying the sex difference in anaphylaxis remains unknown, two main hypotheses have been proposed. First, the COVID-19 mRNA vaccines contain a polyethylene glycol (PEG)-conjugated lipid derivative, which is known to cause allergic reactions through both IgE- and non-IgE-mediated pathways (Laisuan 2021). Prior exposure to PEG may be more common in females due to exposure to PEG-containing products, such as contraceptive injections and certain cosmetic products, leading to hypersensitivity (Somiya et al. 2021). Lending credibility to this hypothesis is the finding that females are more likely to have serum anti-PEG IgM than males (Yang et al. 2016). Second, sex steroid hormones are known to modulate drug-induced anaphylactic reactions, which become more common in females after puberty (Norton and Broyles 2019). Treatment of female mice with an estrogen receptor antagonist or ovariectomy reduced the severity of anaphylaxis, while estradiol supplementation of ovariectomized animals restored the severity (Hox et al. 2015). Mechanistically, high doses of estrogen are known to promote helper T cell type 2 (Th2) responses through upregulation of nitric oxide production leading to the vascular permeability that promotes anaphylaxis and through stimulation of mast cell degranulation (Norton and Broyles 2019; Risma et al. 2021). In contrast, testosterone inhibits IgE production, promotes regulatory T cells which prevent allergic symptoms, and inhibits mast cell degranulation (Norton and Broyles 2019; Risma et al. 2021). Although more research is needed, it is likely that higher levels of exposure to PEG prior to vaccination coupled with a hormonal environment that promotes allergic reactions have led to the higher prevalence of anaphylaxis following COVID-19 vaccination in females.

4.3.2 Myocarditis

Myocarditis, the inflammation of the myocardium that can cause acute heart failure in adults and children, has been reported following administration of the COVID-19 mRNA vaccines. Of the first 300 million doses administered in the United States, 1226 cases of myocarditis or pericarditis were identified, with the vast majority occurring in males (79%) and in younger individuals (median age of 24) (Bozkurt et al. 2021). It has been estimated that for every million vaccine doses administered to males 12–29 years of age, 11,000 cases of COVID-19, 560 hospitalizations, 138 ICU admissions, and 6 deaths are prevented, while 39–47 cases of myocarditis may occur (Gargano et al. 2021). The benefits of vaccination greatly outweigh the risks, even in younger males, but a better understanding of the mechanisms mediating the sex difference in myocarditis may contribute to lowering the risk of severe adverse events following vaccination.

Bozkurt et al. proposed three mechanisms through which mRNA vaccination may cause myocarditis: (1) The mRNA itself is immunostimulatory, and in some cases (e.g., potentially in those with certain genetic predispositions), the mRNA may be recognized as an antigen and initiate a pro-inflammatory cascade that causes myocarditis; (2) the vaccine may stimulate the production of heart-reactive auto-antibodies, which have previously been implicated in the pathogenesis of

myocarditis; and (3) molecular mimicry between the SARS-COV-2 spike glycoprotein and self-antigens, such as α-myosin, may lead to the production of cross-reactive antibodies that can stimulate pre-existing dysregulated pathways in predisposed individuals, resulting in polyclonal B cell expansion, the formation of immune complexes, and inflammation (Bozkurt et al. 2021). Most data on potential mechanisms for the sex difference in myocarditis, however, come from pre-pandemic research on viral infection-induced myocarditis, where a male predominance also is reported (Fairweather et al. 2013; Fung et al. 2016). In a mouse model of coxsackievirus B3-induced myocarditis, males develop a pro-inflammatory helper T cell type 1 (Th1)-skewed response characterized by IgG2A production and T cell secretion of IFNγ and IL-2, while females mount a Th2-skewed response characterized by IgG1 production and T cell secretion of IL-4 and IL-5 (Huber and Pfaeffle 1994). In this model, treatment of males with estradiol and of females with testosterone alters Th-skewing, pointing to a fundamental role of sex steroid hormones in mediating this sex difference (Huber and Pfaeffle 1994). Further, adoptive transfer of female T cells to male mice suppresses the development of myocarditis (Huber and Pfaeffle 1994). Together, these data suggest that regardless of how the COVID-19 mRNA vaccines may initiate a response that leads to myocarditis in certain predisposed individuals, sex steroid hormones likely exacerbate this response in males, resulting in greater incidence of post-vaccination myocarditis.

4.3.3 Vaccine-Induced Immune Thrombotic Thrombocytopenia

In the spring of 2021, several case series were published identifying cerebral venous sinus thrombosis (CVST) in individuals, predominantly females, who had been vaccinated with either of the adonovirus vector-based vaccines (AZD1222 or Ad26.COV2.S) (See et al. 2021; Schultz et al. 2021; Greinacher et al. 2021a; Scully et al. 2021; Wolf et al. 2021; Tiede et al. 2021). Later renamed vaccine-induced immune thrombotic thrombocytopenia (VITT), several systematic reviews also found that female sex and younger age (< 60 years) are risk factors (Sharifian-Dorche et al. 2021; Rizk et al. 2021; Jaiswal et al. 2022; Palaiodimou et al. 2021). One study comparing cases of CVST identified from 2015–2018 to those identified within 28 days of vaccination between March and June of 2021 found that females acount for the majority of cases in both the post-vaccination and control groups (81 and 70%, respectively) (Kammen et al. 2021). A larger study of 294 cases of VITT in Germany, however, did not find a female bias (Pavord et al. 2021), with several authors commenting that the early female predominance could be explained by females being vaccinated earlier than males (Krzywicka et al. 2021; Epidemiology of VITT 2022). Despite conflicting epidemiological data, the higher rate of thrombotic events in females both pre-pandemic and post-vaccination supports a role for biological sex in the occurrence of VITT.

While the precise mechanism of VITT remains largely unknown, it is thought to follow a similar pathway as heparin-induced thrombocytopenia, which is also more common in females (Warkentin et al. 2006). Briefly, components of the vaccine may

form complexes with platelet factor 4 (PF4) causing neo-antigen exposure and stimulating a B cell response, which is further amplified by the vaccine-induced inflammatory environment, that results in the production of anti-PF4 antibodies (Greinacher et al. 2021b). These anti-PF4 antibodies then activate platelets and granulocytes to produce neutrophil extracellular traps (NETs), which promote coagulation and are characteristic of CVST (Greinacher et al. 2021b). Pre-pandemic research suggests that gender-specific risk factors, such as oral contraceptives, pregnancy, and hormonal replacement therapy, are present in approximately 65% of women who are diagnosed with CVST (Coutinho et al. 2009). It is currently unclear whether the same risk factors predispose women to VITT, or if other mechanisms may contribute, and more research is needed to better understand the epidemiology of this rare adverse event.

5 Pregnancy and COVID-19 Vaccines

Vaccination during pregnancy has several unique considerations regarding safety and efficacy. As discussed above, pregnant people are at greater risk of severe COVID-19 than non-pregnant people, and vaccination can be a tool to protect them and their fetus from adverse consequence of SARS-CoV-2 infection. The safety of medical interventions in pregnant people must be considered for both mother and fetus. Pregnancy also induces changes to the immune system that could in theory alter the efficacy of vaccination or the safety of live vaccines. Pregnant females were not included in the initial trials of the COVID-19 vaccines (Klein et al. 2021).

There is now substantial literature emerging for the mRNA-based vaccines produced by Pfizer (BNT162b2) and Moderna (mRNA-1273) and AstraZeneca's adenovirus-vector-based vaccine (AZD1222) during pregnancy (Hernández et al. 2021), but the immunization guidelines for pregnant people differ from country to country. Development and reproductive toxicology (DART) studies are performed on most therapeutics and are designed to detect adverse effects within a complete reproductive cycle. They are generally performed in rodents (guidelines have been published by the FDA, https://www.fda.gov/media/148475/download). Preliminary DART studies have been performed for all 3 vaccines (Table 2) (Hernández et al. 2021; Stebbings et al. 2021). Various systemic effects are reported including elevated body temperature and increased cytokine production. The mRNA vaccine studies administered the full human dose, while half the human dose of AZD1222 was administered. Overall, the results of the DART data for these COVID-19 vaccines demonstrate no detrimental maternal or fetal adverse outcomes. Following confirmation that COVID-19 vaccines showed no detrimental preclinical DART effects (Table 2), they were rapidly deployed for trial in the public. In the pregnant population, their safety is continually being examined through various studies and self-reporting platforms, and many studies have now reported on their use and safety. One multinational study reported pregnancy outcomes after preconception AZD1222 inoculation (Hillson et al. 2021). Several articles investigated the effect of BNT162b2

or mRNA-1273 vaccines on adverse maternal pregnancy outcomes (Rottenstreich et al. 2022; Bookstein Peretz et al. 2021; Shimabukuro et al. 2021b; Gray et al. 2019; Theiler et al. 2021). Shimabukuro et al. included data on mRNA vaccines from two voluntary surveillance programs, V-safe and VAERS (Shimabukuro et al. 2021b). Magnus et al. (Magnus et al. 2021) investigating BNT162b2, mRNA-1273, and AZD1222 distinguished between vaccine and outcomes, while another study did not (Blakeway et al. 2022). None of the studies demonstrate an increased risk of spontaneous abortion, defined as pregnancy loss before 20 weeks of gestation without medical intervention, in recipients. Similarly, adverse maternal outcomes, including cesarean delivery, preterm delivery, stillbirth, or hypertensive disorders, are not affected by any of these vaccinations. No adverse neonatal outcomes, such as neonatal intensive care unit (NICU) admission, small for gestational age, congenital abnormalities, or neonatal death, are increased in any of the vaccinated groups.

Several clinical trials were started to establish the efficacy of the vaccines in pregnant women (ClinicalTrials.gov identifiers: NCT04754594; NCT04958304; NCT04877743). NCT04754594 that examines the efficacy of BNT16b2 is now complete; all the data have not yet been reported. Completion of NCT04958304 that examines mRNA-1273 is expected in January 2024. NCT04877743 that looked at AZD1222 has been terminated. Even in the absence of the reports from these clinical trials, some publications have already reported beneficial effects of vaccinations. One study showed that mRNA vaccination induced significantly greater titers of antibodies compared to SARS-CoV-2 infection during pregnancy (Gray et al. 2019). Another study, however, reports lower IgG titers in the vaccinated than naturally infected women (Collier et al. 2021). Variable sample collection time and declining immunity may explain this discrepancy. mRNA vaccines have similar immunogenicity in pregnant females compared to non-pregnant females, with equivalent antibody titers (Gray et al. 2019). Vaccinated pregnant females are significantly less likely than unvaccinated pregnant females to contract SARS-CoV-2 before delivery (Theiler et al. 2021). These studies suggest that the vaccines are effective in preventing COVID-19 during pregnancy. COVID-19 vaccines also have the potential to confer passive immunity, as research shows IgG can be transferred to the fetus in utero (Prabhu et al. 2021) and IgG and IgA can be transferred via breast milk (Collier et al. 2021).

Overall, evidence from preclinical and clinical research suggests that COVID-19 vaccination has a negligible impact on adverse pregnancy outcomes. This contrasts with SARS-CoV-2 infection during pregnancy which has significant adverse effects on pregnancy (as discussed above). The risk of adverse long-term neonatal outcomes is unknown, but it is less likely if we extrapolate evidence from vaccines in use for other diseases. This should not be interpreted as opprobrium of vaccines, but rather an opportunity to continue preclinical research and commence long-term epidemiological investigations of COVID-19 vaccines which will provide more thorough reassurance on their safety during pregnancy. The weight of evidence supports that COVID-19 vaccination during pregnancy is safe and beneficial in this vulnerable population when based on individual risk.

Table 2 Primary results of preclinical toxicology studies for selected COVID-19 vaccines[a]

COVID-19 vaccine	BNT16252	MRNA-1273	AZD1222
Animal model	Wistar rats	Sprague Dawley rats	CD-1 mice (Baristaite and Gurwitz 2022)
GLP compliant studies	Not reported	Non-GLP compliant	GLP compliant (using 2 platforms: ChAdOx1 and AdCh63)
Side effects	**Increased**: • Acute phase proteins • Fibrinogen	**Increased**: • Body temperature • Globulin • Fibrinogen • Cytokines	**Increased**: • Body temperature • Globulin
	Decreased: • Albumin-globulin ratio • Hematological parameters	**Decreased**: • Albumin • Albumin-globulin ratio	**Decreased**: • Albumin
Dose administered	30 μg RNA/dose (4 human doses) (Gedeborg et al. 2021)[b]	100 μg RNA/dose (4 human doses) (Schmidt et al. 2021)	2.59×10^{10} viral particles (2 doses) The dose given to mice was half the human dose (Qiao et al. 2020)
DART-EFD	No known direct or indirect adverse effects on pregnancy or embryo, fetal, or postnatal development	No adverse effects on embryo, fetal, postnatal development No dose administered during early organogenesis	No adverse effects observed on pup survival, no abnormal gross pathology, visceral or skeletal findings The definitive study is ongoing in mice[c]
Source	European Commission summary of product characteristics: Comirnaty. Available from: https://ec.europa.eu/health/documents/community-register/2020/20201221150522/anx_150522_en.pdf. (Accessed October 7, 2021)	The American College of Obstetricians and gynecologists COVID-19 vaccines and pregnancy. (Technical document). Available from: https://www.izsummitpartners.org/content/uploads/covid19-vaccines-and-pregnancy.pdf. (Accessed October 7, 2021)	European Medicines Agency. Committee for Medicinal Products for Human Use. Assessment report—COVID-19 vaccine AstraZeneca. Available from: https://www.ema.europa.eu/en/documents/assessment-report/vaxzevria-previously-covid-19-vaccine-astrazeneca-epar-public-assessment-report_en.pdf. (Accessed October 7, 2021)

[a] Adapted from Hernández et al. (2021) and augmented from other sources where available as referenced

[b] If adjusted for body weight, the clinical dose of BNT16262 is over 300 times the human dose in rat models

[c] With the exception of common skeletal variations which routinely resolve without intervention

DART-EFD Developmental and reproductive toxicity-Embryo-fetal development

GLP Good Laboratory Practice

6 Conclusions and Future Directions

The available evidence demonstrates that males are disproportionately affected by SARS-CoV-2, with increased disease severity and poorer outcomes compared with females. The reasons for this are multi-factorial, including physiological, immunological (driven both by hormones and chromosomal dependent mechanisms) as well as behavioral aspects. The logical application of this information is to recognize males as a susceptible group and work to develop improved and even specific male-focused treatment regimens. Fortunately, the benefits of vaccination are clear in both sexes. Curiously, although many countries have prioritized vaccination programs by age, profession, and/or comorbidities, the literature does not suggest that any of the programs have been prioritized by sex. Pregnancy represents a special state in females and is associated with worse outcomes relative to non-pregnant females following infection. However, available evidence supports that vaccines are safe and afford a degree of protection in pregnant people consistent with what is observed in the general population. Clinical trials currently underway should provide stronger data, especially regarding the relative efficacies of each vaccine in pregnant females. The enormous scale of COVID-19 vaccine roll out throughout the world provides an unprecedented opportunity for recruitment, collation of recipients and collection of data regarding long-term safety and efficacy of vaccines for adults and their offspring.

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Biological Sex and Pregnancy Affect Influenza Pathogenesis and Vaccination

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Abstract Males and females differ in the outcome of influenza A virus (IAV) infections, which depends significantly on age. During seasonal influenza epidemics, young children (< 5 years of age) and aged adults (65+ years of age) are at greatest risk for severe disease, and among these age groups, males tend to suffer a worse outcome from IAV infection than females. Following infection with pandemic strains of IAVs, females of reproductive ages (i.e., 15–49 years of age) experience a worse outcome than their male counterparts. Although females of reproductive ages experience worse outcomes from IAV infection, females typically have greater immune responses to influenza vaccination as compared with males. Among females of reproductive ages, pregnancy is one factor linked to an increased risk of severe outcome of influenza. Small animal models of influenza virus infection and vaccination illustrate that immune responses and repair of damaged tissue following IAV infection also differ between the sexes and impact the outcome of infection. There is growing evidence that sex steroid hormones, including estrogens, progesterone, and testosterone, directly impact immune responses during IAV infection and vaccination. Greater consideration of the combined effects of sex and age as biological variables in epidemiological, clinical, and animal studies of influenza pathogenesis is needed.

1 Introduction

Influenza is a significant public health threat, with influenza A viruses (IAVs) causing seasonal epidemics, occasional outbreaks, and sporadic pandemics. Pulmonary disease following infection with IAV is caused by excessive and aberrant inflammatory responses to the virus, which leads to immunopathology and tissue damage (Peiris et al. 2010). Effective therapies for limiting severe pulmonary disease

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S. L. Klein and C. W. Roberts (eds.), *Sex and Gender Differences in Infection and Treatments for Infectious Diseases*, Current Topics in Microbiology and Immunology 441, https://doi.org/10.1007/978-3-031-35139-6_5

following IAV infection often limit the 'cytokine storm' and pulmonary inflammation (Cloutier et al. 2012; Walsh et al. 2011), in addition to controlling viral replication. The severity of influenza disease is typically worse for young children, aged adults, individuals with compromised immune function, and pregnant women. Rarely are biological sex or gender-related factors considered when assessing the pathogenesis of influenza. In this chapter, we aim to illustrate that in humans, the influenza virus strain, age, sex, and reproductive status of an individual contribute to the outcome of IAV infection, but rarely are these viral and host factors considered together in either epidemiological or clinical studies. Furthermore, age, biological sex, and reproductive status are rarely reported or systematically evaluated in studies utilizing animal models. Despite limited data, the available evidence indicates that host factors contribute significantly to influenza pathogenesis and vaccine-induced immunity through multiple pathways (Fig. 1).

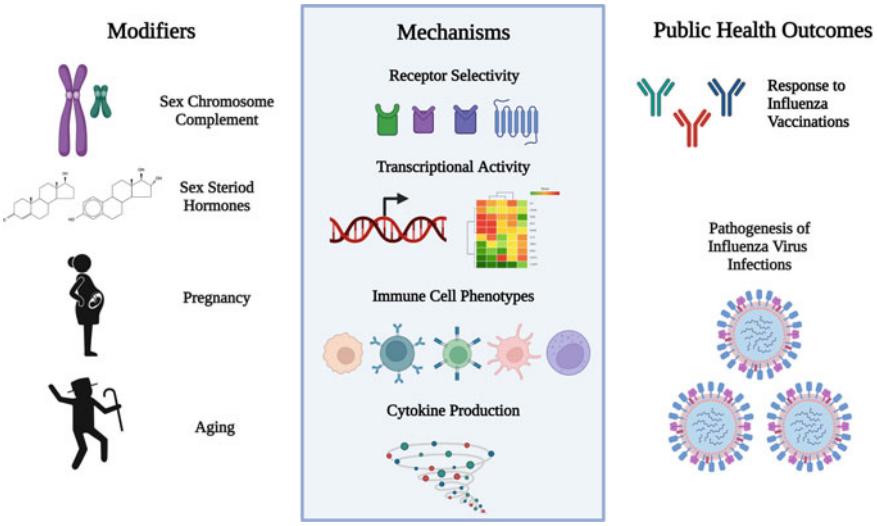

Fig. 1 Biological sex and pregnancy modify immune responses to influenza virus infection and vaccination though multiple mechanisms. Outcomes after influenza virus infection and vaccination are affected by diverse host factor modifiers, such as biological sex, which includes sex chromosome complement, gonadal sex characteristics, and concentrations of sex steroid hormones. Concentrations of sex steroid hormones change dramatically with age and reproductive status (e.g., pregnancy), which can in turn modify influenza outcomes. Host biological factors impact immunity through multiple pathways, including modifying receptor usage and selectivity as well as cellular transcriptional activity to impact downstream immune cell phenotypes and the production of cytokines and antibodies, depending on the cell type affected. As documented in this chapter, the impact of sex differential gene expression and hormonal activity in immune cells results in sex, age, and pregnancy-specific outcomes of influenza virus infection and vaccination

2 Sex Differences in Influenza Pathogenesis

2.1 Seasonal Influenza Epidemics

Because of the transient nature of influenza symptoms in most individuals, most influenza cases are unreported at hospital, national, and continental levels; for the cases that are reported, sex- and age-specific data are sparse. Extracting observations from the limited available data reveals that cases of more severe infection that require hospitalization are greater in prepubertal and older aged males compared with age-matched females (World Health Organization 2020). For seasonal epidemics around the world, most of the reported cases have been of influenza A viruses (IAVs) (H1N1 and H3N2), although influenza B viruses (IBVs) also circulate annually (World Health Organization 2020). There are a few countries that are particularly responsive to analyzing annual and pandemic influenza data through a 'gender lens,' meaning that reported and published clinical and epidemiological data are disaggregated by sex.

In Australia, both IAV and IBV cases requiring hospitalization are greater among males than females prior to puberty and at later decades of life, whereas greater numbers of cases are observed in females than males for ages 20 to 54 years (i.e., reproductive year) (Grant et al. 2018; Wong et al. 2019). Similar observations are reported in Denmark, in which differences between males and females in the risk of hospitalization from seasonal influenza virus shift at puberty. Males are more likely to have severe seasonal influenza illness before puberty, whereas females are more likely to have severe seasonal influenza illness after puberty and before menopause (Jensen-Fangel et al. 2004). In China, pneumonia and influenza death rates are elevated with older age, which is apparent among males aged 65–74 years but not among females until ages $\geq$ 75 years and older (Yu et al. 2015). Similar observations also are reported in Hong Kong, in which among children and older adults, males experience more severe disease (i.e., are hospitalized) than age-matched females not only for IAV but IBV as well (Wang et al. 2015). Taken together, these data illustrate that across diverse countries, social norms, and economies, sex and age differences in the severity of influenza are reported, implicated a shared biological mechanism.

2.2 Outbreaks of Avian Influenza

Avian H5N1 is a highly pathogenic IAV that affects the lower respiratory tract in humans and is primarily transmitted from diseased poultry to humans, with rare person-to-person transmission. Worldwide, the incidence and severity of H5N1 infection and mortality caused by H5N1 infection has been greater among young adult females (10–39 years of age) than males (WHO 2012). There is annual, as well as country, variation in the male–female differences suggesting that gender-related

factors, including occupational exposure, play a significant role. Since the March 2013 outbreak of avian H7N9 influenza, primarily in China with one confirmed case in Malaysia (Xiang et al. 2017, 2016), there have been a total of five epidemics resulting in 1564 confirmed cases up to 2017, with a positive rate of 3.84 and a fatality rate of about 40% (Xiang et al. 2017, 2016; WHO 2022). In this recent 2017 outbreak, 379 cases were laboratory confirmed, all reported cases were severe and required hospitalization, with the median age of 53 years, 52% of the infection resulting in death, and a male-to-female ratio of 2.86 (Wang et al. 2019). The female-biased severity of H5N1 and male-biased severity of H7N9 suggest that age and gender-related variables associated with exposure are likely involved.

2.3 Influenza A Virus Pandemics

The 1918 H1N1 influenza pandemic was the most deadly influenza pandemic to date (Noymer and Garenne 2000). This influenza pandemic was disproportionately fatal in young adult males (i.e., 20–40 years of age) and was exacerbated by co-infection with tuberculosis, which is also considered to be a male-dominant disease (Nhamoyebonde and Leslie 2014; Noymer 2009). Unlike the 1918 H1N1 pandemic, the 1957 H2N2 pandemic was the first pandemic that was lethal without a secondary bacterial infection. The 1957 H2N2 pandemic resulted in higher fatality rates among adult females than males (i.e., < 50 years of age), despite the widespread use of vaccine therapy (Serfling et al. 1967; Kilbourne 2006). Many of the fatal cases during the H2N2 pandemic had underlying cardiac or pulmonary conditions; thus, sex-biased comorbid conditions may have contributed to the increased rates of severe disease and mortality among young adult females during the 1957 H2N2 pandemic (Kilbourne 2006).

During the 2009 H1N1 pandemic in the USA, females were more likely to develop severe disease than males (53.2% female vs. 46.8% male hospitalizations) (Klein et al. 2010a). Age at the time of infection, however, was a strong predictor of the male–female differences in the incidence and severity of 2009 H1N1 pandemic infection and mortality rates. Among adults, females were at a higher risk of hospitalization and death from 2009 H1N1 infection than males (Jacobs et al. 2012). In Japan, a similar female bias was observed in clinical disease following infection with 2009 H1N1 influenza among adults of reproductive ages, which contrasted with the male bias observed prior to puberty (Eshima et al. 2011). In Canada, during the first wave of the pandemic, a majority of critically ill patients with confirmed or probable 2009 H1N1 influenza were young adult females (Kumar et al. 2009). The reason for the greater proportion of hospitalized adult females than males in Canada is not known, but many cases involved comorbid conditions, including chronic lung disease (e.g., asthma), which is typically more severe in young adult females than males (Townsend et al. 2012).

2.4 Animal Models of Sex Differences in Influenza Pathogenesis

Small animal models are instrumental for uncovering mechanisms mediating sex differences in influenza pathogenesis (Robinson et al. 2011a, b; Larcombe et al. 2011; Nguyen et al. 2011; Hoffmann et al. 2015; Tuku et al. 2020). Using different strains mice and IAVs, adult females suffer more severe disease than males (Table 1). Infection of adult C57BL/6 mice with either H1N1 (i.e., A/Puerto Rico/8/34 or mouse-adapted A/California/04/09) or H3N2 (i.e., A/Hong Kong/68) results in increased disease severity in female compared to male mice, as measured by morbidity (i.e., body mass loss and hypothermia) and mortality at lower virus dilutions (Robinson et al. 2011a, b; Avitsur et al. 2011; Lorenzo et al. 2011; Vermillion et al. 2018a, b). Infection of BALB/c mice with some (i.e., A/Hamburg/NY1580/09), but not all (i.e., A/Puerto Rico/8/34), H1N1 strains causes greater disease severity in female compared to male mice (Celestino et al. 2018). In general, female mice experience greater pulmonary inflammation during the acute phase of infection, increased production of proinflammatory cytokines (e.g., IL-6, TNF-α, CCL2), and increased cellular infiltration followed by decreased tissue recovery post infection, despite similar viral clearance from the lungs (Robinson et al. 2011b; Larcombe et al. 2011; Vermillion et al. 2018b). Infection of C57BL/6 mice with mouse-adapted A/California/04/09 induces more robust antibody responses, CD4+ T cell, and CD8+ T cell memory responses in adult females compared to males (Fink et al. 2018). Repair of the damaged lung tissue following IAV infection is generally orchestrated by both immune cells (e.g., regulatory T cells and macrophages) and epithelial cells and involves the production of cytokines and growth factors (Sun et al. 2009; Tate et al. 2011). In response to damage, epithelial cells release factors, including amphiregulin (AREG), that can promote repair and integrity of lung tissue damaged during IAV infection (Monticelli et al. 2011). Expression of AREG is greater in lung tissue as well as in respiratory epithelial cells derived from males as compared with females during IAV (i.e., mouse-adapted A/California/04/09) (Vermillion et al. 2018b). Males also depend on AREG more than females for faster recovery from IAV, because when AREG is deleted from mice, the impact on pulmonary inflammation and function is significantly greater for male than female mice (Vermillion et al. 2018b). Infection of adult female mice with IAVs reduces ovarian function and concentrations of sex hormones (Robinson et al. 2011b; Vermillion et al. 2018a) suggesting that inhibition of sex hormones, including estrogens and progesterone (P4), may contribute to severe outcomes from IAV in females (see below).

Table 1 Mouse models of sex differences in influenza virus pathogenesis and vaccination

Mouse strain	Virus	Pathogenesis	Vaccination
C57BL/6	A/Puerto Rico/8/1934 (H1N1)	M < F (Avitsur et al. 2011; Lorenzo et al. 2011; Robinson et al. 2011a; Robinson et al. 2011b) Gdx_F < Gdx_F + E2 (Robinson et al. 2011b; Robinson et al. 2014)	
	Mouse-adapted A/California/04/09 (H1N1)	M < F (Vermillion et al. 2018b)	F > M (Fink et al. 2018)
	A/Hong Kong/68 (H3N2)	M < F (Lorenzo et al. 2011)	
BALB/c	A/Memphis/1/71 (H3N2)	M < F (Larcombe et al. 2011)	
	A/Puerto Rico/8/1934 (H1N1)	M > F (Celestino et al. 2018)	
	A/Anhui/1/13 (H7N9)	M < F (Hoffmann et al. 2015)	
	A/Hamburg/NY1580/09 (H1N1)	M < F (Hoffmann et al. 2015; Tuku at al. 2020)	
	A/Aichi/2/68 (H3N2)	M < F (Hoffmann et al. 2015)	
	A/Perth/16/2009 (H3N2)		F > M (Arsenović-Ranin et al. 2019)

*Gdx_F: gonadectomized female mice; Gdx_F + E2: gonadectomized female mice given exogenous estradiol

2.5 *Role of Sex Steroids in Modulating Sex Differences in Influenza Pathogenesis*

Estrogens

Estrogens, including 17β-estradiol (E2), occur in higher concentrations in nonpregnant and pregnant adult females than males. E2 is responsible for most of the classical estrogenic effects in both reproductive and non-reproductive tissues. Both cytosolic and membrane-bound estrogen receptors (ERs) are identified in innate and adaptive immune cells (Kovats 2015) (Fig. 2). E2 can have positive or negative regulatory effects on the production of proinflammatory cytokines depending on the cell type, E2 dose, and ER subtype utilized (Robinson et al. 2011b; Kovats 2015; Graham et al. 2020; Fuentes and Silveyra 2019). Physiologic levels of E2 promote the production of proinflammatory cytokines (e.g., IL-1, IL-6, and TNF-α) in response to toll-like receptor ligand stimulation of DCs and macrophages (Laffont et al. 2017).

Conversely, high concentrations of E2, as occurs during pregnancy, promote anti-inflammatory responses (Robinson and Klein 2012). Dose-dependent effects of E2 also result in low E2 concentrations promoting helper T cell type 1 (Th1) responses and cell-mediated immunity and high concentrations of E2 augmenting helper T cell type 2 (Th2) responses and humoral immunity (Straub 2007).

Murine models of IAV pathogenesis illustrate that E2 treatment of females protects against infection-induced morbidity and mortality (Robinson et al. 2011b; Robinson et al. 2014; Pazos et al. 2012). E2 treatment of gonadectomized females reduces pulmonary production of CCL2, a chemokine involved in migration of T cells, and increases production of CCL3 and CXCL1, which are involved in neutrophil recruitment (Robinson et al. 2014). E2 treatment also inhibits pulmonary IFN-γ and TNF-α

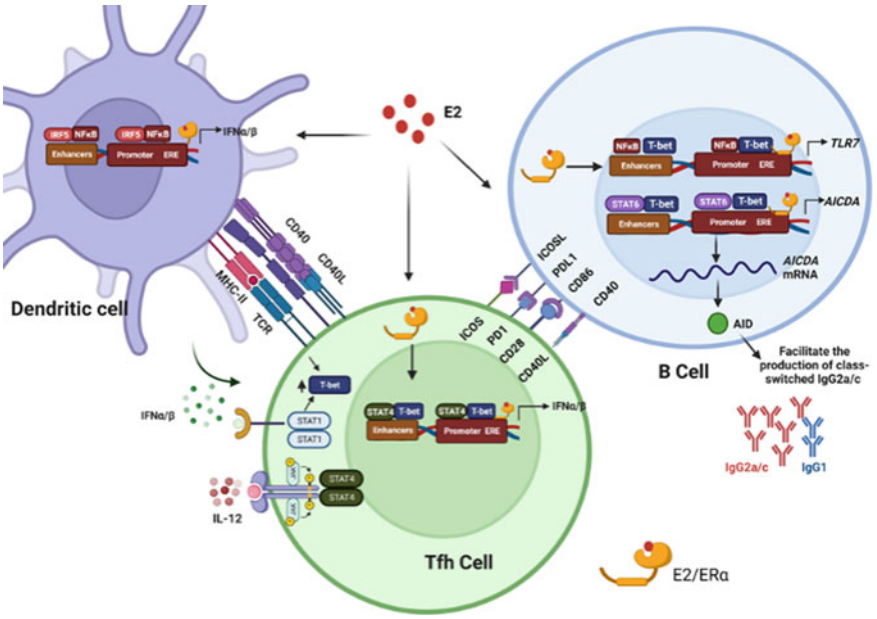

Fig. 2 Sex steroids, including 17β-estradiol (E2), signal through hormone receptors in various immune cell types to cause sex differences in immunity to viruses and vaccines. Estrogens, including 17β-estradiol (E2), transcriptional regulate the activity of innate and adaptive immune cells. Several immune cells, including but not limited to plasmacytoid dendritic cells (pDCs), T cells, and B cells, have intracellular estrogen receptors in the cytoplasm. Estrogen receptor alpha (ERα) is a dominate receptor in immune cells and when E2 binds to ERα, the steroid receptor complex can translocate to the nucleus of these cells to bind to estrogen response elements (ERE) typically in the promotors of responsive genes, resulting in either activation or repression of gene activity. E2 regulates the production of type I interferons (IFNs) via E2/ERα binding to IRF5 and NFκB, the prototypical antiviral transcription factor, in pDCs and via E2/ERα binding to STAT4 and T-bet in helper T cells, including follicular helper T cells (Tfh). In B cells, E2/ERα signaling regulates the transcription of *TLR7* and *AICDA* to facilitate class switch recombination and somatic hypermutation, resulting in greater affinity and diversity of antibody in female compared with male mice

production (Pazos et al. 2012), while increasing cytokine production by virus-specific CD8+ T cells (Robinson et al. 2014). The anti-inflammatory effects of E2 on protection against severe disease are mediated by signaling through ERα and not ERβ (Robinson et al. 2011b). Some (Pazos et al. 2012), but not all (Robinson et al. 2011b; Robinson et al. 2014), studies suggest that E2 treatment decreases type I IFN production and reduces IAV replication in the lungs. Neutrophil depletion in E2-treated females increased morbidity, reduced pulmonary production of chemoattractants for neutrophils, and reduced IFN-γ production by virus-specific CD8 T cells (Robinson et al. 2011b). Neutrophils mediate both inflammation and tissue repair during IAV infection and are regulated by E2 to improve the outcome of influenza in females.

Estriol, a 16α-hydroxylated metabolite of E2 (E3), is produced by the placenta and occurs in high concentrations during pregnancy (Longcope 1984; Zhou et al. 2022). Unlike E2, E3 exhibits weak binding affinity for ERα and greater binding affinity for ERβ (Kuiper et al. 1997). Gonadally intact adult female mice administered E3 after IAV infection have reduced pulmonary inflammation and morbidity as compared to females that received placebo, suggesting that like E2, E3 dampens inflammation and protects against severe influenza (Vermillion et al. 2018a).

Progesterone

Natural P4 is produced by the corpus luteum during the menstrual cycle in nonpregnant females, and its production is sustained at high levels by the placenta during pregnancy (Cable et al. 2022). P4 primarily signals through the progesterone receptor (PR), but can also signal through other steroid receptors including glucocorticoid, mineralocorticoid, and androgen receptors (Hapgood et al. 2014). Progesterone affects the activity of many immune cells, including monocytes, macrophages, dendritic cells, T cells, and B cells (Dressing et al. 2011). In the presence of P4, macrophages and DCs have a lower state of activation, produce greater concentrations of anti-inflammatory cytokines (e.g., IL-10), produce lower concentrations of proinflammatory cytokines (e.g. IL-1β and TNF-α), and decrease nitric oxide production (Butts et al. 2007; Jones et al. 2010; Kyurkchiev et al. 2007; Su et al. 2009). P4 dampens overall CD4+ T cell activation and skews naïve T cells toward a Th2 phenotype (Hellberg et al. 2021; Miyaura and Iwata 2002). As evidence for the regulatory role of P4, the proportion of regulatory T cells (Tregs) increases in response to P4 as well as during the second and third trimesters of human and rodent pregnancies (Lee et al. 2011; Mao et al. 2010; Saito et al. 2010).

Physiological concentrations of P4 protect against both lethal and sub-lethal IAV infection by promoting faster repair of damaged lungs tissue, without altering the viral load. Ovariectomized mice administered exogenous P4 have increased production of tissue repair cytokines, including TGF-β, IL-6, and IL-22, increased numbers of CD39+ Th17 cells, and increased cellular proliferation (Hall et al. 2016). Production of the epidermal growth factor AREG also is increased following P4 treatment, which promotes proliferation and repair of respiratory epithelial cells following IAV infection (Hall et al. 2016). Treatment with either P4 or the synthetic progestin, levonorgestrel also reduces IAV-specific antibody titers as well as IAV-specific memory CD8+ T cells numbers, which results in worse outcome following secondary

IAV challenge in female mice (Hall et al. 2017). While the anti-inflammatory effects of P4-based compounds protect against a primary virus infection, the reduction in memory T cell responses increase susceptibility to secondary IAV challenge. Because P4-based compounds reduce inflammation and expedite repair of lung tissue following primary IAV infection, this may make females less susceptible to secondary bacterial infections, which are the primary cause of mortality following IAV infection in humans.

Androgens

Androgens, including testosterone and dihydrotestosterone (DHT), are secreted from Leydig cells in the testes. Androgens occur in higher concentrations in post pubertal males than females, although circulating testosterone declines with age in both male mice and humans (Potluri et al. 2019; vom Steeg et al. 2016). Androgens signal through androgen receptors (ARs), but if aromatized into E2 also can signal through ER (Davey and Grossmann 2016). AR expression has been detected in various immune cell types, including neutrophils, mast cells, macrophages, B cells, and T cells (Chen et al. 2010; Mantalaris et al. 2001; Viselli et al. 1997). AR signaling in immune cells is generally immunosuppressive (vom Steeg and Klein 2017). Exposure to either testosterone or DHT reduces the production of proinflammatory cytokines, e.g., TNFα, and increases production of anti-inflammatory cytokines, e.g., IL-10 and TGFβ (D'Agostino et al. 1999; Gold et al. 2008; Liva and Voskuhl 2001). Human males with androgen deficiencies have higher concentrations of inflammatory cytokines (e.g., IL-1β, IL-2, TNF-α, and CCL3), leptin, antibody titers, and CD4+:CD8+ T cell ratios as compared to healthy males (Bobjer et al. 2013; Kalinchenko et al. 2010; Kocar et al. 2000; Malkin et al. 2004; Musabak et al. 2003).

The anti-inflammatory environment caused by testosterone protects against sub-lethal IAV morbidity without reducing peak viral titers (vom Steeg et al. 2016), suggesting that the effects are immunomodulatory and involve dampening of inflammation. Males with physiological levels of testosterone as well as castrated males treated with exogenous testosterone have reduced pulmonary pathology and morbidity after sub-lethal IAV infection as compared to either castrated adult males that have surgically reduced concentrations of testosterone or aged males that have reduced naturally occurring concentrations of testosterone (vom Steeg et al. 2016). Testosterone in male mice improves the outcome of IAV infection not by mitigating global pulmonary cytokine production, but by promoting the contraction of pulmonary inflammatory monocytes during peak disease and the frequencies of IAV-specific pulmonary CD8+ T cells and eosinophils in the lungs following control of viral replication (vom Steeg et al. 2020). The protective effects of testosterone on IAV pathogenesis are dependent on AR signaling, which creates a pulmonary environment conducive to reduced pulmonary inflammation. Rather than acting directly on a single cell population, AR signaling has multicellular effects and creates a local environment that promotes accelerated contraction of inflammatory immune cells (vom Steeg et al. 2020). Activation of AR signaling confers protection during IAV

infection by modulating the immune response, which may have therapeutic potential in both male and female patients.

3 Sex Differences in Influenza Vaccine Outcomes

3.1 Human Clinical Data

Of the few human vaccine trials that have considered sex differences in vaccine outcomes, in adults of reproductive ages (18–49 years), females have greater antibody titers (i.e., either neutralizing or hemagglutination inhibition (HAI) titers) than males following receipt of the seasonal influenza vaccine (Potluri et al. 2019; Engler et al. 2008; Furman et al. 2014; Klein and Pekosz 2014). The sex difference is influenced by both age and pre-existing immunity to selected IAV and IBV strain (Furman et al. 2014; Shapiro et al. 2021). During the 2008–2009 influenza season, adult females produced more neutralizing antibodies against the H3N2 and IBV vaccine virus strains, but not the H1N1 vaccine virus strain (Furman et al. 2014). During the 2017–2018 influenza season, adult females mounted more robust neutralizing responses to vaccine H3N2 compared to males (Ursin et al. 2020). In general, sex differences in antibody responses to influenza vaccine viruses are reduced with age (Potluri et al. 2019). Among individual 65+ years of age, antibody titers to both the standard and high dose seasonal influenza vaccine are still often greater in females than males (Falsey et al. 2009), with greater season to season durability of immunity against influenza among older females than males (Shapiro et al. 2021). Both younger age and greater concentrations of circulating E2 are associated with greater monovalent 2009 H1N1 vaccine-induced immunity (Potluri et al. 2019), suggesting that sex steroids and not just chronological aging could reduce immunity following vaccination. Estrogen replacement therapy in postmenopausal women increases antibody responses to seasonal influenza vaccines (Engelmann et al. 2016). Limited data also suggest that sex differences in influenza vaccine-induced antibody responses involve the effects of testosterone on lipid biosynthesis in males (Furman et al. 2014).

Although sex differences in antibody titers are reduced with older age, the quality of the antibody response still differs between the sexes. Older males are at greater risk for severe influenza, despite rates of vaccination being higher for older males compared to older females (Eshima et al. 2011; Kini et al. 2022). In a cohort of adults aged 50–74 years, older females have increased number of antibody secreting cells recognizing H1N1 virus, with greater B cell transcriptional activity (Ovsyannikova et al. 2016). Older females also have greater frequencies of age-associated B cells (ABCs) compared to older males (Cancro 2020). ABCs are a unique B cell population that expand following vaccination against multiple viruses, including influenza (Andrews et al. 2019; Lau et al. 2017). While the function of ABCs post-vaccination and in older individuals is still being investigated, these cells may provide a unique

mechanism by which older females produce greater quality and quantity of antibody responses following influenza vaccination than their male counterparts.

3.2 Animal Models

Animal models have been integral for providing mechanistic insights into sex differences in vaccine-induced immunity against influenza (Fink et al. 2018; Potluri et al. 2019; Ursin et al. 2022) (Table 1). Sex differences in antibody responses are reported in response to an inactivated H1N1 as well as human TIV (Fink et al. 2018; Arsenovic-Ranin et al. 2019). When immunized with live H1N1 or H3N2 influenza virus, adult female mice mount greater neutralizing and total antibody responses than males, which provide greater heterosubtypic protection against IAV viruses (Lorenzo et al. 2011).

After inactivated 2009 H1N1 virus vaccination, young adult female mice develop greater total anti-2009 H1N1 IgG titers, greater neutralizing antibody titers, and are better protected against live virus challenge than their male counterparts (Fink et al. 2018). Through antibody transfer experiments, vaccine-induced antibodies from females can equally protect naïve adult males and females from live virus challenge, illustrating that vaccine-induced antibody is sufficient for protection. Class switch recombination (CSR) of antibody is partially mediated by the expression of the X-linked gene, *Tlr7*, in B cells (Pone et al. 2015). Following vaccination, anti-2009 H1N1 IgG2c, but not IgG1, is greater in females than males. There is greater expression of *Tlr7* in B cells isolated from vaccinated females than males, which is associated with reduced DNA methylation in the *Tlr7* promoter region, greater neutralizing antibody titers, CSR, and antibody avidity in females (Fink et al. 2018). Deletion of *Tlr7* eliminates sex differences in vaccine-induced antibody responses and protection, with a greater impact on females than males.

To determine cross-recognition and cross-protection against IAVs, a panel of 2009 H1N1 viruses that contain mutations in the immunodominant HA protein, including a single amino acid mutant (at position K166Q), a double mutant (K166Q and G157E), a triple mutant (K166Q, G157E, and N211D), and a virus with the K166Q mutation plus an entire antigenic region substituted with a non-human HA were developed (Ursin et al. 2022). Following 2009 H1N1 vaccination, adult females produce greater virus-specific, class-switched total IgG and IgG2c antibodies against the vaccine and all mutant viruses, and antibodies from females recognized more unique, linear HA epitopes than antibodies from males. While females have greater neutralizing antibody titers against the vaccine virus, both sexes show lower neutralization capacity against mutant viruses. After live virus challenge, vaccinated adult females have lower pulmonary virus titers and reduced morbidity than males against the single and double mutant viruses, but not the substitution virus (Ursin et al. 2022), raising questions about sex differences in non-neutralizing antibody functions in protection. Inactivated 2009 H1N1 virus vaccination also causes greater numbers of germinal center (GC) B cells containing superior somatic hypermutation (SHM) frequencies

in vaccinated females than males. Deletion of the gene (*AICDA)* that encodes for activation-induced cytidine deaminase (AID) eliminates female-biased immunity and protection against live virus challenge (Ursin et al. 2022).

There is growing interest in universal influenza vaccines that can provide protection against diverse IAV and IBV strains. Universal influenza vaccine based on an adjuvanted chimeric HA stem (Bernstein et al. 2020) were used to evaluate sex and age differences. Using a prime and boost strategy, while sex differences in vaccine-induced antibody responses are not observed among young adult mice, there is an age-associated decline in immunity, including in non-neutralizing antibody functions (e.g., antibody-dependent cellular cytotoxicity [ADCC]), and protection from infection that is apparent among female but not male mice (Dhakal et al. 2022).

Mechanistically, data illustrate that sex steroids mediate sex differences in vaccine-induced immunity and protection in animal models. For example, following vaccination with an inactivated H1N1, females with higher concentrations of E2 have greater neutralizing antibody responses and protection from challenge among both young adult and aged mice (Nguyen et al. 2011; Potluri et al. 2019) Ovariectomy of female mice reduces and treatment of ovariectomized female mice with exogenous E2 enhances vaccine-specific IgG and neutralizing antibody titers, illustrating that E2 causes greater antibody production following vaccination of females (Potluri et al. 2019). Both aging and reduced E2 are associated with lower antibody responses post-vaccination in humans and reduced protection following live virus challenge in mice (Potluri et al. 2019). Aging (Frasca and Blomberg 2020) and reduced E2 signaling through ERα (Jones et al. 2020) also significantly reduce expression of AID, which have not been evaluated together.

4 Effects of Pregnancy on Influenza Pathogenesis and Vaccine Outcomes

4.1 Influenza Pathogenesis

Pregnancy is a female-specific risk factor for severe disease and death following infection with seasonal and pandemic influenza viruses (Jamieson et al. 2009; Woolston and Conley 1918; Harris 1919; Cox et al. 2006a; Rogers et al. 2010; Mertz et al. 2017). Pregnancy-associated increased mortality rates following influenza infection were noted as early as the 1918 H1N1 pandemic, with 18.1% greater fatality in hospitalized pregnant women versus nonpregnant patients in one hospital, and a 27% mortality rate observed in a survey of 1350 cases of pregnant women (Woolston and Conley 1918; Harris 1919). During the 2009 H1N1 pandemic, pregnant women experienced greater morbidity than the general population and accounted for a disproportionally higher percentage of pandemic influenza deaths. In 2009, pregnant women accounted for 5% of pandemic influenza deaths in the USA, while representing only approximately 1% of the population (Siston et al. 2010; Klein et al. 2010b). Seasonal

influenza viruses, while generally considered less pathogenic than pandemic IAVs, are reported to infect between 483 and 1097 per 10,000 pregnant women annually (Katz et al. 2017), and pregnant women have greater odds of adverse outcomes, including intensive care unit (ICU) admission, acute cardiopulmonary morbidity, and increased hospital length of stay than nonpregnant comparators (Vousden et al. 2021; Neuzil et al. 1998; Cox et al. 2006b).

Risk for severe seasonal and pandemic influenza disease is greater in the third than in other trimesters, in studies that consider gestational age, highlighting that pregnancy is not a static physiological state (Rogers et al. 2010; Neuzil et al. 1998; Rojas-Suarez et al. 2014). Pregnancy induces profound anatomical, physiological, and immunological changes across the gestational trimesters. As gestation progresses, the cardiovascular and respiratory system are altered to accommodate the developing fetus; cardiac output, cardiac stroke volume, pulmonary tidal volume, and systemic demand for oxygen increase, while pulmonary residual capacity is reduced due to the raised diaphragm (LoMauro and Aliverti 2022; Soma-Pillay et al. 2016; Kourtis et al. 2014). Altered cardiopulmonary function during pregnancy are, therefore, hypothesized to increase the risk of hypoxemia and contribute to severe disease during influenza virus infection (Kourtis et al. 2014). Additionally, the immune system during pregnancy undergoes gestational-age-dependent shifts (Abu-Raya et al. 2020), which may increase susceptibility to influenza viruses. Proinflammatory cells and signaling are required during the first trimester for successful implantation and the development of pregnancy-associated tissues such as the placenta and amniotic sac (Mor et al. 2011). Subsequently, an anti-inflammatory state is induced to protect the developing semi-allogenic fetus in the second and third trimester, with increased regulatory T and B cells, reduced cytotoxic T cells and cellular immunity, and a general shift from helper T cell type 1 (Th1) to helper T cell type 2 (Th2) responses (Kourtis et al. 2014; Abu-Raya et al. 2020; Vanders et al. 2013). These immunological shifts are in part supported by the concomitant rise in estrogens and P4 throughout gestation (Tate et al. 2011). While pregnancy-associated hormonal, anatomical, physiological, and immunological changes are hypothesized to contribute to severe disease and death following infection with influenza viruses, the exact mechanisms and individual factors contributing to severe influenza disease during pregnancy in humans remain unknown.

To better understand the contributions of pregnancy to severe disease following influenza infection, murine models have been developed to gain mechanistic insight (Table 2). Murine pregnancy recapitulates many facets of human pregnancy, including similar, but not identical cardiovascular, hormonal, and immunological shifts (Abu-Raya et al. 2020; Arany et al. 2022; Zoller et al. 2007; Malassine et al. 2003). For example, P4 concentrations increase throughout pregnancy in both humans and mice, with secretion by the corpus luteum followed by the placenta in humans and solely the corpus luteum in mice (Malassine et al. 2003). Murine models of pregnancy have limitations, including, but not limited to, shorter (~20 days versus ~ 9 months) gestation period, differing mechanisms of placentation between the species, and the absence of a semi-allogeneic fetus if using inbred mice (Arany et al. 2022; Malassine et al. 2003; Krishnan et al. 2013). Despite these limitations, murine

models of have provided evidence for pregnancy-specific mechanisms of severe influenza disease that would otherwise remain untestable hypotheses in humans.

Studies of influenza virus infection during pregnancy have utilized both pandemic and seasonal influenza viruses, multiple inbred and outbred strains of mice, with infections at timepoints throughout murine gestation (Table 2). Disease following influenza virus infection, as measured by reduced weight gain, clinical signs of respiratory distress, and/or mortality, is consistently greater after infection with pandemic than seasonal influenza viruses and in pregnant compared with nonpregnant females, consistent with human observational studies (Pazos et al. 2012; Kim et al. 2012; Littauer et al. 2017; Marcelin et al. 2011; Engels et al. 2017; Chan et al. 2010; Lauzon-Joset et al. 2019; Swieboda et al. 2020; Kim et al. 2014; Vermillion et al. 2018c). Pregnant dams infected with IAV at embryonic days equivalent to the second or third human trimester have reduced adaptive immune responses to IAV, including reduced systemic CD4+ and CD8+ T cells, IAV-specific antibody titers, and IAV-specific cytotoxic T cell activity, with these effects mediated by the female-sex steroid hormones of progesterone and estrogens (Pazos et al. 2012; Chan et al. 2010; Swieboda et al. 2020). Pregnancy alters local innate immune responses and tissue repair activity with infection of pregnant dams resulting in reduced antiviral IFN responses, increased IL-1β and IL-6), and increased numbers of neutrophils and macrophages in lungs as compared to nonpregnant infected females (Pazos et al. 2012; Marcelin et al. 2011; Engels et al. 2017; Chan et al. 2010; Lauzon-Joset et al. 2019). Proinflammatory responses in the lungs are associated with pulmonary tissue damage, impaired epithelial regeneration, reduced pulmonary P4 concentrations, and a pregnancy-specific shift of alveolar macrophages from a classical (M1) to an alternative (M2) activation state (Marcelin et al. 2011; Lauzon-Joset et al. 2019; Swieboda et al. 2020). Whether viral clearance is diminished during pregnancy remains unclear as reported viral loads between pregnant and nonpregnant mice are contradictory (Kim et al. 2012; Littauer et al. 2017; Marcelin et al. 2011; Chan et al. 2010; Vermillion et al. 2018c). Taken together, murine studies highlight that pregnancy-associated immunomodulation contributes to influenza disease severity.

There is no evidence from either humans or animal models that seasonal or pandemic influenza viruses cross the placenta or infect the developing fetus, yet influenza virus infection during pregnancy is associated with adverse pregnancy and fetal outcomes, including pre-term delivery, fetal growth restriction, and fetal distress (Cox et al. 2006b; Omer et al. 2012; Hartel et al. 2016; Song et al. 2020; Pierce et al. 2009; Naresh et al. 2013; Buchy et al. 2020; He et al. 2017; Dawood et al. 2021; Jacobsen et al. 2021). The 2009 H1N1 pandemic highlighted adverse fetal outcomes. According to a systematic review and meta-analysis of 10 studies from the 2009 H1N1 pandemic, maternal infection resulted in a 71% and 136% increased relative risk of low birth weights and stillbirths, respectively, compared to pregnant women without influenza disease (He et al. 2017). Influenza infection during pregnancy also has been associated with long-term adverse outcomes for offspring, including increased risk of development of metabolic and neuropsychological disorders in adulthood (Song et al. 2020; Brown et al. 2004; Parboosing et al. 2013).

Table 2 Mouse models of influenza virus infection during pregnancy provide mechanistic insights

Influenza virus	Mouse strain(s)	Embryonic (E) age(s) at infection	Maternal outcomes	Offspring outcomes	Mechanistic insights	References
A/Brisbane/59/07 (H1N1)	BALB/c	E12, E14	↓ weight gain ↓ gestation length	↓ offspring size ↑ stillbirths	Disrupted placental architecture ↑ placental inflammation ↑ lung viral load	Littauer et al. 2017
A/California/04/09 (H1N1)	C57BL/6, BALB/c	E10, E13	↓ weight gain ↑ mortality	↑ abortions ↓ offspring size	↑ cytokines, macrophages, and neutrophils in lungs ↓ epithelial regeneration in lungs ↓ serum progesterone	Marcelin et al. 2011; Vermillion et al. 2018c
A/California/07/09 (H1N1)	C55BL/6J, BALB/c	E3.5, E7.5, E12, E12.5	↓ weight gain ↓ gestation length ↑ mortality	↓ offspring size ↓ fetal thymic gene expression	↑ Placental inflammation ↓ labyrinth development ↑ pulmonary T cells ↑ uterine macrophages	Swieboda et al. 2020; Van Campen et al. 2020; Kim et al. 2012
A/Hamburg/NY1580/09 (H1N1)	C57BL/6J females mated with BALB/c males	E5.5, E12.5	↓ weight gain ↑ mortality	↓ offspring growth ↑ vulnerability to infections	↓ pulmonary anti-viral cytokines ↓ hematopoietic development in offspring ↑ morbidity and mortality with allogenic pregnancy	Jacobsen et al. 2021; Engels et al. 2017
A/HK/415742/09 (H1N1)	BALB/c	E12-E14	↑ mortality	↓ fetus mass	↑ severity of pneumonitis ↓ adaptive immune responses ↑ pulmonary proinflammatory cytokines Placental necrosis	Chan et al. 2010

(continued)

Table 2 (continued)

Influenza virus	Mouse strain(s)	Embryonic (E) age(s) at infection	Maternal outcomes	Offspring outcomes	Mechanistic insights	References
A/PR/8/34 (H1N1)	C57BL/6, BALB/c	E9.5, E10.5	↓ weight gain ↑ clinical score	↓ fetus mass	↓ cytotoxic T cell activity Delayed viral clearance ↑ neutrophils in bronchoalveolar lavage Alveolar macrophage phenotype shift	Pazos et al. 2012; Lauzon-Joset et al. 2019
B/Brisbane/60/08 (IBV)	CD1	E14	↓ weight gain	↓ fetus mass	↑ lung viral titers ↓ uterine progesterone ↓ uterine estrogen	Kim et al. 2014
HK-x31 (H3N2)	C57BL/6J, C57BL/6N	E10, E12	↓ weight gain	↓ fetal mass ↑ abortions	↑ vascular oxidative stress ↑ vascular inflammation ↑ immune cells in placenta ↓ placental integrity Evidence of placental and fetal hypoxia	Liong et al. 2020; Oseghale et al. 2022; Antonson et al. 2021

While murine models of influenza virus infection during pregnancy have reported short- and long-term adverse fetal outcomes (Table 2), few studies have focused on uncovering mechanisms. One potential mechanism is placental damage and inflammation, with evidence of disrupted placental architecture, placental necrosis, increased proinflammatory cytokines, and the infiltration of immune cells such as neutrophils in the placenta of dams infected with influenza viruses (Littauer et al. 2017; Chan et al. 2010; Swieboda et al. 2020; Antonson et al. 2021; Van Campen et al. 2020; Liong et al. 2020). Placental damage and inflammation are associated with adverse fetal outcomes in other models, including preeclampsia and infection with 'TORCH' (i.e., *Toxoplasma gondii*, other, rubella virus, cytomegalovirus, herpes simplex virus) microbes (Megli and Coyne 2022; Goldstein et al. 2020; Ilekis et al. 2016). The mechanisms by which infection with influenza viruses causes damage and inflammation in the placenta without direct viral infection or a causal link to adverse fetal outcomes, however, remain unknown.

4.2 Influenza Vaccines

To reduce the risk of severe influenza disease and prevent adverse fetal outcomes, pregnant women are recommended to receive an inactivated influenza vaccine (IIV) at any trimester of pregnancy (Vaccines against influenza WHO position paper 2012). Seasonal and pandemic vaccination with inactivated influenza vaccines is safe during pregnancy, with no reported association with adverse fetal or pregnancy outcomes, immunogenic, and efficacious during pregnancy (Sumaya and Gibbs 1979; Englund et al. 1993; Moro et al. 2011; Christian et al. 2013). Pregnancy does not appear to negatively impact influenza vaccine responses as influenza-specific antibody production and cytokine responses after vaccination with IIV do not significantly differ between pregnant and nonpregnant women (Christian et al. 2013), and vaccine effectiveness for pregnant women after IIV is similar to that of the general population for the influenza season studied (Thompson et al. 2014). Moreover, IIV vaccination during pregnancy is associated with reduced rates of premature and small for gestational age births during influenza season and reduced rates of influenza illness in infants up to 6 months of age (Omer et al. 2011; Steinhoff et al. 2012; Zaman et al. 2008). There is no licensed influenza vaccine for infants less than 6 months of age, and therefore, vaccination during pregnancy and transplacental transfer of influenza-specific antibodies provides valuable protection to this population (Buchy et al. 2020). Maternal vaccination is a method to protect the mother and fetus during pregnancy and the newborn infants in early life from influenza disease.

5 Conclusions and Future Directions

Sex is a biological variable that should be considered in studies of influenza vaccination and pathogenesis. There are gaps in our understanding of the precise mechanisms mediating sex-biased immune responses and how this affects the outcome of influenza virus infection and vaccination. Future research must continue to define the pathways mediating how hormones, as reviewed here, but also genes and even the microbiota differently affect immunity to influenza virus infection and vaccination in males compared with females. Future studies should continue to consider the age and reproductive status of individuals as well as whether females or even males are using exogenous hormones, either through contraceptives or hormone replacement therapy, at the time of infection or vaccination. We recommend that clinicians, epidemiologists, and basic biomedical scientists design experiments that include both males and females, develop a priori hypotheses that the sexes will differ in their responses to and the outcome of influenza virus infection and vaccination across the life course, and statistically analyze outcome data by sex and age. The end goal should be that clinicians and researchers alike consider the sex of their patients or animals when interpreting data pertaining to influenza virus pathogenesis and vaccination.

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Sex and Gender Differences in Tuberculosis Pathogenesis and Treatment Outcomes

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Abstract Tuberculosis remains a daunting public health concern in many countries of the world. A consistent observation in the global epidemiology of tuberculosis is an excess of cases of active pulmonary tuberculosis among males compared with females. Data from both humans and animals also suggest that males are more susceptible than females to develop active pulmonary disease. Similarly, male sex has been associated with poor treatment outcomes. Despite this growing body of evidence, little is known about the mechanisms driving sex bias in tuberculosis disease. Two dominant hypotheses have been proposed to explain the predominance of active pulmonary tuberculosis among males. The first is based on the contribution of biological factors, such as sex hormones and genetic factors, on host immunity during tuberculosis. The second is focused on non-biological factors such as smoking, professional exposure, and health-seeking behaviors, known to be influenced by gender. In this chapter, we review the literature regarding these two prevailing hypotheses by presenting human but also experimental animal studies. In addition, we presented studies aiming at examining the impact of sex and gender on other clinical forms of tuberculosis such as latent tuberculosis infection and extrapulmonary tuberculosis, which both appear to have their own specificities in relation to sex. We also highlighted potential intersections between sex and gender in the context of tuberculosis and shared future directions that could guide in elucidating mechanisms of sex-based differences in tuberculosis pathogenesis and treatment outcomes.

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S. L. Klein and C. W. Roberts (eds.), *Sex and Gender Differences in Infection and Treatments for Infectious Diseases*, Current Topics in Microbiology and Immunology 441, https://doi.org/10.1007/978-3-031-35139-6_6

1 Introduction

Tuberculosis (TB) is a contagious infection that is caused by acid-fast bacilli (AFB) belonging to *Mycobacterium tuberculosis* complex (MTBC). This complex regroups ten closely related bacterial species that infect humans but also wild and domestic animals, namely *Mycobacterium tuberculosis* (MTB), *Mycobacterium africanum* (MAF), *Mycobacterium bovis, Mycobacterium microti, Mycobacterium caprae, Mycobacterium pinnipedii, Mycobacterium canetti, Mycobacterium orygis, Mycobacterium suricattae, and Mycobacterium mungi.* Among them, MTB is the most predominant human-adapted species, while MAF is mostly encountered in West Africa (Silva et al. 2022).

It is estimated that a quarter of the world population is infected with these bacilli in the form of latent TB infection (LTBI) (Houben and Dodd 2016). Infection usually occurs when infectious droplets from the cough or sneeze of a sick person are inhaled by a susceptible host. Bacteria in the droplets can traverse the upper respiratory tract and reach the alveoli of the lungs. Thus, TB is typically considered as a disease of the lungs, but basically any organ of the body can be affected leading to extrapulmonary TB (EPTB). Zoonotic transmission can also occur, mostly through close contact with infected animals, but also by drinking unpasteurized milk and dairy products, or eating raw meats contaminated with *Mycobacterium bovis* or other animal-adapted species of the MTBC (Olea-Popelka et al. 2017).

Following infection, two outcomes are possible (Abel et al. 2014). Most infected individuals will not have any clinical signs of disease despite being infected. Those asymptomatic carriers have LTBI; these individuals are not infectious despite having infection with quiescent bacilli that may remain silent but viable for years. Among the LTBI cases, only 5–10% will develop active disease later during their lifetime due to reactivation of the original infection (Comstock et al. 1974; Abel et al. 2014). These people are symptomatic and infectious. The symptoms include cough, fever, loss of weight, loss of appetite, and night sweats which are hallmarks of active pulmonary tuberculosis (PTB). Conversely, about 5% of infected individuals will develop a "primary" TB disease in the lungs within 2 years of infection (Abel et al. 2014). Primary TB disease mostly occurs at the extremes ages with clinical manifestations often associated with EPTB. Thus, in the absence of TB treatment, around 5–15% of people who are infected with MTB and who have a normal immune system will later develop active TB disease. The later one is one of the deadliest human diseases, ranking among the top ten causes of deaths in low- and middle-income countries (LMICs) (WHO 2020b).

At the global level, TB notifications rates have been heavily impacted by the 2019 Coronavirus Disease (COVID-19) pandemic which diverted health system resources. Therefore, the WHO global TB report based on 2019 estimates which were not affected by reporting bias due to COVID-19 will be used in this book chapter as reference. In that year, an estimated 10 million cases of TB were reported from all regions of the world (WHO 2020a). Most of those cases occurred in Southeast Asia,

Africa, and the Western Pacific, which are also regions where poverty is endemic and rates of HIV infection are high (WHO 2020a).

Active TB disease can be treated and prevented using antibiotics, including isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB) (Ncayiyana et al. 2016). These drugs are used in combination usually in a six-month regimen (a 2-month intensive phase with all four drugs and a 4-month maintenance phase with INH/RIF) in people newly diagnosed with PTB (Ncayiyana et al. 2016). Despite the potency of current TB drugs, primary and secondary drug resistances have been reported which comprise a serious global threat to the WHO's objective of reducing TB incidence by 90% and deaths by 95% by 2035 (WHO 2015). Control of TB is further complicated by the fact that there is only one licensed vaccine, namely Bacillus Calmette Guerin (BCG), which was shown to be most useful in preventing severe forms of TB disease, especially in children (Olea-Popelka et al. 2017).

An important yet understudied aspect of TB is that more males are affected by TB than females. The sex ratio on incident cases of TB has consistently shown higher rates in men, as reviewed elsewhere (Neyrolles and Quintana-Murci 2009). In 2019, adult males accounted for 56% of all incident TB cases compared to 36% in adult females and 12% in children (WHO 2020a). Similarly, the TB mortality rate is also higher in males compared to the opposite sex across the six WHO regions of the world. Along the same lines, treatment success rates and severity of disease appear to differ by sex (Dabitao et al. 2022; Chidambaram et al. 2021). These strong epidemiological and clinical observations have led to the question whether males are more vulnerable to TB than females. This chapter discusses the current state of knowledge on sex differences in TB. The chapter consists of 5 sections, not including the Introduction and Conclusion, encompassing important aspects of TB research such as epidemiology, treatment outcomes, and pathogenesis. Specifically, we will review biological factors that are potentially contributing to male bias in TB burden. We will also present non-biological gendered factors that may be interacting with sex-driven biological differences to amplify the male bias in TB.

2 Sex Differences in the Epidemiology of Tuberculosis

2.1 Latent Tuberculosis Infection

Latent tuberculosis infection (LTBI) is a form of tuberculosis characterized by a persistent *Mycobacterium tuberculosis*-specific T cell response, yet with no sign of clinical disease (Mack et al. 2009). In 2014, it was estimated that 23% of the world population has LTBI (Houben and Dodd 2016). Among those, about 5–10% will develop active tuberculosis during their lifetime and hence contribute to the global burden of TB (Comstock, Livesay, and Woolpert 1974). Thus, identification of the risk factors that promote LTBI is important to achieving TB control. Traditionally, individuals working in healthcare settings (Joshi et al. 2006), prisoners (Margolis

et al. 2013), and miners (Hanifa et al. 2009) have been considered high-risk groups for LTBI, likely because of their increased odds of being frequently exposed to cases of PTB. Nowadays, additional risk factors are included to enable more comprehensive management of LTBI. Among those, the impact of patient sex on the risk of acquiring LTBI and, once acquired, to progress to active disease is increasingly being investigated. This research theme takes its roots on the evidence that males are more likely to develop PTB than females (Borgdorff et al. 2000; Nhamoyebonde and Leslie 2014). Likewise, males receiving TB therapy have lower sputum conversion rates after initiating treatment suggesting a less favorable treatment outcomes relative to females (Chidambaram et al. 2021; Dabitao et al. 2022). Despite these observations, it is still unclear whether males are more susceptible to LTBI than the opposite sex. In addition, the relationship between patient sex and progression from LTBI to active TB has not been clearly defined. In this section, we will present key studies which have focused on the impact of sex on LTBI burden (prevalence and incidence) as well as LTBI treatment outcomes.

The impact of sex on LTBI prevalence has been investigated mostly in Asia, where most of the high-burden TB countries are also located. For example, a study conducted in China to determine risk factors for LTBI among healthcare workers (HCW) reported a prevalence of LTBI of 46% and an incidence rate of 19.1% (He et al. 2015). This was achieved by using the Quantiferon TB Gold In-Tube assay (QTF), an IFN-γ-release assay, which detects memory immune responses to mycobacterial antigens and is more specific than the classical Tuberculin Skin Test (TST) (Farhat et al. 2006). Risk factors associated with LTBI status among these HCWs included being male (aOR = 2.17, 95%CI 1.63–2.89), education level below college, and work experience in healthcare setting of greater than or equal to 25 years. In this study, the occurrence of new cases of LTBI (i.e., incidence of LTBI, as determined by conversion from negative to positive QTF test during one-year follow-up) was not associated with sex in multivariate analyses; variables that were associated with QTF conversion were the amount of time spent with a TB patient (more than 15 min per day) and absence of BCG scar. The lack of a sex difference in the LTBI incidence rate/QTF conversion rate in this study could be function of the small sample size, the type of population studied, namely HCWs, but also the relative short follow-up time (one year). In a similar study, Chen et al. addressed the same question, but this time by focusing on a general population from Eastern China (Chen et al. 2015). The prevalence of LTBI defined by QTF positivity with no clinical and laboratory evidence of TB was 19.98% which as expected was lower than that of the previous study conducted in HCWs. After adjusting for potential confounders, male sex was significantly associated with LTBI risk (aOR: 1.32, 95% CI 1.11–1.58). Additional risk factors were age, smoking, obesity, alcohol use, and close contact with TB patients. Protective factors were primarily found to be higher levels of education and BCG vaccination.

An additional important question is whether sex influences progression from LTBI to PTB. The most compelling data addressing this question are derived from a population-based, multicenter, prospective study conducted at four rural sites in China (Gao et al. 2017). In this study, 7505 LTBI individuals identified by either QTF

or TST were examined and followed for 2 years. During that period, 83 new cases of active TB occurred among the individuals who initially tested positive for both QTF and TST, yielding an incidence rate of 0.82 per 100 person-years. Remarkably, only two factors were identified as being significantly associated with development of active disease, and these were male sex (aHR: 2.36, 95% CI 1·30–4·30) and history of TB (aHR: 5.40, 95% CI 3·34–8·71) (Gao et al. 2017).

Since those studies were conducted mostly in Asia, it is important to examine whether there is a relationship between sex and risk of LTBI acquisition in Africa, a continent harboring more than half of the thirty highest TB burden countries (WHO 2020a). In South Africa, the LTBI prevalence in the general population of Johannesburg, the capital city, was reported to be 34.3% by Ncayiyana et al. using the TST test (Ncayiyana et al. 2016). As in Asia, the risk of infection was associated with male sex (aOR = 2.70, 95% CI = 1.55–4.70) and age but also with marital and socio-economic status, and these factors were independent of HIV status (Ncayiyana et al. 2016). The authors attributed the increased risk of LTBI in males to behavioral factors such as social gathering in informal townships bars which appear to be more frequently used by males. However, the association of male sex and risk of LTBI persisted in settings where there is no apparent difference between men and women in terms of TB exposure such as the healthcare setting. For instance, in Morocco, the prevalence of LTBI by QTF was estimated to be 40.7% in HCWs. The risk factors for LTBI were male sex (OR = 2.21; 95%CI 1.40–3.49), increased age, family history of TB, and working in the department of pulmonary diseases (Sabri et al. 2019). This suggests that the difference in LTBI risk might not solely be explained by behavioral differences between men and women.

A further question is whether there is a sex bias in LTBI rates in low TB burden settings such as in North America and Western Europe. This question has been addressed by Reichler et al., as part of the research activities of the Tuberculosis Epidemiologic Studies Consortium Task Order 2 (Reichler et al. 2020). The study consisted of a prospective cohort study of laboratory-confirmed TB cases and their close contacts at nine health departments in the US and Canada. Consistent with reports in high TB burden countries in Asia and Africa, they found that LTBI detection was associated with male sex (aOR: 1.4, 95%CI: 1.1, 1.6), age of 5 years or older, shared bedroom with a TB case, and exposure to more than one index case (Reichler et al. 2020).

Despite the growing evidence revealing a male sex bias for the development of LTBI in both high and low TB burden settings, it should be noted that there are also reports indicating no differences between the sexes in LTBI incidence. For instance, in Taiwan, Ting et al. conducted a hospital-based cross-sectional study by enrolling adult patients considered to be at high risk of contracting LTBI and to progress from LTBI to active disease (Ting et al. 2014). LTBI diagnosis was based on the QTF test. The participants consisted of close contacts of TB cases, HCWs, and subjects with chronic diseases such as cancer, renal disease, liver cirrhosis, or autoimmune diseases. A total of 1,113 subjects were enrolled. The LTBI rate was 26.6% with a male predominance of 32.6% versus 25.2% among women ($p = 0.010$). However, a male bias in LTBI prevalence was not confirmed in multivariate analyses after adjusting for

age, smoking status, and clinical status. Rather the authors found that age ($p = 0.014$), smoking ($p = 0.048$), and fibro-calcific lesions on chest radiograph ($p = 0.009$) were independent risk factors for LTBI while sex was not (Ting et al. 2014). The lack of a significant sex difference in this study may have been due to heterogeneity among participants since they consisted of healthy subjects (TB contacts and HCW) alongside individuals with major disease states and comorbidities. In addition, some of diseases among study participants, such as cancer and autoimmune diseases, are inherently influenced by sex which introduces a pre-existing bias for the relationship between sex and LTBI in this study. This is further supported by key differences in baseline characteristics between males and females. As noted by the authors, males were older, more likely to smoke, to have malignancies and fibro-calcific lesions by chest radiograph. Conversely, they were less likely to be HCWs, to have had past contact with TB cases, and to have autoimmune diseases. Thus, future studies in homogenous populations with similar baseline characteristics, irrespective of sex, are warranted.

Likewise in Southern Ethiopia, a cross-sectional study in a pastoral community (i.e., nomads living with herds of animals) found a prevalence of LTBI of 50.5% by QTF among adults with no apparent difference between the sexes and no relationship between age and risk of LTBI (Teklu et al. 2018). In contrast, being a resident of a specific Ethiopian region (Dasanech District), consumption of raw meat, and family size greater than 5 persons were associated with risk of having LTBI. The finding of the association between eating behavior and LTBI suggests that *Mycobacterium bovis* or other animal-adapted species might be the dominant cause of TB in this population and that TB is being transmitted as a zoonotic infection in this setting. Thus, it is difficult to extrapolate findings derived from such rural African communities to the general population where human-to-human transmission is predominant. Nevertheless, a recent prospective, multicenter cohort study in Brazil also investigated the possibility of sex differences in LTBI risk among close contacts of TB cases (Wada et al. 2022). In a cohort of 1093, more females were QTF positive (46%) compared to the opposite sex (40%), which was the reverse of what has been reported in the literature so far. After adjusting for covariates in multivariate analysis, the models used did not reveal significant differences in LTBI acquisition by sex (aOR: 1.14, 95% CI: 0.92–1.41) (Wada et al. 2022). As before, the findings reported here are not aligned with earlier studies which found sex-based differences in LTBI risk.

In addition to risk of LTBI acquisition or progression from LTBI to active disease, an outstanding question is whether males and females with LTBI who receive TB preventive treatment have differential outcomes. This is an important research question for effective public health interventions in countries, where identifying and treating people with LTBI are established practices. Treatment of LTBI in countries of low incidence of TB is currently based on 3 drugs, namely isoniazid alone, isoniazid plus rifapentine, or rifampin alone (CDC 2020). Thus, understanding the impact of sex on LTBI treatment outcomes may also involve the question of sex-based differences in the half-lives and metabolism of these three drugs. Unfortunately, studies focused on this question are rare. Nonetheless, the Tuberculosis Epidemiologic Studies Consortium (TBESC) based in the US and Canada has published

two articles on this question. In the first one, they reported that females with LTBI are more likely to discontinue isoniazid treatment because of adverse reactions (RR 1.67, 95% CI 1.32–2.10) (Pettit et al. 2013). The finding was based on a large cohort of 1,306 individuals. Drinking alcohol was also an independent predictor of treatment discontinuation in addition to female sex. In the second study, the authors focused on factors that are associated with LTBI treatment completion. At baseline, being male (aRR 1.19, 95% confidence interval [CI] 1.08–1.31), foreign birth, having health insurance, and not being worried about taking TB treatment were independently associated with treatment completion (Hirsch-Moverman et al. 2015). To understand the observed sex differences in adverse reactions to isoniazid, two independent factors were proposed, namely body weight and polymorphisms in genes involved in isoniazid metabolism (Pettit et al. 2013). For the first one, the authors speculated that there may have been females in the study weighing less than 60 kg who may have unintentionally received full-dose isoniazid (300 mg) instead of a weight-adjusted dose, thereby increasing the likelihood of adverse reactions among females. For the second one, the role of genetic polymorphisms on the activity of N-acetyltransferase-2 (NAT-2), an enzyme responsible for metabolizing isoniazid, has been proposed. NAT-2 activity is thought to be determined by its genotype (Ohno et al. 2000; Parkin et al. 1997). Polymorphisms that are linked to slow acetylation capacity have been associated with hepatotoxicity and peripheral neuropathy upon treatment with isoniazid. However, the study was unable to offer insight on these two hypotheses since body weight was not collected among participants and genotyping of NAT-2 polymorphisms was not performed.

In summary, there is a growing body of evidence coming from various geographic regions of the world and socio-professional categories suggesting that male sex is a risk factor for acquisition of LTBI, progression to active disease, but favorable LTBI treatment outcomes. There are also a handful of reports suggesting the contrary. This means that future studies, including experimental approaches, are needed to better clarify the presence or absence of a relationship between sex and LTBI incidence and outcomes.

2.2 Active Pulmonary Tuberculosis

According to the World Health Organization (WHO), PTB is defined as "any bacteriologically confirmed or clinically diagnosed case of TB involving the lung parenchyma or the tracheobronchial tree" (WHO 2013). Classical symptoms of PTB include a cough lasting more than 2 weeks, chronic unexplained fever, weight loss, pleuritic chest pain, loss of appetite, and night sweats. The diagnosis is based on the detection of lung lesions by chest X-ray or computerized tomography (CT) scan and in the laboratory by sputum smear microscopy, sputum culture on solid or liquid media, or molecular tests (Ryu 2015).

PTB is the most common form of TB disease globally and the most studied in terms of sex differences. In 2019 before the COVID-19 pandemic, PTB represented 84%

of the 7.1 million new cases of TB worldwide (WHO 2020a). Among the six world regions defined by WHO, namely the African Region (AFR), the Region of the Americas Region (AMR), South-East Asian Region (SEAR), the European (EUR) Region, the Eastern Mediterranean Region (EMR), and the Western Pacific Region (WPR), most PTB cases occurred in the SEAR, AFR, and WPC regions (WHO 2020a). Meanwhile, the EUR region had the lowest incidence of PTB. At the country level, 30 countries accounted for 86% of TB burden in 2019, among which eight countries comprised the epidemiologic epicenter, namely India, China, Indonesia, Philippines, Pakistan, Nigeria, Bangladesh, and South Africa (WHO 2020a). In countries like South Africa, the situation is further complicated by the dual HIV and TB epidemics. For example, 58% of people treated for TB disease by the National TB Program are co-infected with HIV in South Africa (Sizulu et al. 2018). By way of comparison, the global rate of HIV and TB co-infection is 8.2%.

The most reported risk factors of PTB are undernutrition, HIV infection, alcohol use, smoking, and diabetes (Hayashi and Chandramohan 2018; Imtiaz et al. 2017; Lonnroth et al. 2010; WHO 2022). As in LTBI, additional risk factors are being considered; these include virulence of the infecting mycobacterial strain, age, ethnic background, and the sex of the infected person. For the latter one, an excess of PTB in adult males when compared to females has been consistently reported in TB notification rates of the WHO's Global TB Reports as indicated in Fig. 1. Notably, in 2019, 56% of new cases of TB occurred in adult males, while 32% and 12% were in adult females and children, respectively (WHO 2020a). In most countries, males are on average more than two times more likely to develop PTB than women (Horton et al. 2016; Neyrolles and Quintana-Murci 2009). Specifically, the male-to-female sex ratio in term of TB incidence is consistently in favor for males across all WHO regions irrespective of socio-economic status or HIV disease burden (WHO 2020a). These observations thus raise the question of whether male sex is an independent risk factor for developing PTB. Below, we will describe some of the seminal papers that demonstrate an increased susceptibility of males in developing PTB compared to the opposite sex.

In 1929, 251 newborns were accidentally infected with a BCG vaccine that was contaminated with a virulent strain of *Mycobacterium tuberculosis* during a TB prevention campaign at the Lübeck General Hospital in Germany (Fox, Orlova, and Schurr 2016). A total of 228 children (90.8%), developed radiological or clinical evidence of TB, among which 72 died from a TB-associated illness. After one year of follow-up, it was found that males were more likely to die from TB compared to females (36.5% versus 24%; relative risk [RR] 1.6, 95% CI 1.0–2.3) (Fox, Orlova, and Schurr 2016). Since the "Lübeck disaster" occurred before availability of antibiotics to cure TB, it provides one of the strongest pieces of evidence that sex has a biological impact on the natural history of TB disease progression.

In addition to the Lübeck disaster, a few rigorous investigations were also conducted to determine whether sex differences in TB notification rates are due to epidemiological biases or artifacts such as under-reporting or underdiagnosing in females or whether they are due to fundamental biologic differences. Firstly, Borgdoff et al. analyzed 29 prevalence surveys focused on smear positive TB cases from 14

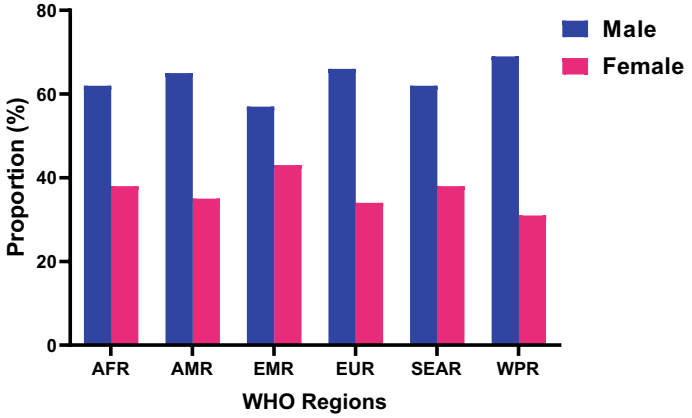

Fig. 1 Proportion of adult males and females among incident TB cases reported across the six WHO regions in 2019 (Source: WHO 2020a) Note: AFR: African region, AMR: American Region, EMR: Eastern Mediterranean Region, EUR: European Region, SEAR: South-East Asia Region; WPR: Western Pacific Region

countries and compared those data to the notification rates of individual countries (Borgdorff et al. 2000). In this seminal paper, the authors showed that the female-to-male prevalence ratios were consistently below 1 across the 14 countries studied, but with varying intensities (Borgdorff et al. 2000). Interestingly, the prevalence ratios were very similar or lower to those found from the notification rates, suggesting that the difference observed between the sexes is a true epidemiological difference and not a bias introduced because of reduced access to care for women in these countries. Secondly, one of the best pieces of evidence demonstrating an increase susceptibility of males to TB is a population-wide prevalence survey conducted in Bangladesh (Hamid Salim et al. 2004). A total of 266,189 people from the general population were selected by two-stage random sampling to participate in the study, among whom 7001 TB suspects were identified based on interviews conducted to detect the presence or absence of symptoms indicative of active TB. A morning sputum sample of each suspected case was later tested in the laboratory by Ziehl–Neelsen sputum smear microscopy to confirm the diagnosis of PTB. By this strategy, 64 PTB cases (48 males and 16 females) were detected, yielding a substantially higher prevalence rate among males (75%). By virtue of how subjects were selected in this study, the results also indicate that the sex difference in PTB prevalence is not due to differences in access to healthcare services between men and women. Lastly, an investigation led by Horton et al. suggested that men, rather than women, are less likely to be diagnosed in a timely manner (Horton et al. 2016). In fact, Horton and colleagues conducted a systematic review and meta-analysis of more TB surveys conducted in adult populations from low- and middle-income countries from 1993 to 2016. They confirmed that the TB prevalence among males was more than two times higher than for females even in regions where HIV infection rate is high. In addition, the male-to-female ratio of prevalent-to-notified cases (average time to diagnosis) based on

39 surveys was 1.5 times higher in males than females, demonstrating that a barrier to healthcare services for women in LMICs is unlikely to explain the male bias in TB prevalence.

The evidence reported in those seminal papers raises the question of whether males develop worse PTB disease severity and whether the male's response to TB treatment is inferior to that of females. We will be focusing on the influence of sex on disease severity in this paragraph, while the relationship between sex and treatment outcomes will be discussed in a separate section. We have shown that newly diagnosed males with PTB who are naïve to therapy have a higher bacterial load compared to females, irrespective of the mycobacterial genotype (Dabitao et al. 2022). These findings were based on both sputum smear grade and time to culture positivity which is defined as the time it takes (in days) for a mycobacterial clinical isolate to grow ex vivo (Dabitao et al. 2022). The higher smear bacterial grade in males suggests that males have more extensive lung involvement relative to females, before commencing treatment. This hypothesis is supported by two independent studies conducted in newly diagnosed cases with no history of smoking and who had no history of prior TB treatment. Computed tomography (CT) images revealed more severe pulmonary lesions suggestive of a higher bacterial burden in males compared to females (Chu et al. 2019). Scoring of CT images at baseline and after treatment also indicated a faster response to treatment in females than males (Tan et al. 2018). In addition to lung lesions, other markers indicative of disease severity such as C-reactive protein, a marker of systemic inflammation, were elevated in males (Chu et al. 2019). This has led us to propose that there are sex-specific, host–pathogen interactions during PTB (Dabitao et al. 2022), but the mechanism(s) remain to be defined.

Therefore, regarding active PTB, unlike in LTBI, we have a wealth of epidemiological, clinical, and microbiological pieces of evidence indicating that males are more vulnerable than females. These observations have raised the question as to what factors drive the sex difference, and two dominant theories have been proposed in the literature. The first hypothesis is that non-biological factors such as access to health care, health-seeking behaviors, smoking, substance abuse, professional exposure, non-compliance, and comorbidities are the true drivers of male TB sex bias (Feng et al. 2012; Watkins and Plant 2006; Weiss et al. 2006). Notably, a multicenter study conducted by the WHO regarding treatment adherence during TB in four countries, namely Malawi, Bangladesh, India, and Colombia, found a higher dropout rate in men during the course of TB treatment (Weiss 2006). The second hypothesis is that biological factors such as sex hormones and genetic differences drive the male sex bias (Neyrolles and Quintana-Murci 2009; Nhamoyebonde and Leslie 2014; Gupta et al. 2022). A discussion of the data supporting or challenging these two prevailing theories based on published animal and human studies will be included later in this chapter.

2.3 Extrapulmonary Tuberculosis

Active TB disease can also affect non-pulmonary tissues of the body. This is known as extrapulmonary tuberculosis (EPTB). According to the WHO, any forms of tuberculosis that occur in tissues outside the lung parenchyma are defined as EPTB (WHO 2013). EPTB usually occurs when mycobacteria reach the bloodstream and the lymphatic system to invade non-pulmonary tissues. Thus, virtually any tissue of the body can be impacted (Kulchavenya et al. 2019). Clinical forms of EPTB include lymph node, pleural, osteoarticular, urogenital, cerebral, abdominal, and cardiac disease. The treatment of EPTB and PTB is similar, as they are both based on the same antibiotics, which for most forms are recommended to be given during six months. The duration of treatment, however, should be increased to 9 to 12 months with certain forms of cerebral or bone and joint manifestations of TB (Lee 2015; Nahid et al. 2016). Also, in TB meningitis, adjunctive corticosteroids are recommended at the initiation of treatment for a duration of 6–8 weeks (Nahid et al. 2016).

In 2019, the WHO estimated that EPTB represents on average 16% of the 7.1 million incident tuberculosis cases worldwide (WHO 2020a). However, the rates vary substantially by WHO region from 8% in the Western Pacific Region to 24% Eastern Mediterranean region. The disparity persists even in countries from the same geographic region. For example, EPTB rates range from 5.8% to 44% between European countries (Sandgren et al. 2013). The reason for these variations between countries is unclear, but the impact of HIV infection, socio-economic factors, and cessation of BCG vaccination in newborns in many countries are suspected factors. Another important aspect of EPTB epidemiology is the fact that the absolute number of EPTB cases is not declining as observed for PTB, and hence, the proportion of EPTB cases among all forms of TB is rising. For example, EPTB constituted only 7.6% of all TB in 1962 in the US, but this rate went up to 21% in 2006 (CDC 2007). Similarly, EPTB makes up 50% of all forms of active TB disease in Australia (Barry et al. 2012). In contrast to developed countries, the epidemiology of EPTB in Africa is poorly described mainly because of the challenge to diagnose certain clinical forms. However, it is recognized that the overall drop in incidence of TB in many African countries is not evident for certain forms of EPTB such as TB meningitis and TB osteomyelitis in countries like South Africa (Hoogendoorn et al. 2017). The increase of the EPTB proportion among TB cases has prompted investigations seeking to identifying risk factors promoting progression to EPTB.

In the literature, host factors are commonly reported as risk factors influencing the pathogenesis of EPTB, but the mechanisms remain ill-defined. The factors include race, HIV infection, extremes of age, and female sex. In 2010, Fiske et al., reported that men and women of African ancestry were the most affected by EPTB in the US (Fiske et al. 2010). The study also revealed that black people also have a higher risk of developing EPTB than white people after adjusting for covariates (OR: 1.82 and 1.54) (Fiske et al. 2010). Similarly, it has been established that HIV positive individuals have a higher risk of developing EPTB than HIV negative individuals in

the US (Yang et al. 2004). Also, both young infants and the elderly constitute high-risk groups for EPTB. For example, post-menopausal women have been hypothesized to be more likely to develop EPTB due to lower estrogen levels and sex hormone effects on the immune system (Lin et al. 2013). Equally, the most common forms of TB in prepubescent children are TB meningitis and miliary TB (Katsnelson 2017). We will focus on the role of sex as risk factor of EPTB in next paragraphs.

Historically, one of the most ancient cases of TB was found in a submerged archeological site in the Mediterranean Sea in a prehistoric village called Atlit Yam near Haifa, Israel (Hershkovitz et al. 2008). The skeletons of a woman and infant dated to be more than 9000 years old were confirmed to both have bone lesions caused by *Mycobacterium tuberculosis*, confirming presence of EPTB. Similarly, a meta-analysis of more than 31 published papers conducted between 2010 and 2016 in TB high-burden countries has reported a female-to-male ratio of EPTB of 2.9:1, 1:9:1, and 1:7:1, respectively, in Pakistan, Afghanistan, and Bangladesh (Mehraj et al. 2016). These observations among other have led to the hypothesis that females are more vulnerable to extrapulmonary forms of TB than males. The hypothesis also suggests a reversal of the sex bias that has been consistently observed in cases with PTB.

The increased risk of EPTB in women has been questioned by some research groups on the grounds of variable sex ratios in TB between countries. For example, the sex ratio of EPTB was 0.6:1 in favor of males in India, one of the hotspots for TB in the world (Mehraj et al. 2016), but in contrast EPTB was associated with female sex in the US (Fiske et al. 2010; Yang et al. 2004), the Netherlands (te Beek et al. 2006), Turkey (Musellim et al. 2005), Nepal (Sreeramareddy et al. 2008), China (Pang et al. 2019), and North Africa (Mened et al. 2019).

The reason for an excess of EPTB cases in females compared to the opposite sex is unknown. Biological factors such as sex hormones are potentially involved and will be discussed in a dedicated section below. Indeed, the current evidence from animal studies suggests that estradiol might have a protective role during TB, while testosterone has the opposite effect (Hertz and Schneider 2019). Similarly, there may be genetic influences that confer a predisposition to EPTB since people of African or Asian ancestries are more likely to develop EPTB than those of European ancestry (Fiske et al. 2010; Hayward et al. 2021; Asghar et al. 2008). Although genomic loci or genomic variants responsible for increased susceptibility in black ethnicity have not been clearly identified yet, a pilot genome-wide study has identified two single-nucleotides polymorphisms (SNPs), which are associated with EPTP but not PTB (Oki et al. 2011). Thus, future studies are needed to determine the impact sex hormones and genetic factors in EPTB pathogenesis and their intersection with of mycobacterial strains and route of infection. In addition, since there are various forms of EPTB, studies looking at each clinical form in relation to sex could provide insights.

On the other hand, while non-biological factors (healthcare access or differences in exposure) remain a potential argument for the male bias in PTB, it seems unlikely that these same non-biologic differences could simultaneously predispose females to EPTB but not to PTB. For example, a retrospective study conducted in Afghanistan

found that women were more likely to report that their TB-like symptoms existed for more than a year prior to seeking care than men (Fader et al. 2010). Thus, delay in receiving appropriate care has been proposed to explain higher rates of EPTB in females, while underdiagnosis of PTB in females is also considered as driver of excess of PTB in males. This seems to be a weak and counterintuitive argument since sex bias in EPTB rates is observed even in high-income countries where there is no apparent socio-cultural barrier to accessing care and TB diagnosis. A delay in diagnosis for females also may not be a solid argument because Horton et al. have shown that males are less likely to be diagnosed with TB than females (Horton et al. 2016). In addition, women gain unique access to healthcare services for their prenatal and postnatal care and are more likely to be the adult who seeks pediatric care for children, both which would argue for greater access than men. If reduced access to health care was the driving factor of higher risk of EPTB in females, the same trend would also be expected for other endemic infectious diseases such as HIV. However, this is not the case, since it is well established that more than 60% of incident HIV cases in developing countries in sub-Saharan Africa occur among women and young girls (UNAIDS 2021), suggesting that they have access to HIV diagnosis, a component of most national healthcare systems as is TB. Nevertheless, differential exposure to mycobacteria between the sexes, for example, by the means of zoonotic infections, primarily with *Mycobacterium bovis,* should be explored. It is known that TB infection can occur by eating contaminated food and via aerosols from infected livestock. So, it is possible that mycobacterial strains and route of transmission alongside other biological variables may be at play.

In summary, the rising proportion of EPTB among active TB cases is concerning given the challenges to establish an accurate TB diagnosis in LMICs. In stark contrast to PTB which occurs more frequently in males, the current global epidemiology suggests that EPTB is more common in females. However, at this stage, we do not have enough data to explain why females seem to be more vulnerable to EPTB than males and whether the excess in females is universal across all clinical manifestations of EPTB or just restricted to specific forms of EPTB. Future studies are indeed needed to investigate this underexplored phenomenon.

3 Sex Differences in Tuberculosis Treatment Outcomes

Sex differences in the burden of TB have been shown in most countries (Borgdorff et al. 2000; Nhamoyebonde and Leslie 2014). However, whether a patient's sex influences TB treatment outcomes is still an area of active research. In this section, we will review publications from the last 20 years (2001–2021) focusing on this topic to summarize key findings and discuss their implications for the clinical management of TB. As a note, studies examining risk factors for poor treatment outcomes in general were not included in this section. Similarly, those investigating TB outcomes primarily in HIV and TB-infected individuals or efficacy of investigational TB drugs in randomized trials were also not included. Our search strategy identified

13 peer-reviewed publications specifically designed to address the impact of sex on active PTB treatment outcomes during the last two decades (2001–2021). The study consisted of 4 prospective cohort studies, 9 retrospective studies, and one cross-sectional survey (Table 1). They were conducted in 11 countries covering four major populations living in Asia, Africa, America, and Oceania.

Treatment outcomes were determined with at least one of the following characteristics: smear and/or culture conversion at two months, treatment failure at five months, treatment success at six months (cure), treatment default (3 months or more delay in starting treatment after being diagnosed), retreatment, and mortality. In all studies, the male-to-female ratio showed a higher TB rate in men, with an average of 2.3. Similarly, the prevalence of TB was on average higher in males (66%) confirming sex bias in TB burden across study sites. Overall, 10 out of 13 studies (~ 77%) found sex-based differences in at least one of the outcomes listed above, suggesting that males and females do not respond equally to TB treatment. Below we will summarize key findings from these studies, as presented in Table 1 of this book chapter. We will also discuss potential reasons for some of the inconsistencies between studies and important clinical outcomes that are so far missing in the literature.

3.1 Studies Finding Sex-Based Differences in TB Treatment Outcomes

In 2003, a retrospective study from Syria on newly diagnosed TB cases revealed that male sex was significantly associated with treatment failure (2.9-fold increased risk) after adjusting for confounding variables such as socio-demographic factors, health-seeking behavior, and treatment compliance (Bashour and Mamaree 2003). Two large retrospective analyses conducted in India and Bangladesh subsequently reached similar conclusions (Balasubramanian et al. 2004; Karim et al. 2008). In addition to differences in cure rates between males and females, males were more likely not only to default in treatment (Balasubramanian et al. 2004), but also to die from TB (Hazard Ratio in the Bangladesh study: 2.3, $p = 0.007$) (Karim et al. 2008). An increase in mortality rates, but not cure rates, among males was also reported in similar studies conducted in Australia and Ethiopia (Dale et al. 2017; Ramos et al. 2020). Since retrospective studies have inherent limitations such as incomplete datasets to allow for robust multivariate analyses controlling for effects of confounding variables such as age, bacterial load, drug resistance, smoking status, alcoholism, HIV, and other comorbidities, independent prospective cohort studies were conducted to address the question whether males respond less favorably to TB therapy. In Mexico, a prospective cohort study conducted on a well characterized cohort of 623 TB cases reported that male sex was associated with poorer TB treatment outcomes, including higher retreatment and treatment default rates and higher odds of death after adjusting for potential covariates including diabetes and drug resistance (Jimenez-Corona et al. 2006). Likewise, in a prospective study in Taiwan, Feng and al. found that being of

Table 1 Overview of two decades of research (2001–2021) on the relationship between patient sex and TB treatment outcomes

First author	Continent	Country	Year	Study design	Sex ratio	Prevalence in males (%)	Multivariate analyses	Outcomes
Sex-based differences in tuberculosis treatment outcomes								
Bashour, H	Asia	Syria	2003	Prospective	1.97	66.3	Yes	Worse for males
Balasubramanian, R	Asia	India	2004	Retrospective	2.70	73.3	Yes	Worse for males
Jimenez-Corona, M	America	Mexico	2006	Prospective	1.58	59	Yes	Worse for males
Karim, F	Asia	Bangladesh	2008	Retrospective	2.46	71	No	Worse for males
Feng, J.Y	Asia	Taiwan	2012	Prospective	3.41	77.3	Yes	Worse for males
Mukherjee, A	Asia	India	2012	Retrospective	2.25	69.3	No	Worse for males
Oshi, S.N	Africa	Nigeria	2015	Retrospective	1.40	57.7	Yes	Worse for males
Dale, K	Oceania	Australia	2015	Retrospective	1.20	54.5	Yes	Worse for males
Jmaa, M.B	Africa	Tunisia	2020	Retrospective	1.20	54.4	Yes	Worse for males
Ramos, J.M	Africa	Ethiopia	2020	Retrospective	1.09	52	Yes	Worse for males
Chidambaram, V	Asia	Taiwan	2021	Retrospective	2.12	68.2	Yes	Worse for males
Lack of sex-based differences in tuberculosis treatment outcomes								
Begum, V	Asia	Bangladesh	2001	Retrospective	2.84	74	No	Similar outcomes
Kamel, M	Africa	Egypt	2008	Prospective	2.20	69.2	Yes	Similar outcomes
Belo, M.T	America	Brazil	2010	Cross-sectional	1.99	66.6	No	Similar outcomes

male sex was independently associated with lower rate of sputum culture negativity at two months, which corresponds to the end of the intensive phase of therapy (OR, 1.96; 95% CI, 1.12–3.41) (Feng et al. 2012). Also, male sex was associated with older age, comorbidities, and worse treatment outcomes in the same study (Feng et al. 2012).

It is worth noting that those studies revealing sex-based differences in TB treatment outcomes were mostly conducted in Asia except for the two studies performed in Mexico and Australia. Thus, it was not clear whether the magnitude of sex-based differences in outcomes varies between geographic regions; for example, differences in circulating mycobacterial strains, human genetics, and socio-economic and behavioral factors could influence the differences based on geography. Further to this point, our group has shown that infection with *Mycobacterium africanum* (MAF), which is a species of the MTBC mostly restricted to the West African region, is associated with a delay in the onset of TB symptoms, including cough, when compared to infection with *Mycobacterium tuberculosis* (Baya et al. 2020). Accordingly, a retrospective study conducted in Nigeria, a West African country, also found that being an urban male was associated with unsuccessful TB treatment outcome after adjusting for socio-demographic and clinical factors (Oshi et al. 2015). However, there was no difference in mortality rates between males and females in the Nigerian study (Oshi et al. 2015), which is in sharp contrast to studies conducted in Asia and similar studies conducted in North and East Africa, namely in Tunisia and Ethiopia (Ben Jmaa et al. 2020; Ramos et al. 2020). These latter two studies reported an increased odds of males succumbing from TB after adjusting for covariates (Ben Jmaa et al. 2020; Ramos et al. 2020). Differences between the Nigerian's study and the other studies conducted in the African continent warrant future investigations.

We recently published a paper focused on a cohort from Mali in West Africa. Our data are more in line with the Nigerian study. Specifically, we did not find a relationship between sex and on-treatment mortality, but we rather found that males had lower smear conversion rates during the intensive phase of treatment but not at later time points of the six-month TB regimen (Dabitao et al. 2022). Though, in 2021, Chidambaram and al. conducted a retrospective analysis of a dataset generated in Taiwan which was combined with data from systematic review and meta-analysis of 398 articles (Chidambaram et al. 2021). The retrospective arm of the study found that male sex was associated with higher culture positivity at two months of treatment and higher adjusted hazard ratio (aHR: 1.53) for all-cause mortality at 9 months after initiating therapy. Meanwhile, mortality risk as function of patient sex was highly heterogenous within the meta-analysis, requiring sub-group analysis in terms of severity of disease (use of intensive care unit versus general setting) (Chidambaram et al. 2021).

Together, among studies reporting sex bias in treatment outcomes, none of them found unfavorable PTB outcomes in females compared to males. Thus, the preponderance of published data points to male sex as an independent predictor of worse TB treatment outcomes.

3.2 Studies Finding Lack of Sex-Based Differences in TB Treatment Outcomes

Although most of the studies reveal differences in TB treatment outcomes, in terms of success rate, treatment failure, retreatment, and mortality, we cannot ignore studies that fail to replicate these findings. In 2001, Begum et al. published a retrospective analysis conducted in a public health sector in Bangladesh based on a large registry of confirmed and suspected TB cases (Begum et al. 2001). The treatment success rate was similar between males and females, 86% and 84%, respectively, based on univariate analysis, suggesting lack of difference between the sexes following TB treatment. Similar findings were subsequently reported by a WHO-funded study in Egypt with a relatively small cohort of 334 individuals and by a cross-sectional and questionnaire-based survey in Brazil (Kamel et al. 2003; Belo et al. 2010). The lack of sex-based difference in TB outcomes in the Egyptian study may be explained by the fact that it included not only newly diagnosed TB cases but also relapse and treatment failure cases. Thus, it is likely that a mixture of patients was sampled including those with drug-sensitive and resistant *Mycobacterium tuberculosis* complex and those receiving different drug regimens, and this may have diluted out sex-based differences in TB outcomes (Kamel et al. 2003). A similar limitation may impact the first study by Begum et al., as drug resistance status was not reported and data were not adjusted for potential confounders (Begum et al. 2001). Likewise, a relatively high rate of missing data among males may have masked unfavorable treatment outcomes (like mortality) in the study conducted in Brazil (Belo et al. 2010).

Together, failure to find differences between the sexes in terms of TB treatment outcomes might be explained by the inherent limitations of retrospective study design and/or the quality of data collected. For future studies, we strongly recommend careful prospective collection of socio-demographic, behavior, clinical, microbial, and compliance data to gain a full picture of this phenomenon. In terms of clinical data, it may be valuable to assess lung function in addition to the classical chest X-ray in TB cases. Similarly, in addition to drug resistance testing, measuring TB drug levels or their active metabolites may identify differences in treatment compliance between the sexes, as compared to self-reported treatment compliance, which is inherently biased.

In summary, the current literature suggests that males and females do not similarly respond to TB treatment. Specifically, being male appears to be an independent risk factor for poor treatment outcomes. These findings require public health consideration by designing strategies to closely monitor treatment in males, and they also suggest that sex-specific evaluation be included in clinical trials in which dose and duration of TB treatment is being studied. This could offer an opportunity of decreasing cost of treatment, but also increase compliance in males.

4 Sex Differences in Experimental Animal Models of Mycobacterial Infection and Disease

Despite compelling epidemiological data that consistently show that tuberculosis (TB) occurs more frequently in males than females (Borgdorff et al. 2000; Nhamoyebonde and Leslie 2014), most investigations of TB pathogenesis and response to treatment or immunization have been performed in experimental animal models exclusively of one sex. This important omission has been highlighted, in part because of a growing body of studies suggesting that males and females do not respond in a similar manner to infectious and non-infectious diseases (Klein and Flanagan 2016), as well as recent recommendations for all preclinical animal studies to be conducted in both sexes from the National Institutes of Health (NIH) and top research journals (Clayton and Collins 2014). In this section, we will present studies which have been conducted in experimental animal models of TB to address the need of determining the role of sex in TB pathogenesis. The research articles presented here have been summarized in Table 2**.**

Table 2 Summary of key studies focusing on sex differences in experimental models of tuberculosis

First author	Journal	Year	Mouse strains	Mycobacterial strains	Major findings
Brown, I.N	Immunology	1987	A/J, BALB/c,CBA/Ca, C3H/He, C57BL, C57BL/10ScSn(B10), C57L, DBA/2, C57L (Ity^r/ Lsh^r/ Bcg^r)$^-$ B10.LLsh^r, B10 x B10 LLsh^r	*Mycobacterium lepraemurium (Douglas strain)*	Sex influences resistance to infection, given that males were more susceptible than females in both resistant and susceptible mice strains
Yamamoto, Y	American Review of Respiratory Disease	1990	Balb/c	*M. intracellular N-260 smooth T variant*	Mycobacteria grow more rapidly in male's macrophages compared to females
Yamamoto, Y	Infection and Immunity	1991	C3H/He, A/J, BALB/c, DBA/2, B10.A, C57BL/6	*M. marinum*	Castration increases male resistance to infection, and this effect was reversed by testosterone treatment

(continued)

Table 2 (continued)

First author	Journal	Year	Mouse strains	Mycobacterial strains	Major findings
Tsuyuguchi, K	Clinical Experimental Immunology	2001	DBA/2	*M. avium*	Lack of estrogen accelerated disease progression, as evidenced by increased number of bacteria in the lungs of ovariectomized mice compared to sham-operated animals
Bini, E.I	PLOS One	2014	BALB/c	H37Rv	Increased mortality and morbidity in non-castrated males relative to females and castrated males
Dibbern, J	Scientific Reports	2017	C57BL/6	H37Rv	Increased susceptibility to disease and death in males compared to females upon aerosol challenge
Hertz, D	Scientific Reports	2020	C57BL/6	HN878 Beijing strain and H37Rv	Impaired B cell follicles formation in the lungs is associated with accelerated disease progression in males

4.1 Infections with Non-tuberculous Mycobacteria

The first evidence of differential susceptibility to TB by sex was reported in studies of murine models of non-tuberculous mycobacterial (NTM) infection, namely *Mycobacterium lepraemurium* (Brown and Glynn 1987; Curtis and Turk 1984). The studies, conducted by two independent groups, initially sought to identify key genes involved in host resistance to mycobacterial infection, but also revealed that male mice were more vulnerable to productive mycobacterial infection than females, as

evidenced by the number of acid-fast bacilli (AFB) found in the spleen, draining lymph nodes, and footpads of infected animals (Brown and Glynn 1987; Curtis and Turk 1984). The two studies were descriptive, thus did not address the mechanistic basis of the observed sex-dependent phenotype. This was subsequently investigated by Yamamoto and collaborators through their examination of mice infected with *Mycobacterium intracellulare* and *Mycobacterium marinum* (Yamamoto et al. 1990, 1991). They found that the macroscopic lesions after intravenous (IV) injection of the *M. intracellulare* smooth T variant were more extensive in visceral organs of males compared to females and that significantly higher numbers of mycobacteria were recovered from the lungs of infected male mice relative to females, reinforcing the initial observation that male mice are more susceptible to infection than female mice. Furthermore, peritoneal macrophages from males were more permissive to productive infection than those from the opposite sex suggesting more potent antimicrobial activity of female compared to male macrophages. Since establishing that susceptibility to TB is influenced by host genetics, the same group of investigators performed a series of experiments using *Mycobacterium marinum* to infect common strains of mice including susceptible strains such as BALB/c and resistant ones like DBA/2 (Yamamoto et al. 1991). Once again, in both resistant and susceptible strains, males were found to be more susceptible to infection than females. Specifically, 90% of males succumbed to infection within the first 41 days of infection, while all females survived up to 80 days of infection. Combined, these results indicate that biological sex can influence mycobacterial disease outcomes and should be considered when conducting investigations in the context of TB. To determine whether sex hormones are involved in this process, Yamamoto et al. compared the phenotypes of castrated male mice to non-castrated male and female mice following mycobacterial infection and found that castration increased male resistance to infection to similar level as that seen in females with the effect of castration being reversed by exogenous testosterone treatment. A key outcome of testosterone treatment was reduction of mycobacteria in the lungs and kidneys suggesting a pathogenic role for testosterone in TB infection. Furthermore, resistance to infection in female mice was explained by a more potent immune response that is dependent on effective T cell responses and macrophage recruitment to the lungs of infected animals. Although these experiments were conducted mostly with the use of IV injections of high doses of mycobacteria with relatively few details provided regarding tissue pathology and the cell types involved in the local immune responses, they have nevertheless helped to lay the foundation to explore sex-dependent effects on innate and adaptive immune responses to TB.

Approximately one decade after these early studies Tsuyuguchi et al. revealed, in 2001, for the first time a protective role of estrogen during *Mycobacterium avium* complex (MAC) pulmonary infection in mice (Tsuyuguchi et al. 2001). MAC is an NTM microbe that typically causes lung disease in patients with immunodeficiencies or underlying structural lung disease, and MAC lung disease has long been known to be more prevalent in older populations and in women. The Tsuyuguchi et al. study was based on the prevailing theory that older women were more susceptible to *M. avium infection* due to a reduced level of estrogen during menopause.

Ovariectomized and sham-operated DBA/2 female mice were infected by intra-tracheal instillation of *M. avium*. Three weeks after infection, the mycobacterial burden in the lungs of ovariectomized mice was found to be significantly higher than in sham-operated mice, and treatment of the ovariectomized mice with estradiol (E2) reduced colony forming units (CFUs) to the same level as in sham-operated mice. In addition to these in vivo data, in vitro studies of murine macrophages showed that E2 pre-treatment of interferon-γ-activated macrophages significantly increased their antimicrobial activity, as evidenced by augmented production of reactive oxygen intermediates. While mouse studies such as this underscored the protective effects of estrogen against MAC infection in mice, it is important to recognize that the increased disease severity of NTM infections in male mice does not match the epidemiology of NTM lung disease in humans where women are disproportionately affected. This discrepancy is likely due to the fact that these mouse models do not account for underlying lung disease, typically bronchiectasis not in the context of cystic fibrosis (non-CF bronchiectasis), which occurs with greater severity in women and is an important risk factor for NTM lung disease (Morrissey and Harper 2004; Vidaillac et al. 2018).

4.2 Infection with Mycobacterium tuberculosis Complex

The common theme of these studies was their focus on NTM infection. It was, however, unclear whether the findings would be reproducible in animals infected with *Mycobacterium tuberculosis*, specifically by aerosol challenge, which more closely replicate natural infection. Work published by Bini et al. in 2014 (Bini et al. 2014) showed for the first time that BALB/c male mice infected by intra-tracheal instillation of the *M. tuberculosis* H37Rv strain were more susceptible to disease than females, as evidenced by higher mortality and mycobacterial burden in the lungs of male mice. Consistent with previous reports on NTM, the increased susceptibility of male mice was prevented by castration indicating again a detrimental role for testosterone upon exposure with pathogenic mycobacteria. Substantially higher mRNA expression of inflammatory markers such as TNF-α, IFN-γ, IL-12, iNOS, and IL-17 were found in females and castrated male mice when compared to non-castrated male animals. Granulomas found in females appeared to be larger than in males, reinforcing the hypothesis that sex differences in immune responses to *M. tuberculosis* mediate disease outcomes in experimental animal models of TB. Recently, a group led by Sneider et al. addressed this hypothesis using the C57BL/6 mouse strain previously known to be more resistant to mycobacterial disease compared to BALB/c mice (Brown and Glynn 1987). Their findings confirmed previously described findings of accelerated disease progression in males infected by aerosolized *M. tuberculosis* H37Rv, including increased morbidity and mortality compared to females (Dibbern, Eggers, and Schneider 2017). They also found higher numbers of CFUs and reduced numbers of lymphocytic aggregates in the lungs of males versus females. In contrast to the Bini's study in BALB/c mice, there was an increased expression of iNOS in

the lungs along with sustained increase of pro-inflammatory cytokines (IFN-γ, IL-6, and IL-1β) and chemokines (RANTES, Eotaxin, MCP-1, etc.) in infected C57BL/6 males relative to the opposite sex. Notably, heightened cytokine levels correlated with the higher lung CFU counts and more aggressive lung pathology observed in males. The differential outcome was independent of *M. tuberculosis* strain since the same phenotype was found with infection by a more pathogenic strain, namely the Beijing lineage strain HN878 (Hertz et al. 2020). Mechanistically, the authors proposed in a subsequent publication that impairment in B cell follicle formation in the lungs occurs with accelerated disease progression in males and may be causally associated with the more aggressive disease seen in males. Indeed, they also found a reduced expression of cytokines of the Th17 axis (i.e., IL-23, IL17A, and IL-1β) after infection with HN878 in males, as well as a decrease in the expression of chemokines (CXCL13, CCL19) known to be involved in lymphocytic trafficking to infected lungs.

These findings, together, strongly suggest differential disease outcomes between males and females following experimental murine infection with mycobacteria (Table 2). They also support a predominant role for biological factors in mediating sex differences in TB, as animal studies are not impacted by the effects of non-biological factors that are inherent to human studies. However, many unanswered questions still need to be addressed. For example, none of the studies have fully characterized the immune cells that mediate this sex-specific phenotype, nor have they established immune correlates for relative resistance to disease in females. Since both innate and adaptive immune responses are responsible for successful control of *M. tuberculosis* infection, the cell-type-specific immune responses that govern sex differences during TB need to be determined. Moreover, most studies were conducted in animal models of chronic mycobacterial infection, and we need to determine if similar phenotypes occur in experimental models of acute infection. Similarly, the capacity for production of antimicrobial peptides in infected animals by sex has not been investigated. Lastly, the impact of other potential biological factors such as genetic factors, as many immunologically important genes reside on the X chromosome, has not yet been fully worked out, indicating that more studies are indeed needed to better understand why male animals are more susceptible to TB infection than females.

5 Potential Mechanisms Mediating Sex Differences in Tuberculosis Pathogenesis

Based on current literature, two main biological factors appear to mediate sex differences in TB pathogenesis. The first one is sex hormones, and the second one is genetic factors (Gupta et al. 2022). Thus, we will be discussing them in this section and present additional putative factors that could be contributing to the male bias in TB.

5.1 Sex Hormones

Hormones are organic compounds, primarily secreted by endocrine glands, which function to regulate physiological and metabolic activities of cells and tissues (Starka and Duskova 2020). They can be classified in two major categories based on their chemical structure, namely the steroid hormones and the non-steroid hormones. Steroid hormones are all synthesized from cholesterol and are lipid-soluble which facilitates their transport outside cells via the plasma membrane to the blood circulation (Holst et al. 2004). There are three types of steroid hormones, specifically glucocorticoids, mineralocorticoids, and sex hormones (Holst et al. 2004). This section will discuss effects of latter one on TB pathogenesis.

Sex hormones are produced by the testis, ovaries, adrenal glands, or by conversion of sex steroids in specific tissues (Hiller-Sturmhofel and Bartke 1998; Holst et al. 2004). Their effects on target cells are mediated through genomic regulation upon binding to their nuclear receptors in the cytoplasm followed by nuclear translocation or via non-genomic mechanisms by binding to their cognate membrane-associated receptors and triggering downstream signaling cascades (Hiller-Sturmhofel and Bartke 1998; Holst et al. 2004). Sex hormones include androgens, estrogens, and progestogens. Androgens are generally considered to be male hormones because of their effects in promoting masculinization, while estrogens and progestogens are typically considered to be female hormones because estrogens promote feminizing effects, while progestogens are mainly involved in pregnancy (Hiller-Sturmhofel and Bartke 1998). It should be noted that they are all found in both sexes, yet at different quantities. The most predominant forms of these hormones in humans are testosterone, estradiol (E2), and progesterone. Below, we will discuss how these hormones could contribute to immunity in general, then specifically to sex differences in TB pathogenesis.

The effects of sex hormones on immunity have been extensively reviewed (Cervantes et al. 2022; Wilkinson et al. 2022; Klein and Flanagan 2016), but here we will summarize the main effects which are relevant to anti-mycobacterial immune responses. In humans, reports have shown that the sex hormones, including androgens (e.g., testosterone and dihydrotestosterone), estrogen (17β-estradiol), and progesterone, are able to modulate the recruitment and function of various immune cell types, as these cells express cognate receptors for the sex hormones (Klein and Flanagan 2016; Fish 2008). This is thought to be achieved by discrete, overlapping, and sometimes opposing effects of the hormones on immune cells. The general consensus is that testosterone and progesterone have immunosuppressive activities, while the effect of estrogen is more complex and likely dose-dependent. Although this may be an oversimplistic way for describing effects of these hormones on immunity, it can help our basic understanding of the role of sex hormones in the pathogenesis of TB and provide the foundation for future mechanistic studies. For example, low levels of estradiol promote Th1 responses, while high levels of 17β-estradiol favor Th2 immunity (Straub 2007). In humans, estrogen has been shown to induce the production of pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-12 (all key

effector cytokines for Th1-induced immunity) while blocking secretion of IL-10 (a potent anti-inflammatory cytokine) (Fox, Bond, and Parslow 1991; Straub 2007). The ability of estrogen to skew immune responses is thought to be largely due to its capacity to regulate the transcriptional activity of cytokine genes. In fact, IFN-γ and its associated transcription factor T-bet have estrogen response elements in their promoter (Fox, Bond, and Parslow 1991; Karpuzoglu et al. 2007). In contrast, testosterone has been shown to have the opposite effect on these cytokines (Janele et al. 2006; Lotter et al. 2013; Pinzan et al. 2010). Specifically, testosterone diminished production of IFN-γ, TNF-α, and reactive oxygen species by macrophages (D'Agostino et al. 1999; Janele et al. 2006; Lotter et al. 2013). For example, men with androgen deficiencies, which are manifested by low levels of circulating testosterone, have higher levels of pro-inflammatory cytokines when compared to men with normal androgen levels (Bobjer et al. 2013; Musabak et al. 2003). In addition to Th1 cells, activation of macrophages enabling their mycobactericidal ability, which is pivotal for TB containment, is increased by estradiol (Calippe et al. 2008), while testosterone suppresses macrophage activation via downregulation of pathogen recognition receptors (PRRs) such as Toll-like receptor 4 (TLR4) (Fish 2008).

With regard to TB, one of the earliest pieces of evidence supporting the contribution sex hormones in TB pathogenesis in humans was reported by Hamilton and colleagues in 1969 (Hamilton and Mestler 1969). In this study, the authors revealed that eunuchs (i.e., castrated men with very low testosterone levels) were less likely to die of TB (8.1%) compared to males with no castration (20.6%) (Hamilton and Mestler 1969), suggesting a detrimental role for testosterone during TB. This data has been replicated in mice by independent groups as discussed earlier (Dibbern et al. 2017). In addition, the global TB report has consistently shown that the male bias in TB burden (incidence and prevalence) does not start until puberty. For instance, the sex ratio of the number of TB cases in boys compared to girls who are less than 15 years was 1.09 in 2019 (WHO 2020a). This ratio increased to 1.72 after puberty, which is clearly a major sex-specific change in disease risk that correlates with the surge of testosterone levels in males during puberty.

To date, direct mechanistic effects of the sex hormones on immunity against TB in humans have not been defined. Nevertheless, a growing body of knowledge exists in experimental models of TB as reviewed above and on the impact of sex hormones on the inflammatory cytokine responses known to be associated with TB disease. In 2007, Rey and colleagues studied the relationship between endocrine responses, including pituitary, adrenal, gonadal, and thyroid hormones, and inflammatory cytokines responses in HIV negative males presenting with different degrees of pulmonary TB who were naïve to treatment (Rey et al. 2007). They found that testosterone and dehydroepiandrosterone (DHEA) levels were significantly reduced, while IFN-γ, IL-10, and IL-6 were all increased in these men with active TB. In contrast, estradiol, cortisol, prolactin, and thyroid hormone were modestly increased in the same male patients (Rey et al. 2007). Additionally, when PBMCs from these males were stimulated with *Mycobacterium tuberculosis* (MTB) antigens, supernatants were collected from these cells inhibited the production of DHEA by the adrenal cell line NCI-H295-R in vitro suggesting that soluble mediators from immune

cells of TB-infected males play a role reducing androgen secretion by adrenal cells (Rey et al. 2007). A similar reduction of testosterone level in male TB patients and a reduced level of pro-inflammatory cytokine levels was later confirmed by Bini and al. (2015). In that study, the authors also demonstrated a reduction of testosterone levels following treatment of Leydig cells with TNF-α, TGF-β, and IFN-γ in vitro, suggesting that gonadal androgen release may be inhibited by inflammatory cytokines in males with TB (Bini et al. 2015). Dysregulation of hormonal responses during TB was also detected in post-menopausal women (defined as not having menstruation for the past 12 months) with TB in Turkey (Erbay et al. 2016). Specifically, estrogen levels were found to be significantly higher in post-menopausal women with TB compared to those with no TB. In contrast, in a cohort of 100 pre-menopausal women (mean age ~ 36 years) with PTB from Egypt and 50 healthy controls, sex hormones, including progesterone and estradiol were significantly reduced in PTB cases when compared to the healthy controls (Magdy et al. 2019). The opposite was observed with follicle stimulating hormone (FSH) and luteinizing hormone (LH). Remarkably, there was a positive correlation between bacterial load and sex hormones levels; women with the highest smear grade of 3 + had the lowest progesterone and estradiol levels, but had higher FSH and LH concentrations (Magdy et al. 2019). Thus, active TB in pre-menopausal females is associated with reduced estrogen levels, but this effect seems to be reversed (higher estrogen levels) in post-menopausal women. This observed reduction in estradiol and progesterone also suggests that PTB may have a negative impact on reproductive function in females. In keeping with this, a different report from Egypt revealed that 67.2% of males with PTB had erectile dysfunction (Magdy et al. 2019). As mentioned earlier, mean testosterone levels are significantly decreased in males with PTB, a factor which may explain the observation of erectile dysfunction in males with active TB. It is important to note that these hormonal profile alterations during PTB appear to be reversible as testosterone, estradiol, DHEA, and cortisol levels were found to return to normal levels with treatment (Tsegaye et al. 2022).

In addition to the interplay between endocrine and cytokine responses, other important host defense mechanisms appear to be modulated by the sex hormones. For example, DHEA, a precursor of testosterone and estradiol, was shown to promote autophagy in MTB-infected macrophages, hence reducing intracellular bacterial burden (Bongiovanni et al. 2015). In contrast, DHEA had no observable effects on cytokine production nor on the anti-inflammatory effect induced by cortisol treatment of MTB-infected macrophages in the same cells (Bongiovanni et al. 2015). Based on this study, DHEA-induced autophagy appears to be a host-beneficial hormonal mechanism that may contribute to control MTB growth. In contrast to the findings on DHEA, Gan et al. reported that estradiol treatment of human bronchial epithelial cell lines infected by MTB inhibited autophagy and reduced the levels of reactive oxygen species (ROS), and this was accompanied by a reduction of MTB growth in infected cells (Gan et al. 2022). Thus, whether induction or inhibition of autophagy by sex hormones contributes to sex differential effects in TB in vivo remains to be clarified. Nevertheless, the role of sex hormones on host responses to MTB is not limited to

autophagy alone; for example, DHEA has been also shown to promote the production of antimicrobial peptides such as cathelicidin and human β-defensin (HBD)-2 and HBD-3, and the elevated levels of these antimicrobial peptides correlated with a decrease of intracellular bacterial burden in both macrophages and epithelial cells (Marin-Luevano et al. 2021).

Along the same line, the impact of sex hormones on immune responses to BCG immunization against TB has also been investigated. Neonatal BCG vaccination has been shown to protect infants from childhood respiratory tract infections and to reduce overall childhood mortality in a sex-dependent manner with girls showing greater benefit than boys (Biering-Sorensen et al. 2018; Roth et al. 2005; Stensballe et al. 2005). This ability of BCG to prevent heterologous infections has been termed "trained immunity" and can be modeled in vitro by exposing monocytes to BCG (or other infectious agents or antigens), allowing the cells to rest, and then stimulating with a heterologous infectious agent or antigens followed by measurement of pro-inflammatory cytokines. Trained immunity, as such, represents a type of innate immunological memory primarily in myeloid cells (Netea et al. 2011). In 2018, a research group from the Netherlands has studied the impact of sex hormones on BCG-induced trained immunity ex vivo (de Bree et al. 2018). Co-treatment of monocytes derived from males with BCG and dihydrotestosterone (DHT) led to significant reductions of TNF-α and IL-6 production (de Bree et al. 2018). Meanwhile, addition of estradiol and BCG to monocytes collected from females inhibited production of only TNF-α but not IL-6. In contrast, neither DHT nor estradiol altered BCG-induced training of monocytes from both sexes 6 days prior to a second stimulation with LPS. The authors concluded that sex hormones do not play a significant role in the known sex-based differences observed after immunization with BCG (de Bree et al. 2018). In a second study, the same group evaluated 303 healthy, BCG vaccinated subjects at the time of BCG vaccination and 90 days later (Koeken et al. 2020). They showed while monocytes from BCG vaccines had enhanced cytokine responses in vitro to BCG restimulation, there was on overall reduction of serum inflammatory markers 90 days after vaccination compared with 14 days post-vaccination. The reduction in systemic inflammation was significantly greater in men who received BCG than in women (Koeken et al. 2020). Thus, BCG appears to have a heterologous protective effect against non-specific infections in childhood that is stronger in females than males, but also an anti-inflammatory effect which is stronger in males.

In summary, much remains to be learned about the effects of sex hormones on the male bias associated with TB. Our basic understanding of the role of sex hormones in the pathogenesis of TB is derived primarily from animal studies. Future studies using well-defined clinical cohorts of both sexes covering the spectrum of TB diseases (i.e., latent, active, and extrapulmonary TB) are warranted to unravel this phenomenon.

5.2 Genetic Factors

Twin studies, family-linkage evaluations, and candidate-gene studies have all suggested that genetic factors contribute to susceptibility to TB (Abel et al. 2014). In a seminal study, Kallmann et al. reported that the proportion of twin siblings of index cases developing active disease was 66.7% in monozygotic (identical) twins versus 23% in dizygotic twins (Kallmann and Reisner 1943). The finding that monozygotic twins were at higher risk for TB than dizygotic twins strongly suggested that having a different assortment of parental genes was protective for the dizygotic twins and strongly suggested a genetic component to TB susceptibility. Likewise, the diameter TST responsiveness, which is the amount of variability in skin induration following intradermal injection of tuberculin, was found to correlate to a higher degree in monozygotic twins than in dizygotic in a study from The Gambia, West Africa (Jepson et al. 2001). In addition to human studies, murine models have also demonstrated that host genetic background mediates disease outcome (O'Garra et al. 2013), notably certain mice strains have been shown to be relatively resistant to MTB disease, while others are highly susceptible. This led to the identification of the *Bcg* locus on chromosome 1 that was later found to harbor a polymorphic gene *Nramp1* which has been shown to promote resistance to intracellular pathogens, including mycobacteria (Vidal et al. 1995). Additional murine genes that have alleles which appear to confer susceptibility to TB include *HLA* class II, *VDR*, *MAL/ TIRAP*, *DC-SIGN*, *MCP-1*, and *TLR8* (Neyrolles and Quintana-Murci 2009). Human studies have implicated some of these genes in partial susceptibility to TB, and this supports a model of human TB susceptibility being dependent on the cumulative effects of many genetic variants.

The introduction of unbiased genomic strategies such as genome-wide association (GWAS) has expanded the list of genetic polymorphisms associated with TB. The goal of this strategy is to systematically detect genomic loci with substantial positive associations on having active TB. Genetic variants have been identified in diverse ethnicities including African, Europeans, and Asian. However, GWAS data in TB have been challenging to replicate in different settings, perhaps because of the low frequency of the variants detected in certain populations combined with need to have a large sample size to reach genome-wide significance. In addition to these limitations, whether the variants detected by GWAS contribute to sex-specific differences in TB incidence has not been rigorously investigated. The only GWAS study that found a variant on the q arm of the X chromosome (Xq) in Africa was not replicated in other studies (Bellamy et al. 2000). Also, GWAS studies in TB are usually not stratified by sex, and most of them have focused on autosomal chromosome analysis only thereby excluding the X and Y chromosomes from consideration. To our knowledge, the only study that has addressed this oversight using a clinical cohort from South Africa did not find any signification associations in any of the 22 autosomal chromosomes or in the X chromosome based on sex-stratified or combined analysis (Schurz et al. 2018). This lack of finding human polymorphisms on the X chromosome that correlate with TB suggests, but does not prove, that the sex chromosomes play a minimal role in

contributing to the male bias for active TB. Nevertheless, there is additional evidence from other approaches that the sex chromosomes do influence TB susceptibility, and these will be described below.

Sex determination in mammals is governed by the two sex chromosomes: X and Y. The X chromosome is present in both sexes and encodes approximately 1100 annotated genes, whereas the Y chromosome is present only in males and contains only around 100 genes (Libert et al. 2010). The fact that males have only one X chromosome leads to an evolutionary disadvantage when recessive mutations occur in genes located on the X chromosome. For this reason, most of the X-linked primary immunodeficiency diseases (PID) do not affect females or cause less severe disease manifestations. The Mendelian susceptibility to mycobacterial diseases (MSMD) gene set represents a collection of known primary human immunodeficiencies that predispose to mycobacterial diseases (Bustamante et al. 2014). Typically, children with mutations in MSMD genes present with opportunistic infections by BCG or to non-tuberculous mycobacteria (NTM), and the known MSMD genes currently comprise a group of genetic mutations in genes (Bustamante et al. 2014). Among these genes, seven are autosomal genes (*IFNGR1*, *IFNGR2*, *IL12B*, *IL12RB1*, *STAT1*, *IRF8*, and *ISG15*) and two are X-linked, namely NEMO (NF-kB essential modulator) and CYBB (cytochrome b-245 beta chain) and hence responsible for X-linked recessive MSMD (Bustamante et al. 2014; Schurz et al. 2019).

The NEMO (NF-kappa-B essential modulator) protein comprises a regulatory subunit of the inhibitor of NF-κB (IκB) kinase (IKK) (Bustamante et al. 2014; Schurz et al. 2019). Null mutations in the NEMO gene abolish NEMO-dependent NF-κB activation causing in *utero* lethality in males (Libert et al. 2010; Smahi et al. 2000). Conversely, polymorphic mutations in the NEMO gene are associated with impairment of NF-κB signaling, specifically in CD40- and c-Rel/NF-κB-mediated production of IL-12 by myeloid cells, leading to a rare congenital disease in male patients characterized by abnormal development of hair, teeth, and nails and an increase in susceptibility to a wide range of pathogens, including NTM (Filipe-Santos et al. 2006; Libert et al. 2010). Meanwhile, the CYBB protein, commonly known as GP91-PHOX, NADPH oxidase, or NOX2, is an essential component of the microbicidal phagocytes oxidase system (Libert et al. 2010; Smahi et al. 2000). CYBB deficiency impairs superoxide production in phagocytic cells leading to an inability to kill pathogens present in phagocytic vacuoles. This genetic defect is responsible for a PID named X-linked chronic granulomatous disease (X-CGD) (Libert et al. 2010). X-CGD patients are affected by recurrent and severe bacterial and fungal diseases, mostly due to *Staphylococcus* and *Aspergillus but also upon* exposure to BCG (Bustamante et al. 2007). A recent genetic analysis of 636 Chinese TB patients compared with 608 healthy control subjects identified the rs5917471 T allele as being protective against active TB with a stronger association for males compared to females (Liu et al. 2015). Thus, in addition to the strong X-linked male TB predisposition to mycobacterial diseases for loss-of-function mutations in NEMO and CYBB, it also appears non-loss-of-function mutations at least in the CYBB gene that may contribute to the male sex bias for TB.

Another layer of genetic difference between males and females in terms of X-linked genes is the fact that one X is inactivated in females. This mechanism is known as X chromosome inactivation (XCI) and results in random silencing of one maternal X chromosome (Schurz et al. 2019). XCI therefore comprises an important mechanism to maintain equal gene expression dosage of X-linked genes in males and females. XCI is thought to be maintained by epigenetic mechanisms during cellular division to ensure a balanced gene expression of X-linked genes. However, some genes can escape inactivation or silencing, and it has been estimated that 15% of X-linked genes escape inactivation (Fish 2008). Genes that are more likely to escape silencing are located at the distal end of the X chromosome (Xp) and are enriched with markers that are associated with active gene expression (i.e., open chromatin marks). Escape from XCI has, indeed, been shown to play a protective role for females in certain cancers often by enabling expression of a second tumor suppressor gene (Fish 2008). In light of this, there is speculation that escape from XCI in X-encoded immune response genes in females could translate to protection of females against infections such as TB. Example of immune genes that were found to escape silencing is tissue inhibitor of metalloproteinase 1 (TIMP1), dual specificity phosphatase 21 (DUSP21), GATA-binding protein 1 (GATA1), NF-κB activating protein (NKAP), and IL-1R-associated kinase (IRAK1) (Libert et al. 2010).

While the question of escape from XCI as a mechanism to explain the male sex bias in TB remains an open question, there have been several circumstantial clues suggesting that escape from XCI may play a role. It has been noted that many X chromosome-encoded immune genes are in the distal regions of the Xp arm where they are predicted to have a higher chance of escaping XCI (Schurz et al. 2019). Examples of such distally encoded genes include CYBB, Toll-like receptor (TLR) 7, and TLR8. TLR8 is of considerable interest in terms of genetic predisposition to TB because of reports that polymorphisms in TLR8 are associated with differential susceptibility to TB between males and females (Schurz et al. 2019). Four single-nucleotides polymorphisms (SNPs) have been described in *TLR8*, namely rs3764879, rs3788935, rs3761624, and rs3764880 (Davila et al. 2008; Schurz et al. 2019). These SNPs were associated with increased TB risk in males, but not in females in Indonesia and Russia (Davila et al. 2008). In a subsequent study from South Africa, however, Salie et al. found that rs3761624 increased the risk of TB in South African females only, while rs3764879 and rs3764880 were associated with TB susceptibility in both sexes yet with differential effects (Salie et al. 2015). Inconsistencies between those studies may be due to the influence of ethnicity and/or differences in the virulence of MTB strains in the two regions.

In addition to X-linked protein coding genes, the X chromosome also encodes an estimated 112 micro-RNAs (miRNA) compared to just two on the Y chromosome (Nhamoyebonde and Leslie 2014). Specifically, miRNA are small non-coding RNAs usually consisting of 18–24 nucleotides which are involved in regulation of gene expression at the post-transcriptional level (Sinigaglia et al. 2020). They perform their regulatory activity by binding to the 3' untranslated region (UTR) of their target messenger RNA leading to its degradation or to inhibition of protein synthesis. Many miRNAs have immunoregulatory activities and govern gene expression in most if not

all immune cells. miRNAs are thought to regulate immune function by four mechanisms: (i) inhibition of innate immunity, (ii) suppression of inflammatory responses, (iii) inhibition of phagosome maturation and autophagy, and (iv) inhibition of apoptosis (Sinigaglia et al. 2020). Examples of X-linked microRNA are miRNA-223, miRNA-448, miRNA-18b, miRNA-106a, miRNA-503, miRNA-424, and miRNA-105-2 (Schurz et al. 2019). Like the X-linked protein coding genes, miRNAs can theoretically escape XCI which may also lead to consequences on target gene expression. Dysregulation of miRNA expression has been associated with many infections including TB as well as certain non-infectious diseases. For example, miR-146a, miR-21, miR-132, and miR-155 are known to suppress TLR-mediated inflammatory responses in myeloid cells which could promote *Mycobacterium tuberculosis* (MTB) survival (Kim et al. 2017). Conversely, downregulation of let-7f, miR-20b-5p, and miR-142-3p was associated with suppression of inflammation during TB (Sinigaglia et al. 2020). Additionally, mice lacking the X-linked miRNA-223 fail to control MTB infection due to a massive recruitment of neutrophil and excessive inflammation in the lung which suggests miR-223 has a protective effect during TB by regulating chemotaxis and inflammatory responses (Dorhoi et al. 2013).

In addition to the roles of miRNA in TB pathogenesis, a growing body of evidence suggests their potential usefulness as biomarkers for TB diagnosis or to distinguish active TB disease from LTBI. Several approaches including microarrays, RNA-Seq, and RT-PCR on serum, plasma, PBMCs, specific cell subsets, or exosome vesicles have been used to exploit miRNA expression for diagnostic purposes. For instance, miR-29a-3p and a group of six exosomal micro-RNAs (miR-20a, miR-20b, miR-26a, miR-106a, miR-191, and miR-486) were found to be significantly overexpressed in active TB patients relative to healthy controls (Ndzi et al. 2019; Hu et al. 2019). Two of these miRNAs (miR-29a and miR-26a) are also known to target the IFNγ-dependent immune axis, hence their relevance in TB pathogenesis (Sinigaglia et al. 2020). Similarly, Latorre and al. have found that expression of three miRNA (miR-150, miR-21, and miR-29c) is dysregulated in active TB cases when compared to LTBI and healthy subjects (Latorre et al. 2015). Likewise, upregulation of miR-29a-3p was successfully used to discriminate active TB versus LTBI in Cameroon, Central Africa (Ndzi et al. 2019).

In summary, the role of genetic factors in sex-specific susceptibility to TB has been underexplored. Current data on the role of X-linked genes and miRNA and their silencing in TB pathogenesis are promising but lack clear mechanistic insights. New studies are needed to address this oversight.

5.3 Putative Biological Factors

Besides hormonal and genetic factors and their underlying effects on immunity, additional intrinsic biological differences between males and females do exist in terms of lung anatomy and respiratory function and metabolism which also require consideration. These biological factors, that are known to be influenced by sex at

a physiological level, have not been rigorously investigated in the context of TB. Inherent sex differences in the lung anatomy and physiology between males and females are known to exist from infancy to adulthood and have been reviewed by Townsend et al. (Townsend et al. 2012). For example, females are known to have a smaller lung dimensions and central airway diameters than males, and this is thought to have a beneficial effect on lung function in terms of respiratory mechanics and airflow (Dominelli et al. 2018; Townsend et al. 2012). However, to date little research exists on sex-specific alterations in lung function in individuals affected with PTB before and after TB treatment by combining standard measures of pulmonary function such as spirometry, 6-min walk test, chest X-ray, and/or high-resolution CT. Such an approach could certainly help tease out the impact of biological differences between the sexes during TB stemming from intrinsic difference in lung anatomy between the sexes.

Regarding metabolism, the impact of metabolic pathways in regulating immune responses to TB is a growing field of research, but the potential influence of biological sex in these processes has not been well-studied. Nevertheless, reports have revealed substantial metabolic changes in host cells after mycobacterial infection, as reviewed by Park et al. (2021). For example, macrophages were shown to exhibit an increased in glucose consumption and lactate production in the presence of oxygen early after being infected with MTB suggesting an interplay between metabolic pathways and immune responses to MTB (Cumming et al. 2020). In a clinical cohort from South Africa, Weiner et al. reported a decrease in metabolites such as histidine, cysteine, glutamine tryptophan, citrulline, and creatine in the serum of active TB patients, while levels of kynurenine, phenylalanine, and pyroglutamine were increased (Weiner et al. 2012). The same research group was also able to identify a TB-specific metabolic profile which the authors suggest may be useful for predicting the onset of clinical disease for up to a year prior diagnosis (Weiner et al. 2018). Indeed, metabolic alterations during TB have been noted across several species including humans, mice, and zebrafish suggesting their usefulness as biomarkers of TB disease progression (Ding et al. 2020). Similarly, TB-associated metabolic changes were not also substantially impacted by infecting MTB strains as revealed by Tientcheu et al. in a study conducted in The Gambia. Specifically, infection with MTB and MAF was both associated with similar metabolic changes including a rise in the levels of histidine, ornithine, tryptophan, and leucine (Tientcheu et al. 2015). Conversely, MTB itself can also change its metabolic state to a persister state in which the bacilli are refractory to antibiotic killing (Ehrt et al. 2018). Whether sex hormones such as testosterone and estradiol are sensed by the bacteria leading to effects on MTB-specific metabolic changes such as entry into the persister state remains unknown.

6 Gender-Based Factors

According to WHO, gender refers to characteristics that are defined by society to distinguish men from women, as well as the relationship between these two groups of individuals (WHO 2019). Those characteristics include cultural norms, behaviors, and roles in the society that are typically associated with being a man or a woman. Thus, the contribution of gender in disease risks or outcomes is challenging to measure as it appears to be both context- and time-dependent. This is further complicated by the fact that gender can intersect with sex, which refers to biological differences between males and females in terms of genetics (sex chromosomes), sex hormones, and anatomy (reproductive organs) (Klein and Flanagan 2016). In this section, we will attempt to summarize our view regarding the potential role of gender in TB burden, by discussing existing theories and relevant studies conducted on this topic.

6.1 Access to Health Care and Health-Seeking Behaviors

Male bias in TB is traditionally thought to be primarily driven by gendered factors which are non-biological factors. We are just beginning to understand the potential effects of biological factors in sex differences during TB. A recurring argument to explain the excess of cases in males is the possibility of under-reporting or under-diagnosis of TB in women due to difference in healthcare access or health-seeking behaviors between men and women (Thorson 2015). We think this argument is weak since sex bias in TB burden does exist in high-income countries, where universal health care is fully in place and women are keenly aware about their rights and benefits when it comes to health care. Conversely, certain clinical presentations of TB which occur outside the lung's parenchyma (i.e., extrapulmonary TB) were found to be more prevalent in women compared to men as discussed earlier (Katsnelson 2017; Padberg et al. 2015). If differences in health-seeking behaviors or access to health care were responsible of sex bias in TB notification rates, one would also expect to see male bias in most forms of EPTB, which is clearly not the case. As mentioned earlier, in a meta-analysis of surveys of 28 countries spanning from 1993 to 2016, Horton and colleagues have revealed that adult men leaving in LIMCs are less likely to be timely diagnosed than the opposite sex (Horton et al. 2016), which suggest that women are not disadvantaged in seeking or getting access to TB clinics in those settings. A recent study conducted in West Africa has also ruled out the impact of sex on TB diagnosis delay since low education level, social factors, and lack of consideration of TB as potential etiology by healthcare practitioners rather than patient's sex were independently associated with TB diagnosis delay (Soumare et al. 2022). Thus, differential access to health or difference in health-seeking behaviors might not explain the global male bias in TB burden and treatment outcomes.

6.2 Smoking, Professional Exposure, and Overcrowding

However, the impact of other non-biological factors such as smoking, alcohol abuse, high-risk profession, and overcrowding may be more nuanced, hence requiring in-depth investigation for full understanding. Globally, men are more likely to smoke and abuse alcohol and illicit substances than women (GBD 2018, 2021). In 2019, tobacco was the leading risk factor of men deaths around the world (GBD 2021). Along those lines, an ecological study has shown that 30% of the differences between males and females in terms of TB risk can be attributed to smoking (Watkins and Plant 2006). However, sex differences in TB outcomes persisted when the impact of smoking on TB outcomes was controlled in clinical cohort studies by performing multivariate regression analysis (Feng et al. 2012; Chidambaram et al. 2021). Still, TB disease severity in the lungs was more pronounced in males with no history of smoking relative to females (Chu et al. 2019). Additionally, human studies were recapitulated in laboratory animals infected with MTB, which are not exposed to tobacco smoke or environmental factors (Hertz and Schneider 2019; Gupta et al. 2022). Another argument is that men tend to enroll in high-risk professions (such as mining) and frequent crowded spaces with poor air ventilation (such as bars and prisons) that might increase the likelihood of acquiring TB or other respiratory infections relative to women. This hypothesis is further supported by studies conducted during the COVID-19 pandemic, revealing that men are less likely to wear masks and adhere to preventing measures such as social distancing to minimize transmission (Galasso et al. 2020; Looi 2022). Yet, we also know that nearly 20% of TB exposure occurs in the household, independently of professional exposure and casual community contacts (Martinez et al. 2017). For these reasons, gendered factors (i.e., smoking, high-risk profession, overcrowding, social behaviors) alone might not adequately explain the global male bias observed in TB. However, we hypothesize that they could be interacting with biological factors to promote sex-based differences in TB pathogenesis and treatment outcomes. For example, a higher treatment dropout rate in men compared to women has been reported in independent studies (Weiss et al. 2006; Dabitao et al. 2022), suggesting that men are less compliant than women during treatment of PTB.

Nevertheless, we do acknowledge that TB has serious negative consequences in women's lives and well-being because of the stigma, societal issues (discrimination), psychological distress associated with having active TB disease in many LMICs (UNOPS 2020). A study conducted in India has shown that 25% of women diagnosed with TB feel rejected and discriminated by people in their community (Srivastava et al. 2018). Likewise, 10% of the marriages end up in divorce after diagnosis (Srivastava et al. 2018). Worse, TB has serious adverse maternal and perinatal outcomes during pregnancy. A meta-analysis has demonstrated a 4.2-fold increase in odds of perinatal deaths in pregnant women who have PTB (Sobhy et al. 2017). Thus, it is logical to continue to assess the impact of gender in all aspects of TB care, particularly on treatment compliance, pregnancy as well as socio-economic impacts of active TB disease.

In summary, we are just beginning to appreciate that separating the effects of gender from biological factors is complex in the context of TB. Succeeding this quest might require setting up well-designed experimental animal studies, which are presumably not impacted by human-specific characteristics/behaviors (addiction, professional exposure, environmental factors, health-seeking behaviors, etc.). Nonetheless, animal works will be the most useful if complemented by solid evidence derived from humans using samples from robust longitudinal clinical cohorts representing the spectrum of TB (i.e., latent TB infection, active pulmonary TB, and extrapulmonary) in both sexes.

7 Conclusion and Future Directions

A growing body of literature is revealing the contribution of biological sex in TB pathogenesis and treatment outcomes. Animal and human studies suggest that biological factors such as sex hormones and human genetics are modulating immune responses, yielding sex-specific differences in disease outcomes. Non-biological factors, which are influenced by gender, are also likely involved but to a lesser extent than biological factors. The relationship with sex and risk of PTB is evident across regions of the world, as males are substantially more at risk for developing PTB than females and have poor treatment outcomes. However, the impact on sex on the risk of acquiring LTBI and progressing to EPTB is not yet clear, hence requiring conducting large-scale cross-sites human studies. Indeed, we do not have an answer to several important research questions that are prerequisites to elucidate this phenomenon as outlined below:

- Can sex alone mediate differentially susceptibility to LTBI, independently of non-biological factors?
- Why are females more affected by certain clinical manifestations of extrapulmonary TB than men?
- What cell-type-specific immune response to MTB infection mediates differential susceptibility between the sexes?
- Which pathways are triggered or inhibited by sex hormones to mediate sex-specific PTB outcomes?
- What is the contribution of X-linked immune genes and micro-RNAs in the pathogenesis of TB?
- Why is immunization with BCG more protective in girls than boys?
- What is the impact of age and comorbidities such as HIV and diabetes on sex differences in TB?
- What is the intersection between sex and gender that drives poor treatment outcomes in men?
- What gender-based factors promote non-compliance to TB treatment in men?

- Do mycobacterial-specific factors (differences in virulence genes, resistance to drugs, persistence) contribute to sex differences in TB outcomes?

Those questions are of course not exhaustive but could provide mechanistic insights for the better understanding of the impact of sex on TB acquisition, progression to disease, response to treatment, and immunization. In the long term, this emerging theme of research could facilitate rational design of sex-specific therapeutics, identification of novel biomarkers to monitor treatment success or failure, but also increased treatment compliance rates to end the global TB epidemics.

Acronyms and Abbreviations

TB	Tuberculosis
AFB	Acid-Fast Bacilli
MTBC	*Mycobacterium tuberculosis* Complex
MTB	*Mycobacterium tuberculosis*
MAF	*Mycobacterium africanum*
LTBI	Latent tuberculosis infection
EPTB	Extrapulmonary Tuberculosis
PTB	Active pulmonary TB disease
LMICs	Low- and middle-income countries
COVID-19	2019 Coronavirus Disease
INH	Isoniazid
RIF	Rifampicin
PZA	Pyrazinamide
EMB	Ethambutol
BCG	Bacillus Calmette Guerin
HCW	Healthcare Workers
QTF	Quantiferon TB Gold In-Tube assay
TST	Tuberculin Skin Test
TBESC	Tuberculosis Epidemiologic Studies Consortium
NAT-2	N-acetyltransferase-2
WHO	World Health Organization
CT	Computed tomography
HIV	Human Immunodeficiency Virus
NTM	Non-tuberculous mycobacteria
MAC	*Mycobacterium avium* Complex
E2	Estradiol
CFUs	Colony Forming Units
DHEA	Dehydroepiandrosterone
FSH	Follicle stimulation hormone
LH	Luteinizing Hormone
HBD-2	Human β-defensin-2
HBD-3	Human β-defensin-3
PID	Primary immunodeficiency diseases
MSMD	Mendelian susceptibility to mycobacterial diseases

NEMO	NF-kB essential modulator
CYBB	Cytochrome b-245 beta chain
TLR	Toll-like receptor
miRNA	Micro-RNAs

Acknowledgements The authors gratefully acknowledge the support of NIH grants K43 TW011426, D43 TW010350, and R01 AI167750.

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Sex-Linked Differences in Malaria Risk Across the Lifespan

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Abstract Despite the high burden of malaria worldwide, there is surprisingly scarce research on sex-based differences in malaria outside of pregnancy. A more thorough understanding of sexual dimorphism in malaria, and what underlies these sex-based differences, could elucidate the underlying mechanisms driving malaria pathogenesis and has the potential to inform malaria control efforts, including new vaccines. This review summarizes our current understanding of sex-based differences in the epidemiology of malaria across the lifespan, potential sex- or gender-based mechanisms driving these differences, and the knowledge gaps that need to be addressed.

1 Introduction

Malaria remains a major global health problem with nearly 609,000 deaths and 241 million cases annually estimated in 2021 (World Health Organization 2022). Malaria is caused by parasites of the genus *Plasmodium*, which are transmitted to people through the bites of *Plasmodium*-infected mosquitoes. The life cycle of malaria parasites involves several stages involving both the mosquito vector and human host (Fig. 1). Most malarial morbidity and mortality is due to *Plasmodium falciparum*, although other species, including *Plasmodium vivax*, account for significant morbidity worldwide. The development of an effective and widely available malaria vaccine, and novel therapeutics that reduce disease severity, represent critical steps

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S. L. Klein and C. W. Roberts (eds.), *Sex and Gender Differences in Infection and Treatments for Infectious Diseases*, Current Topics in Microbiology and Immunology 441, https://doi.org/10.1007/978-3-031-35139-6_7

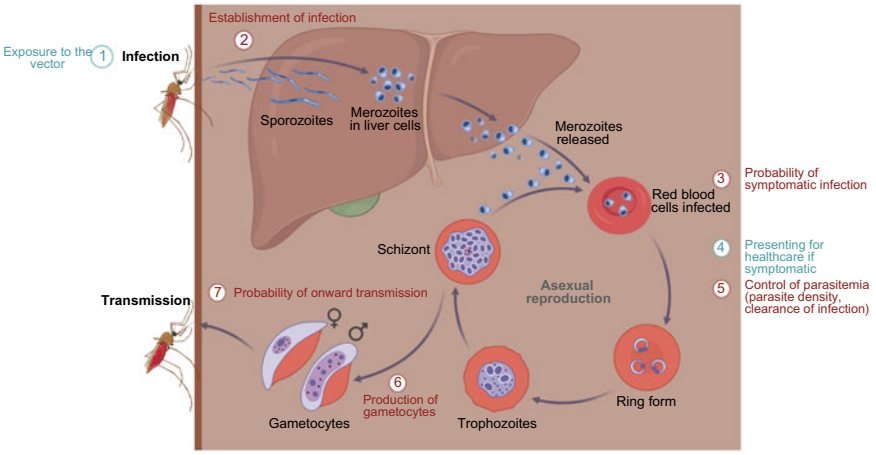

Fig. 1 Malaria parasite life cycle. Numbers and text in teal indicate where gender-based behavioral differences may impact observed sex-specific differences in malaria epidemiology or pathogenesis; numbers and text in dark red indicate where biological sex may impact observed sex-specific differences in malaria epidemiology or pathogenesis

toward the control of malaria. However, a major challenge is the knowledge gap about mechanisms underlying protective immunity and pathogenesis in humans.

In malaria endemic settings, individuals are often repeatedly exposed to the bites of *Plasmodium*-infected mosquitoes, with many individuals infected hundreds of times per year. In these settings, the burden of disease falls mainly on young African children, with 77% of deaths reported in African children less than 5 years of age (World Health Organization 2022). With increasing age and following repeated malaria episodes, individuals gain protection against severe disease, followed eventually by protection against symptomatic illness. This age- and exposure-dependent naturally acquired immunity consists of two distinct components: "anti-parasite" immunity, or partial control of blood-stage parasite densities, and "anti-disease" immunity, or the ability to tolerate higher parasite densities without fever (Rodriguez-Barraquer et al. 2018). However, the precise determinants driving these distinct components of naturally acquired antimalarial immunity remain poorly understood.

Despite the high burden of malaria worldwide, there is surprisingly scarce research on sex-based differences in malaria outside of pregnancy. Sex-based differences in the distribution and outcome of various infectious diseases are widely acknowledged. For example, there is a clear male bias in tuberculosis burden and mortality, and amebic liver abscess due to *Entamoeba histolytica* and *Leishmania* infection are both more common in post-pubertal males (Bernin and Lotter 2014; Nhamoyebonde and Leslie 2014). These sex-based differences in infectious diseases are not always clear-cut; for example, after HIV infection, women maintain lower viral loads on average compared to men, but progress more quickly to AIDS at the same viral load (Addo and Altfeld 2014). One of the earliest studies to show that there may be sex-specific differences in malaria prevalence and immunologic response was the Garki

project in Nigeria, which demonstrated higher malaria prevalence in post-pubertal males and higher antibody titers in females (Molineaux et al. 1980). Unfortunately, many studies that followed did not report data disaggregated by sex, and for years research into sex-based differences in malaria was focused primarily on the increased susceptibility of pregnant women to infection and severe disease. A more thorough understanding of sexual dimorphism in malaria, and what underlies these sex-based differences, could elucidate the underlying mechanisms driving malaria pathogenesis and has to the potential to inform malaria control efforts, including new vaccines. This is particularly important as we enter the era of vaccines for malaria prevention with the 2021 WHO recommendation for the use of the RTS,S/AS01 vaccine in moderate and high *P. falciparum* burden regions (World Malaria Report 2021).

Sex is defined here by biology, in terms of differential chromosomes, reproductive organs, and sex steroids, while gender is defined by cultural perceptions and behavioral norms superimposed by society. Biological sex can affect immune responses, resulting in differential susceptibility to pathogens, ability to control or clear infections, and capacity to gain protective immunity (Klein and Flanagan 2016). Gender may influence an individual's exposure to specific pathogens and their likelihood to seek medical care, which could affect the course of infection. Therefore, sex-related biological differences may modulate a person's ability to become infected with malaria parasites, mitigate symptoms, and/or clear infection, while behavioral differences between genders may influence both exposure to malaria-carrying mosquitoes and the likelihood of being diagnosed with clinical malaria.

It is likely that both sex- and gender-dependent differences contribute to the observed differences in the prevalence and incidence of malaria in males versus females. This review will provide a summary of the available data on sex-based differences in malaria epidemiology and pathogenesis. Specifically, this review will address the following questions: (1) Is there an observed difference in the prevalence and/or incidence of malaria between males versus females among different age groups? (2) How might different behaviors related to gender influence the risk of exposure to malaria parasites? (3) What biological factors related to sex might impact the risk of infection, the severity of the disease once infected, and/or rates of parasite clearance?

2 Epidemiology of Sex-Specific Differences in Malaria Infection, Disease, and Vaccination

Sex differences in malaria in children

Several studies have explored whether malaria prevalence, incidence, or severity differs in children, who suffer the highest morbidity and mortality from malaria (Tables 1 and 2). The hallmark Garki project was a longitudinal study conducted in the lowlands of northern rural Nigeria from 1969 to 1976; this study documented malaria prevalence due to different *Plasmodium* species through repeated cross-sectional

surveys of certain villages and grouped the results of these surveys together to analyze the data by sex (Molineaux et al. 1980). In this high-transmission setting, there was no apparent sex difference in *P. falciparum* or *Plasmodium malariae* prevalence in children prior to 5 years of age (Molineaux et al. 1980). A cross-sectional survey in Brazil from 1989 to 1991, a hypoendemic area where *P. vivax* predominates over *P. falciparum*, showed a higher prevalence in males in age group 0–10 but did not further stratify to children less than 5 (Camargo et al. 1996). However, a study in India by Pathak et al. in a similarly low-transmission setting utilized household-to-household active surveillance to test those with symptoms and found no difference in parasite prevalence between males and females < 10 years of age (Pathak et al. 2012). In Madagascar, a nationwide cross-sectional survey across a range of transmission intensities found higher parasite prevalence in females at age 0–1 year and higher prevalence in males from 2 years and older (Kesteman et al. 2014). A study in Ghana showed higher prevalence in females aged 6–7 years than in males (Landgraf et al. 1994). Overall, there is insufficient evidence to claim significant sex-based differences in malaria prevalence in young children.

In the same study by Pathak et al. above, records from public and private medical centers for the years 2002–2007 of cases of slide-confirmed malaria showed no sex-based differences in the incidence of *P. vivax* malaria in children < 15 and no differences in the incidence of *P. falciparum* malaria in children < 10 (Table 2) (Pathak et al. 2012). This is consistent with data from Thailand that showed no sex-based difference in malaria cases under the age of 5 (Luxemburger et al. 1997). Two studies in Sudan with different methodologies, one based on case report data and one from data generated during a longitudinal study with a village health team, reported a higher incidence in males < 5 years old compared to females (Creasey et al. 2004; Abdalla et al. 2007). In Uganda, a health facility-based study found a higher incidence in females < 15 years compared to males, and a study in Sri Lanka found higher incidence in females compared to males at ages < 6 years (Mendis et al. 1990). An observational prospective fever cohort study conducted in Jinja, Uganda, that enrolled febrile children aged 2 months to 5 years admitted to the hospital examined whether sex was a determinant of disease severity or diagnosis in their cohort (McDonald et al. 2022). There was no evidence of differences by sex in disease severity scores generally or in the frequency of diagnosis of malaria. As with prevalence, there is insufficient evidence for sex-specific differences in the incidence of malaria in children.

Sex differences in malaria risk in adolescence and adulthood

Although sex-specific differences in malaria prevalence have not been uniformly described in younger children, several studies have reported a male bias in malaria prevalence in older children and adults (Table 1). The Garki Project found that after 5 years of age, males have higher malaria prevalence than females; for *P. falciparum,* the differences were most significant in age groups 9–18 years and 19–28 years (Molineaux et al. 1980). In the cross-sectional survey in Brazil from 1989 to 1991 referenced above, malaria prevalence was higher in males in all age groups (0–10, 11–16, 16–40, and > 40 years) (Camargo 1996). Similarly, the national cross-sectional

Table 1 Studies that investigated sex-based differences in malaria prevalence

Study	Country and year	Method	Transmission intensity and *Plasmodium* spp.	Results	Confounding factors
Briggs et al. (2020)	Uganda, 2017–2018	Active surveillance for asymptomatic infection by quantitative PCR (qPCR) in a cohort study	Previously hyperendemic but with recently lower malaria transmission after vector control *P. falciparum*	Higher overall prevalence in males, with relative differences most pronounced in those 15 years and older. No sex-based difference in parasite density by qPCR (adjusted for age)	No evidence of behavioral trends that would result in more infections in males; older females most likely to travel in this cohort. Possible that males were differentially exposed in the recent past, which could affect immunity
Carmargo et al. (1996)	Brazil, 1989–1991	SPR by cross-sectional survey	Hypoendemic *P. vivax* > *P. falciparum*	Age groups (0–10, 11–16, 16–40, and > 40): Higher prevalence in males in all age groups	11% of females and 40% of males worked outdoors; more adult males worked outside the town limits
Golassa et al. (2015)	Ethiopia, 2012	SPR by cross-sectional survey	Hypoendemic *P. falciparum* > *P. vivax*	Higher prevalence in males compared to females; no age stratification	Attributed to occupational differences between males and females

(continued)

Table 1 (continued)

Study	Country and year	Method	Transmission intensity and *Plasmodium* spp.	Results	Confounding factors
Houngbedji et al. (2015)	Cote d'Ivoire, 2011–2012	National, cross-sectional school-based survey; prevalence assessed by microscopy or RDT. Geometric mean parasite density (GMPD) calculated from microscopy	Hyperendemic *P. falciparum*	Higher prevalence in males compared to females; no age stratification. No sex-based differences in GMPD	None noted
Kesteman et al. (2014)	Madagascar, 2012–2013	National cross-sectional survey; prevalence by RDT	Several transmission zones (Hyperendemic, seasonal, hypoendemic) *P. falciparum*	Prevalence was higher in males in all age categories except in children under 2 years	Posited that lower serum iron in females may be protective, or there may be gender-based differences in behavior
Landgraf et al. (1994)	Ghana, 1994	SPR by cross-sectional survey; GMPD calculated from microscopy	Holoendemic *P. falciparum*	Ages 6–7: Higher prevalence in females Ages 8–17: Higher prevalence in males. GMPD lower in females compared to males	No difference in social, religious, cultural, or clothing factors that could contribute to these sex differences. No mosquito bed nets used in the area. No difference between sexes in chloroquine in urine

(continued)

Table 1 (continued)

Study	Country and year	Method	Transmission intensity and *Plasmodium* spp.	Results	Confounding factors
Molineaux et al. (1980)	Nigeria, 1969–1976	Longitudinal cohort study that analyzed prevalence by SPR with repeated cross-sectional surveys	Hyperendemic *P. falciparum* > *P. malariae*	Age > 5 years: Higher prevalence in males. Statistically significant differences in age group 9–18	None noted
Mulu et al. (2013)	Ethiopia, 2006	Cross-sectional survey of samples collected at a health facility; prevalence by SPR, and parasitemia per uL blood by microscopy	Hyperendemic *P. falciparum* > *P. vivax*	Higher prevalence of malaria in males across all spp.; no age stratification. Higher mean parasitemia in females compared to males (not statistically significant)	None noted
Pathak et al. (2012)	India, 2000–2009	Active surveillance household-to-household in those reporting fevers or other symptoms; prevalence by SPR	Hypoendemic *P. vivax* > *P. falciparum*	No difference in SPR by sex when stratified by age in the active surveillance dataset (very low SPRs)	Males could be more likely to spend more time outdoors; however, their data showed no changes in age and sex distribution patterns of parasite positivity by active surveillance due to the wet season, when there is less movement out of the home. Post-pubertal females may be more likely to be anemic due to nutritional deficiencies. Changes in male behavior like increasing tobacco and alcohol consumption could contribute to increased attractiveness to mosquitoes

(continued)

Table 1 (continued)

Study	Country and year	Method	Transmission intensity and *Plasmodium* spp.	Results	Confounding factors
Tiedje et al. (2017)	Ghana, 2012–2013	Prevalence by SPR and PCR from serial cross-sectional surveys	Hyperendemic *P. falciparum*	Microscopic and submicroscopic prevalence higher overall in males; statistically significant in those ages 20+	None noted

Table 2 Studies that investigated sex-based differences in malaria cases or incidence

Study	Country and year	Method	Transmission intensity and *Plasmodium* spp.	Results	Confounding factors
Abdalla et al. (2007)	Sudan, 2002	Analyzed local studies and reports for reported cases and adjusted for under-reporting and positive predictive value of microscopy. Age–sex ratios were used to disaggregate the overall incidence in each region and the incidence in each age–sex group was pooled to estimate the age–sex specific incidence in all Sudan	Hypoendemic, mesoendemic, and hyperendemic areas *Plasmodium* spp. not specified	Males had higher incidence of malaria in all age groups	None stated
Briggs et al. (2020)	Uganda, 2017–2018	Passive surveillance for symptomatic infection confirmed with microscopy in a cohort clinic	Previously hyperendemic but with recently lower malaria transmission after vector control *P. falciparum*	No difference in incidence by sex both unadjusted and adjusted by age	None stated
Creasey (2004)	Sudan, 1990–2000	Percent age group diagnosed with CIM as observed in a longitudinal study run by a village health team	Hypoendemic *P. falciparum* > *P. vivax*	Averaged over 10 years, males had higher incidence than females in all age groups (<1 years, 1–5 years, 6–10 years, and 11–15 years)	None stated

(continued)

Table 2 (continued)

Study	Country and year	Method	Transmission intensity and *Plasmodium* spp.	Results	Confounding factors
Luxemburger, et al. (1997)	Thailand, 1997	Hospital admissions data	Hypoendemic *P. falciparum* > *P. vivax*	Higher number of malaria cases in males in children > 5 and adults; no sex differences in incidence of severe malaria after adjusting by age	More women in the camp
Mendis et al. (1990)	Sri Lanka, 1986–1988	Incidence of malaria cases confirmed by microscopy using passive case detection	Endemic *P. vivax*, "unstable" transmission with epidemics of *P. falciparum* During the period of this study, *P. falciparum* > *P. vivax*	Higher overall malaria incidence in females than males at ages < 6. Similar incidence rates for ages 6–15 and in those 51+. Higher incidence in males at ages 16–25 and 26–50	Occupational movement may contribute to working age males having a higher incidence of disease
Okiring et al. (2022)	Uganda, 2020–2021	Incidence of confirmed malaria (by RDT or microscopy) at 12 health facilities	Hyperendemic *P. falciparum*	Higher incidence in females, with larger differences in those 15–30 years and > 39 compared to those under 15 years	Females were more likely to present for care for non-malarial illnesses compared to males. Among those tested for malaria, post-adolescent males had a higher probability of testing positive than females

(continued)

Table 2 (continued)

Study	Country and year	Method	Transmission intensity and *Plasmodium* spp.	Results	Confounding factors
Olson et al. (2010)	Brazil, 2006	Slide-confirmed malaria cases reported through both passive and active surveillance	Hypoendemic *P. vivax* > *P. falciparum*	No sex-based differences found; no age stratification	4% more males in the region
Pathak et al. (2012)	India, 2002–2007	Slide-confirmed malaria cases from public and private medical centers	Hypoendemic *P. vivax* > *P. falciparum*	No sex-specific difference at ages < 15. Higher incidence among males ages 15+ for *P. vivax* and *P. falciparum*	Males were more likely to seek care at the health facilities. Additional possible factors listed in Table 1
Sumner et al. (2021)	Kenya, 2017–2019	Active surveillance by quantitative PCR (qPCR) for asymptomatic infection followed by passive surveillance for symptomatic infection confirmed with RDT and qPCR	Hyperendemic *P. falciparum*	Elevated risk of symptomatic malaria in those with asymptomatic infection in the last month. This association was modified by sex, with males having a lower risk of symptomatic malaria following asymptomatic infection	None stated

survey in Madagascar found higher prevalence in males in all adolescent and adult age categories (Kesteman et al. 2014). Though Landgraf et al. found female bias in malaria prevalence in Ghana between ages 6 and 7, prevalence was higher in males ages 8–17 (Landgraf et al. 1994). They also noted a sex-based difference in geometric mean parasite densities; males had a higher GMPD than females, but only between 8 and 16 years of age. The Garki project also noted higher parasite densities in males after 5 years of age (Molineaux et al. 1980). Finally, several studies that do not stratify by age and sex also show an overall male bias in malaria prevalence. In Ghana, the presence of parasites was assessed by microscopy and quantitative PCR

in serial cross-sectional surveys; both microscopic and submicroscopic *P. falciparum* parasite prevalence was higher in males in both surveys (Tiedje et al. 2017). A national cross-sectional school-based survey in Cote d'Ivoire also showed a higher overall prevalence in males (Houngbedji et al. 2015). In addition, two studies in Ethiopia in regions of different transmission intensity both found a male bias in the prevalence of malaria (Golassa et al. 2015; Mulu et al. 2013).

Though there is a preponderance of evidence that there is a sex bias in malaria prevalence in adolescence and adulthood, the data regarding a sex-based difference in malaria case counts or incidence is more mixed (Table 2). In Sudan, two different studies, one based on reported cases across the country and one based on a longitudinal cohort in a hypoendemic area, reported that males had a higher incidence of malaria in older children and adults (Creasey et al. 2004; Abdalla et al. 2007). Similarly, Pathak et al. found a higher incidence of both *P. vivax* and *P. falciparum* malaria among males ages 15 years and older in India (Pathak et al. 2012), and Luxemburger et al. found a higher number of malaria cases in males in children > 5 and adults in Thailand (Luxemburger et al. 1997). In Sri Lanka, a study from 1986 to 1988 in an area with endemic *P. vivax* and epidemics of *P. falciparum* reported higher incidence of slide-confirmed malaria in males at ages 16–25 and ages 26–50 compared to females, but no differences for those ages 6–15 and in those 51 years and older (Mendis et al. 1990). However, Olson et al. found no sex-based differences in the incidence of slide-confirmed malaria cases in a hypoendemic region of Brazil (with no age stratification performed) (Olson et al. 2010). In Uganda, a study based on diagnostic-confirmed *P. falciparum* malaria incidence showed a higher incidence in females compared to males, with larger differences in those 15–30 years and > 39 years compared to those < 15 years (Okiring et al. 2022). While Pathak et al. in India noted that males were more likely to present for care at health facilities (Pathak et al. 2012), in Uganda females were more likely to present for care. In addition, the study in Uganda noted that among those tested for malaria, post-adolescent males had a higher probability of testing positive than females (Okiring et al. 2022).

Because prevalence of infection is a function of both the number of new infections occurring and the duration of those infections, it is possible that sex-based differences in the duration of infection (or clearance of infection) could explain the male bias in malaria prevalence, even if exposure to new infections is the same. In a cohort study in eastern Uganda where transmission was recently low after multiple rounds of indoor residual spraying of insecticides, it was noted that there was no sex-specific difference in the incidence of malaria overall or in gender-based risk factors for malaria; however, males had a higher prevalence of *P. falciparum* by microscopy (2.9% prevalence in males compared to 1.4% prevalence in females across all age categories) (Briggs et al. 2020). To test the hypothesis that sex-based differences in host–parasite interactions could explain the difference in prevalence, researchers intensively followed *P. falciparum* infections in this cohort and estimated both force of infection (FOI) and rate of clearance using amplicon deep-sequencing of AMA-1. They found no evidence of differences in FOI by sex, but did find that females cleared asymptomatic infections at nearly twice the rate as males in multivariate models adjusted for age, timing of infection onset, and parasite density. This provided

evidence that biological sex-based differences may be an important factor in the host response to the malaria parasite.

Another study, conducted in a high-transmission region of Kenya, evaluated the likelihood of symptomatic malaria following an asymptomatic *P. falciparum* infection. Compared to being uninfected, asymptomatic infections were associated with an increased one-month likelihood of symptomatic malaria, and this association was modified by sex, with females at higher risk for symptomaticity than males (Sumner et al. 2021). At first glance, the observations from these two studies seem to conflict. However, in the Uganda cohort, the incidence of symptomatic malaria for participants over 10 years of age was over twice as high in females compared to males, although malaria was uncommon and the difference between females and males was not statistically significant (Briggs et al. 2020). Therefore, one hypothesis that synthesizes the results from the two studies is that males are less able to control parasite densities or clear infections, leading to higher parasite prevalence among males, and females are less able to tolerate parasites without fever, leading to a higher probability of symptoms once infected. In addition, these two cohort studies were performed in areas with different transmission intensities, which could also have an effect on the natural history of infections. Reconciling the sex-based findings from these studies will require additional investigation and highlights the need to include sex as an effect modifier in descriptive malaria epidemiology studies, in those exploring the impacts of interventions relevant to prevention and control, and studies evaluating pathophysiologic differences in host immune responses.

The unique role of pregnancy and sex-specific differences in infants following in utero *malaria exposure*

Although individuals acquire protection against malarial morbidity with increasing age and following repeated *Plasmodium* infections, the risk of *P. falciparum* morbidity increases during pregnancy (Rogerson et al. 2018; Desai et al. 2007). The increased susceptibility of pregnant women to malaria is due, at least in part, to a unique physiologic niche provided by the human placenta. During pregnancy, *P. falciparum* expression of variant surface antigens allows the parasite to bind to chondroitin sulfate A (CSA) on the placental syncytiotrophoblast, leading to placental sequestration of parasites, or placental malaria. These infected red blood cells then stimulate maternal mononuclear cells to express chemokines that enable recruitment of maternal histiocytes, leading to substantial inflammation and intervillositis. This can result in a number of adverse events for the mother, including but not limited to anemia and hypertension. Placental malaria has also been associated with adverse outcomes for the infant including: (1) miscarriage, still birth, and intrauterine growth retardation and (2) low birthweight and fetal anemia (Rogerson et al. 2018; Harrington et al. 2019).

Malaria prevalence is highest in the first and second trimesters of pregnancy and is more common in women who are pregnant for their first time (primigravidae) versus women who have had multiple pregnancies (multigravidae). The reason for this gravidity dependent protection is thought to be due in part to the acquisition of antibodies that block adhesion of infected red blood cells to CSA and other adhesion

molecules in the placenta (Rogerson et al. 2007). Similar to non-pregnant women, *P. falciparum* infection prevalence in pregnancy is affected by season, geographic location, and receipt of malaria prevention including insecticide-treated bed nets and intermittent preventive treatment of malaria in pregnancy (IPTp) with sulfadoxine–pyrimethamine. IPTp-SP remains one of the main tools recommended by the World Health Organization for reducing malaria-associated adverse birth outcomes (WHO Guidelines for malaria). However, the effectiveness of SP as an antimalarial is threatened by wide-spread antifolate resistance of *P. falciparum* in East and Central Africa (Flegg et al. 2022). In studies investigating alternative drugs for IPTp, dihydroartemisinin–piperaquine (DP) has shown promising results. Compared to IPTp-SP, IPTp-DP was associated with a lower risk of clinical malaria, parasitemia during pregnancy, and placental malaria at delivery (Kakuru et al. 2016; Mlugu et al. 2021; Kajubi et al. 2019). However, in the majority of these studies, IPTp-DP was not associated with improved birth outcomes compared to IPTp-SP, with some suggestion that SP might confer some benefits in pregnancy independent of its antimalarial properties (Roh et al. 2020; Waltmann et al. 2022). Trials testing novel IPTp regimens for malaria, combining agents with both malarial and non-malarial mechanisms of action, are ongoing.

An additional concern of placental malaria is the effect that this exposure may have on the development of the infant's immune system (Harrington et al. 2019; Jagannathan 2018). Several observational studies have reported associations between PM and the risk of malaria, non-malarial febrile illnesses, and malaria in infancy, possibly due to immune tolerance induced by in utero exposure to malaria antigens (Hesran et al. 1997; Schwarz et al. 2008; Malhotra et al. 2009; Jagannathan et al. 2018). It has been unclear whether infant sex may modify these associations.

It has been observed that male infants may experience more negative outcomes as a result of exposure to adverse events in utero as compared to female infants (Hernández-Julián et al. 2014; Kent et al. 2012). In a recent study, male infants born to mothers with a history of severe placental malaria had a higher incidence of malaria in infancy (adjusted incidence rate ratio (aIRR) 2.17, 95% CI 1.45–3.25, $p = 0.002$); no significant difference was observed among female infants (aIRR 0.74, 95% CI 0.46–1.20, $p = 0.22$) (Kakuru et al. 2020a). Similarly, male infants born to mothers with severe placental malaria had a higher incidence of complicated malaria and prevalence of parasitemia in infancy in comparison with those born to mothers without placental malaria, with no significant differences with these outcomes observed in female infants (Kakuru et al. 2020a). In another study, effective prevention of malaria in pregnancy with IPTp-DP was associated with a lower risk of malaria in infants compared to IPTp-SP; however, this effect was significantly modified by infant sex (p-value for interaction 0.02) (Kakuru et al. 2020b). Male infants born to mothers given IPTp-DP had a significantly lower incidence of malaria compared with male infants born to mothers given IPT-SP, (IRR 0.75, 95% CI 0.58–0.98, $p = 0.03$). There was no significant association between IPTp-DP and a female infant's risk of malaria (IRR 0.99, 95% CI 0.79–1.24, $p = 0.93$) (Kakuru et al. 2020b). Together, these data suggest that there are sex-specific consequences of in utero exposure to malaria parasites in pregnancy.

Regarding mechanisms mediating these sex-specific differences, one study evaluated maternal fetal transfer of antimalarial antibodies and found no significant differences between the sexes (Clifton 2010). Other possibilities for these differences may be due to sex-specific differences in glucocorticoid receptor expression, fetal-placental responsivity to cortisol, or neonatal and infant immune responses, including responses to toll-like receptor ligands and induction of regulatory T-cell populations (Fish 2008; Prahl et al. 2017; Fischinger et al. 2019).

Sex differences in response to RTS,S/AS01 malaria vaccine

Sex-based differences in vaccination-induced immunity have been described for both inactivated and live vaccines (Klein and Flanagan 2016; von Seidlein 2019). Antibody responses to vaccination are typically higher in females compared to males; females also report higher rates of local and systemic adverse reactions. Therefore, it is important to consider sex-based differences in response to the malaria vaccines under development. RTS,S/A01 is the only licensed antimalarial vaccine as of publication and was recently recommended by the WHO for prevention of malaria in moderate and high *P. falciparum* burden regions. The vaccine antigen is made from the carboxy-terminal end of the circumsporozoite (CSP) protein of *P. falciparum* strain NF54 and the hepatitis B virus surface protein (RTS,S Clinical Trials Partnership 2015). The multicenter phase 3 trial of three doses of RTS,S/AS01 at months 0, 1, and 2 and a booster dose at month 20 in children (age 5–17 months) showed a vaccine efficacy of 36.3% (95% CI 31.8–40.5) for clinical malaria and of 32.2% (13.7–46.9) for severe malaria with a median follow up of 48 months (https://fctc.who.int/publications/i/item/who-position-paper-on-malaria-vaccines). Analysis of vaccine efficacy by sex showed that it was slightly higher among boys than girls 5–17 months of age in the 3-dose group (37% versus 32%) and in the 4-dose group as well (43% versus 35%) (White et al. 2015). In a subsequent analysis of the phase 3 trial data, anti-circumsporozoite antibodies were shown to be a surrogate of protection against clinical malaria, but no sex-based difference was seen in titer levels after vaccination (Klein et al. 2016).

In an unplanned post-hoc analysis of the phase 3 RTS,S/A01 trial data, an imbalance in mortality among girls was noted, with approximately twofold higher deaths among girls who received RTS,S/A01 than those who received comparator vaccines ($p = 0.001$) (https://cdn.who.int/media/docs/default-source/immunization/mvip/full-evidence-report-on-the-rtss-as01-malaria-vaccine-for-sage-mpag-%28sept2021%29.pdf). In contrast, the ratio of deaths among boys was slightly lower in the RTS,S/AS01 arm versus the control arm. The authors of this study pointed out that rather than dispense with these findings as "due to chance" or due to the low female mortality in the control arm, this finding should be further investigated, as other non-live vaccines have greater detrimental effects for girls compared to boys (https://cdn.who.int/media/docs/default-source/immunization/mvip/full-evidence-report-on-the-rtss-as01-malaria-vaccine-for-sage-mpag-%28sept2021%29.pdf). During the Malaria Vaccine Pilot Evaluation (MVPE), in which the RTS,S/AS01 vaccine was rolled out in Ghana, Kenya, and Malawi to determine vaccine efficacy in real-world settings, WHO recommended the pilot of the 4-dose schedule

should include sufficiently large populations of children 5–17 months of age to address safety concerns. In April 2021, after 24 months of data had been collected, the results showed no evidence of an excess of gender-specific mortality comparing age-eligible children living in implementation areas with those in the comparison areas (https://cdn.who.int/media/docs/default-source/immunization/mvip/full-evidence-report-on-the-rtss-as01-malaria-vaccine-for-sage-mpag-%28sept2021%29.pdf).

Unfortunately, the full evidence report on the RTS,S/AS01 MVPE program does not report vaccine efficacy by sex (https://cdn.who.int/media/docs/default-source/immunization/mvip/full-evidence-report-on-the-rtss-as01-malaria-vaccine-for-sage-mpag-%28sept2021%29.pdf). There is a newer vaccine in development named R21, which is also CSP-based but uses a different adjuvant (Matrix M). R21 has shown higher efficacy in the field in phase 2 and 3 studies compared to RTS,S/A01, but none of the published data on this vaccine have been disaggregated by sex (Datoo et al. 2022; Baume and Marin 2007).

3 Gender-Based Differences in Malaria Exposure and Care-Seeking May Partially Explain Observed Sex-Specific Differences in Malaria Epidemiology

Given that there is a known increased risk of malaria infection in pregnancy, the studies showing a male bias in malaria prevalence even in age groups with women of childbearing age is an unexpected finding. Behavioral gender-based differences have been posited to explain the male bias in malaria prevalence seen in many studies, since males may spend more time outdoors and therefore have increased exposure to the malaria vector *Anopheles* (Camargo 1996; Pathak et al. 2012; Kesteman et al. 2014, Mendis et al. 1990; Golassa et al. 2015). For example, in Brazil, Carmargo et al. noted that 40% of males worked outdoors compared to 11% of females, and more adult males worked outside the town limits (Camargo 1996). However, most studies that posited this as an explanation did not assess gender-based differences in behavior. Another way behavior may modify risk of infection is if there are any gender-based differences in perceived risk of infection leading to differential use of protective measures, such as sleeping under a long lasting insecticide-treated net (LLIN), as is recommended in endemic countries. One study discussed that females may be more likely to sleep under bed nets, which would reduce their likelihood of being bitten by mosquitos (Lefèvre et al. 2010).

There may also be gender-based behavioral differences that increase attractiveness to the vector because mosquitoes travel by olfactory cues and pheromones to locate their targets. Males are more likely to consume alcohol in sub-Saharan Africa (Kalichman et al. 2007; Frei et al. 2017), and a study in which male participants imbibed a home-brewed beer made from sorghum demonstrated that the odor emitted by a beer drinker increases their attractiveness to mosquitoes (Kalichman et al.

2007). Males working outside may also increase their susceptibility to malaria due to increased sweating. It has been repeatedly shown that microbial growth enhances the attractiveness of human sweat to the *Anopheles* vector, and one study showed that mosquitoes had no preference for male versus female sterile sweat but preference for male sweat over female once it was incubated with common axilla bacteria (Braks et al. 2000; Smallegange et al. 2011). Additionally, males tend to sweat more while exercising (Lindsay et al. 2000). In females, it is well-established that pregnant females are twice as attractive to a mosquito as a non-pregnant females (Ansell et al. 2002; Himeidan et al. 2004; Tanner and Vlassoff 1998). Therefore, gender-based differential attractiveness to mosquitoes could contribute to being bitten more frequently by malaria-infected mosquitoes.

Furthermore, behavioral patterns related to gender may increase or decrease a person's likelihood of seeking care if they have malaria-like symptoms (Tolhurst et al. 2008), which can bias observational studies looking for true differences in reported malaria cases or malaria incidence. For example, adult males might be less likely to seek medical treatment if they live in a culture where male strength is highly valued, or if they are the primary breadwinner and do not have time off work to seek care. Gender inequalities and power dynamics in domestic partnerships might also result in delayed ability to seek care for women. Mothers' independent access to resources has been identified as an important factor influencing treatment seeking for children, as well as access to male-controlled resources (vom Steeg and Klein 2016). On the other hand, women are often in caretaker roles, which means they may be more likely to attend health facilities to seek care for sick children and therefore have more opportunities to seek care for themselves. This was the case in a recent study in Uganda in which malaria incidence was higher in females in all age groups at 12 public health facilities across Uganda (Okiring et al. 2022). Females were more likely to present for care for non-malarial illnesses compared to males, and in a cross-sectional survey of the communities surrounding the health centers, the risk of reporting a fever in the past 2 weeks and seeking care at the local health facility was higher in females compared to males in those 15–39 years of age and over 39 years.

Nevertheless, gender-specific behavioral and sociocultural differences may not fully explain the sex-specific differences in the incidence of malaria. During the Garki project, no villagers slept under LLINs, and there was still an observable sex-specific difference in malaria infection as assessed by cross-sectional survey (Molineaux et al. 1980). In addition, some studies have shown no differences in the utilization of LLINs in men versus women (Briggs et al. 2020). As pointed out by Pathak et al., if increased time in the outdoors was responsible for increased vector exposure, one would expect the age and sex distribution patterns of parasite positivity to be different for the wet season versus the dry season, and their study found no evidence of this (Pathak et al. 2012).

4 Detailed Investigations into Sex-Based Differences in Malaria Pathogenesis

While gender may explain differences in malaria prevalence or incidence via differences in exposure to infected mosquitos and/or care-seeking behavior, there is substantial signal that biological differences between the sexes factor into the host response to the malaria parasite. Sex-specific differences have long been observed in the pathogenesis of infectious diseases, and, in particular, the host immune response to infection (Klein and Flanagan 2016). In general, females are thought to mount more vigorous innate and adaptive immune responses, both humoral and cell-mediated, clearing infectious disease faster than their male counterparts. Immune cell activation, cytokine production, circulating T lymphocytes, and response to polyclonal stimulation are all higher in females compared with males (Klein and Flanagan 2016). There are many possible reasons for these differences, including sex-specific differences in immune cell gene expression, incomplete X chromosome inactivation, and Y chromosome-encoded immunoregulatory function (Klein and Flanagan 2016; Chan et al. 2022). Furthermore, sex hormones including estrogens, progesterone, and testosterone also have direct effects on immune cell function, and variation in concentrations of these hormones over the lifespan of individuals would predict significant interaction between age, sex, and infection outcomes.

Sex-specific differences in the host immune response to *Plasmodium* might modify the risk of infection, the response to infection, and/or parasite clearance. Relatively, few studies have been conducted evaluating sex-based differences in the human immune response to *Plasmodium* infection. The Garki project, a cohort study which observed that females had lower prevalence of *Plasmodium* infection than females, also evaluated *Plasmodium*-specific antibodies in a seroprevalence survey. Females, on average, had higher levels of anti-*P. falciparum* antibodies (IHA and IgM) (Molineaux et al. 1980). The finding of higher malaria-specific antibodies has also been observed in more recent studies, including a study of children in Eastern Uganda (Apio et al. 2020), a study in school children living in Northern Uganda (Kc et al. 2022), and a meta-analysis of adolescents and adults $\geq$ 11 years of age enrolled in clinical trials of whole sporozoite vaccine (Sanaria PfSPZ) (Kurtis et al. 2001). In the latter study, there were no sex-specific differences in malaria-specific antibody responses observed among children < 11 years of age (Kurtis et al. 2001), consistent with the epidemiologic data presented earlier. However, despite elevated antibody levels seen in post-pubertal females, this study did not observe that this translated to protection from infection (Kurtis et al. 2001). Whether malaria-specific antibodies accelerate clearance remains uncertain.

Regarding sex hormones, a study in Kenya investigated dehydroepiandrosterone sulfate (DHEAS, a pubertal steroid) levels and resistance to *P. falciparum* malaria among 248 males ages 12 to 35 years old males. When stratified by age group (12–14, 15–20, and 21–35), higher DHEAS levels were significantly correlated with lower mean parasitemia and a greater resistance to malaria in both of the older age groups (Leenstra et al. 2003). Another study observed a correlation between higher DHEAS

levels and decreased parasite density among 12–18 year-old school females in a hyperendemic region of western Kenya (Cernetich et al. 2006). Increasing DHEAS levels also correlated with an increase in mean hemoglobin levels (Cernetich et al. 2006).

In animal models, sex-specific differences have long been observed among C57BL6 mice upon infection with the murine malaria strain, *Plasmodium Chaubaudi* (Wunderlich et al. 1991; Benten et al. 1997; Krücken et al. 2005). In these studies, despite having comparable infection duration and parasite levels during infection, wild-type male mice were significantly more likely to die following *P. Chaubaudi* infection than wild-type females. Males also exhibited slower recovery and had lower immune responses that were sex-hormone responsive than female mice. In one study, gonadally intact females had higher expression of several cytokines and chemokines following *P. Chaubaudi* infection, including IFNɣ and interleukin-10, as well as higher levels of anti-*P. Chaubaudi* IgG antibodies, than males (Wunderlich et al. 1991). This study also utilized immunodeficient knock-out models to assess the roles of various immune compartments in mediating these sex-specific differences. While adaptive immune responses and IFNɣ were critically important for parasite clearance and recovery from infection, sexual dimorphisms persisted even in the absence of T or B cells, suggesting an important role for the innate immune response in mediating sex-specific differences in disease outcomes (Wunderlich et al. 1991).

Murine models have also tested the role of sex hormones in malaria pathogenesis. Administration of pharmacologic doses of testosterone suppressed expression of immune-related genes in the livers of female *P. Chaubaudi*-infected mice, and also reduced antibody production (Klein et al. 2008). Gonadectomy of females reversed the sexual dimorphic response to *Plasmodium* infection, suggesting that estradiol may also play an important role in these sex-specific differences (Wunderlich et al. 1991). Indeed, in a follow-up study, administration of estrogen in female C57BL6 mice mitigated weight loss, RBC loss, and hypothermia compared with female mice administered placebo and was associated with higher IFNɣ and interleukin-10 during peak parasitemia as well as higher anti-*P. Chaubaudi* IgG antibodies (vom Stegg et al. 2019). Together, these data suggest that sexually dimorphic differences in hormone production and host immune responses may drive differences in malaria pathogenesis in mice.

Murine models have also tested whether there are sex-specific differences in response to *Plasmodium* vaccination. In one study, adult and juvenile male and female mice were vaccinated with irradiated transgenic *Plasmodium bergheri* sporozoites and then challenged with *P. berheri* 45 days post-vaccination (vom stegg et al. 2019). Adult females were more protected from infection than males, which was associated with higher anti-circumsporozoite (CSP) antibody titers and avidity, as well as some differences in CD8+ T cell populations. However, no sex differences in either these adaptive immune responses or in protection from infection were observed in mice vaccinated prior to puberty. Because this finding suggested a role for steroid hormones, the authors depleted the mice of their respective sex steroids. Depletion

of testosterone in males resulted in greater protection from infection, while depletion of estrogens in female mice did not alter protection. However, although differences in anti-CSP antibody titers were also observed in human trials of irradiated SPZ vaccination, these differences were not associated with heightened protection against infection (Kurtis et al. 2001). Therefore, although murine models recapitulate some of the sex-specific observations seen in humans, such as differences in antibody responses following infection or vaccination, more research is needed to more fully understand mechanisms underlying sexually dimorphic responses following *Plasmodium* infection and vaccination in humans.

5 Summary and Conclusion

Overall, there do appear to be sex-based differences in the prevalence of malaria infection, although this differs across the lifespan. While there appears to be no sex-based difference in malaria prevalence in young children, the preponderance of the existing literature shows that post-pubertal males have a higher prevalence of malaria infection compared to similarly aged females. Data on differences in the clinical incidence of malaria is more mixed, which may be due to a true lack of sex-based differences, differences in how incidence or clinical cases are defined across studies, or differences in care-seeking that may differ across cultures and countries. Recent studies have suggested that post-pubertal females may have a superior ability to clear parasites from the bloodstream (anti-parasite immunity) but may also have a higher risk of becoming symptomatic once infected (anti-disease immunity). It is also clear that pregnancy represents a unique time of vulnerability for adult females, during which the mother is at higher risk for malaria morbidity and mortality compared to non-pregnant females of the same age. Furthermore, in utero malaria exposure may result in sex-specific differences in infant malaria risk, although more definitive data on this is needed.

Both gender and sex may contribute to the observed difference in malaria prevalence in males compared to females. It is possible that behavioral differences related to gender, such as increased time working outdoors or less uptake of household-level malaria control interventions such as bed nets, may increase the risk of malaria exposure in males. While these gender-based differences are often hypothesized, data to support these conjectures is scarce. Sex-specific biological differences likely also contribute to the higher prevalence of malaria infection in males, although mechanisms for this remain unclear. Potential mechanisms discussed in this review that could influence these differences include (but are not limited to): (1) sex-specific differences in host immune responses, including both innate and adaptive immune responses; (2) sex-specific hormonal differences including DHEAS, progesterone, estrogen, and testosterone, (3) heterogeneity of expression of genes on the X-chromosome, and (4) sex-specific differences in glucocorticoid receptor expression. It is still unclear which of the proposed mechanisms above (alone or in combination) is responsible for the sex-specific differences in malaria prevalence seen in humans,

though the strongest evidence to date is for sex-specific differences in host immune responses to infection.

Future directions to explore sex-based difference in malaria infection might include longitudinal cohort studies to study how sex hormones (e.g., estrogen/testosterone within males/females) and antimalarial immune responses (e.g., innate immune recognition, malaria-specific T and B cell responses and antibody responses) are associated with malarial outcomes, including risk of infection, risk of symptomatic disease, severity of symptoms, and ability to control and clear infection. In addition, mechanistic studies are needed to determine how in utero exposure and malarial vaccines may differentially impact males versus females. Though there is currently little evidence in humans to suggest that there are sex-based differences in vaccine efficacy in regard to the leading malaria vaccine candidates, as RTS,S/AS01 and other vaccines are rolled out it will be important to disaggregate vaccine efficacy and other outcomes by sex. Furthermore, as new candidates emerge, sex-based differences should be carefully assessed for and, if discovered, may be useful in deepening our understanding of malaria pathogenesis. In sum, a more thorough understanding of sex-specific differences in malaria pathogenesis may provide opportunities for novel treatment and vaccination strategies, and this should be an active area of future research.

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Sex Difference in Amebiasis

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Abstract Infection with the protozoan parasite *Entamoeba histolytica* is much more likely to cause severe, focal liver damage in males than females, although the infection rate is the same in both sexes. The differences in disease susceptibility may be due to modulation of key mechanisms of the innate immune response by sex hormones. Complement-mediated mechanisms and estrogen-dependent activated natural killer T cells lead to early elimination of the parasite in females, whereas a pathological immune axis is triggered in males. Testosterone, which is generally thought to have more immunosuppressive properties on cells of the immune response, leads to overwhelming activation of monocytes and host-dependent destruction of liver tissue in males resulting in worse outcomes.

1 General Introduction

Prevalence and morbidity of parasitic infections are often greater in men than in women, although exceptions certainly occur (Klein 2004; Bernin and Lotter 2014). Sex differences in the incidence of parasitic disease depend not only on biological sex, but often on sociocultural and behavioral factors that may influence exposure. However, even once relevant studies are adjusted for these factors, there are still hormonal and chromosomal sex-related factors that influence the immune system and thus have an impact on the outcome of infections.

Amebiasis is a prototypical example for a parasitic disease that occurs with a clear sex bias toward males. This human parasitosis is widespread in tropical and

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S. L. Klein and C. W. Roberts (eds.), *Sex and Gender Differences in Infection and Treatments for Infectious Diseases*, Current Topics in Microbiology and Immunology 441, https://doi.org/10.1007/978-3-031-35139-6_8

subtropical areas and affects approximately 50 million people (Zulfiqar et al. 2022), resulting in 55,000 deaths annually (Shirley et al. 2018). Although several Entamoeba species like *Entamoeba (E.) dispar* or *E. moshkovskii* are able to infect humans, only *Entamoeba (E.) histolytica* is able to induce severe clinical symptoms of disease.

Infections with this protozoan parasite occur through ingestion of cysts via contaminated food or drinking water. In the intestine, trophozoites emerge from these cysts and colonize the colon system, where they can persist for several months or even years without causing signs of disease. In about 10% of cases, the parasite invades the mucosa, thereby causing severe symptoms of invasive amebiasis, like dysentery or hemorrhagic colitis. If the parasite penetrates the mucosal wall, it can spread to different organs, like the liver, the lung or the brain, via the bloodstream. In the liver in particular, massive destruction of the liver tissue, known as an amebic liver abscess, is observed. While amebic dysentery affects both men and women, more than 80% of amebic liver abscesses occur in adult men, while the disease is rare in children and women (Blessmann and Tannich 2002). In an epidemiological study from Vietnam, it was shown that the risk of developing an amebic liver abscess increases significantly after puberty in boys. The peak incidence was found in adult men around 40 years of age, with a male-to-female ratio of 7:1 (Blessmann and Tannich 2002). Thereafter, the incidence decreases steadily with age, suggesting a causal relationship with the male sex hormones testosterone and dihydrotestosterone circulating in the serum (Travison et al. 2017). In women, the incidence remains stable but increases slightly with age. Interestingly, the sex difference exists despite higher parasite prevalence in the intestines of women (Blessmann et al. 2003). The sex-specific occurrence of amebic liver abscesses is observed not only in a wide variety of areas around the world (Acuna-Soto et al. 2000), but also in travelers visiting endemic areas (Cordel et al. 2013).

2 Interactions of *E. histolytica* and Host Innate Immunity

To date, the transition between asymptomatic colonization of the intestine and invasion through the intestinal wall by the parasite is not fully understood. The parasite possesses a broad spectrum of effector molecules that form the prerequisite for its pathogenicity, and many of these factors exert an influence on the innate immune system. Since there are considerable general sex-specific differences in the activation of immune cells (Klein 2012; Markle and Fish 2014), the initial contact with the parasite can be decisive for the further course of the disease. Early studies have shown that when the parasite encounters intestinal epithelial cells, the surface galactose/N-actelygalactosamine (GalNAc)-inhibitable lectin initiates a cascade of events (Begum et al. 2020), starting with the production of proinflammatory cytokines and the recruitment of innate immune cells, i.e., neutrophils and macrophages, that are able to amplify and extend the initial immune response by secreting reactive oxygen species, nitric oxide, and further chemokines (Guerrant et al. 1981; Denis 1989a,

b). Moreover, binding of GalNAc lectin exerts a direct enhancing effect on NF-κB-dependent mRNA expression of Toll-like receptors (TLR2 and TLR4) by host macrophages (Kammanadiminti et al. 2004). Regardless of whether these findings were collected in vitro or in vivo, there are many avenues through which sex-specific influences could mitigate or support infection at very early stages. However, at the time these studies were conducted, the sex of the host cells engaging with the parasite was not taken into account. As known so far, 17ß-estradiol increases the numbers and antimicrobial activity of neutrophils, represented by higher degranulation and release of elastase (Markle and Fish 2014; Klein and Flanagan 2016), and the production of protective interferon gamma (IFNγ by natural killer T (NKT) and natural killer (NK) cells (Gourdy 2005; Manukyan et al. 2020), which might contribute to female resistance at the initial site of infection. Another component of the innate immune response, the complement system, is also of high relevance for parasite survival within the host during its spread via the host's blood stream. *E. histolytica* is highly sensitive for complement-mediated lysis, which has been shown to be mediated by putative serine/threonine kinase (Walderich et al. 1997; Urban et al. 1996). Interestingly, serum samples from female individuals were significantly more effective in lysing *E. histolytica* trophozoites than those from male individuals. Reduction in complement activity induced by heat-inactivation of the serum or addition of EDTA inhibited the killing and abolished this sex difference (Snow et al. 2008). However, further studies to identify the relevant complement component are needed, and similar studies using other microbial agents have not yet been performed.

3 Direct Effect of Hormones on *E. Histolytica*

There are several examples showing that sex hormones directly affect parasite growth and reproduction and, conversely, parasites themselves are able to affect host sex hormone levels, either by production or consumption of sex hormones (Klein 2004). In the majority of reports, the female sex hormone estradiol exhibits a more positive effect on parasites in vitro, as it increases the growth and reproductive capacity of the tapeworms *Taenia crassiceps* and *Taenia taeniaformis* (Escobedo et al. 2004) and also directly enhances the numbers of gametocytes from *Plasmodium falicparum* (Lingnau et al. 1993). The male sex hormones testosterone or dihydrotestosterone, on the other hand, seem to have a more negative effect on parasites. In case of the tape worms, in vitro treatment with testosterone affected DNA integrity, presumably by inducing apoptotic effects (Escobedo et al. 2004). In its sulfated and unsulfated form, the precursor of male sex steroids dehydroepiandrosterone (DHEA) is the most abundant hormone circulating in the blood (Kamin and Kertes 2017) that exerts antiparasitic effects that exert antiparasitic effects on a variety of parasites. Treatment with DHEA or its analogs inhibits the gametocyte formation of *P. falciparum* (Freilich et al. 2000), the viability and fertility of *Schistosoma ssp.* and has been shown to exhibit toxoplasmicidal activity in vitro (Morales-Montor et al. 2001; Remoue 2002; Muniz-Hernandez et al. 2021). Interestingly, DHEA has also been

shown to impede adherence, proliferation and motility of *E. histolytica* in vitro and causes lysis of trophozoites in a dose-dependent manner. As determined through patterns of DNA fragmentation and TUNEL assays, trophozoite lysis was presumably mediated by necrotic rather than apoptotic processes (Carrero et al. 2006). In contrast, treatment with 17β-estradiol, progesterone, or testosterone had only minor effects on *E. histolytica* trophozoites (Carrero et al. 2006).

The capacity of parasites to modulate host sex hormone levels might also stem from the expression of androgen and estrogen receptors, as has been shown for *Taenia solium* and other helminths (Escobedo et al. 2004; Aguilar-Diaz et al. 2018; Wu and Loverde 2011), or the production of sex hormones by helminth parasites, as known for *Taenia ssp.* (Romano et al. 2003). To date, neither sex hormone receptors or analogs, nor production of sex hormones have been found in human protozoan parasites.

In summary, parasite viability appears to depend on sex hormones. Parasites are able to modulate host sex hormone levels and thus influence the effect of sex hormones on cells of the immune system and thus the success of their survival in the host.

4 Sex Differences in Human Immunity During Amebiasis

4.1 Innate Immunity in Human Amebiasis

Early studies in the past century have shown that virulent *E. histolytica* trophozoites are able to ingest and kill neutrophilic granulocytes in vitro in a contact-dependent manner (Guerrant et al. 1981). However, previous activation of neutrophils or macrophages with IFNγ, tumor necrosis factor alpha (TNF), amebic extracts, or the Gal/GalNAc lectin give these cells the ability to kill amebic trophozoites, presumably mediated by nitric oxide (NO) (Salata and Ravdin 1985; Salata et al. 1987; Denis and Chadee 1989a, b; Seguin et al. 1997; Ghadirian and Salimi 1993). However, sex-specific influences on these cells were never addressed at the time. We recently studied differences in the activation of blood monocytes isolated from men and women (Sellau et al. 2020). Three major monocyte subpopulations have been described for humans: classical CD14+CD16-, intermediate CD14+CD16+, and non-classical CD14-CD16+monocyte subpopulations (Geissmann et al. 2003; Auffray et al. 2009; Ziegler-Heitbrock et al. 2010). Although there were no differences in the percentage of these populations in the periphery, classical monocytes from men exhibited a higher expression of the C-C- chemokine receptor 2 (CCR2) on the surface. This was interesting because we had previously shown that out of a large group of serum chemokines, only the ligand for this receptor, CCL2, was more highly expressed in men infected with *E. histolytica* trophozoites in the intestine than in women (Bernin and Lotter 2014). Isolation of classical monocytes from men and women and subsequent stimulation with amebic antigens revealed that CCL2 and,

in particular, CXCL1, a second chemokine involved in the recruitment of monocytes and neutrophils to a site of infection, were more highly expressed by monocytes from men than from women. Furthermore, it has been shown that CXCL1 production is further increased in the presence of the highly potent androgen dihydrotestosterone (Sellau et al. 2020). Monocytes and macrophages are a major source of tumor necrosis factor alpha (TNFα) (Trinchieri 1991). Upon LPS or amebic antigen stimulation, CD14+CD16- monocytes from men produced slightly higher amounts of TNFα than those from women. Initial in vitro experiments on isolated CD14+CD16- monocytes did not reveal a substantial influence of dihydrotestosterone on TNFα production by monocytes. However, in LPS-stimulated PBMCs from men who underwent testosterone treatment during sex reassignment, testosterone treatment increased the production of TNFα, but also of CCL2 and CXCL1 (Sellau et al. 2020). TNFα seems to be of particular importance for immunopathological mechanisms in invasive amebiasis. For example, in an epidemiological study, TNFα was shown to modulate the outcome of amebic colitis in children. A concentration-dependent increase in 1000 pg/mL TNFα levels correlated with an 18% increased likelihood of developing *E. histolytica* diarrhea (Peterson 2010).

In addition to excessive recruitment and TNFα-dependent activation of monocytes and neutrophils and their release of nitric oxide (NO) and reactive oxygen species (ROS) to combat the invading parasite, host tissue may also be inadvertently damaged (Moonah 2013). Accordingly, blocking TNFα in two mouse models of amebic colitis and hepatic amebiasis, respectively, reduced inflammation and damage to the intestine and liver (Zhang et al. 2003; Helk et al. 2013). Overall, male sex hormones have an impact on recruitment and activation of innate immune cells, particularly monocytes and macrophages, and thus may potentially contribute to immune-mediated tissue damage in amebiasis.

Natural killer T cells (NKT cells) bridge the innate with the adaptive immune response and are able to mount fast and considerable production of Th1 and Th2 cytokines, thus playing an important regulatory role in a variety of infectious diseases (Godfrey and Kronenberg 2004; Tupin et al. 2007). They express an invariant T cell receptor (TCR), consisting of the Vα24-Jα18/Vβ11 chains in humans and Vα14-Jα18/Vβ8.2 chains in mice, that recognizes lipid antigens in a CD1d-dependent manner with alpha-Galactosyl Ceramide (αGalCer) being the most potent stimulant (Sullivan and Kronenberg 2005). Based on their expression of CD4 and CD8, NKT cells can be divided into three subpopulations: CD4+, CD8+, and CD4−CD8− (double negative; DN) (Godfrey 2010). Several studies have described higher amounts of circulating NKT cells in women compared to men (Singh et al. 2022; Kee et al. 2012; Bernin et al. 2016). Furthermore, stimulation of iNKT cells with αGalCer induced higher production of intracellular IFNγ, IL-4, IL-17, and TNFα by CD4+ and DN+NKT cells from women compared to men (Bernin et al. 2016). Interestingly, *E. histolytica* trophozoites possess a molecule in their membrane that has been shown to trigger CD1d-dependent production of cytokines in human and murine iNKT cells, the *E. histolytica* lipopeptide phosphoglycan (*Eh*LPPG) (Lotter et al. 2009). Compared to αGalCer, *Eh*LPPG induces weaker cytokine production with no sex differences in terms of IFNγ or IL-4, but a strong tendency toward a higher

production of IL-17A in NKT cells from women compared to men (Bernin et al. 2016). Whether this contributes to the sex differences during ALA in human remains to be explored in further epidemiological investigations. However, at least in mice, it has been demonstrated that NKT cells are responsive to female sex hormones, in that estradiol treatment increases IFNγ production upon αGalCer stimulation (Gourdy et al. 2005).

5 Adaptive Immune Responses to Infections with *E. histolytica*

5.1 Humoral Immunity

Severe invasive courses of amebiasis, as well as asymptomatic colonization of the intestine, robustly lead to the induction of a humoral immune response in the host. This includes serum and secretory IgA, IgM, and the four IgG subclasses IgG1, IgG2, IgG3, and IgG4 (Haque et al. 2001; Moonah et al. 2013; Bernin and Lotter 2014; Kaur et al. 2004).

Secretory IgA is one of the most effective immunoglobulin classes elicited against the intestinal form of amebiasis. Protection from amebic infections was found to be associated with mucosal IgA directed against Gal/GalNAc lectin, with adherence mediating the pathogenicity factor of *E. histolytica* in a cohort of preschool children in Bangladesh (Haque et al. 2001), as well as in patients who had recovered from hepatic amebiasis. Here, high levels of anti-lectin IgA were associated with reduced susceptibility toward subsequent amebic infections in the intestine; however, in all of these studies, the samples were not stratified by sex-specific criteria (Ravdin et al. 2003; Haque et al. 2006; Abd-Alla et al. 2006).

In contrast, no correlation with protection was found with serum IgG titers against the lectin (Haque et al. 2001). In adult ALA patients or asymptomatic carriers, serum anti-amebic IgA was detected only at low titers with no differences between men and women (Bernin and Lotter 2014). However, so far, no association between IgG subclasses and protection has been reported (Moonah et al. 2013). On the contrary, IgG against the Gal/GalNAc lectin favored re-infection in children, and previous episodes of ALA in adults in Vietnam led to 2.7-fold re-infection rates compared to the control group (Blessmann et al. 2003). Analysis of the humoral immune response within this collective revealed that ALA infection induced higher IgG titers in ALA patients compared to asymptomatic controls. Stratified by sex, the IgG and IgG subclass titers were significantly higher in males compared to females between these groups, including serum IgA. Interestingly, in asymptomatically infected carriers, total IgG and IgG1 antibodies were significantly higher in women compared to men (Bernin and Lotter 2014). Because ALA occurred at a male-to-female ratio of 7:1 in this study population from an amebiasis-endemic area in Vietnam (Blessmann and Tannich 2002), it could be hypothesized that the spread of *E. histolytica* was reduced

in females who already possessed higher antibody titers. Since *E. histolytica* infection induces long-lasting anti-amebic antibody titers, it is unclear whether the high titers in asymptomatically infected women are a remnant of controlled pre-infections, or due to continuous challenges to the immune response through subclinical mucosal lesions. Considering the strong complement sensitivity of amebic trophozoites, the high IgG1 levels, as the strongest complement activator among the IgG subclasses, could further explain the previously mentioned higher capacity of female serum to induce complement-mediated lysis of trophozoites (Snow et al. 2008). This, among other mechanisms, may provide another explanation for the resistance toward ALA in women.

5.2 T Cell-Mediated Immunity

In contrast to antibody-dependent immune responses, studies examining CD4 and CD8 T cell-dependent immune responses in invasive amebiasis are very limited and none of these previous studies considered sex-specific differences.

Studies from the 1990s showed that patients with amebic liver abscesses have lower T4-to-T8 ratios compared to age and sex-matched controls. This was thought to be due to a decrease in T4 helper cells and an increase in T8 suppressor cells. In addition, in vitro the proliferative response of the patients' T lymphocytes to the plant mitogen concanavalin A (Con A) was decreased, suggesting immunosuppressive mechanisms in the T cell response during active hepatic amebiasis. Following therapy for amebic hepatic abscess, at least four weeks were necessary for the development of an optimal T cell proliferative response (Salata et al. 1986). An increase in CD8+ cells and a decrease in CD4+ cells in hepatic amebiasis, either in the peripheral blood of patients (Taher uz et al. 1993) or in immunohistochemical studies of human liver sections (Ventura-Juarez et al. 1997) has also been observed in other studies. In non-diseased, but anti-amebic antibody-positive individuals, a high proliferation rate and IL-2 production were observed in T lymphocytes after stimulation with the Gal/GalNAc lectin (Schain et al. 1992). Besides IL-2, cell-mediated IFNγ appears to be effective against amebiasis, not least through its ability to induce amebicidal activity in neutrophils and macrophages. In a prospective study, high IFNγ-production by pediatric peripheral blood mononuclear cells (PMBCs) following stimulation with soluble ameba extract positively correlated with a significantly lower incidence of future amebic diarrhea episodes in children (Haque 2007). Protection against intracecal *E. histolytica* infection by passive transfer of IFNγ and other proinflammatory cytokines, including IL-17-producing T cells, to naive animals later confirmed these findings (Guo et al. 2009; Guo et al. 2011).

6 Host Genetic Influences on Susceptibility to Invasive Amebiasis

Mounting evidence suggests that host genetic factors influence the frequency and severity of *E. histolytica* diarrhea episodes. The HLA class II allele DQB1*0601 and the haplotype DQB1*0601/DRB1*1501 are associated with drastically decreased rates of *E. histolytica* infection (Duggal et al. 2004). More recently, a 12-year study of the Mirpur cohort has shown that a single amino acid replacement (Q223R) in the extracellular domain of the leptin receptor encoded by a SNP is associated with a nearly fourfold increase in susceptibility to infection by *E. histolytica* as well as an overall decrease in time to infection. The effects of the mutation were confirmed only in a population of adult male ALA patients, where homozygosity for the allele encoding arginine at position 223 was associated with twofold increased susceptibility to extraintestinal disease. These observations highlight the importance of leptin signaling and point to a specific mutation that increases susceptibility to *E. histolytica*, decreases time to infection, and increases the likelihood of developing extraintestinal amebiasis, but unfortunately these studies were performed only conducted on male, not female patients (Duggal et al. 2011).

7 Sex Differences in Mouse Models for Amebic Colitis

Innate immunity plays a major role in the control of intestinal and hepatic amebiasis. Most of the findings in this area come from murine models for the disease. Detailed studies on sex-dependent influences arise mostly from the model for hepatic amebiasis. However, there are studies showing that the outbreak rate of amebic colitis appears to be higher in men than in women (Acuna-Soto et al. 2000). In their work, two groups put forward a mouse model for amebic colitis in which they transfer the trophozoite form of *E. histolytica* into the caecum of anesthetized mice (Hamano et al. 2008) (Houpt et al. 2002). Interestingly, this form of amebic colitis induction can only be performed in a limited number of mouse models, as the commonly used mouse strains C57BL/6 and BALB/c appear to have a natural resistance to intestinal infection with *E. histolytica.* The CBA and/or C3H mouse line shows the most promising results in terms of infection rate, therefore, when implementing the amebic colitis model in the laboratory, one should focus on working with these mice (Ghosh et al. 1994) (Ghosh 2000). The group of Hamano et al. (2008) investigated this resistance phenomenon and created an F1 hybrid mouse consisting of B6 and CBA DNA. These hybrid mice show similar resistance to amebic infections in the gut as the B6 mouse. They even went one step further and backcrossed the F1 mouse with the B6 parent to create the N2 mouse (25% CBA; 75% B6). Strikingly, a sex difference in infection rate was observed in these N2 mice, with male offspring being significantly more likely to develop amebic colitis (42%) than female N2 offspring (15%). In addition, the group linked the resistance to some mutations and SNPs on

chromosomes 1, 2, 4, 7, and 17 that affect the development of infections, parasite load, and inflammation. (Hamano et al. 2008). When establishing this kind of a mouse model, a reliable scoring method and a series of assays to determine the severity of colitis in the infected mice are needed. A relatively good method is immunohistochemistry of paraffin-embedded caecum fractions, which can be used to detect the amount of trophozoites and infiltrating immune cells. Another appropriate method is the detection of *E. histolytica* antigen in mouse feces using a specific ELISA test method. One can also assess mucosal thickness and colon length to distinguish between infected and uninfected mice. In addition, some markers, in the form of cytokines and antibodies which are associated with amebic colitis, exist; the mucous membranes of infected mice tend to produce more IL-4, IL-13 and IFNy, and significantly higher levels of IgA antibodies can also be detected in blood serum (Houpt et al. 2002). All in all, there are very few studies and groups work with this mouse model for amebic colitis and sex-specific aspects have not been considered.

8 Sex Differences in the Murine Model for Hepatic Amebiasis

Hepatic amebiasis leading to the development of amebic liver abscess is one of the most common symptoms of invasive amebiasis following infection with *E. histolytica* (Gathiram and Jackson 1987). Since humans are the only relevant host for *E. histolytica*, the development of a suitable immune-competent animal model has been challenging. However, multiple passages through the livers of mice rendered a human isolate strain, HM1: IMSS (Davis et al. 2007), capable of inducing ALA lesions in C57BL/6 mice (Lotter et al. 2006). Most interestingly, this model reflected the same sex differences observed in humans, with larger abscesses and longer times needed for parasite clearance in male mice compared to female mice. However, in contrast to the human disease, mice were able to control abscess development within the first three weeks after infection (Lotter et al. 2006). Upon histological examination, many similarities between humans and rodents in the composition of abscess lesions were found, specifically regarding immune cell infiltrates and central areas of necrosis (Ventura-Juarez et al. 2003). Hormone exchange studies revealed that gonadectomy and testosterone supplementation increased the size of liver abscesses in female mice, while gonadectomy and estradiol treatment of male mice rendered these mice more resistant to ALA development (Lotter et al. 2006; Carrero et al. 2006). IFNγ, as mentioned above, plays a crucial role in the immune control of amebic liver abscess. Hence, abscess size is increased in SCID mice with targeted disruption of the IFNγ receptor or upon antibody-mediated immune depletion of IFNγ, as is the susceptibility to ALA in female mice (Seydel et al. 2000; Lotter et al. 2006). Furthermore, the female resistance toward ALA development is mirrored by increased production of IFNγ in spleen cells of female mice compared to those of male mice, while larger quantities of non-protective IL-4 are produced in spleen cells

of male mice (Lotter et al. 2006). As noted above, NKT cells are highly abundant in the liver and are an important source of early IFNγ (Godfrey et al. 2004; Tupin et al. 2007). *Eh*LPPG induces IFNγ in NKT cells from the liver in a sex-dependent manner (Lotter et al. 2013). It has been shown that NKT cells from female mice, isolated from the liver during amebic infection, produce more IFNγ following stimulation with *Eh*LPPG or the stimulant αGalCer compared to NKT cells from male mice. Moreover, it could be demonstrated that this IFNγ production was reduced in female mice upon testosterone treatment. Conversely, NKT cells from gonadectomized male mice demonstrated higher IFNγ production compared to non-gonadectomized mice (Lotter et al. 2013; Gourdy et al. 2005).

Using this C57BL/6 model for amebiasis, it was possible to characterize the composition of infiltrating innate immune cells in the early phase of abscess development and their contribution to liver damage (Helk et al. 2013). Antibody-mediated depletion of neutrophils or monocytes resulted in moderate to considerable reduction of abscess sizes, respectively. The use of C-C chemokine receptor 2 (CCR2) knockout mice, in which egress and recruitment of monocytes, is hampered (Shi et al. 2011) as well as adoptive transfer experiments revealed that inflammatory, CCR2+Ly6C^{hi} monocytes substantially contribute to ALA development (Helk et al. 2013).

Furthermore, clodronate-dependent depletion of liver-resident Kupffer cells as well as antibody-dependent depletion of TNFα further confirmed that immunopathological mechanisms underly ALA development in mice (Helk et al. 2013). On the other hand, regenerative and anti-inflammatory Ly6C^{lo} monocytes were found to promote tissue regeneration after ALA induction through expression of the cytokine IL-13 and induction of arginase 1 (Noll 2016). In addition to greater abscess formation in males, significantly higher levels of TNFα, CCL2, and CXCL1, another chemokine involved in the recruitment of neutrophils and monocytes, were detected in the sera of male mice compared to female mice during the course of infection. One major source for these cytokines was found to be Ly6C^{hi} monocytes, and unsurprisingly, a higher percentage of these cytokine-producing monocytes was found in the livers of male mice compared to female mice following amebic infection. Most interestingly, following testosterone substitution in gonadectomized male mice, not only did the amount of Ly6C^{hi} monocytes increase substantially compared to placebo-treated, gonadectomized male mice, but also the percentage of TNFα, CCL2, and CXCL1-producing Ly6C^{hi} monocytes (Sellau et al. 2020).

In addition, activation of the pathological IL-23/IL-17 immune axis prior to recruitment of monocytes via the chemokine CCL2 could be identified as a factor in the early infection phase (Noll et al. 2016; Gaffen et al. 2014). A lack of IL-23 or IL-17 significantly reduced ALA formation in male mice. IL-17 influences the migration of neutrophils and monocytes into inflammatory tissue by inducing CCL2 expression (Shahrara et al. 2009). TH_{17} cells have been identified as a major source of IL-17, and a sex bias has been found in terms of the size of this cell population (Groneberg et al. 2022). As counterparts of TH_{17} cells, T_{reg} cells contribute to an anti-inflammatory milieu and are present in greater numbers in female mice. During the polarization of a naïve T cell into a TH_{17} or T_{reg} cell, the cytokine IL-6 acts as the main modulator determining the outcome of the polarization. However,

the expression of IL-6 is influenced by the amount of available oxygen, which is present in lower quantities under pathophysiological conditions (such as ALA) and therefore leads to hypoxia in the tissue. Under such conditions, monocytes express more IL-6 (Matsui et al. 1999), which in turn leads to an increase in the TH_{17} population. Hypoxia is associated with overexpression of the protein hypoxia-inducible factor 1 (HIF-1α). HIF-1α can directly activate the TH_{17}-specific transcription factor retinoid-related orphan receptor-γτ (RORγτ), converting naive T cells into the TH_{17} phenotype (Dang et al. 2011; Shi and Pamer 2011). The impact of HIF-1α on the effects of ALA in mice, particularly in males, is significant enough that deletion of this protein even completely abrogates sex-specific abscess formation (Groneberg et al. 2022). An overview of all the mechanisms and pathways described above that lead to the sex differences in ALA formation is shown in Fig. 1. Translating certain findings to humans, increased CXCL1 levels can be observed in stimulated peripheral blood lymphocytes from men undergoing sex reassignment treatment through administration of testosterone as well as in isolated classical CD14+ monocytes (Sellau et al. 2020). Overall, the ALA mouse model has provided relevant insights into immune mechanisms underlying abscess formation, the composition of the immune cells involved and cytokine expression. Future studies could translate further findings from this animal model to human ALA patients or monocyte-mediated diseases and potentially give rise to new, personalized treatment strategies.

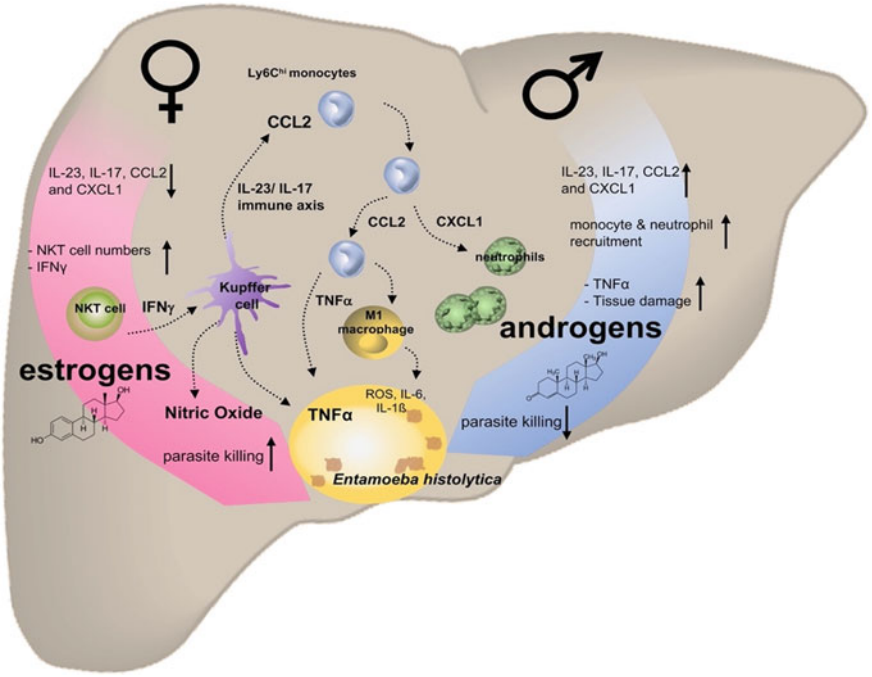

Fig. 1 Sex differences in immune mechanisms underlying *E. histolytica*-induced liver damage

Intrahepatic infection with *E. histolytica* trophozoites triggers the pathological IL-23/IL-17 immune axis, leading to production of CCL2 and recruitment of TNFα- and CXCL1-producing inflammatory monocytes. The enhanced recruitment of monocytes and neutrophils in males is modulated by androgens and leads to the sex difference in abscess development, while estrogen-dependent IFNγ production by NKT cells contributes to higher resistance in females.

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Zulfiqar H, Mathew G, Horrall S (2022) In: *StatPearls*

Sex-Differential and Non-specific Effects of Vaccines Over the Life Course

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Abstract Biological sex and age have profound effects on immune responses throughout the lifespan and impact vaccine acceptance, responses, and outcomes. Mounting evidence from epidemiological, clinical, and animal model studies show that males and females respond differentially to vaccination throughout the lifespan. Within age groups, females tend to produce greater vaccine-induced immune responses than males, with sex differences apparent across all age groups, but are most pronounced among reproductive aged individuals. Females report more adverse effects following vaccination than males. Females, especially among children under 5 years of age, also experience more non-specific effects of vaccination. Despite these known sex- and age-specific differences in vaccine-induced immune responses and outcomes, sex and age are often ignored in vaccine research. Herein, we review the known sex differences in the immunogenicity, effectiveness, reactogenicity, and

The original version of this chapter was revised. The incorrect citation with the author's name Clair, LAS has now been corrected as St. Clair, LA. The correction to this chapter is available at https://doi.org/10.1007/978-3-031-35139-6_11

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S. L. Klein and C. W. Roberts (eds.), *Sex and Gender Differences in Infection and Treatments for Infectious Diseases*, Current Topics in Microbiology and Immunology 441, https://doi.org/10.1007/978-3-031-35139-6_9

non-specific effects of vaccination over the lifespan. Ways in which these data can be leveraged to improve vaccine research are described.

1 Introduction

Biological sex (i.e., differences between males and females based on sex chromosome complement, reproductive tissues, and sex steroid concentrations) and age contribute significantly to differences in vaccine acceptance, induced immune responses, and clinical outcomes (Klein et al. 2010). Although females are often less likely to accept vaccines (Pulcini et al. 2013), they tend to develop greater antibody and cell-mediated immune responses following vaccination than males (Klein et al. 2010; Umlauf et al. 2012; Zhang et al. 2008) (Fig. 1). After vaccination against influenza, yellow fever, rubella, measles, mumps, hepatitis A and B, herpes simplex 2, rabies, smallpox, dengue viruses, and SARS-CoV-2, protective antibody responses can be as much as twice as high in adult females as compared with males (Klein et al. 2010; Nam et al. 2022). Females are also more likely to develop severe adverse reactions, including local and systemic pain, inflammation, fever, and allergic reactions (Furman et al. 2014; Klein et al. 2010; Poland et al. 2009). It has often been assumed that greater adverse events among females reflects a gender difference (i.e., sociocultural construct that defines behavioral, occupational, and even decision-making strategies) rather than a biological difference, as data on adverse events are often obtained through passive reporting, and females may be more likely to report adverse events than males (Klein and Morgan 2020, Morgan and Klein 2019). Data for COVID-19 mRNA vaccination illustrate that females are more likely to report non-serious adverse events and experience more local reactions (e.g., rash at injection site) and anaphylaxis than males (Blumenthal et al. 2021; Shimabukuro et al. 2021). In response to COVID-19 adenovirus-vectored vaccines, females < 50 years of age are more likely to experience thrombosis and thrombocytopenia (Lai et al. 2021; Li et al. 2022) and males < 50 years of age are more likely to develop myocarditis (Pepe et al. 2021). Many of these more serious adverse events cannot merely be attributed to reporting biases and likely reflect biological differences between the sexes. Furthermore, studies from low-income settings with high infectious disease pressure have revealed that routine childhood vaccines may affect all-cause mortality in such settings, to a degree that is not explained by the vaccine-specific effects, i.e., vaccines also have so-called non-specific effects ("off-target" or "heterologous" effects). Intriguingly, these effects differ greatly between boys and girls, both beneficial and harmful non-specific effects being strongest for girls. These differences are indicative of biological differences in vaccine response already in childhood.

The biology of males and females is not static throughout the life course, but instead reflects the changes that occur in genetic and epigenetic factors, hormones, and environmental interactions over the lifespan (Bronikowski et al. 2022, Fischer et al. 2018, He et al. 2021, Klein and Flanagan 2016); however, minimal consideration is given to how these sex-specific shifts in biology over the life course contribute to

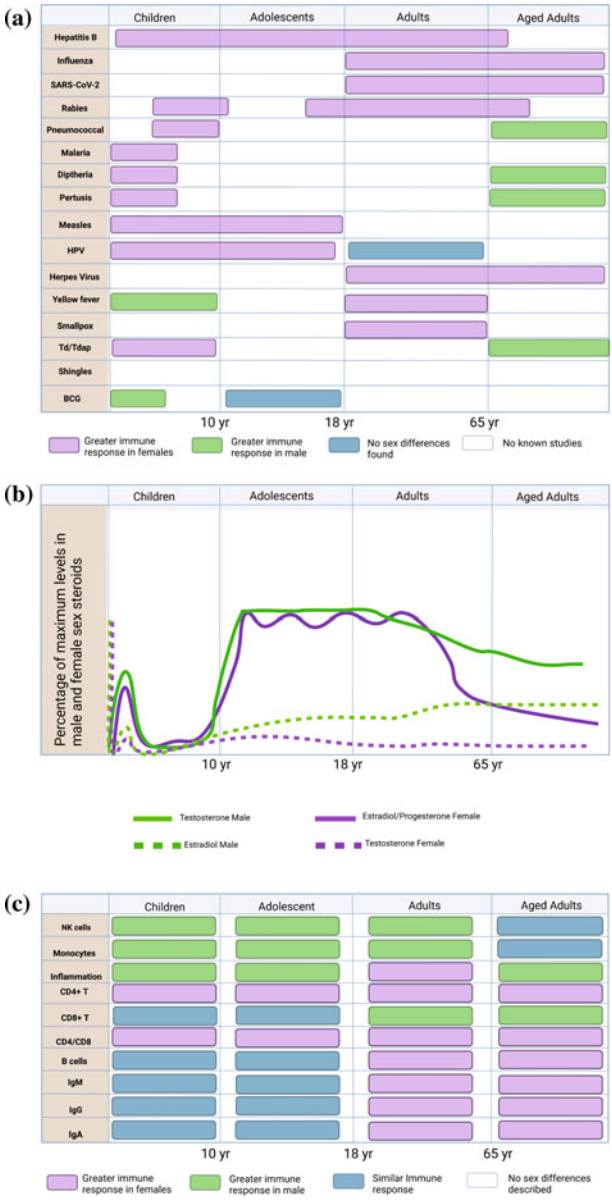

Fig. 1 Vaccine-induced immunity, sex steroids, and immunological responses change over the lifespan. **a** Immunization tables recommend that certain vaccines are given during particular ages, with greater antibody responses observed in males (green) or females (purple) at different life stages. Concentrations of circulating sex steroids (**b**) and sex differences in cell-mediated immune responses (**c**) also change over the life course, reflecting two possible biological mechanisms that impact sex differences in vaccine-induced immunity

vaccine-induced immunity (Fig. 1). The goal of this review is to highlight the breadth of sex differences in vaccine-induced immunogenicity, reactogenicity, and protection against vaccine-preventable diseases as well as non-specific effects on other diseases and all-cause mortality over the life course and provide recommendations to improve pre-clinical and clinical vaccine trials. We propose that all future vaccine studies should evaluate the impact of sex and age on immunological responses and vaccination outcomes.

2 Effects of Biological Sex on Vaccine Responses Over the Life Course

2.1 Birth to 5 Years of Age

As the immune system is not fully developed in neonates, they are particularly vulnerable to microbial infections. Infants have an anti-inflammatory skewed immune response that is characterized by high levels of circulating IL-10, adenosine, and regulatory T cells (Tregs); suboptimal immunological memory; reduced reactivity of innate immune cells; and a Th2-biased adaptive immune response (Burl et al. 2011, Kollmann et al. 2012, Levy et al. 2006, Ndure et al. 2014, Noho-Konteh et al. 2016, Papaioannou et al. 2019, Tsafaras et al. 2020, Zazara and Arck 2019).

Neonates and infants are the target recipients of many vaccines (Fig. 1a). Starting in 1974, the World Health Organization initiated the Expanded Program on Immunization (EPI), through which they have developed standardized vaccination schedules recommended for neonates, infants, and children (World Health Organization 2021).

Antibody responses to many childhood vaccines are greater in females with some exceptions (Flanagan et al. 2015). Females who are vaccinated with the hepatitis B vaccine (HBV) or the combined measles, mumps, rubella vaccine (MMR) by one year of age, but not after, showed greater and longer lasting antibody responses compared to males (Flanagan et al. 2015; Kontio et al. 2016; Trevisan et al. 2020). No sex differences are observed in antibody response to HBV and MMR when children were vaccinated at 5 months or at 18 months of age, respectively (Kontio et al. 2016; Trevisan et al. 2020). Greater antibody responses to diphtheria, pertussis, hepatitis A, HBV, pneumococcus, rabies, human papilloma virus (HPV), and rubella vaccines as well as to the candidate malaria vaccine RTS,S/ASO2 are observed in females compared to males (Boef et al. 2018, Flanagan et al. 2015, Noho-Konteh et al. 2016). In a meta-analysis of individual data from vaccine trials, antibody responses to *Haemophilus influenzae* type b (Hib) and tetanus toxoid vaccines were found to be comparable among younger aged males and females under 3 years of age (Voysey et al. 2016). Individual studies, however, reveal that females following infant vaccination have moderately higher IgG levels against Hib and tetanus compared to males, while no sex differences are observed in antibody responses to diphtheria and

pertussis vaccines (Boef et al. 2018). In a meta-analysis of the published clinical trial data on the quadrivalent HPV vaccine (qHPVV), antibody titers are greater in children (<16 years of age) than in adults, and qHPVV is more immunogenic in females across all age groups than in males (Aldakak et al. 2021). Further, stronger sex differences are observed in vaccine responses against low-risk HPV strains (6 and 11) compared to high-risk strains (16 and 18) (Aldakak et al. 2021).

Compared to humoral responses, data regarding the effect of biological sex on cell-mediated responses to childhood vaccination lag further behind and show no clear trend in sex-specific responses. Studies have reported a male bias in cell-mediated immune responses as evidenced by larger bacille Calmette-Guérin (BCG) scars and tuberculin skin test reactivity in infants under one year age (Burl et al. 2010; Diness et al. 2007) and greater rubella-specific lymphoproliferation (Mitchell 1999) in males aged 15 years old as compared to females. Females have greater herpes simplex 2 virus (HSV-2)-specific CD4+ T cell responses following HSV-2 vaccination (Zhang et al. 2008). Studies of tuberculosis and measles, however, report no sex differences in either *M. tuberculosis*-specific Th1 or Th2 responses (Sartono et al. 2010) or in measles-specific in vitro cytokine responses (Noho-Konteh et al. 2016).

Limited data are available on adverse events following childhood vaccination (Weber et al. 2014). BCG-associated vaccine adverse events such as osteitis/osteomyelitis and suppurative lymphadenitis are rare, but higher in males compared to females and in individuals vaccinated at age < 6 months compared to children vaccinated at a later age in Japan (Okuno et al. 2022). In general, MMR vaccines cause more adverse events, such as fever, parotitis, meningitis in infant females (Khalil et al. 2003), but more males are affected with MMR-associated thrombocytopenic purpura among children < 5 years old (France et al. 2008). The available data suggest that the sex and age of children can affect the adverse events following childhood vaccination.

Although sex differences in antibody responses following childhood vaccination are reported, it is unclear whether these differences have any clinical relevance as differences in antibody titer may not matter once protective levels are achieved. While many studies of vaccine efficacy in children include equal numbers of both sexes, very few studies stratify their data according to sex. In Guinea-Bissau, measurements of hospitalization rates due to measles showed that measles vaccination was more efficacious in female children below 4 years old compared to males following infant vaccination (Martins et al. 2014). There are contradictory reports on sex differences in the efficacy of the HBV vaccine. In one study, male sex was found to be associated with non-responsiveness to HBV (Zuckerman 2006), while a retrospective follow-up study of Gambian adults vaccinated against HBV during infancy showed no evidence of sex differences in vaccine efficacy (Peto et al. 2014). Another study found that only infant females produced anti-HBV antibody responses above the threshold for protection, predicting that females were likely to be better protected than males (Bocsan et al. 2005). A study in Spain suggested that BCG vaccination efficacy is greater in infant males than females (Altet et al. 1992); there were, however, no sex differences in protective efficacy against TB during a 15-year follow-up study in India (Narayanan 2006). These results highlight a need for sex-disaggregated

clinical data on vaccine responses, efficacy, and non-specific effects in children as it may inform both sex-specific timing and sequencing for childhood vaccines and the use of sex-specific doses.

There are limited data available regarding the immunogenicity, reactogenicity, and effectiveness of COVID-19 vaccines in children and infants. Moreover, no studies have presented sex-disaggregated data, so it is unclear whether sex differences in response to COVID-19 vaccination exist in these age groups (Frenck et al. 2021; Sacco et al. 2022). One complication arising from childhood infection with SARS-CoV-2 that is specific to children is that they are at risk of developing multi-systemic inflammatory syndrome (MIS-C), with males having a higher risk than females for MIS-C. Two studies that evaluated the risk of multi-systemic inflammatory syndrome (MIS-C) in hospitalized children report that vaccination with either mRNA-1273 or BNT162b2 is associated with > 90% effectiveness against developing MIS-C (Levy et al. 2022; Zambrano et al. 2022); however, these studies did not disaggregate their data based on sex, so it is unclear if vaccination equally protects male and female children against MIS-C. It is evident that adult females mount greater antibody responses and report more adverse events to the SARS-CoV-2 vaccines than males; whether COVID-19 vaccine-induced immunity and reactogenicity also differs between the sexes at different childhood ages, must be considered.

2.2 Puberty and Adulthood

In contrast to the limited number of sex-stratified studies of vaccination in children, sex differences in vaccine responses after puberty and adulthood are well documented (Fig. 1a). Following vaccination against influenza virus, measles, mumps, rubella, hepatitis A and B, yellow fever virus, dengue viruses, herpes simplex 2, rabies, and COVID-19, greater antibody responses in females compared to males have been reported (Cook 2008; Klein et al. 2016; Nam et al. 2022). In the context of influenza vaccines, females (18–49 years of age) who received either a full or half dose of the trivalent-inactivated vaccine (TIV) had higher neutralizing antibody and hemmaglutination inhibition (HAI) antibody titers against H1N1, H3N2, and influenza B antigens than males (Engler et al. 2008; Furman et al. 2014). In fact, females who received the full dose are observed to have HAI titers that are twice as high as males, while the antibody titers produced by females who received the half-dose vaccine are equivalent to males who received the full-dose vaccine (Engler et al. 2008). This suggests that the effective vaccine dose for influenza is lower in females compared to males and that sex-specific vaccine dosing regimens warrant additional evaluation. In The Netherlands, a large retrospective cohort study found that in healthy adult workers (16–70 years of age) who received the standard three-dose vaccination regimen of HBV, females had higher rates of seroconversion post-vaccination, while males had a greater prevalence of non-response to HBV (Vermeiren et al. 2013). Similar studies in Serbia, Pakistan, and Brazil all found that rates of non-response to HBV were greater among males compared to females (Motta-Castro et al. 2009;

Rosic et al. 2008; Zeeshan et al. 2007). In a study of healthy volunteers vaccinated against yellow fever virus, adult females had greater transcriptional activity of toll-like receptor signaling-associated genes than males (Klein et al. 2010). In response to infection, it is well documented that adult females tend to produce more robust antibody responses than adult males (Ursin et al. 2021). Taken together, vaccination may elicit stronger innate and adaptive immune responses in females compared to males.

While most vaccination studies highlight a predominance of female-biased greater vaccine-induced immunity, there are documented instances in which either a male-bias is reported or where conflicting data exist. In a US meta-analysis of smallpox vaccination, males have approximately 27% greater antibody titers after smallpox vaccination than females (Troy et al. 2015), while another study reported that males had greater vaccinia-specific cytokine responses following vaccination compared to females (Haralambieva et al. 2013). In Thailand, vaccination against Japanese encephalitis virus (JEV) with the mouse brain-derived inactivated JEV vaccine (MBDV) was introduced as part of the National Immunization Program beginning 1990 (Sudjaritruk et al. 2022). In an age-stratified seroepidemiological study, despite vaccination, JEV seroprotection, as measured by neutralizing antibody responses, only persists in ~ 50% of the population (Sudjaritruk et al. 2022). Moreover, in adults (21–67 years of age), males have greater neutralizing antibody responses than females (Sudjaritruk et al. 2022). Adolescent males (10–20 years of age) and older males (>51 years of age) have greater neutralizing antibody responses as compared to younger adult males (21–50 years of age) reflecting a pattern of high antibody responses following vaccination, waning immunity, and boosted responses in older adults from JEV exposure (Sudjaritruk et al. 2022). Interestingly, following implementation of JEV vaccines in Thailand, the age distribution of JEV cases shifted from children to adults, likely due to the lower levels of seroprotection among young adults (Sudjaritruk et al. 2022). In a cohort of 748 adolescents (11–19 years of age) who received two doses of the MMR-II vaccine, greater neutralizing antibody titers were observed in females compared with males (Riggenbach et al. 2022). In contrast, isolated peripheral blood mononuclear cell (PBMC) cultures from male participants that were stimulated ex vivo with mumps virus secreted greater levels of inflammatory cytokines (e.g., MIP-1α, MIP-1β, TNFα, IL-6, INF-γ, and IL-1β) than PBMCs isolated from female participants (Riggenbach et al. 2022). These results not only suggest that humoral and cell-mediated immunity may be regulated by different processes, but also suggest that they are regulated in a sex-dependent manner (Riggenbach et al. 2022).

From the onset of the COVID-19 pandemic it was clear that, on a global scale, males were more likely to experience severe disease, require ICU admission, require invasive ventilation, and were 30% more likely to die due to COVID-19 than females, all effects that were exacerbated with increasing age (Jacobsen et al. 2021, The Sex and Gender COVID-19 Project 2022). This resulted in two major shifts in the approach to studying sex differences in response to COVID-19 vaccines. The first is that each of the major manufacturers of COVID-19 vaccines (i.e., Moderna—mRNA-1273, Pfizer-BioNTech—BNT162b2, Johnson & Johnson—Ad26.COV2.S,

and AstraZeneca—ChAdOx1) ensured that their clinical trials included an equal number of male and female participants (Jensen et al. 2022). BNT162b2, mRNA-1273, and Ad26.COV2.S vaccine trials included sex-disaggregated efficacy data showing no statistical differences between males and females (Jensen et al. 2022). Sex- and age-disaggregated data, however, are only reported for the BNT162b2 and mRNA-1273 vaccines (Baden et al. 2021; Polack et al. 2020). The second shift came from within the research community as an unprecedented number of peer-reviewed studies evaluated sex- and age-specific efficacy, safety, and durability of SARS-CoV-2-specific immunity post-vaccination and boosting as new SARS-CoV-2 variants emerged. Noteworthy, adverse event data were not reported by sex.

Overall, adult females have been shown to have greater COVID-19 vaccine-induced antibody titers and durability of immunity against both mRNA and adenovirus-vectored vaccine platform; mRNA vaccines and heterologous vaccine schedules elicit greater antibody responses in both males and females than adenovirus-vectored platforms (Collatuzzo et al. 2022; Hvidt et al. 2022; Nam et al. 2022; Steensels et al. 2021). Furthermore, adult individuals who receive the mRNA-1273 vaccine compared to BNT162b2 mRNA vaccine consistently have greater antibody responses both post-initial vaccine dose and post-boosting, likely attributable to the greater mRNA dose in the former vaccine (Collatuzzo et al. 2022; Hvidt et al. 2022; Lo Sasso et al. 2021; Nam et al. 2022; Steensels et al. 2021).

Reports of sex-specific adverse events in adults following COVID-19 vaccination have been varied. In general, greater reactogenicity (i.e., local reactions and systemic pain, fever, headache, and fatigue) following both the first and second doses of the BNT162b2, mRNA-1273, and ChAdOx1 vaccines is reported for adult females compared to adult males (Hoffmann et al. 2021, Ogawa et al. 2022). In one study, younger adults (median age of 33 years) reported greater adverse events than middle-aged adults (median age of 43 years) (Wi et al. 2021). While serious adverse reactions are rare following COVID-19 vaccination, some studies have shown that 70% of serious adverse reactions are reported in females and that females are at greater risk for development of anaphylaxis (Blumenthal et al. 2021; Shimabukuro et al. 2021; Somiya et al. 2021) and vaccine-inducted thrombotic thrombocytopenia (VITT) (Greinacher et al. 2021; Lai et al. 2021). The observation of VITT is primarily associated with the adenovirus-vectored vaccines ChAdOx1 and Ad26-COV2.S (Greinacher et al. 2021; Li et al. 2022). Despite a predominance of adverse events reported in females, adolescent and young adult males (12–39 years of age) are at 10 times greater risk for developing myocarditis and pericarditis following vaccination with COVID-19 mRNA vaccines (Bozkurt et al. 2021; Klein et al. 2021; Pepe et al. 2021). Given the extent and range of sex-specific immunogenicity and reactogenicity of each COVID-19 vaccine platform, each platform must be carefully evaluated for sex- and age-associated efficacy and safety.

In addition to COVID-19 vaccination, adverse events are generally more common in adult females than males for several vaccines, including influenza, hepatitis B, and yellow fever vaccines (Klein et al. 2015). Females requiring yellow fever, diphtheria, tetanus toxoids, oral polio, and oral typhoid vaccines prior to traveling report more adverse events than males (Philipps et al. 1996). While generally considered safe, the

prevalence of death due to viscerotropic disease following yellow fever vaccination is higher in young adult females (19–34 years of age) than males (Seligman 2011). However, analysis of reporting data in the US from 2000 to 2006 found that males reported more serious adverse events following yellow fever vaccination (Lindsey et al. 2008).

While vaccine-induced antibody production is often utilized as a correlate of protection (Klein et al. 2010), it is important to note that vaccine-induced immune responses and vaccine efficacy are not the same. One example of this difference is evident from two double-blind phase 3 randomized efficacy trials of the herpes simplex type 2 (HSV-2) glycoprotein-D-adjuvant subunit vaccine with adult participants (>18 years of age) from four countries (Stanberry et al. 2002). In this study, researchers reported no sex differences in humoral and cell-mediated immune responses following vaccination, but initially found that the vaccine was not efficacious against HSV infection when the data were not stratified by sex and prior exposure to HSV (Stanberry et al. 2002). However, further analysis determined that the vaccine was effective at preventing HSV infection in ~ 70% of females that were seronegative for both HSV-1 and HSV-2, but was not effective at preventing infection in seronegative males (Stanberry et al. 2002). In combined analysis of four influenza virus challenge studies, individuals were assessed for pre-existing antibody titers to influenza prior to viral challenge; while females had overall higher pre- and post-challenge HAI and neutralizing antibody titers compared to males, males had consistently higher neuraminidase inhibition (NAI) titers than females both pre- and post-challenge (Giurgea et al. 2022). Importantly, females were more likely to develop symptoms than males post-challenge (Giurgea et al. 2022). Thus, these studies highlight that while immunogenicity may be either higher in one sex or equivalent between sexes, efficacy can also vary which adds an additional layer of complexity to these issues.

The use of animal models can provide insights into sex differences in vaccine efficacy. Adult females of diverse species tend to have greater antibody responses than males (Klein et al. 2016). In adult non-human primates, following vaccination against simian immunodeficiency virus (SIV), females show increased local IgA antibody titers, numbers of memory B cells, and numbers of plasma cells as compared to males (Mohanram et al. 2016; Tuero et al. 2015). SIV-vaccinated females also have elevated levels of virus-specific IgG1, IgG2, and IgG3 antibodies as compared to males, with anti-SIV IgG3 antibody levels correlating with antibody-mediated cytotoxicity and phagocytic activity, suggesting that the quality of the antibody response is greater in females than males (Mohanram et al. 2016). Studies in mice further illustrate that following vaccination or challenge, females develop greater systemic and mucosal antibody responses than males (Dhakal et al. 2021; Lorenzo et al. 2011). When immunized with an H1N1 or H3N2 influenza virus, adult female mice mount greater neutralizing and total antibody responses than males (Lorenzo et al. 2011). Following vaccination, female mice are better protected against lethal challenge with a novel influenza strain than males (Lorenzo et al. 2011, Ursin et al. 2022).

2.3 Aged Individuals

Overall, antibody responses tend to be lower in aged adults (>65 years of age) than younger adults (18–64 years of age) (Haq et al. 2014; Poland et al. 2014; Shapiro et al. 2022a, b). The occurrence and magnitude of sex differences in vaccine responses in aged individuals, however, differ greatly by vaccine antigen. Aged females (65–74 years of age) have greater antibody responses following vaccination with the seasonal and pandemic 2009 H1N1 (pH1N1) influenza vaccines than males (Booy et al. 2011; Furman et al. 2014; Khurana et al. 2012; Loeb et al. 2020; Talaat et al. 2010) and are reported to have seroconversion and seropositivity rates 2–3 times higher than age-matched males (Kao et al. 2010; Moehling et al. 2020). While aged females had greater antibody responses to the pH1N1 vaccine, males generated antibodies with greater avidity (Khurana et al. 2012). Among aged as well as adult females, greater seroconversion of neutralizing antibody responses to the pH1N1 vaccine is associated with greater circulating concentrations of estradiol (Potluri et al. 2019). In a longitudinal study of adults over 75 years of age who were vaccinated with a high-dose influenza vaccine in at least four out of six influenza seasons, pre-vaccination HAI titers against H3N2 and influenza B (but not H1N1) decreased with age in males and remained constant in females (Shapiro et al. 2021a, b). This decline in the durability of humoral immune responses in males may explain why males are more susceptible to influenza B infection and hospitalization (Wang et al. 2015; Wong et al. 2019) and highlights a need to consider sex-specific vaccine strategies in older adult populations (Klein and Pekosz 2014).

In contrast to influenza vaccination, aged males produce greater antibody responses to pneumococcal vaccines than females (Brandão et al. 2004; Cook et al. 2007; Goldblatt et al. 2009). In older adults (50–80 years of age) who received the two-dose seven-valent pneumococcal vaccine, aged males not only had higher serotype-specific IgG antibodies following each dose than females, but also had a greater increase in their antibody response following receipt of the second dose than females (Goldblatt et al. 2009). In long-term care residents who received the 23-valent pneumococcal vaccine, IgG antibody responses against all four of the pneumococcal serotypes analyzed were greater in aged males than females (Brandão et al. 2004). Greater male responses to Td/Tdap vaccines (tetanus, diphtheria, pertussis) in aged individuals has also been reported (Bayas et al. 2001; Hainz et al. 2005; Marlovits et al. 2000).

Whether there are sex differences in aged individuals in response to COVID-19 vaccines is not clear as there are several conflicting reports. In a study that compared immune responses of long-term care residents (median age of 88 years) in Ontario, Canada, who received two doses of either the BNT162b2 or mRNA-1273 vaccine, females had greater anti-spike IgG, anti-spike RBD IgG, and neutralizing antibodies titers following the initial vaccine dose than males, but this sex difference was not apparent following the second dose (Abe et al. 2021). Notably, individuals who received the mRNA-1273 had greater neutralizing antibody titers (2.95-fold) to the

vaccine strain and broader neutralization capacity against other SARS-COV-2 variants than those who received the BNT162b2 (Abe et al. 2021). One key difference between the mRNA-1273 and BNT162b2 vaccines is that the mRNA-1273 contains a higher dose of mRNA (i.e., 100 μg) than the BNT162b2 vaccine (i.e., 30 μg) (Centers for Disease Control and Prevention 2022). Similar to influenza vaccination, high-dose COVID-19 vaccines may be more effective for people over 65 years of age. In a study comparing SARS-CoV-2 naïve healthcare workers (HCWs, median age of 48 years) and SARS-CoV-2 naïve long-term care residents (median age of 76 years) in Ohio, long-term care residents had lower post-vaccination titers following receipt of the second dose of BNT162b2 compared to HCWs (Canaday et al. 2021). No sex differences were noted following receipt of the initial vaccine doses, but a strong negative correlation between age and antibody titers was observed (Canaday et al. 2021). In aged individuals who received both doses of the BNT162b2 and also had prior SARS-CoV-2 infections, post-vaccination antibody levels were greater in females than males (Canaday et al. 2021). This study also found that older individuals who were assessed as being frail produced 75% lower antibody responses than healthy, aged adults (Canaday et al. 2021). In contrast, in a study examining vaccine responses following three dose of either mRNA-1273 or BNT162b2 in older adults (75–98 years of age) compared to younger healthcare workers (18–74 years of age) in the Baltimore area, it was reported that aged females produce greater anti-spike IgG, anti-spike RBD IgG and neutralizing antibody titers than aged males following receipt of the initial vaccine series (Shapiro et al. 2022a, b). When results were evaluated to determine the sex-specific effects of age on humoral responses, age-associated reductions in humoral immune responses are greater in males than females following receipt of the first two vaccine doses (Shapiro et al. 2022a, b). Receipt of the third booster dose eliminated these sex differences (Shapiro et al. 2022a, b).

Among aged individuals, females aged 65 and older are more likely to report systemic adverse reactions (i.e., fever, headache, myalgia, redness, swelling, and injection site pain) in response to the pneumococcal vaccines, the herpes zoster vaccine, influenza vaccines, Td/Tdap, and COVID-19 vaccines (Fink et al. 2015, Hoffmann et al. 2021, Klein et al. 2021). It is unclear whether these differences in adverse reactions reflect a sex difference in reactogenicity or a gender bias in reporting. Interestingly, reporting of serious adverse reactions following COVID-19 vaccination is higher in younger adults than older, and serious adverse reactions are more common in adult females than males (Xiong et al. 2021). However, while fewer serious adverse reactions are reported in aged individuals, men have more serious adverse reactions (including permanent disability and death) than females for individuals 65 years of age and older (Xiong et al. 2021).

There are a limited number of studies evaluating vaccine efficacy in the elderly that include sex-disaggregated data. In a study of older community-dwelling adults in Taiwan who received the seasonal influenza vaccine, an association between higher HAI titers and lower rates of hospitalization and reduced risk of mortality was found in females (Wang et al. 2002). Influenza vaccine effectiveness, determined by all-cause mortality, is also reportedly higher in aged females than males in Spain (Vila-Córcoles et al. 2007), England (Fleming et al. 1995), and the USA (Nichol et al. 2007).

Similarly, several retrospective analyses have shown that among older adults who received herpes zoster or pneumococcal vaccines, aged females are better protected against disease and mortality than males (Hillebrand et al. 2015). In Germany, hospitalization rates for herpes zoster infection were found to be higher in older vaccinated males than females (Hillebrand et al. 2015). A study evaluating the cause of death registered on the US death certificates discovered that there was a significant decline in pneumonia-associated mortality among aged females compared to males following the introduction of the 23-valent pneumococcal vaccine in 1983 (Soneji et al. 2011). Similarly, older females were reported to be better protected against hospitalization due to community-acquired *Streptococcus pneumoniae* infection in the USA and Europe (Wiemken et al. 2014). While the amount of sex-disaggregated data on vaccine efficacy are certainly limited, these studies seem to suggest that vaccine efficacy is generally higher among aged females than males.

3 Sex Differences in Non-specific Effects of Vaccines

3.1 Epidemiological Studies

The sexes differ in the non-specific effects (NSEs) (also called off-target or heterologous effects) following vaccination. NSEs of vaccines are best defined as effects on morbidity and mortality (either increase or decrease) that are unrelated to prevention of the disease targeted by that vaccine (Aaby et al. 2014). Many studies have demonstrated sex-differential NSEs of vaccines, and in keeping with the trends showing greater antibody responses and adverse events in females following vaccination, females are generally more susceptible to NSEs (Aaby et al. 2020, Aaby et al. 2014, Benn et al. 2020, Flanagan et al. 2017, Flanagan et al. 2013).

The first evidence of NSEs of vaccines was discovered in randomized controlled trials (RCTs) of the high-titer measles vaccine (HTMV) that occurred in the late 1980s (Aaby et al. 2003). This vaccine was administered to infants (4–6 months of age) in measles endemic areas to determine whether the HTMV vaccine was as effective in younger infants as the standard dose MV that was given to infants > 9 months old (Aaby et al. 2003). While initial reports showed that the HTMV vaccine-induced antibody responses that were as effective as the standard dose given after 9 months of age, additional studies found a two-fold increase in the mortality among female recipients of HTMV compared to male recipients (Aaby et al. 2003; Knudsen et al. 1996). This was the first evidence that a vaccine could be protective against its targeted pathogen while have non-specific deleterious effects on susceptibility to other pathogens in a sex-differential manner (Aaby et al. 2003).

Subsequent studies evaluating other childhood vaccines have shown that many vaccines have sex-differential NSEs. Overall, the current evidence suggests that live vaccines are associated with beneficial NSEs with improved morbidity and mortality, while non-live vaccines are associated with deleterious NSEs (Aaby et al. 2014,

Flanagan et al. 2017, Flanagan et al. 2013). Both the beneficial and the deleterious NSEs are generally most pronounced in females. The live BCG vaccine is one of the best characterized vaccines in relation to NSEs. A number of observational studies and RCTs have shown that neonatal BCG vaccination is associated with reduced overall infant mortality as compared to infants who did not receive the vaccine (Flanagan et al. 2013; Shann 2010). Several recent RCTs in Guinea-Bissau of BCG vaccination of low-birth weight neonates, a group normally excluded from BCG vaccination, found BCG associated with a 38% (CI = 17–54%) decrease in mortality in the first four weeks of life (Biering-Sørensen et al. 2017). It was determined that BCG vaccination protected these neonates from respiratory infections and sepsis which are the most common causes of neonatal mortality among low-birth weight children in low-income countries (Aaby et al. 2011). A recent Ugandan RCT showed that neonatal BCG vaccination reduced physician-diagnosed non-tuberculous infectious disease incidence by 29% (95% CI = 5–47%) in the first 6 weeks of life as compared to BCG naïve infants (Prentice et al. 2021). Interestingly, the studies in Guinea-Bissau and another study in The Gambia found that the decrease in mortality following BCG vaccination was greater in females compared to males (Aaby et al. 2011; Garly et al. 2003). The male mortality reduction followed a different time-course to the female reduction, being in the first week of life for males but weeks 2–4 for females at which time the effect had waned for males (Biering-Sørensen et al. 2018). However, a systematic review by the Strategic Advisory Group of Experts (SAGE) on vaccination at the WHO indicated that the BCG effects were not convincingly sex-differential (Higgins et al. 2016). A fascinating observation is that maternal BCG status, as evidenced by the presence of a maternal BCG scar, provides sex-differential vertical protection to the newborn with improved male survival, particularly against sepsis (Schaltz-Buchholzer et al. 2022).

Live standard dose measles vaccine has consistently been associated with stronger reductions in all-cause mortality in females than in males. The systematic WHO/SAGE review analyzed two RCTs and ten observational studies and concluded that measles vaccine was associated with a 54% (95% CI 22–94%) more beneficial effect on all-cause mortality in females than in males (Higgins et al. 2016). Another live vaccine, smallpox vaccine, has also been associated with stronger NSEs in females than in males. The presence of a smallpox vaccination scar is linked with protective NSEs with improved long-term survival in Danish (Rieckmann et al. 2017) and West African adults (Jensen et al. 2006). The latter study showed a greater survival benefit to females, and another smallpox scar study also showed that smallpox vaccination was associated with protection against HIV-1 infection in women but not in men (Rieckmann et al. 2019).

The live oral polio vaccine (OPV) is the only live vaccine which seems to have more beneficial NSEs in males. It has been linked with sex-differential NSEs with lower mortality in boys receiving OPV plus pentavalent vaccine as compared to unvaccinated infants (Pfeiffer et al. 2017). The same more beneficial effect of OPV in males vs. females has been seen in studies of OPV campaigns (Andersen et al. 2021, 2018). In an RCT of BCG + OPV vs. BCG-alone at birth, following children until the time of OPV campaigns, receiving OPV-BCG vs. BCG-alone was associated

with 32% (95% CI 0–55%) reduction in all-cause mortality, the beneficial effect being separately significant for males (45% 95% CI 5–68%) (Lund et al. 2015). In a Russian RCT of 1,115 adult volunteers, OPV protected against laboratory confirmed COVID-19 as compared to the placebo group (Yagovkina et al. 2022). The data were not analyzed by sex. In a Guinean RCT of 3,726 individuals aged 50 years and older, OPV was associated with a 29% (95% CI = 2–49%) reduction in the risk of mortality, admissions, and consultation for infections in males during the COVID-19 pandemic, whereas it had no effect in females (Fisker et al. 2022). Thus, while the live BCG, MV, and smallpox vaccines seem to be associated with more beneficial NSEs in females than in males, OPV seems to be associated with more beneficial NSEs in males.

Several inactivated vaccines including diphtheria-tetanus-whole cell pertussis (DTwP), HBV, and inactivated polio vaccine (IPV) have been associated with harmful non-specific effects (Aaby et al. 2012, 2007, 2016; Garly et al. 2004). Observational studies have shown an increase in mortality in children who received the DTwP vaccine, especially in females compared to males (Aaby et al. 2012; Shann 2010), although a more recent study found no such effect, possibly due to the confounding effects of lower mortality rates, high BCG coverage, and OPV campaigns (Sørensen et al. 2022).

The RTS,S malaria vaccine, which is based on hepatitis B surface antigen plus the malarial circumsporozoite protein in ASO1 liposome-based adjuvant, was found in phase III clinical trials to be associated with higher all-cause mortality in girls (mortality ratio 1.91, 95% CI 1.30–2.79, $p = 0.0006$), but no such effect was observed in boys (mortality ratio 0.84, 95% CI 0.61—1.17, $p = 0.33$) in two age groups (6–12 weeks and 5–17 months). This highly significant sex-differential effect ($p = 0.001$) suggests that the vaccine has deleterious NSE in female recipients in keeping with other non-live vaccines and warrants further investigation given its relatively poor efficacy of 18–36% against clinical malaria (Klein et al. 2016).

The deleterious effects of DTP and other non-live vaccines in females are seen as long as the vaccine is the most recent vaccine. Upon scrutiny, it was discovered that the HTMV vaccine itself was not deleterious to females, but that the combination of receiving HTMV and then subsequent vaccination with either the DTwP vaccine, IPV, or the DTwP-IPV vaccine is what led to the increased risk of mortality (Aaby et al. 2003). The findings that the NSEs are sex-differential and strongest as long as a given vaccine is the most recent vaccine open the possibility that males and females may benefit from different routine vaccination schedules. A schedule that ensures that females in particular have a live vaccines for most of their childhood would likely be associated with lower all-cause mortality alongside specific protection against the vaccine-targeted diseases (Shann 2021).

3.2 Immunological Mechanisms for Sex-Differential NSE of Vaccines

The biological and immunological mechanisms that underpin sex differences in NSEs of vaccines have been unclear (Flanagan et al. 2017; Flanagan et al. 2013), but mechanistic explanations are starting to emerge in the literature (de Bree et al. 2018a, b). Biologically, it makes sense that NSEs of vaccines would be sex-differential given the myriad of X-linked immune response genes and microRNAs (Fish 2008); the opposing effects of male and female sex hormones on the immune system (Klein and Flanagan 2016); and the finding that even the microbiome is sex-differential (Vemuri et al. 2019).

Several studies have shown that BCG vaccination causes epigenetic and metabolic reprogramming leading to enhanced innate immunity, a process called trained immunity (Bekkering et al. 2021; Netea et al. 2020). Epigenetic effects of BCG vaccination in humans were initially demonstrated in adults (Kleinnijenhuis et al. 2012) but have also been confirmed in neonates. An Australian study showed epigenetic remodeling of circulating monocytes persisting to greater than 1 year post-BCG vaccination (Bannister et al. 2022), and a Ugandan neonatal study found significantly lower histone trimethylation at the TNF promoter in BCG-vaccinated infants compared to a BCG naïve group (Prentice et al. 2021). An immunological study of low-birth weight infants immunized with BCG in Guinea-Bissau found that the protective NSEs of BCG were associated with enhanced innate immune responses at 4 weeks of age, with better enhancement in infant females compared to males (Jensen et al. 2015), at the age when females were observed to benefit most from BCG (Biering-Sørensen et al. 2018). A study of Australian newborns found that BCG + HBV vaccination at birth led to decreased IFN-γ in males 7 days after vaccination as compared to unvaccinated infants and increased IFN-γ production in female neonates (Nissen et al. 2018; Pittet et al. 2022). A study of the impact of the sex hormones estrogen and dihydrotestosterone on the training of monocytes by BCG suggests that they are unlikely to play a role in innate immune training, and thus, other mechanisms for sex-differential NSE of BCG should be sought (de Bree et al. 2018a, b).

A Gambian infant study found that measles vaccination increased pro-inflammatory innate immune responses in male infants, whereas DTwP vaccination suppressed T cell and innate immune responses in infant females but not males (Noho-Konteh et al. 2016). In a study in Guinea-Bissau, infant females vaccinated against measles were found to have increased plasma concentrations of IL-1ra, CXCL8, and CCL2 compared to males (Jensen et al. 2014). Whether DTwP and measles vaccines cause epigenetic changes has not been explored but clearly warrants investigation. In a small study, oral polio revaccination was associated with changes in gut and upper respiratory microbiomes of infants (Medeiros et al. 2022)**.** The study was too small to assess sex differences, but the findings may help to explain why the OPV-induced immune response seems to play a role against pathogenic gut bacteria by reducing etiology-specific bacterial diarrhea in male infants (Upfill-Brown et al.

2017). Taken together, these results highlight the fact that vaccines can lead to sex-differential innate and adaptive immunological changes which can account for the observed NSE. These warrant further urgent investigation to understand the biological interactions that govern sex-specific NSEs. Harnessing the mechanisms whereby vaccines augment heterologous immunity could be used to improve vaccine design and vaccine campaign strategies leading to better protective immunological responses in both adults and children. Indeed, BCG and OPV were both studied in the early stages of the COVID-19 pandemic for their potential to provide non-specific protection against the disease prior to the advent of effective COVID-19 vaccines (Aaby et al. 2022, O'Neill et al. 2020). BCG failed to protect healthcare workers against COVID-19 in a trial conducted in South Africa (Upton et al. 2022), but may have reduced all-cause mortality among participants across trials (Aaby et al. 2022). None of these studies reported by sex, but there is every reason to believe that the effects would be sex-differential and many of the BCG studies investigating their protective effect against SARS-CoV-2 are yet to report their findings The beneficial effects of live vaccines therefore have the potential to be harnessed for future pandemic infections before specific vaccines can be developed. Importantly, understanding why some vaccines induce deleterious NSEs is critical for finding ways to overcome or circumvent these undesirable effects.

4 Concluding Remarks

It is evident that over the life course, males and females experience differences in the immunogenicity, reactogenicity, protection, and NSEs following vaccination. While the overall trend is that young adult females produce greater antibody responses, have higher correlates of protective immunity, and report more adverse events following vaccination compared to males, older adults, and children, this is not true in all instances. In fact, collapsing these observations of sex differences in response to vaccination over the life course in this way is almost as problematic as the lack of sex-disaggregated data that examines immune responses to vaccination over the lifespan. In the data that we have summarized, it is clear that nuance exists based on the type and dose of vaccine and antigen, the age of the vaccinee, prior exposure to either infection or repeated (seasonal) vaccination, and perhaps other host factors that include, but are not limited to, circulating sex steroid concentrations, genetics, and comorbidities. Differences between males and females in circulating sex steroid hormones (Fig. 1b) as well as immune responses (Fig. 1c) are dynamic and the nature of these sex differences changes both between and within the sexes throughout the lifespan. There is a greater need for research (including pre-clinical and clinical trials) that evaluates vaccine responses and vaccine efficacy as well as non-specific effects of vaccines to include sex and age as biological variables instead of controlling for them (Shapiro et al. 2021a, b). While the mechanisms mediating sex differences in immunological responses are still unknown, vaccine design and vaccine strategies

should be sex- and age-specific in order to both improve immunogenicity, mitigate adverse events, promote beneficial NSEs, and limit detrimental ones.

Disclosure Statement The authors have nothing to disclose.

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Immunology of Pregnancy and Systemic Consequences

Fiona M. Menzies

Abstract Pregnancy is an immunological paradox, with renowned Nobel Prize winning transplantation biologist Sir Peter Brian Medawar being the first to introduce this concept back in 1953. This concept considers how the maternal immune system can tolerate the developing fetus, which is 50% antigenically foreign to the uterus. There have been significant advances in our understanding of the immune system in regulating fertility, pregnancy and in complications of these, and what was once considered a paradox can be seen as a highly evolved system. Indeed, the complexity of the maternal–fetal interface along with our ever-advancing knowledge of immune cells and mediators means that we have a better understanding of these interactions, with gaps still present. This chapter will summarise the key aspects of the role of the immune system at each stage of pregnancy and highlight the recent advances in our knowledge.

1 Introduction

The female reproductive tract is abundant with immune cells and mediators, and there are many factors which can modulate their production, recruitment and mechanism of action. In the absence of pregnancy, the immune system serves to protect the female reproductive tract from infection. In every region of the female reproductive tract and at every stage of the uterine cycle (Monin et al. 2020), as well as in response to coitus, through pregnancy, to post-partum uterine involution, components of the immune system are present and function to provide an environment suitable for the establishment of a successful pregnancy and ensure the return of the uterus to a pre-pregnancy state.

Recently, the concept of the pregnant uterus as a sterile environment has also been challenged. For decades, it has been assumed that the developing fetus does not have a natural microbiome and the presence of microorganisms within the uterus

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S. L. Klein and C. W. Roberts (eds.), *Sex and Gender Differences in Infection and Treatments for Infectious Diseases*, Current Topics in Microbiology and Immunology 441, https://doi.org/10.1007/978-3-031-35139-6_10

before birth is associated with disease and creation of a potential threat to the success of pregnancy. In recent times, this has been questioned with the investigation of the presence of some microorganisms within the amniotic fluid, meconium and placenta. Table 1 summarises some of the key studies in this area, highlighting the sample types studied and major organism types identified. Indeed, this is an interesting and developing area of reproductive research, with consideration now being given to the possibility that the microbiome of the uterus can influence fertility, conception and pregnancy success (Bardos et al. 2019; Chen et al. 2017; Moreno et al. 2016). This, however, remains a controversial and ongoing area of study with debate around true detection of species versus contamination of samples (Blaser et al. 2021; Fricke and Ravel 2021; Bushman 2019; Perez-Munoz et al. 2017).

It is therefore widely acknowledged that the female reproductive tract is a unique mucosal environment, but we must also consider the fluctuation in immune function which arises in response to cyclical changes in hormonal levels. In Chap. 2, we learned about the influence of sex hormones in driving differential responses in immunity between females and males. For the first 8 weeks of pregnancy, progesterone, which is often described as the pregnancy hormone, is produced by corpus luteum of the ovary. After this time, progesterone production is taken over by the placenta and its concentration continues to rise dramatically in maternal serum until birth. Estrogens

Table 1 Studies examining detection of microorganisms within the pregnant uterus

Sample type	Bacterial species	References
Amniotic fluid	Proteobacteria	Collado et al. (2016)
	Propionibacterium	Collado et al. (2016), Stinson et al. (2019)
	Streptococcus (low abundance)	Collado et al. (2016)
	Lactobacillus	Collado et al. (2016)
	None detected	Lim et al. (2018)
Meconium	*Lactobacillus*	Collado et al. (2016)
	Proteobacteria	Collado et al. (2016)
	Propionibacterium	Collado et al. (2016)
	Streptococcus (low abundance)	Collado et al. (2016)
	Pelomonas puraquae	Stinson et al. (2019)
Placenta	Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes, Fusobacteria	Aagaard et al. (2014)
	Lactobacillus crispatus	Prince et al. (2016)
	Lactobacillus	Collado et al. (2016)
	Proteobacteria	Collado et al. (2016)
	Propionibacterium	Collado et al. (2016)
	Streptococcus (low abundance)	(Collado et al. 2016
	None detected	Lauder et al. (2016), Leiby et al. (2018), Theis et al. (2019), de Goffau et al. (2019)

are produced primarily by developing follicles and the corpus luteum. Estradiol is the main estrogen in women of fertile age, estriol is produced by the placenta during pregnancy and estrone is produced in women of menopausal age (Kuijper et al. 2013). Levels of estrogens in maternal serum and secreted in urine increase dramatically during pregnancy, but fall after delivery of the fetus (Kuijper et al. 2013). In this chapter, we will also consider how these powerful systemic increases in estrogens and progesterone, required to sustain pregnancy, can act as a mechanism to modulate the maternal immune system (Oertelt-Prigione 2012; Pennell et al. 2012; Robinson and Klein 2012; Menzies and Henriquez 2009; Druckmann and Druckmann 2005). While these hormonal changes occur to support pregnancy, they can have consequences for the pathogenesis of infections or autoimmune diseases.

2 The Impact of Seminal Fluid on the Uterine Environment

The delivery of spermatozoa to the female reproductive tract is aided by the seminal plasma in which they are found. Seminal plasma is much more than a transport medium, and it makes up 95–98% of semen contents (Rodriguez-Martinez et al. 2011), providing nourishment and protection for sperm, and containing a multitude of peptides and proteins that influence sperm function and prepare the uterine epithelia for pregnancy (Schjenken and Robertson 2020; Rodriguez-Martinez et al. 2011) through inducing changes in gene expression and cellular composition (Robertson 2005). Seminal plasma contains an array of biologically and immunologically active compounds including hormones, such as estrogen and progesterone as well as prostaglandin E2 and an array of cytokines (Hampl et al. 2013; Robertson 2005; Maegawa et al. 2002), including interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNFα), interleukins (IL)-1β, -6, -8, -10, -12 and TGF-β (Bromfield 2014). Most notably, the cytokine TGF-β, which is abundant within seminal plasma of both mice (Tremellen et al. 1998) and humans (Loras et al. 1999), is present in all three isoforms (TGF-β1, TGF-β2, TGF-β3) and can induce proinflammatory cytokine production by cervical cells (Sharkey et al. 2012a). Interestingly, studies have shown that levels of TGF-β in human seminal plasma fluctuate, with sexual abstinence impacting on content of TGF-β1 and TGF-β2 and TGF-β1 correlating inversely with age (Sharkey et al. 2016). These findings, along with the suggestion that other semen contents may also demonstrate patterns of fluctuation, have potential implications for semen analysis and understanding the content and quality of semen for assisted conception practices.

The presence of immunomodulatory molecules within seminal plasma is required for the success of pregnancy, whether it is via natural conception or assisted reproduction. Indeed, the importance of the inflammatory response to mating is demonstrated by studies showing that In Vitro Fertilisation (IVF) is more successful in women who are exposed to semen at the time of embryo transfer (Tremellen et al. 2000). Identifying the role of individual components of seminal plasma, and their role in immunomodulation of the uterus for pregnancy, is an area of fertility research which

has gained considerable ground in recent years. For example, recently, it has been shown in an in vitro study, that expression of CD25 and IL-10 by female T cells is modulated by exposure to seminal plasma, with differences observed between seminal plasma donated from males who experience recurrent pregnancy losses and their control counterparts (du Fosse et al. 2022).

In humans, deposition of semen results in the influx of macrophages, dendritic cells (DCs), and T cells into the epithelial and stromal compartments of the ectocervix, accompanied by an increase in gene expression for the cytokines granulocyte macrophage colony stimulating factor (GM-CSF), IL-1α, IL-6 and the chemokine CXCL8 (Sharkey et al. 2012b). Macrophages and DCs remain within the stromal region of the cervix, while increases in T cells are found in both the epithelium and stroma, with a greater proportion of these being CD8+ than CD4+ (Sharkey et al. 2012b).

This immunomodulation of the uterine epithelium prepares the site for implantation, but also serves to prime the uterus and create a hospitable environment for the introduction of paternal antigens. After mating, there is a substantial increase in the cellularity of the uterine draining (para-aortic) lymph nodes (dLNs) in mice (Johansson et al. 2004), the main site of cross-presentation of paternal antigen (Moldenhauer et al. 2009). Recent studies have shown that in mice, the DC populations within the uterus and dLNs change significantly in the period between mating and implantation (Yasuda et al. 2020). Interestingly, these studies suggest that most of the DCs found within the uterus migrate to the site just prior to implantation. As summarised in Fig. 1, seminal plasma drives the activation and expansion of specific CD4+ and CD8+ T cells, with maternal Antigen-Presenting Cells (APCs) cross-presenting paternal antigens to CD8+ T cells in a TAP-dependent manner (Moldenhauer et al. 2009). Another T cell population considered to be important for the establishment of tolerance is T regulatory cells (Tregs) (Aluvihare et al. 2004). Studies in mice show that as the female approaches the estrus phase of the cycle, Tregs accumulate in the uterine horn, and this is accompanied by expression of T cell-specific chemokines CCL3, CCL4, CCL22 and CX3CL1 (Kallikourdis and Betz 2007). More specifically, studies have shown that CCL19 is likely to be a key recruiter of Tregs to the uterus (Guerin et al. 2011), and this is driven by seminal fluid (Shima et al. 2015; Kahn and Baltimore 2010; Robertson et al. 2009a) and priming by tolerogenic APCs in the uterus (Shima et al. 2020). While Treg populations increase both systemically and locally during pregnancy in humans, paternal-antigen-specific T cells have not yet been identified (Tsuda et al. 2019). The importance of Treg cells in maintaining an environment suitable for pregnancy, as it progresses, will be discussed in later sections.

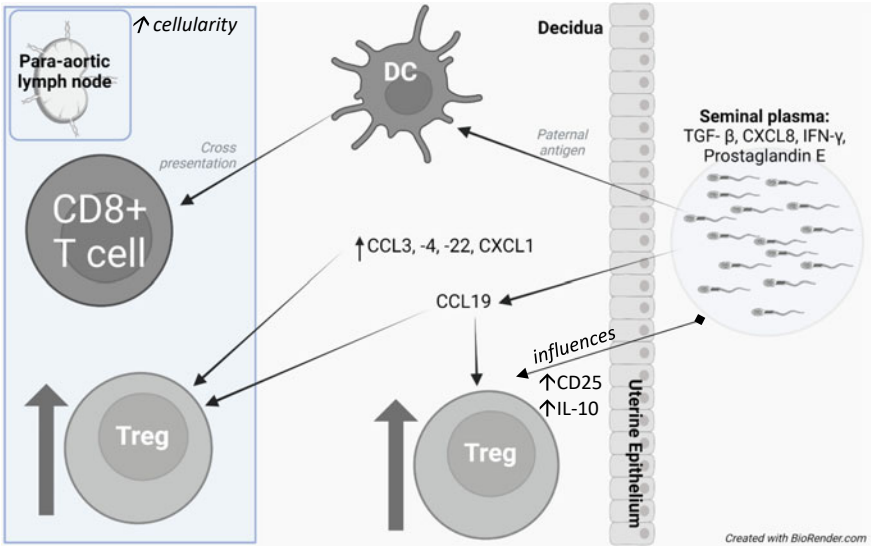

Fig. 1 Female response to seminal plasma. The uterine epithelium responds to immunomodulatory molecules (e.g. TGF-β, CXCL8, IFN-γ, Prostaglandin-E) within seminal plasma by producing T regulatory-attracting chemokines. Dendritic cells cross present paternal antigens to CD8+ T cells within the para-aortic lymph nodes, which drain the uterus, and are known to increase in cellularity post-mating. These mechanisms serve to prepare the maternal immune system for the development of a semiallogeneic fetus (du Fosse et al. 2022; Guerin et al. 2011; Robertson et al. 2009a, b; Kallikourdis and Betz 2007; Aluvihare et al. 2004)

3 Maternal Rejection of Semen

The importance of the immunomodulation induced by seminal plasma within the female uterus is highlighted by consideration of the consequences of the rejection of semen. Immune infertility is estimated to impact 20% of couples of reproductive age (Brazdova et al. 2016) with many potential underlying causes, affecting both male and female reproductive systems. One such cause impacting fertility is the production of anti-sperm antibodies (ASAs) which can cause infertility (Brazdova et al. 2016). Indeed, fertile women (and men) are also thought to produce ASAs as a normal physiological process; however, this does not necessarily lead to infertility (Vickram et al. 2019). In some women, for unknown reasons, there can be a failure of tolerance to the complex antigenic milieu of the sperm, leading to pathological production of ASAs and therefore fertility issues. Pathological ASA production by women impacts on several aspects of sperm function. Incubation of ASA from infertile women with normal sperm for 1 h in vitro leads to a reduction in sperm motility, viability and membrane integrity (Pujianto et al. 2018). The negative impact of ASA on pregnancy is sufficient that recent studies are now considering the potential of ASAs to create vaccines that can be utilised as a form of contraception (Vickram et al. 2019; Baldeon-Vaca et al. 2021).

4 Mechanisms Involved in Preventing Fetal Rejection

4.1 What Is the Implantation Window?

In humans, implantation occurs approximately 8–10 days post-ovulation (Wilcox et al. 1998), with the chance of conception approximately 30% per cycle (Zinaman et al. 1996). With such a low chance of success, it is unsurprising that around 75% of early losses are attributed to implantation failure (Wilcox et al. 1998). Indeed, it can be considered that early pregnancy loss (before 10 weeks' gestation) is the default consequence of fertilisation (Annual Capri Workshop 2020). As advances in reproductive technology have developed, so too has our understanding of the underlying molecular, biochemical and immunological mechanisms associated with successful pregnancy outcomes and our appreciation that uterine and embryological and factors equally contribute to success.

The uterine endometrium goes through a remodelling process of decidualization to become receptive to the blastocyst, creating a period of 48 h within the luteal phase of the ovarian cycle known as the "implantation window". What is interesting to note is that decidualization occurs irrespective of the presence of a blastocyst. During decidualization, endometrial fibroblast-like stromal cells change to become larger, more rounded decidual stromal cells (DSCs) (Dunn et al. 2003), which can be characterised by their secretion of prolactin (Telgmann and Gellersen 1998; Wu et al. 1995; Daly et al. 1983), insulin-like growth factor binding protein-1 (Matsumoto et al. 2008) and tissue factor (Lockwood et al. 1993) as well as expression of transcription factors such as Fox01 (Adiguzel and Celik-Ozenci 2021). The timing of the expression of these molecules is also crucial, as exemplified by studies showing that premature expression of prolactin is associated with repeated implantation failure (Berkhout et al. 2020). Adequate decidualization is a critical point for the fate of the pregnancy. Either decidualization continues in preparation for blastocyst implantation, or it breaks down to begin menstruation. Creation of the environment in preparation for the implantation window is dependent on a number of morphological, biochemical and hormonal changes (Aghajanova et al. 2008; Ng et al. 2020), but is also influenced by the immune environment (Murata et al. 2021).

4.2 Implantation as an Immunological Event

Implantation requires inflammation as a normal adaptation, involving various immune cells and mediators to support the preparation of the endometrial lining for receiving the blastocyst (Sehring et al. 2022). Contrary to this, excessive or aberrant inflammation can lead to implantation failure or miscarriage (Pantos et al. 2022). To understand the role of inflammation during implantation, it is first necessary to consider the presence of immune cells in the non-pregnant uterus.

4.3 Macrophages During Implantation

Macrophages are mononuclear phagocytic cells which are key players of the innate immune system. Macrophages constitute approximately 20% of the total number of decidual leukocytes at the maternal–fetal interface (Zhang et al. 2017; Ning et al. 2016), playing a number of key roles at implantation. In addition, these cells are highly responsive to the female sex hormones, adapting their role and distribution accordingly even in the non-pregnant state (De and Wood 1990; Ning et al. 2016). Both progesterone and estrogen module the inflammatory activities of macrophages (Jones et al. 2008; Menzies et al. 2011; Liu and Wang 2013).

At implantation, macrophage numbers increase within the decidua to aid in the clearance of cellular debris (Abrahams et al. 2004) and degradation of artefacts present after mating (De et al. 1991). Murine studies utilising a macrophage depletion model have confirmed the necessity for these cells at implantation through disruption of progesterone production by the corpus luteum (Care et al. 2013). Subsequent studies have shown that during the implantation period, macrophages of the M2 subtype are present in the stromal region and in close proximity to the lumen and glands (Ono et al. 2020).

Beyond implantation, macrophages are also found within the placenta as two distinct populations: decidual macrophages and Hofbauer cells (Mezouar et al. 2021) with the ability to isolate and study these independent cell types only recently becoming a possibility (Lasch et al. 2022). Classification of placental macrophages as M1 or M2 is proving somewhat more difficult for researchers to ascertain; however, studies suggest that first and early second trimester macrophages resemble M1-types macrophages (Zhang et al. 2017) and late second and third trimester macrophages resemble the M2-type (Gustafsson et al. 2008). CD14+ macrophages within the term placenta are of both maternal (30%) and fetal (70%) origin (Mezouar et al. 2019) and exhibit characteristics of M1 macrophages (Mezouar et al. 2021; Gustafsson et al. 2008).

4.4 Tregs as Master Regulators Within the Pregnant Uterus

Tregs have been the subject of much study and review in both humans and mice over the past 15 or so years and are considered to be pregnancy-protective (Muralidhara et al. 2022; Tsuda et al. 2021, 2019; Krop et al. 2020; Shigeta et al. 2020; Zhang and Sun 2020; Jorgensen et al. 2019; La Rocca et al. 2014; Teles et al. 2013; Leber et al. 2010; Zenclussen et al. 2010; Guerin et al. 2009; Aluvihare et al. 2004). As mentioned earlier, Tregs accumulate within the endometrium during the pre-implantation period (Kallikourdis and Betz 2007) and offer a protective role at the implantation site. Murine studies of allogeneic and syngeneic matings demonstrate Tregs which become activated against self-antigens early after embryo implantation to create a tolerant environment (Chen et al. 2013). Fetal-antigen-specific Treg cells

are not present at embryo implantation; however, their number increases as pregnancy progresses (Rowe et al. 2012). Decidual Tregs increase with advancing gestation (Somerset et al. 2004), and there is preferential recruitment of fetus-specific Treg cells from the maternal blood to the decidua (Tilburgs et al. 2008). Interestingly, decidual Treg cell numbers are reduced in pregnant women with pre-eclampsia compared to levels found in normal pregnant women (Quinn et al. 2011). This suggests a key role for Treg cells in regulation of the maternal–fetal interface and is now a developing area of research (Robertson et al. 2019).

This leads to the question as to how Tregs cells regulate the immune response during pregnancy? Treg cells modulate the activities of T cells. Culturing T cells in the presence of medium conditioned by placental trophoblasts demonstrated a skewing towards the production of Th2-associated transcription factors and cytokines and inhibition of those associated with Th1 and Th17 cells (Liu et al. 2011). Traditionally, pregnancy was considered a Th2-associated phenomenon, with the placenta and uterine environment skewed towards an abundance of Th2-type cytokines (Lin et al. 1993; Wegmann et al. 1993); however, this well-accepted paradigm has now been amended to include Th17 and Tregs (Saito et al. 2010). Indeed, the balance between Th17 cells and Treg cells is critical for the outcome of pregnancy in humans. During healthy pregnancy, the ratio of circulating Treg cells to Th17 cells is increased significantly compared to non-pregnant controls; however, in pre-eclamptic women, this skewing away from Th17 cells is not observed (Santner-Nanan et al. 2009).

4.5 The Specialities of Uterine Natural Killer (NK) Cells

Natural Killer (NK) cells are a type of innate lymphoid cell (ILC) crucial in the early innate defences against various pathogens, viruses in particular. In humans, two main populations of blood, or peripheral, NK cells can be defined based on expression of the surface molecule CD56. About 90% of peripheral NK cells express relatively low levels of CD56 and are defined as $CD56^{dim}$, and these cells are characterised by a high level of spontaneous lytic activity (Dosiou and Giudice 2005). Contrary to this, $CD56^{bright}$ NK cells, so called as they express relatively high levels of CD56, have little lytic activity. A third type of NK cells can be identified, unique to the uterus. The phenotype and regulation of uterine NK (uNK) cells within the pregnant uterine environment are crucial for pregnancy success (Faas and de Vos 2017; Giuliani et al. 2014; Manaster and Mandelboim 2010; Vacca et al. 2013, 2011). This is demonstrated by the link between recurrent pregnancy loss and dysregulation between uNK cells and peripheral NK cells (Mahajan et al. 2022; Giuliani et al. 2014; Tang et al. 2011).

uNK cells differ from both of the peripheral NK phenotypes, $CD56^{dim}$ and $CD56^{bright}$, but have more in common with this latter phenotype, including weak lytic activity and lack of expression of FCγRIII (CD16) (Manaster and Mandelboim 2010). Binding of IgG to CD16 results in activation of antibody-dependent cell-mediated cytotoxicity (ADCC) by NK cells (Bryceson et al. 2006), and so the absence of CD16 on uNK cells demonstrates another tolerogenic feature of these

cells. Endometrial uNK cells are fairly inactive in the non-pregnant uterus (Manaster et al. 2008). uNK cells constitute 50–70% of immune cells within first trimester decidua near to the blastocyte implantation site (Manaster and Mandelboim 2010; Bulmer et al. 2010; Mahajan et al. 2022). These decidual uNK cells still express many activating NK receptors, but rather than inducing cytotoxicity and potentially a reaction towards the fetally derived cells, these uNK cells act to support trophoblast invasion and angiogenesis (Hanna et al. 2006) as well as regulation of Th17 cell function at the fetal–maternal interface (Fu et al. 2013).

4.6 Cytokines and Chemokines in the Pregnant Uterus

So far, we have considered some of the key immune cells which are involved in the tolerance of fetally derived cells during pregnancy. The trafficking of cells and their actions are influenced by key chemokines and cytokines which may be produced by these cells or act on these cells. With regard to the uterus during pregnancy, there are a few cytokines, namely LIF and IL-15, which stand out as having key roles in modulating the immune environment.

The non-pregnant human endometrium of non-pregnant humans expresses several chemokine receptors (e.g. CXCR1, CXCR2, CCR5, CXCR4, CCR2, CCR3, CXCR3 and CX3CR1) and produces a number of chemokines during the implantation window (e.g. CCL2, CCL4, CCL5, CCL7, CCL11, CCL14, CCL16, CCL21, CCL22, CXCL8, CX3CL1), all of which have been extensively reviewed within the literature (Ramhorst et al. 2016; Du et al. 2014; Park and Yang 2011; Red-Horse et al. 2004, 2001). Many of these chemokines are involved in the trafficking of immune cells, including macrophages, Tregs and uNK cells to the decidua. It has been suggested that chemokines and their receptors may also play other significant roles during the establishment of pregnancy, with receptors found on cells of the blastocyst and extravillous trophoblasts (Dominguez et al. 2003a, b; Dimitriadis et al. 2005; Hannan and Salamonsen 2008, 2007; Hannan et al. 2006). As will be discussed later, chemokines and their receptors have also been extensively studied for their roles in driving inflammation at the other end of the gestational period—at the point of labour.

One of the most characterised factors involved in implantation is Leukemia Inhibitory Factor (LIF), and its role during pregnancy has been extensively reviewed in the literature (Pantos et al. 2022; Li et al. 2020; Winship et al. 2015; Moberg et al. 2015). LIF is a highly glycosylated 40–50 kDa glycoprotein that functions as a pleiotropic anti-inflammatory cytokine, part of the IL-6 family. Studies using LIF-deficient mice have shown it that is essential for successful implantation (Stewart et al. 1992) through binding the LIF-receptor (LIFR) and inducing STAT3 activation (Suman et al. 2013; Poehlmann et al. 2005) as well as inducing both autocrine and paracrine signalling pathways in the endometrium to facilitate implantation (Cullinan et al. 1996; Dominguez et al. 2002). LIF is expressed during the mid-late phase of the menstrual cycle and early stages of pregnancy, in both the glandular and luminal

epitheliums (Pantos et al. 2022; Markert et al. 2011). The importance of LIF in this scenario is demonstrated by studies showing that women diagnosed with recurrent implantation failure have significantly less LIF in their endometrial glandular epithelium than normal pregnant women (Mariee et al. 2012). By contrast, it was found that women with recurrent implantation failure have higher levels of the cytokine IL-15 in the stroma (Mariee et al. 2012) and in the placenta (Toth et al. 2010) than in control women with normal pregnancies. IL-15 is a 14–15 kDa cytokine that is a member of the IL-2 family of cytokines. IL-15 has the ability to influence both innate and adaptive arms of the immune response, by stimulating NK cells, T cells and NKT cells. IL-15 transcripts are strongly expressed during the secretory, or luteal, phase of the cycle in humans a time in which a large number of NK cells are present within the uterus (Gordon 2021; Chegini et al. 2003; Kitaya et al. 2000). IL-15 is important for the differentiation of uNK cells within the endometrium during regular regeneration activities and during pregnancy (Strunz et al. 2021), but the placenta has low expression of IL-15 in the early development stages, but this gradually increases throughout pregnancy, with a peak in levels at the point of labour (Gordon 2021). Furthermore, placental explant studies have shown that IL-15 production is significantly lower in pre-eclamptic placenta samples than healthy term placentas (Agarwal et al. 2001).

5 Altered Human Leukocyte Antigen Expression by Trophoblasts

The placenta is an organ unique to pregnancy and crucial for its success. The process of placentation begins when the blastocyst implants into the decidua, with extravillous trophoblasts (EVT) making their way into the uterine wall. A number of pregnancy complications, such as placenta accrete, as serious condition when the placenta grows too far into the uterine wall, and recurrent spontaneous abortions, have clear links with dysregulation of trophoblast invasion (Illsley et al. 2020; Moser et al. 2018). Given the invasive nature of this process, tight regulation is required in order to prevent attack of the trophoblasts by maternal leukocytes.

What mechanisms are in place to prevent attack of these fetally derived, invasive cells? Interestingly, EVT cells have a unique expression of Human Leukocyte Antigen (HLA). EVTs do not express the polymorphic MHC class I molecules HLA-A and HLA-B or the class II HLA-D molecules, but do display the class I molecules HLA-C, HLA-E, HLA-F and HLA-G (Apps et al. 2009; Hackmon et al. 2017). Expression patterns are summarised in Table 2.

HLA-C, HLA-E and HLA-F expression by EVTs provides a mechanism of protection for the fetus through modulation of activities of maternal NK cells (Papuchova et al. 2019). HLA-C can bind the NK cell inhibitory receptor KIR, thereby acting to "switch off" these cytotoxic cells which may react against fetally derived antigens (Sharkey et al. 2008). HLA-C is thought to play a key role in early implantation through controlling the depth of invasion of trophoblasts (Hackmon et al. 2017). In

Table 2 HLA-C, -E, -F, -G expression patterns

Molecule	Expression pattern	References
HLA-C	Surface EVT expression	Papuchova et al. (2019), Hackmon et al. (2017), Tilburgs et al. (2017), Apps et al. (2008, 2009), King et al. (2000b)
HLA-E	EVT expression, ligand for CD94/NKG2 NK cell receptor Strong first trimester expression	Hackmon et al. (2017), Cai et al. (2014), Apps et al. (2009), Ishitani et al. (2003), King et al. (2000a)
	Co-expressed with HLA-G on placental cells Human umbilical cord lining epithelial cells	
HLA-F	Surface EVT expression Highly expressed in cytotrophoblast and syncytiotrophoblast layers in first trimester	Hackmon et al. (2017), Shobu et al. (2006), Ishitani et al. (2003)
	EVT expression, mainly in cytoplasm during first trimester and then surface during second and third trimesters	
HLA-G	Surface EVT expression, strong levels across gestation	Hackmon et al. (2017), Cai et al. (2014), Apps et al. (2009), Ishitani et al. (2003), King et al. (2000b), Blaschitz et al. (1997), Hammer et al. (1997), Yang et al. (1996)
	Membrane bound HLA-G expressed on EVT, and sHLA-G on all placental trophoblasts	
	Placental Hofbauer cells	
	Endothelial cells of chorionic villi	
	Amniotic cells Human umbilical cord lining epithelial cells	

EVT—extravillous trophoblasts; HLA—Human Leukocyte Antigen; NK—Natural Killer; sHLA—soluble Human Leukocyte Antigen

addition, maternal T cells may specifically recognise fetal HLA-C on trophoblast cells, and that this activation promotes Treg differentiation (Tilburgs et al. 2009). The surface expression of HLA-C is known to be regulated at a transcriptional and post-transcriptional level (Papuchova et al. 2019).

HLA-E is expressed by EVTs and this has been shown to be very much restricted to first trimester (Hackmon et al. 2017). This molecule regularly forms complexes with HLA-G, and together, these mediate the inhibitory actions of uNK cells (King et al. 2000a). HLA-G is the most studied of the placenta-associated HLA molecules, with seven isoforms identified: HLA-G1, HLA-G2, HLA-G3, HLA-G4 being membrane bound and HLA-G5, HLA-G6, HLA-G7 being soluble (Hviid 2006; Hunt and Langat 2009). The trophoblast-bound molecules have roles in binding inhibitory NK cell

receptors to limit their action and also to control the degree of trophoblast invasion into the uterine wall. The soluble forms have been associated with modulation of T cell function through impairment of expression chemokine receptors (Morandi et al. 2010). The function of HLA-F has only been more recently explored, and it has been found that it is found on the cell surface of actively migrating EVTs and this supports their specific role in early EVT invasion and interactions with uNK cells (Hackmon et al. 2017).

6 Immunomodulation by Placental Exosomes

Exosomes are defined as membrane-bound extracellular vesicles (EVs) with a size of ~ 40–160 nm in diameter, originating from the endosome of a cell (Kalluri and LeBleu 2020). Interest in the immunomodulatory functions of exosomes has grown significantly in recent years, and studies into the impact of exosomes derived from the placenta have also created much interest. In fact, the identification of placental EVs was reported as far back as 1998 (Knight et al. 1998). Exosomes derived from the placenta have been associated with crucial immunomodulatory functions for the successful maintenance of pregnancy (Bai et al. 2021; Nair and Salomon 2018; Tong et al. 2018).

Throughout pregnancy, the placenta continually sheds EVs into the maternal bloodstream, with the syncytiotrophoblast layer being the main source of these (Salomon et al. 2017; Tannetta et al. 2017; Dragovic et al. 2015). Placental EVs contribute to the modulation of the maternal immune environment for the protection of the fetus, by altering the actions of immune cells. For example, activation of the potentially cytotoxic NK cells through the NKG2D activating receptor is reduced by the binding to NKG2D ligands on placental EVs (Hedlund et al. 2009). More recently, it has been shown that placental EVs can secrete HLA-E, promoting the secretion of IFN-γ and VEGF by decidual NK cells (Jiang et al. 2021) at implantation. Indeed, placental EVs increase significantly through the first trimester (Sarker et al. 2014; Kshirsagar et al. 2012). In addition, placental EVs can modulate the differentiation, activation and subtype polarisation of decidual macrophages to favour pregnancy (Aldo et al. 2014). One way in which this can occur is through driving the induction of the proinflammatory cytokine IL-1β from macrophages (Atay et al. 2011). Studies have shown that placental EVs lead to downmodulation of T cell activity, and thereby protection of pregnancy, through inhibiting T cell proliferation and cytotoxicity, driving Treg differentiation and T cell apoptosis (Stenqvist et al. 2013).

7 The Role of the Maternal Immune System During Parturition and Post-partum Involution

The initiation of labour, or parturition, in humans is complex, and the underlying mechanisms are not yet fully understood; however, many pathways have been considered such as the activation of the hypothalamic–pituitary–adrenal axis, prostaglandin production, an increase in the responsiveness to oxytocin and the functional withdrawal of progesterone (Smith 2007).

In addition to these physiological mechanisms, the infiltration of inflammatory cells and their mediators is now considered key in driving normal labour at term. Indeed, the impact of inflammation within the uterus on labour initiation has been eluded to for some time, with infection being responsible for 30–50% of cases of preterm birth (Goldenberg and Thompson 2003; Goldenberg et al. 2000; Romero et al. 2006). Normal labour is associated with an influx of inflammatory cells into the uterus, driven by an increase in chemokine and proinflammatory cytokine production at this site. Labour is associated with inflammation and this is characterised by an influx of macrophages, neutrophils and T cells to the myometrium and cervix (Osman et al. 2003; Thomson et al. 1999) along with an increase in the production of proinflammatory cytokines, including IL-1β, TNF-α and IL-6 (Osman et al. 2003; Young et al. 2002) and chemokines such as CXCL8 (IL-8), CCL2, CCL3 and CCL5 (El-Azzamy et al. 2017; Gomez-Lopez et al. 2010). Indeed, the utilisation of chemokine inhibitors to halt preterm birth has recently been investigated (Shynlova et al. 2021; Coleman et al. 2020). Until recently, it was considered that inflammation within the myometrium was a driver of labour induction; however, it is now recognised that it is more likely that inflammation is a consequence of labour induction, designed to prepare the uterus for post-partum remodelling (Singh et al. 2021, 2017).

Post-partum uterine repair and involution are a highly efficient process, required for the success of future pregnancies. Studies into the underlying molecular and immunological mechanisms of human post-partum repair have been slow to make it into the scientific literature, mainly due to the practical and ethical issues involved in collecting appropriate tissue samples. Much of what we do know about post-partum involution has come from other models of tissue repair (Paliulyte et al. 2017) use of mouse and rat models. Repair of the uterus requires extracellular matrix remodelling, proliferation, apoptosis and breakdown of collagen (Salamonsen 2003; Hsu et al. 2014).

8 Consequences of Pregnancy for the Immune System

8.1 Impact of Pregnancy Hormones on the Immune System

The female body significantly changes its hormonal balance for pregnancy maintenance. The sex hormones progesterone and estrogens can have many effects on the cells of the immune system (Wira et al. 2015; Menzies and Henriquez 2009; Robinson and Klein 2012) which can have wider implications. For example, pregnancy affects maternal autoimmune diseases and the outcome of infectious diseases.

The number and activation status of circulating leukocytes changes in the mother during normal pregnancy. There is an increase in the total leukocyte count during pregnancy, with an increase in the number of peripheral monoyctes and granulocytes (Belo et al. 2005; Luppi et al. 2002a, b), but a decrease in the number of lymphocytes (Castilla et al. 1989). In pregnant women, monocytes are activated, producing the proinflammatory cytokines IL-1β and IL-12 (Luppi et al. 2002a) but by comparison neutrophils appear to have reduced effector functions, with a reduction in chemotaxis and microbial killing ability (Kindzelskii et al. 2004; Crouch et al. 1995; El-Maallem and Fletcher 1980).

While the role of T cell subsets has been studied for some time and discussed earlier within this chapter, data about the impact of pregnancy on B cells are only just emerging in the literature. While B cells constitute 5–15% of circulating lymphocytes, the number of these changes significantly in response to pregnancy. Numbers of B cells generally fall in late pregnancy and the post-partum period (Lima et al. 2016). B cells are now characterised as different subsets, B1 and B2 B cells. B1 cells produce autoantibodies, which are detrimental to pregnancy, whereas B2 cells are responsible for production of asymmetric antibodies (IgG molecules with a modified Fab region), which can be protective for pregnancy (Muzzio et al. 2013; Gutierrez et al. 2005; Zenclussen et al. 2001; Barrientos et al. 2009). We previously discussed the importance of Tregs for the maintenance of pregnancy, but what about B regulatory cells (Bregs)? Much of what we know so far about Bregs has come from studies into autoimmunity, tolerance and cancer (Guzman-Genuino and Diener 2017) and is considered to be mediators of immunosuppression, so this would suggest a key role in pregnancy protection. Bregs are a major source of the immunosuppressive cytokine IL-10, and murine studies have shown that Bregs are required for pregnancy tolerance, with IL-10 being a main mediator of this (Rolle et al. 2013; Jensen et al. 2013).

8.2 Impact of Pregnancy on the Response to Infection

It is well established that generally, women exhibit much more vigorous humoral responses than men, with higher serum levels of IgG and total IgM (Eidinger and Garrett 1972; Giltay et al. 2000). Sex hormones play a critical role in modulating

immune function, and the importance of understanding these influences is highlighted by consideration of the sex bias in the susceptibility to a number of infections. The same can be said for pregnancy, and it is well known that the altered immune environment leads to pregnant women being more susceptible to some infections.

An example of this is the fact that the pregnant women are more susceptible to the impacts of the protozoan parasite *Toxoplasma gondii*. The change in the uterine immune environment described within this chapter, that is the development of a Th2 dominant milieu, favours the transmission and replication of this parasite, which is normally controlled by a Th1-type response (Roberts et al. 1995). Transmission of the parasite leads to congenital toxoplasmosis (Gomez-Chavez et al. 2019; Singh 2016; Hampton 2015; Torgerson and Mastroiacovo 2013), which can have a range of detrimental outcomes for the fetus, dependent on the stage of pregnancy at which transmission occurs.

Due to the immunological modifications which occur systemically during pregnancy, it is therefore unsurprising that pregnant women are generally more susceptible to respiratory infections (Englund and Chu 2018), including COVID-19 (Forestieri et al. 2022) and influenza (Mertz et al. 2019), leading to a higher risk of hospitalisation.

8.3 Autoimmune Disease During Pregnancy

As well as infections, the sex and pregnancy-associated hormones have been shown to have a significant impact on the incidence and progression of autoimmune disease. With global ageing populations and increasing incidence of autoimmune disease, it may be necessary to explore personalised treatment options.

The significant improvement in symptoms experienced by Rheumatoid Arthritis (RA) patients during pregnancy allows us to hypothesise that treatment options based on pregnancy-associated immune modifications could be useful. Women are three times more likely to develop RA than men; however, approximately 75% of women with RA experience an improvement in their symptoms as a direct consequence of the systemic effects of pregnancy (Forger and Villiger 2020). This effect is not long-lasting, and within 3 months post-partum, typical RA symptoms return (Adams Waldorf and Nelson 2008). While much remains to be learned about the underlying mechanisms for the disease amelioration, recent studies have eluded to the down-regulation of effector T cells, upregulation of Tregs and alterations to antibodies as potential reasons. More specifically, RA is associated with changes in the glycosylation of the IgG Fc region; however in pregnancy, it has been found that galactosylation of these immunoglobulins is related to the symptom improvement (Bondt et al. 2013, van de Geijn et al. 2009).

Multiple Sclerosis (MS) is characterised by the immune attack of the myelin sheath surrounding nerves. Generally, women are three times more likely to develop MS as men (Disanto and Ramagopalan 2013) with women more likely to carry the associated HLA DRB1 allele (Bove and Chitnis 2014); however, men are more likely

to develop more progressive disease with poorer recovery after attacks. Similar to RA, pregnancy is associated with a reduction in the incidence of relapses, but a return of the condition in the months following delivery (Confavreux et al. 1998). Progesterone, estriol and estradiol may play an important role in the preventing relapses in sufferers of this condition (El-Etr et al. 2005; Kim et al. 1999). More recently, a Danish nationwide cohort study has shown no links between MS and recurrent pregnancy loss (Mikkelsen et al. 2022).

Another autoimmune disease where pregnancy impacts on the disease is systemic lupus erythematosus (SLE); however, unlike with RA and MS, symptoms are not improved with pregnancy, in fact in many cases symptoms worsen. Women with SLE are at a much greater risk of developing pregnancy complications and fetal loss (Moroni and Ponticelli 2016; Foocharoen et al. 2009; Kalok et al. 2019). SLE is defined immunologically by the production of autoantibodies against nuclear antigens (Crispin et al. 2010). More specifically, 45% of SLE patients exhibit autoantibodies to the estrogen receptor, ER-α, which interferes with T cell homeostasis (Colasanti et al. 2012).

9 Conclusion

As we learn more about the human immune system, we have to explore how these immune functions are modulated during pregnancy and in turn how this impacts on the ability to deal with infections and other immune-related diseases. In this chapter, we have explored the key mechanisms involved in protection of the fetus from the maternal immune system and eluded to how this immunomodulation can impact on other aspects of the mother's health, namely, response to infection and autoimmune disease. Much remains to be determined about how we can apply this increased knowledge of the immune adaptations to pregnancy for clinical use, to find more efficient ways of predicting, diagnosing and treating both fertility issues and pregnancy complications.

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Correction to: Sex-Differential and Non-specific Effects of Vaccines Over the Life Course

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Correction to:
Chapter "Sex-Differential and Non-specific Effects of Vaccines Over the Life Course" in: S. L. Klein and C. W. Roberts (eds.), *Sex and Gender Differences in Infection and Treatments for Infectious Diseases*, Current Topics in Microbiology and Immunology 441, https://doi.org/10.1007/978-3-031-35139-6_9

This chapter was inadvertently published with the incorrect citation with the author's name Clair, LAS. This has now been corrected in the chapter as St. Clair, LA. The correction chapter has been updated with the change.

The updated version of this chapter can be found at https://doi.org/10.1007/978-3-031-35139-6_9

S. L. Klein and C. W. Roberts (eds.), *Sex and Gender Differences in Infection and Treatments for Infectious Diseases*, Current Topics in Microbiology and Immunology 441, https://doi.org/10.1007/978-3-031-35139-6_11