Chapter 10
Prenatal Alcohol Exposure: Impact on Neuroendocrine–Neuroimmune Networks

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10.1 Introduction

Alcohol exposure *in utero* can have numerous adverse effects on a developing fetus. The term fetal alcohol spectrum disorder (FASD) refers to the broad spectrum of structural, neurocognitive, and behavioral abnormalities or deficits that can occur following prenatal alcohol (ethanol) exposure (PAE). At the most severe end of the spectrum is fetal alcohol syndrome (FAS), which involves the complete phenotype of characteristic facial anomalies, growth retardation, and central nervous system (CNS) abnormalities. Alcohol exposure at levels that result in some but not all components of the facial, growth, and CNS deficits, and with evidence of neurobehavioral abnormalities, is termed partial fetal alcohol syndrome (PFAS). In the absence of any facial anomalies or growth deficits, a range of effects can occur that may be primarily physical, termed alcohol-related birth defects (ARBD), or primarily neurological and/or neurobehavioral, termed alcohol-related neurodevelopmental disorder (ARND) [1].

Following the description of FAS by Jones and Smith in 1973, there has been extensive interest in investigating the effects of PAE on the function of the immune system. In general, studies on children exposed to alcohol prenatally have demonstrated impairments in immune competence in both innate and adaptive immunity. Adaptive immunity is MHC (major histocompatibility complex) restricted and can be classified as either cellular immunity, mediated by T lymphocytes, or humoral immunity, mediated by B lymphocytes. Innate immunity is not MHC restricted. Through phagocytes such as monocytes, macrophages, and polymorphonuclear

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leukocytes (PMNL), as well as natural killer cells and mediators such as complement and C-reactive protein, innate immunity provides a first line of defense against many common pathogens. Importantly, interactions between the innate and adaptive components of the immune system are necessary to launch effective immune responses.

In this chapter, we will review the normal development of the immune system, a complicated and multisystem process, to provide the context for understanding how and when PAE can impact immune function. We will then review data from both clinical studies and animal models that have demonstrated adverse effects of PAE on neuroendocrine activity and immune competence. We will describe the sexual dimorphism of the normal immune response and discuss sex differences in alcohol's adverse effects on both neuroendocrine and immune functions. Examination of mechanisms underlying alcohol's adverse effects will focus primarily on neuroendocrine–neuroimmune interactions and will probe how PAE alters the interaction of hormonal and immune systems. Included in this section is a discussion of recent work from our laboratory utilizing an animal model of human rheumatoid arthritis, which reveals an increased susceptibility to inflammation in PAE offspring. Finally, we will examine fetal programming of stress and immune function by PAE and consider possible mechanisms mediating alcohol-induced fetal programming, including epigenetic mechanisms.

10.2 Normal Ontogeny of the Immune System

10.2.1 Timeline of Development

Although development of the immune system takes place throughout life, the basic framework of the immune system and immune competence develops before birth (see Fig. 10.1). The normal newborn is not immunologically naïve. Indeed, data have shown that neonatal T and B cells are matured to a stage that they can mount an antigen-specific response to antigens encountered *in utero*, such as the parasite *Ascaris* and tetanus toxoid vaccination [4, 5] (Table 10.1).

The first stage of fetal hematopoiesis involves the formation and development of hematopoietic stem cells in the yolk sac over the first month of fetal life. These stem cells migrate to the liver 3–4 weeks later and subsequently migrate to the thymus and spleen. Stem cells can be found in the bone marrow at 11–12 weeks of gestation [6, 7], and differentiated T and B cells can be identified in fetal blood [8] and spleen [9] as early as 14 weeks of gestation. Environmental influences can affect lymphopoiesis and cell migration as early as 7–10 weeks postconception [10]. The spleen is considered completely immunocompetent by 18 weeks of gestation. At this time, splenic T cells have adult levels of expression of CD3, CD4, and CD8 and are able to respond to mitogens [11], and splenic accessory cells are fully functional in delivering co-stimulatory signals [7]. However, fetuses still have fewer memory T cells than adults at this time [7]. Yolk-sac-derived pro-B cells develop in the liver and acquire surface immunoglobulin (Ig) M, IgD, and CD20 expression by 10–13 weeks of gestation [12]. B cells are detectable in lymph nodes from 16 to

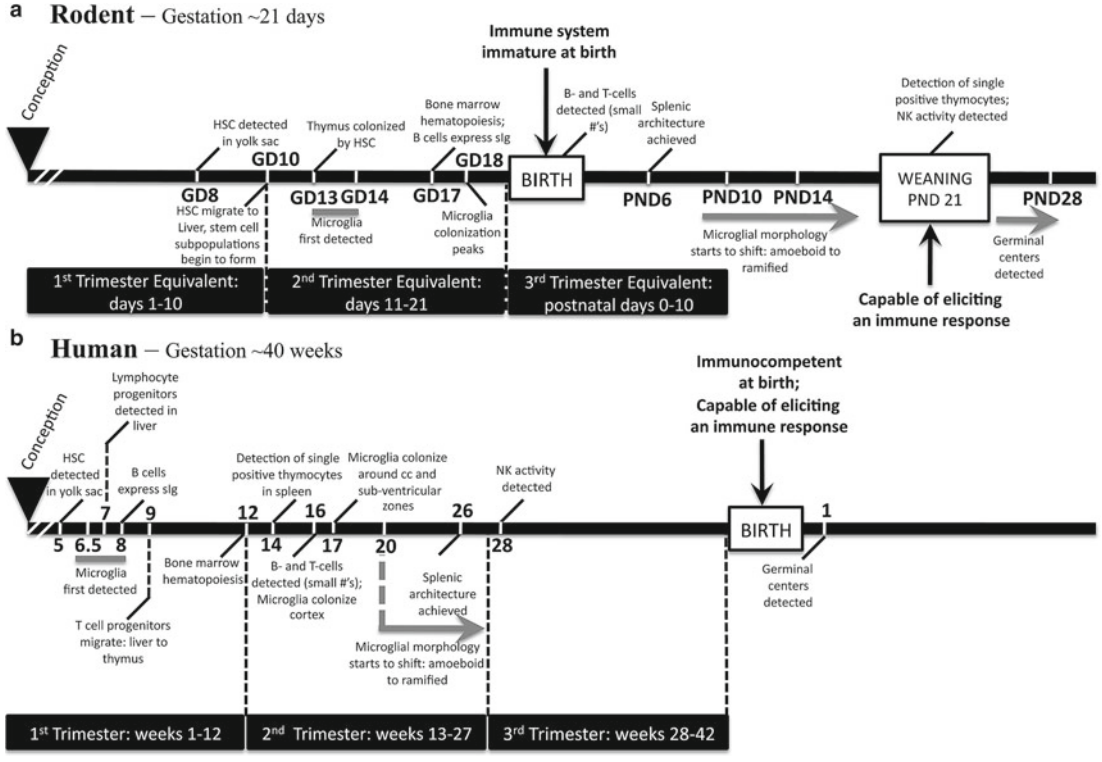

Fig. 10.1 Comparison timelines of key developmental immunological milestones in rodents and humans. Timelines showing many of the important immunological milestones in the rodent (**a**) and the human (**b**) fetus and newborn are illustrated [figure modeled on (Holsapple et al. [2])]. *HSC* hematopoietic stem cells, *sIg* surface immunoglobulin, *NK* natural killer cells, *CC* corpus callosum, *splenic architecture* delineation of *red* and *white* pulp areas (Information in this figure is drawn from a number of sources including Holladay and Smialowicz [250]); Holsapple et al. [2]; Bilbo and Schwarz [3]; Schwarz [213])

Table 10.1 Common immune terminology.

Immune term	Definition
Adaptive immune response	The response of specialized immune cells to foreign antigen. Adaptive immunity is activated approximately 3–7 days postinfection, allowing for the targeted elimination of pathogens as well as immunological memory. The adaptive immune system can be subdivided based on how immunity was achieved: (1) *naturally acquired immunity* develops following a natural occurring infection; (2) *artificially acquired immunity* develops as a result of an external or artificial challenge, as in the case of vaccination; (3) *passive immunity* develops through the transfer of antibodies and/or activated immune cells from a host, for example, from mother to infant through breast milk
Antibody	Protein produced by plasma B cells in response to infection or vaccination. Every antibody has a unique structure, allowing it to bind to a specific target substance, known as the *antigen*. Antibodies bind to antigens, neutralizing them and targeting them for elimination by phagocytic cells
B lymphocyte (B cell)	A subset of white blood cell that mediates humoral immunity. B cells are generated in the bone marrow, and each B cell has a unique B cell receptor, allowing it to bind to a particular antigen. Prior to activation, B cells play an immune surveillance role and do not start producing antibodies until they become fully activated. There are many types of B cells, each having a different function; these include memory B cells, plasma B cells, B-1 cells, and B-2 cells
Cell-mediated immunity	Includes the aspects of the adaptive immune response in which antigen-specific T cells have the primary role
Cluster of differentiation	Groups of antibodies that identify cell-surface markers. This method of identifying cell-surface markers is important for immunophenotyping of cells. The marker to which the antibody binds is termed CD, followed by a number (e.g., CD4, CD8). The surface markers expressed by a cell are informative as to the lineage and function of the cell. Thus, cell populations can be categorized based on the CD molecules they express. The symbols "+" and "−" are used to indicate whether the cell type expresses a given surface marker or not CD45+: all hematopoietic cells CD45+, CD3+: thymocytes, T cells CD45+, CD3+, CD4+: T helper cells CD45+, CD3+, CD8+: cytotoxic T cells CD45+, CD19+ or CD45+, CD20+, CD24+: B cells (CD20 is not expressed during the first and last stage of B cell development) Other important CD antigens: CD7: expressed by pluripotential hematopoietic cells, thymocytes, and T cells CD25: expressed by activated T cells, B cells, and monocytes CD43: expressed by leukocytes (except resting B cells)
Cytokines	Signaling molecules secreted by immune system cells as a means of cellular communication. Cytokines include proteins, peptides, and glycoproteins, and their defining feature is that they have a wide array of immunomodulating effects, including pro- and anti-inflammatory effects

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Table 10.1 (continued)

Immune term	Definition
Humoral immunity	Includes the antibody-mediated aspects of the adaptive immune response. The main cell types involved in humoral immunity are cells of B lymphocyte lineage. Humoral immunity, unlike cell-mediated immunity, can be transferred to a recipient via serum (cell-free)
IgE	Class of immunoglobulin involved in defending the body against parasites; also implicated in allergic reactions
IgG	Class of immunoglobulin produced by plasma B cells; most abundant serum immunoglobulin in humans
IgM	Class of immunoglobulin produced by B cells; first antibody to be secreted by B cells following exposure to an antigen
Immunoglobulin (Ig)	Antibody molecules (plasma proteins) belonging to the immunoglobulin superfamily, which includes an array of proteins with immunoglobulin domains and includes T and B cell receptors, antibodies, and MHC molecules. Immunoglobulins can exist in both membrane-bound and soluble forms. The specific antigen receptor on B lymphocytes is an example of a membrane-bound immunoglobulin. Soluble immunoglobulins are generally composed using variations of the same building blocks, known as heavy chains and light chains, with two heavy and two light chains per antibody Key examples of secreted immunoglobulins: *IgG*, *IgM*, *IgE*
Inflammation	Mechanism of the innate immune response resulting in the accumulation of fluid and white blood cells in response to physical trauma, infection, or irritation. Inflammation is a protective response designed to remove the noxious agent and initiate healing. Inflammation can be categorized as (1) *acute*, the early and transient response to harmful stimuli resulting in a cascade of responses including movement of plasma and white blood cells to the site of trauma/infection, and (2) *chronic*, persistent inflammation following persistent challenge or with autoimmune diseases and is associated with a shift in the immune cells present within the tissue as well as tissue destruction in addition to healing
Innate immune response	The nonspecific immune mechanisms that act as a first line of defense against pathogens. Innate immunity is a critical component of the immune response; however, it does not increase with repeated exposures to a pathogen, and it is not long lasting or protective against future encounters with the same pathogen
Leukocytes	White blood cells; critical cells of the immune system involved in defending the body against pathogens. Leukocytes include lymphocytes, monocytes, and polymorphonuclear leukocytes
Lymphocytes	A class of white blood cells involved in mediating adaptive immune responses. Lymphocytes can be divided into two main categories—*B lymphocytes* (B cells) and *T lymphocytes* (T cells)
Macrophage	Phagocytic cells that are of key importance in the innate immune response. Macrophages engulf foreign antigens and necrotic tissue, removing them from the environment. Macrophages also participate in the development of inflammation

(continued)

Table 10.1 (continued)

Immune term	Definition
Major histocompatibility complex (MHC)	Cluster of genes encoding cell-surface molecules (membrane glycoproteins) that mediate the interactions between leukocytes and other cells (includes other leukocytes)
Microglia	Nonneuronal cells within the brain. These cells are the resident macrophages of the central nervous system, scavenging the brain and spinal cord for infectious agents and damaged cells. Microglia also play many important roles during brain development including participating in neuronal pruning, phagocytosis of dying neurons, and producing cytokines
Monocyte	A type of leukocyte; the precursor to macrophages. Monocytes are attracted to damaged tissue from the circulation, and as they enter the tissue, through a process called leukocyte extravasation, they undergo changes, developing into macrophages
Pathogens	Infectious agents, including viruses, bacteria, or fungi that can cause disease in a host (human, animal, or plant)
T lymphocyte (T cell)	A subset of white blood cell that mediates cell-mediated immunity. These cells mature in the thymus and express unique cell-surface receptors (T cell receptors), allowing them to bind and recognize antigens. There are many different subsets of T lymphocytes, each undertaking specific functions in the cell-mediated immune response; these include T helper cells, cytotoxic T cells, and memory T cells
T_H1 cells	A subset of CD4+ T cells that are of key importance in adaptive immune functions including activating macrophages. These cells produce T_H1 cytokines, which are involved primarily in cell-mediated immunity and tend to produce proinflammatory responses. T_H1 cytokines include IL-2 and IFN-γ
T_H2 cells	A subset of CD4+ T cells that are of key importance in adaptive immune functions including stimulating B cells to produce antibody. These cells produce T_H2 cytokines, which are involved primarily in humoral immunity, and tend to be more anti-inflammatory. T_H2 cytokines include IL-3, IL-4, IL-5, and IL-13

17 weeks, in spleen at 16–21 weeks, and are abundant in bone marrow at 16–20 weeks of gestation [10].

10.2.2 Development of the Thymus

The structure of the human thymus develops as a result of epithelial–mesenchymal interactions. The endoderm of the third pharyngeal pouch differentiates into thymic epithelium. Neural crest cells then migrate and become the mesenchyme that will form the layers around the epithelial primordium of the thymus and the connective tissue framework [13]. T cell progenitors arrive in the thymus from the liver during the ninth week of gestation, and at about 10–12 weeks, a cortex and a medulla become

differentiated [10]. Prothymocytes express CD7 and CD45 in the fetal liver at 7 weeks and CD3 at 8–9 weeks of gestation, when these cells migrate to the thymus [7]. Expression of the T cell receptors, CD4 and CD8, occurs around 12 weeks of gestation, and mature CD4+ and CD8+ (single positive) T cells leave the thymus after week 13. At 13–14 weeks, thymocytes acquire the ability to proliferate to most mitogens, and the T cell pool expands rapidly during 14–16 weeks of gestation [11, 14]. During the second trimester, susceptibility to environmental factors can disrupt the thymic development and T cell proliferation and lead to a defective T cell repertoire.

10.2.3 Comparison of Rodent and Human Timelines

Rodent gestation (~3 weeks) is substantially shorter than human gestation (~40 weeks) and as such, newborn rat and mouse pups are born developmentally and immunologically immature, at a time equivalent to the end of the second trimester in humans [15]. Therefore, studies in rodent models of PAE have focused primarily on the early events in ontogeny of the immune system, that is, during the late gestational period and early postnatal life (the first 10 days of postnatal life are roughly equivalent to the third trimester of human gestation). Typically, these models involve alcohol exposure for the full period of gestation. As the formation of a functional immune system requires sequential, yet synchronized, developmental events, including the coordinated development of immune cells and organs, which begins during early embryonic development, administration of alcohol to the rat or mouse throughout gestation encompasses all of the key early developmental events. Figure 10.1 presents a comparative timeline of immune system development in humans and rodents.

As shown in this figure, human gestation is approximately 40 weeks in duration with many of the key steps involved in maturation of the immune system being initiated in the first and second trimesters.

- At birth, the human neonate is immunocompetent and capable of initiating an immune response. However, the neonate is immunologically naïve, having yet to encounter antigens. Thus, while an immune response can be elicited, the strength of the response has not yet achieved adult levels at birth.
- In contrast, gestation in the rodent is approximately 21 days in duration, and in general, shorter gestation periods are associated with relatively immature immune systems at parturition [250]. At birth, the rodent immune system is very immature, and the pre-weanling rodent is incapable of initiating an immune response.
- Many key developmental milestones in the rodent are achieved during the equivalent of the third trimester in the human, which is approximately the first ten postnatal days in the rodent. By weaning (approximately postnatal day 21), an immune response to pathogenic challenge can be initiated; however, maturation of the immune system is still occurring, and an adult level response cannot yet be achieved.

- Of note, both the rodent and human fetus are released from intrauterine maternal influences at birth, notably the restraint on Th1 responses. However, maternal influences persist through lactation. As such, the maternal immunological milieu and response system can impact development of the fetal as well as the neonatal immune system.

10.3 Adverse Effects of PAE on the Immune System

10.3.1 Deficits in Immune Competence in Children with FASD

Clinical data on deficits in immune competence in children with FASD remain somewhat limited. An early review of 13 documented cases of FAS in infants and children by Johnson and colleagues [16] showed a higher incidence of minor infections, such as recurrent otitis media and respiratory infections, as well as major life-threatening bacterial infections, in alcohol-exposed compared to nonexposed children.

Inborn errors of immunocompetent cells in children with FAS result in immunodeficiency disorders or increased susceptibility to infections. Recurrent opportunistic infection and infection caused by ubiquitous microorganisms, such as bacteria, viruses, and fungi, typically occur with deficits in cell-mediated immunity, whereas deficits in B cells, immunoglobulins, complement, and phagocytes usually lead to infection by encapsulated and pyrogenic bacteria, such as *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*. In children with FASD, both types of recurrent infections have been reported, suggesting that both T and B cell-mediated immunities are compromised [16].

Subsequent studies confirmed an increased vulnerability to recurrent serious otitis media and recurrent upper respiratory infections, both of which have significant implications for hearing loss and, consequently, speech and language problems, as well as learning disabilities in children with FAS [17]. *In utero* alcohol exposure has also been shown to have an adverse impact on numerous parameters of immune function, including decreased eosinophil and neutrophil cell counts and leukocyte responses to mitogens, resulting in an increased incidence of hypogammaglobulinemia [16]. Interestingly, a study by Ammann and colleagues [18] described a number of characteristics of patients with the DiGeorge syndrome (a congenital immune deficiency syndrome) that were paralleled in children with FAS. Children with both syndromes show deficits in immune function, including altered T cell function. Immune status was also assessed in subjects who were part of a longitudinal cohort of 320 mother–child pairs [19]. Alcohol-exposed teens did not appear to have an increased susceptibility to infectious diseases compared to controls. However, adolescents showing dysmorphic features appeared to have increased rates of asthma (threefold), allergic rhinitis, and persistent skin rashes and/or eczema. As well, absolute lymphocyte counts, including counts of T cells (both T helper and T cytotoxic/suppressor) and NK cells, but not B cells, were increased in alcohol-exposed adolescents, regardless of

dysmorphia or gender. It was suggested that this increase might have resulted from the acute stress of neuropsychological testing that had occurred during the morning, just prior to the blood draw. We will come back to this issue of stress, neuroendocrine activity, and immune function in FASD in Sects. 10.12 and 10.13 below.

A more recent study by Gauthier and colleagues [20] lends additional support to the early findings of alterations in immune competence following *in utero* alcohol exposure in the human population. This study addressed whether maternal alcohol use would increase the risk of sepsis in very low-birth-weight, alcohol-exposed newborns. It has been well established that premature newborns are at a higher risk of infection in early life, and despite improvements in neonatal intensive care, the rate of infant death due to sepsis remains high [21]. In addition, research in animal models suggests that lung development may be impaired following *in utero* alcohol exposure (see Sect. 10.7), and as a result, alcohol-exposed neonates may be at a higher risk for the development of infection during early life. This was substantiated by the findings of Gauthier and colleagues [20], which indicated that very low-birth-weight newborns exposed to alcohol *in utero* have a 15-fold higher incidence of early-onset sepsis (defined as presence of microorganisms in blood cultures collected within the first 72 h post-birth) as compared to the matched control group [20]. It was hypothesized that this increased risk in the alcohol-exposed neonates is due not only to immune alterations in the neonate but also to alcohol-induced impairments in immune function in the mother [20]. Adult alcohol exposure has been shown to impair immune function at many levels including negatively impacting the activation and response of macrophages and impairing lymphocyte proliferation and antibody production [22]. As a result, the immune milieu may also be significantly altered in the alcohol-consuming pregnant female, which may impact development, including development of the immune system in the offspring (see Sect. 10.2).

Gauthier and colleagues [23] have also examined the risk of infection in newborns of alcohol-consuming and control mothers in one of the largest clinical studies of this type ($n = 872$). Due to the large sample size, timing and degree of alcohol consumption before and during pregnancy could be assessed and linked to neonatal infection risk. The level of alcohol intake was found to be an important factor in predicting neonatal infection risk, with increasing levels of alcohol consumption by the mother 3 months prior to conception or during the first, second, or third trimesters of pregnancy resulting in a significantly increased risk. In addition, binge drinking, defined as consumption of seven or more drinks per week during the 3 months prior to conception or the second or third trimesters of pregnancy, was found to increase the risk of neonatal infection by approximately fourfold. When controlling for many possible confounds including maternal smoking, low maternal income, and being small for gestational age, high levels of maternal drinking (binge drinking), specifically during the second trimester, were found to increase the risk of infection by approximately fourfold, compared to that in unexposed newborns. Thus, while maternal drinking is often associated with factors such as concomitant smoking, drug use, or low socioeconomic status, which could themselves affect health outcomes, this study points toward a direct link between maternal alcohol consumption and decreased immune competence in the neonate. Interestingly, the second trimester appears to be particularly sensitive to the immunoteratogenic

effects of alcohol, perhaps as during this period many immunological milestones are achieved in the fetus. As such, alcohol exposure during the second trimester may permanently impact the fetal immune system, resulting in an increased risk of infection at birth and potentially extending to an increased infection risk in later life.

While studies to date on children with FASD have provided clear evidence of alterations in immune function, detailed information regarding the effects of alcohol on the developmental time course of the naïve immune system, including effects on T and B cell development, as well as susceptibility to infections and autoimmune disorders can be difficult to obtain in human studies. Human studies are also limited by the lack of information regarding the level, timing, and duration of alcohol consumption, as well as confounding factors such as concomitant exposure to maternal smoking, drugs of abuse, and stress, all of which have been shown to impact development of the immune system [24, 25]. Animal models, which allow for the precise control of genetic and environmental factors, are as important for research on the immune system as they are for research on other aspects of alcohol's adverse effects.

10.3.2 Studies in Rodent and Primate Models Demonstrate Impaired Development and Function of the Immune System

To date, a large proportion of the research examining the effects of maternal alcohol consumption on immune competence has been conducted in rodent models, in which there is extensive knowledge of physiology, pharmacokinetics, and behavior. Rodent models have been invaluable in addressing fundamental immunological questions and have served as an important tool in shaping our understanding of autoimmune disorders, transplant rejection, and immune responses to infectious agents [26]. Additionally, many inbred, mutant, and congenic rodent strains have been established to address immune system function, and microsurgical techniques have been tailored for immunological probing [26].

Compared to rodent models of FASD, nonhuman primate models have distinct advantages due to the high degree of physiological, developmental, reproductive, and phylogenetic similarities to the human, making them especially important and relevant in assessing immunomodulatory agents [27, 28]. Nonhuman primates are also very similar to humans with regard to development and composition of the immune system. For example, important lymphoid tissues such as the spleen, tonsils, and thymus follow very similar developmental timelines in humans and nonhuman primates (reviewed in [2]). Additionally, humans and nonhuman primates are highly comparable in early postnatal development of the immune system, in that newborns of both species display lower levels of memory T cells and higher levels of naïve T cells, compared to adults [29]. This indicates that the immune systems of newborn humans and nonhuman primates are developmentally mature at birth but that antigenic contact has yet to occur in the protective environment of the womb in both species [29]. In addition, both susceptibility to infectious agents and the

immune response are relatively conserved between humans and nonhuman primates. Species-specific functional differences in immune responses do exist, due to the fact that these responses are quite malleable and may evolve relatively rapidly alongside the rapidly evolving microbe environment, an environment that differs between species [30]. Nonetheless, due to the strong phylogenic similarities between humans and nonhuman primates, functional overlap of the immune systems is expected to be relatively high.

10.3.2.1 Rodent Models

Research using rodent models has confirmed the clinical findings of alterations in immune function following *in utero* alcohol exposure (reviewed in [31, 32]) and has increased our understanding of both the spectrum of effects in different organ systems and the mechanisms mediating the immunoteratogenic effects of alcohol. Interestingly, deficits in innate immunity have typically not been observed in animal studies. In contrast, marked deficits in adaptive immunity have been reported consistently in PAE offspring. PAE alters development of the thymus in both rats and mice. In a mouse model, maternal consumption of an ethanol-containing diet (25% ethanol-derived calories (EDC), BALs ~90 mg/dl) delayed development of the thymus [34], decreased thymus cell numbers, and diminished mitogen-induced cell proliferation in late-term fetuses [35]. Decreased thymus weight, size, and cell counts have also been observed at birth in a rat model (liquid diet exposure, 35% EDC, BALs ~79–127 mg/dl) [36]. These changes have been shown to persist throughout the preweaning period and into adolescence [35, 37–39], although data on mice suggested that recovery or catch-up in total thymocyte numbers may occur [37]. Decreased mitogen-induced proliferation of thymic cells was reported to persist until weaning in PAE male rats [36], but paradoxically, proliferation was shown to be greater than control levels in adolescence [40, 41]. The mechanisms underlying this increased thymocyte proliferation remain to be elucidated.

The adverse effects of alcohol on development of the thymus were confirmed by in vitro studies using organotypic cultures. Alcohol-treated organ cultures (alcohol concentrations of 0.2–0.8%) showed a concentration-dependent decrease in total cell numbers and percentages of immature fetal thymocytes (CD4+/CD8+) [42] likely due to increased apoptosis [34, 42–44]. Furthermore, thymic cell counts, total numbers of CD4+ and CD8+ cells, and numbers of immature CD8+/TCR+ and CD8+/CD45RC+ thymocytes were decreased postweaning and into early adolescence [39], suggesting that prenatal exposure to alcohol alters the later stages of thymocyte maturation.

The adverse effects of PAE last well into adulthood and appear to involve primarily alterations in cell-mediated rather than humoral immunity. PAE animals, typically exposed via maternal consumption of ethanol-containing liquid diets (~35–36% EDC), have decreased numbers of Thy1.2+, CD4+, CD8+, and IgG+ splenocytes [38, 45, 46]. In addition, data from both rodent and primate studies indicate that splenic lymphocytes taken from PAE males from adolescence through

young adulthood show decreased proliferative responses to mitogens [36, 47–53], although in some studies, it was shown that the proliferative response may normalize by 8 months of age [51, 54]. Furthermore, deficits not only in the response of freshly isolated splenic T cells but also in the response of T lymphoblasts (obtained following treatment with concanavalin A [Con A]) to IL-2 or further Con A stimulation have been observed in PAE rats [49, 52, 54, 55]. In contrast, the changes in mitogen-induced proliferation of thymocytes reported in PAE animals appear to normalize in young adulthood [40].

Increased susceptibility to infections following PAE has also been reported in rodent models, in parallel with the human data, and provides further evidence of alterations in immune competence. For example, work by McGill and colleagues [56] found that mice exposed to alcohol during gestation and lactation (10–12% ethanol in drinking water) showed enhanced disease severity as well as increased and sustained pulmonary viral titers following influenza virus infection. Similarly, in a study of *Macaca nemestrina*, 4 of 18 (22%) animals exposed to once weekly oral doses of alcohol died or were euthanized after infectious disease or failure to thrive during the first year of life, whereas none of the controls died [33].

As noted, humoral immunity appears to be less affected by fetal alcohol exposure than cellular immunity. For example, in both rats [51] and nonhuman primates [33], the serum immunoglobulin response to immunization was shown to be unaffected by prenatal exposure to alcohol. On the other hand, abnormal development of B cell lineages in mouse bone marrow, spleen, and liver has been reported. Decreased numbers of splenic B cells and decreased B cell proliferative response to LPS (liquid diet, 25% EDC) [57], as well as delayed B cell maturation [58], have been observed. In addition, numbers of both immature and mature B cells in spleen and bone marrow were found to be decreased at birth, although most recovered to normal levels by 3–5 weeks after birth [59, 60].

10.3.2.2 Primate Models

Studies using nonhuman primates, including pigtailed macaques [61–63], vervet monkeys [64], and *M. nemestrina* [65], have demonstrated significant teratogenic effects of prenatal exposure to alcohol on numerous systems and behaviors (ethanol doses ranging from 0.3 to 4.1 g/kg maternal weight; BALs ~24–550 mg/dl). In general, alcohol-exposed offspring showed neuroanatomical, neurological, developmental, and facial anomalies similar to those seen in human FAS, with severity increasing as dose of alcohol increased [61–65]. Numerous cognitive and behavioral abnormalities were also observed, even in the absence of physical or facial anomalies, which increased with increasing dose of exposure, and were greater with earlier than with later gestational exposure [61]. At higher doses, facial features characteristic of FAS (1.8 g/kg) and microcephaly (4.1 g/kg) were observed [66].

In contrast to these important studies, few researchers have utilized nonhuman primate models to assess immune status following *in utero* alcohol exposure. A study by Grossmann and colleagues [33] was the first to examine immune function in the nonhuman primate *M. nemestrina*. Interestingly, the initial focus of the

study was not immunological, but the focus shifted following death of a large proportion (4 of 18) of their alcohol-exposed animals from infectious disease. This high mortality rate from an otherwise treatable infection suggested profound alterations in immune competence in this population and prompted a review of immune status [33]. Exposure to alcohol (mean maternal BALs ~220 mg/dl) throughout gestation was shown to decrease T cell proliferative responses to tetanus toxin and result in lower tetanus toxoid titers after both initial vaccination and a booster, suggesting a deficit in the development of immunological memory. This finding clearly has important implications for the long-term health of the animals. In contrast, however, there were no significant effects of *in utero* alcohol exposure on total numbers of white blood cells, leukocyte subsets or monocyte phagocytic activity compared to that in control subjects.

10.4 Immunity at the Fetal–Placental Interface

Alterations in immunity at the fetal–placental interface could contribute to the increased incidence of spontaneous abortions and premature births that occur with maternal alcohol consumption. Under normal condition, immunity at the fetal–placental interface is biased toward humoral immunity, and cellular immunity is suppressed to prevent fetal rejection. In addition, cell-mediated immunity is skewed toward Th2 responses and production of cytokines such as IL-3, IL-4, IL-5, and IL-13, rather than toward Th1 responses and production of cytokines such as IL-2 and IFN-γ [67]. Th1/Th2 responses refer to the functionally polarized responses by CD4+ T helper (Th)- and CD8+ T cytotoxic (Tc)-cell subsets that depend on the cytokines they produce. Th1 cytokines are involved primarily in cell-mediated immunity and tend to produce proinflammatory responses, whereas Th2 cytokines are involved primarily in humoral immunity and tend to be more anti-inflammatory and to counteract Th1-mediated responses. The Th1/Th2 concept suggests that modulation of the relative contribution of Th1- or Th2-type cytokines regulates the balance between protection and immunopathology, as well as the development and/or the severity of some immunologic disorders.

It has been suggested that progesterone and CD4+ regulatory T cells are key factors enabling the fetus to evade immune rejection by the pregnant female and thus allowing the female to maintain pregnancy. Interestingly, progesterone appears to suppress cytotoxic activity of lymphocytes from pregnant women via interaction with progesterone receptors [68]. As well, CD4+/CD25+ regulatory T cells, which are suppressive in nature, may act at the interface between the fetus and the pregnant female to suppress allogeneic responses directed against the fetus [68–70]. *In utero* exposure to alcohol could alter the balance between regulatory T cells and T effector cells and, thus, contribute to the increased incidence of spontaneous abortions and premature births. This possibility is supported by observations of elevated cord-blood IgE concentrations in alcohol-exposed infants, indicating increased activity of Th2-type responses [71].

10.5 Lactational Transfer of Immunity

Human and animal milk contains cytokines and immunoglobulins that can pass to the newborn and likely play a role in augmenting immune defenses of neonates who are born with relatively immature immune systems [72, 73]. Alcohol consumption during pregnancy and/or lactation is known to alter maternal immune function. Of particular relevance, maternal alcohol consumption can result in alterations in the structure of the mammary gland and/or immune cells and soluble immune factors in the milk. A series of studies, using liquid diets with 36% EDC, have reported numerous alterations in the maternal mammary gland, including lower mammary gland weight and length, and histological changes such as a decreased percentage of alveolar epithelium, an increased percentage of connective tissue and alterations in the density of the mammary epithelium [74, 75]. Similarly, Vilaro and colleagues [76] reported that alcohol consumption decreased both absolute and relative mammary gland weight and mammary tissue protein content and reduced daily milk production. Immune components of the milk are also altered by alcohol consumption. Changes include altered numbers and distribution of T and B lymphocytes, primarily within the connective tissue compartment of the lactating mammary gland [77], increased IL-2 production by milk cells stimulated with Con A [78], decreased milk NK cell levels, and reduced specific IgG antibody levels following challenge with the intestinal parasite *Trichinella spiralis* [79].

Alterations in immune function of offspring nursed by alcohol-consuming dams have been reported. In a mouse model, with alcohol exposure (20% EDC, BALs ~250–290 mg/dl) during pregnancy and lactation or lactation only, offspring showed reduced numbers of splenic lymphocytes overall and, particularly, Thy 1.2+, CD4+, CD8+, and IgG+ lymphocyte subsets [38]. Deficits in lactational transfer of immunity have also been observed in the rat model utilizing *T. spiralis* challenge. Under normal conditions, T cell-mediated immunity to *T. spiralis* can be transferred from dam to neonate during lactation [251]. However, PAE neonates exhibited increased intestinal worm counts in response to *T. spiralis*, suggesting a decreased capacity to mount an immune response to the pathogen [79–81]. Abnormalities in the offspring involved depressed T and B cell-mediated responses such as lower serum IL-2 and tumor necrosis factor (TNF) contents and lower IgM and IgG antibody titers. Alterations in immune cells and soluble immune components in milk, as described above, could have direct effects on postnatal development and function of the offspring immune system and play a role in the depressed lactational immune transfer observed [79, 82]. Moreover, the detrimental effects of alcohol appear to increase across generations. That is, second-generation PAE pups mothered by first-generation PAE adults who themselves consumed alcohol during pregnancy showed significantly reduced proliferative responses to both *T. spiralis* antigen and stimulation with Con A, lower titers of serum antibodies, and lower percentages of T cells and cytotoxic T cells, compared to the first-generation PAE and pair-fed groups [83]. Of note, treating ethanol-consuming dams with an immunostimulatory drug, levamisole, during pregnancy and lactation was shown to reverse some of the deleterious effects of lactational transfer of immunity to suckling rat pups [84].

Rodent models such as these, in which alcohol exposure is extended to include the lactation period, are important in that they address one of the key limitations of rodent models in general, the fact that rodents are born developmentally immature, at a stage of development equivalent approximately to the end of the second trimester, compared to humans. The third trimester in the human is a sensitive period characterized by key development events including the development of immunocompetence [2]. The third trimester equivalent in the rodent is approximately the first 10 days postnatal. The finding that alcohol exposure during lactation (through maternal milk) results in long-term deficits in cellular immunity, in parallel with the human situation, supports the finding of high sensitivity to alcohol's immunoteratogenic effects during the third trimester of human gestation. These data have important implications for understanding possible deficits in immune function that might occur in children of women who consume alcohol in the late gestation period.

10.6 PAE and Increased Susceptibility to Tumors

One index of immune function in children following *in utero* alcohol exposure is the incidence of various forms of cancer. Studies have reported, for example, an increased incidence of malignancies, particularly those of embryonic origin, including neuroblastoma, ganglioneuroblastoma, medulloblastoma, and embryonal rhabdomyosarcoma in children ranging from birth to 12 years of age [53, 85], although a large case–control study found no evidence of an association between maternal smoking or drinking alcohol and risk of childhood germ cell tumors [86]. One case report found an association between the development of an adrenal cortical carcinoma and a history of FAS [87], and alcohol consumption has been identified as a prenatal risk factor for testicular cancer [88]. With only a few exceptions [89, 90], studies have reported an association between maternal alcohol consumption and an increased risk for or incidence of infant or childhood leukemia. A comprehensive review and meta-analysis of case–control studies found a link between alcohol consumption during pregnancy and increased risk of childhood leukemia, particularly acute myeloid leukemia (AML) [91]. Associations between maternal drinking and both acute lymphoblastic leukemia (ALL) [92] and acute nonlymphocytic leukemia [93] have also been reported. As well, positive dose–response associations between maternal alcohol consumption and both AML [92, 94] and ALL [94] have been observed. Interestingly, data suggest that a possible interaction between PAE and polymorphisms of carcinogen-metabolizing genes may influence the risk for ALL [95].

Increased susceptibility to malignancies has been demonstrated in animal models of FASD as well [53]. For example, susceptibility to an estrogen-induced prolactin-secreting tumor was increased in adult males prenatally exposed to alcohol (liquid diet, 35% EDC), as assessed by greater anterior pituitary weights and serum prolactin levels compared to those in control males [96]. Interestingly, whereas there were no effects of PAE on sensitivity to nitrosomethylbenzylamine (NMBA)-induced esophageal tumors, PAE males showed a marked decrease in thymus to body weight ratio as well as adrenal gland hyperplasia compared to controls,

suggesting altered immune function [96]. Research by Hilakivi-Clarke and colleagues [75] investigated the effects of maternal alcohol consumption [liquid diet, low (7% EDC) and moderate (15% EDC) alcohol concentrations] on pregnancy estradiol levels and risk of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in female offspring. Somewhat surprisingly, they found that estradiol levels were significantly higher in dams in the 7% but not the 15% EDC diet condition compared to control dams. Female offspring from both alcohol groups had a significantly greater incidence of tumors than controls, and females from the 15% EDC diet condition had greater tumor multiplicity (number of palpable tumors/animal) and higher mammary gland estrogen receptor-α (ER-α) expression than control offspring. While the role of estradiol in the increased risk for mammary tumors in PAE females requires further investigation, these data suggest that PAE may increase breast cancer risk. Recent findings by Polanco et al. [97] examining susceptibility to *N*-nitroso-*N*-methylurea (NMU)-induced tumors in PAE offspring support and extend these findings. Consistent with Hilakivi-Clarke et al. [75], these investigators found higher tumor multiplicity and decreased tumor latency in PAE females (dams consumed liquid ethanol diet, 35% EDC). In addition, PAE female offspring had more ER-α negative tumors and higher circulating estradiol and insulin-like growth factor (IGF)-1 levels compared to control females. As well, IGF-binding protein-5 (IGFBP5) mRNA and protein were decreased in tumors of PAE compared to control females. These findings suggest a more malignant tumor phenotype in PAE compared to control females.

Together, these data demonstrating a relationship between PAE and carcinogenesis have important implication for understanding health risks of individuals prenatally exposed to alcohol. The mechanisms underlying the increased cancer risk remain to be determined. Estradiol and components of the IGF system may be involved. In addition, compromised immune status of fetal alcohol-exposed individuals may play a role [98]. A depressed immune system may impair the body's ability to destroy cancer cells. Alternatively, defective neuroimmune feedback mechanisms may fail to control the immune response to challenge, leading to uncontrolled lymphocyte proliferation and increased risk for lymphomas and other hematopoietic cancers. Fetal alcohol-induced alterations in hormone levels or in neuroendocrine–neuroimmune interactions could also play a role in tumorigenesis. Furthermore, it may be that alcohol itself does not act as a carcinogen but rather acts as a cocarcinogen with a variety of chemical or hormonal agents and that susceptibility to cancer is increased in the context of a depressed immune system. Future work in this area is important for elucidating mechanisms underlying the increased risk for cancers in individuals prenatally exposed to alcohol.

10.7 Effects of PAE on the Pulmonary Immune System

Recent data suggest that maternal alcohol use increases the risk of extreme premature delivery by 35-fold, with many of these infants born with underdeveloped lungs [99]. Extensive investigation into the functioning of the vulnerable pulmonary

system following *in utero* alcohol, particularly in the context of prematurity, has been conducted. The respiratory tract, being the conduit for vital oxygen to enter the lungs, is constantly challenged with foreign antigens, which are drawn into the system along with oxygen. As a result, proper functioning of the respiratory system requires both pro- and anti-inflammatory responses, both of which must be tightly controlled in order for the primary function of the respiratory system to be maintained.

Given the high rate of premature births in alcohol-exposed infants, it is not surprising that the incidence of sepsis and infection is higher. However, even when one controls for gestational age, alcohol-exposed newborns remain at a higher risk for infection compared to unexposed newborns [20, 23]. Recently there has been extensive investigation into the immune function of the developing lung, with the hypothesis that *in utero* alcohol exposure may alter the immune status of the lung, potentially resulting in an increased risk of early-life infection. Late gestational alcohol exposure has been shown to impact the immune status of the fetal ovine lung including decreasing surfactant levels and altering extracellular matrix composition, which may confer an increased risk of infection after birth [100]. Of note, at 9 weeks of age, many of the deficits associated with *in utero* alcohol exposure in the ovine lung appear to have normalized, suggesting that the lung may be capable of a certain degree of repair [101]. Yet the finding of increased infection risk in early life still warrants further investigation, as early life has been shown to be a critical period during which infection may permanently impact adult immune responses (see Sect. 10.14).

Investigation into the mechanism(s) underlying the altered pulmonary immune competence with *in utero* alcohol exposure has focused on the alveolar macrophage. The alveolar macrophage is the resident inflammatory cell of the lung. Given its connection to the outside world, the lung acts as a portal of entry for infection. Alveolar macrophages provide the first line of defense against foreign and infectious particles in the lung, by initiating and regulating the inflammatory cascade and participating in phagocytosis of infectious particles. Timely clearance of infectious agents by alveolar macrophages is important for decreasing the risk of serious infection. Alcohol-induced disruption of macrophage activity or function could underlie at least some of the immune deficits observed following *in utero* alcohol exposure. Of note, newborns are particularly vulnerable to infection, as neonatal alveolar macrophage numbers are depressed at birth and the existing alveolar macrophages show decreased phagocytic abilities and deficient chemotaxis [102]. As a result, any alcohol-associated impairments in alveolar macrophage function have the potential to significantly impact health during the vulnerable early postnatal period. Indeed, alcohol exposure is known to have a number of adverse effects on macrophage function, which include decreases in both the percentage of cells that phagocytose bacteria and total phagocytosis, increases in expression of early apoptotic markers, and increases in the levels of the fatty acid oxidation product, malonyldialdehyde (MDA), which is indicative of chronic oxidative stress [100, 103, 104]. Overall, these data suggest that *in utero* alcohol exposure results in alveolar macrophage dysfunction, limiting their ability to survive and adequately clear an infection, which would pose a significant health risk to the newborn.

Ping and colleagues [104] propose that decreased availability of the antioxidant glutathione may underlie, at least in part, the increase in oxidative stress in the newborn lung and that this may contribute to alveolar macrophage dysfunction. Glutathione is an essential antioxidant in the lung and is required for optimal functioning of alveolar macrophages [105]. *In utero* alcohol exposure is known to alter glutathione levels in other tissues, including the fetal brain and liver, resulting in increased oxidative stress [106, 107]. It is possible that glutathione levels may also be low in the lung of alcohol-exposed neonates, at a time when glutathione levels are expected to be high [108], and that this may underlie some of the alcohol-associated immune deficits in the lung. This was explored by Ping and colleagues [104] through administration of *S*-adenosyl-methionine (SAM), a key methyl donor in the methionine–homocysteine cycle, to the drinking water of alcohol-exposed and control animals, as SAM has been shown to restore glutathione levels following alcohol exposure [109]. SAM administration was found to significantly ameliorate many of the alcohol-induced deficits including increasing the level of alveolar macrophage phagocytosis per cell, decreasing expression of the early marker of apoptosis, and decreasing MDA, indicative of decreased oxidative stress, all to control levels [104]. The mechanism(s) underlying these SAM-mediated improvements remains to be determined. However, evidence suggests that the methionine–homocysteine cycle is disrupted with PAE and supplementation may be on approach for reversing some of the alcohol-induced deficits (see Sect. 10.16.1 for further discussion). In addition, antioxidant administration (e.g., glutathione) has been shown to restore growth, decrease neuronal apoptosis, and improve hepatic function [109–111] in animal models of PAE.

In a unique parallel to the clinical studies, which found a higher incidence of infection and sepsis in newborns exposed to alcohol *in utero* [20, 23], Gauthier and colleagues [112] examined the response to immune challenge using an *in vivo* model of group B *Streptococcus* pneumonia in a guinea pig model. *In utero* alcohol exposure was found to increase the amount of group B *Streptococcus* detected in the lung, increase systemic sepsis (increased *group B Streptococcus* levels in the blood), and impair phagocytosis of group B *Streptococcus.* Overall, and in support of the clinical findings, this suggests that *in utero* alcohol exposure significantly impairs the lung's ability to defend against bacterial infection in the newborn. Moreover, when SAM is administered to the alcohol-consuming dam, the newborn's response to immune challenge is greatly improved, lending further support to the hypothesis that alcohol exposure disrupts key components of the methionine–homocysteine cycle (see Sect. 10.16.1). Interestingly, Gauthier and colleagues [112] also showed that inhaled glutathione therapy, administered 6 h prior to group B *Streptococcus* challenge, significantly improved health outcome in the pup. Glutathione levels have been supplemented in a similar manner in premature human infants, as low glutathione levels are associated with later chronic lung disease [113]. These results point toward important potential clinical implications. Maternal supplementation with glutathione may be one eventual application, although this would require early recognition of alcohol consumption during pregnancy, which is not generally feasible. More hope, however, stems from the potential for administration of inhaled

glutathione in preterm infants as well as in infants with confirmed alcohol exposure to potentially reduce the incidence of lung infection and adverse health outcomes in these newborns.

10.8 Paternal Alcohol Exposure and Offspring Immune Function

A relatively understudied area is the effects of paternal alcohol exposure on offspring immune function. Reduced birth weight and length, reduced litter size, and increased malformations have been reported in offspring sired by alcohol-consuming males [3, 114, 115], although fetal vulnerability to depressed body weight was shown to have a maternal genetic contribution as well [116]. In addition, data suggest that paternal alcohol exposure, either in combination with maternal alcohol exposure [117] or alone [118], may have adverse effects on offspring function, including immune function. It was shown, for example, that offspring sired by alcohol-consuming males (17.5–35% EDC) exhibit decreased locomotor activity, an effect opposite to that seen with maternal alcohol exposure; show poorer adaptation to stress as measured in the forced swim task; and show increased susceptibility to infection [119]. Utilizing *Pseudomonas aeruginosa*, a Gram negative opportunistic bacterium, and applying it topically to corneas that had been scarified, an increased severity of ocular infection and an increased incidence of perforated corneas were observed in both mice and rats in adulthood [117, 118]. Interestingly, in mice (liquid ethanol diets, 5–25% EDC), the increased susceptibility to infection occurred despite the presence of high titers of antibody specific to pseudomonas [118]. Further research is warranted on the role of paternal alcohol consumption in adverse developmental outcomes of their offspring.

10.9 Sexual Dimorphism in the Immune Response

Sex differences in responsiveness to immune challenges provide an additional level of complexity in understanding the data regarding the adverse effects of PAE. Sexual dimorphism of the immune system is well known [120, 121]. Both humoral and cell-mediated immune responses are more active in adult females than in males [120, 122]. For example, the thymus is larger in female than in male mice, and castration of young males leads to feminization (i.e., increased weights) of the immune organs. In response to immunization, women develop higher antibody titers than males, and they show a higher rate of transplant rejection. Females also represent the majority of patients affected with autoimmune disorders, ranging from 65 to 75% of patients with rheumatoid arthritis to 85% of patients with Hashimoto's thyroiditis and Grave's disease [121]. In humans, circulating levels of IgM are significantly elevated in girls compared to boys as early as 6 years of age, and juvenile rheumatoid

arthritis can appear before 5 years of age. Gonadal hormones clearly play an important role in immune sexual dimorphism both in adulthood and during development. It appears that both the type and concentration of sex hormones within the microenvironment play a key role in lymphocyte maturation [120]. Both the immune organs and lymphocytes have receptors for the sex steroid hormones, linking the endocrine and immune systems [120, 121]. In addition, data suggest that other hormone systems and complex hormonal interactions can affect developing lymphocytes and regulate adult effector cells. Estrogen appears to be particularly important in the development of sexual dimorphism in the immune system, both through direct effects on immune cells and through modulation of other hormone systems, including adrenal glucocorticoids, thyroid hormones, growth hormone, and prolactin. These hormonal systems interact with each other and with the immune system to influence sexually dimorphic immune responses. Thymic hormones and cytokines generated by activated lymphocytes may also play a role in sexually dimorphic immune responses [120].

Sexual dimorphism related to fetal alcohol-associated alterations in behavior [123, 124], HPA function [124–126], neuroimmune interactions [127, 128], and susceptibility to inflammatory disorders [129] have been characterized (see further discussion below). Despite recognition that these sex differences exist, a large proportion of studies using rodent and nonhuman primate models of FASD are conducted only in males or with both sexes but too small a sample size to achieve the statistical power required to detect sexual dimorphism. Lending further support to the importance of considering sex differences are findings of sexual dimorphisms in the immune responses following PAE [49, 127, 128, 130, 131], many of which may also be impacted by fetal alcohol-induced, sex-specific HPA differences.

10.10 Mechanisms Underlying Alcohol Effects on the Developing Immune System

10.10.1 Direct Effects of Alcohol

Alcohol has a number of direct effects on the fetal immune system. Alcohol can directly disrupt the development of the fetal thymus. The thymus has a highly specified microenvironment provided by epithelial and mesenchymal cells. This environment is critical in attracting immature lymphoid precursors and enabling them to be selected and differentiated into mature T cells. Alcohol exposure during the period of thymus development inhibits the ontogeny of the thymic epithelium and disrupts the microenvironment necessary for T cell maturation, which in turn leads to impaired T cell-mediated immunity [13]. These data are interesting in light of the shared clinical characteristics of FAS and DiGeorge syndrome [18]. The latter is a congenital immune deficiency syndrome involving mainly T cells and caused by an abnormality in the development of several components of the thymus [132].

10.10.2 Indirect Effects of Alcohol

Alcohol can also alter immune function through numerous indirect effects.

10.10.2.1 Alcohol Effects on HPA Mediators of Thymic Function

Selective effects of alcohol on thymic corticotropin-releasing hormone (CRH) and pro-opiomelanocortin (POMC) gene expression in male fetuses have been reported. Significant increases in thymic CRH and decreases in thymic POMC gene expression were observed on G19 [133]. These changes appear to be unrelated to corticosterone (CORT) concentrations in the fetus or pregnant female but are possibly induced by the fetal testosterone surge. Similarly, the development of glucocorticoid receptor (GR) sites on thymocytes of PAE animals during the first 2 months of life was shown to differ from that in control animals [40]. This indicates a role for the glucocorticoid hormones and thus a possible role for prenatal or early-life stress in the differential thymic development described in PAE compared to control animals.

10.10.2.2 Altered IL-2/IL-2 Receptor Interactions

Altered IL-2/IL-2 receptor interactions may play a role in the development of altered immune function in PAE animals [49, 134, 135] (these studies utilized liquid diets, 35–36% EDC). For example, although PAE animals have normal levels of IL-2 production, IL-2 receptor expression and distribution, calcium influx into T cells, and binding of IL-2 to its receptor, they show a markedly reduced proliferative response to mitogens. In addition, the internalization and/or utilization of IL-2 by lymphoblasts is reduced, and the half-time for dissociation of IL-2 from its receptor is increased in T cells from PAE animals [135]. Therefore, impaired intracellular signaling events, mediated by altered IL-2/IL-2R interactions, may underlie, at least partly, the immune deficits observed in PAE animals.

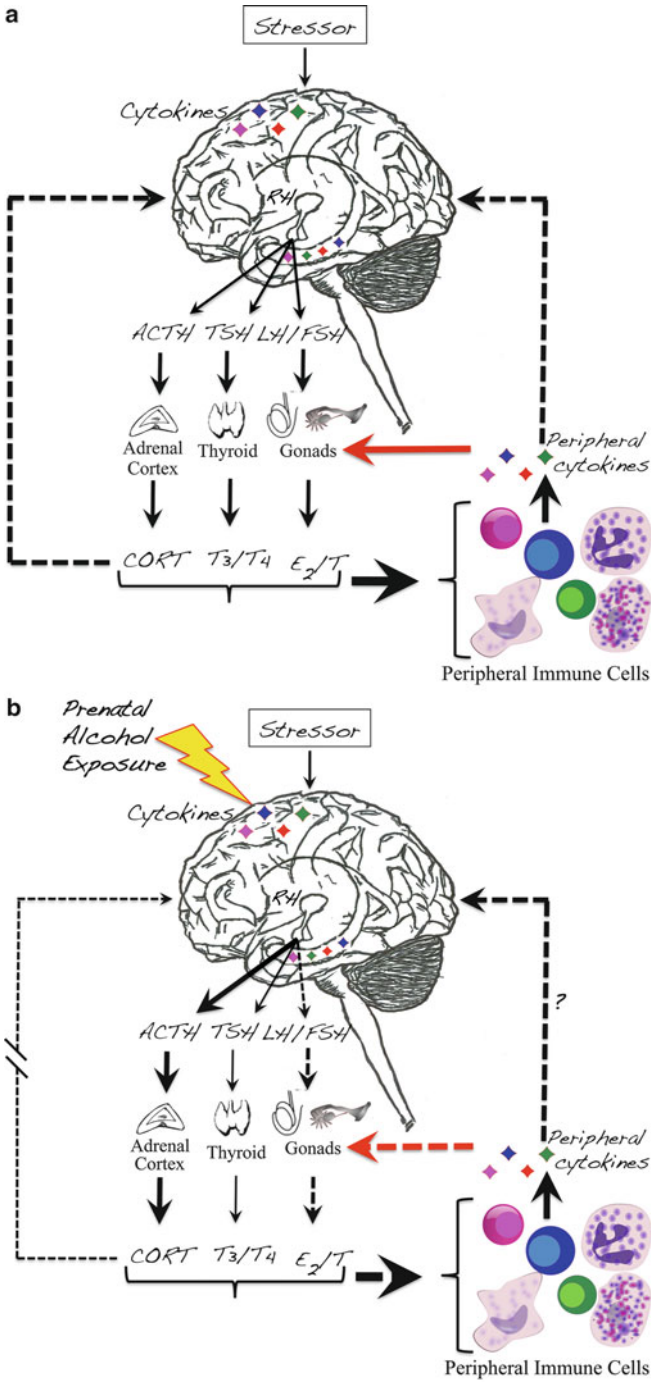
10.10.2.3 Altered Neurotransmitter Function

Altered neurotransmitter regulation of immune function is another factor that could play a role in altered immune function of PAE animals. Prenatal exposure to alcohol (liquid diets, 36% EDC) was found to decrease concentrations of norepinephrine (NE) and β-adrenoreceptors in the lymphoid organs and diminish synaptic transmission in the spleen and thymus, but not the heart [55]. Altered noradrenergic synaptic transmission, including a higher rate of NE turnover leading to reduced NE concentration and reduced β-adrenoceptor density, could affect immune capacity, in terms of NE-mediated IL-2 secretion and cytotoxic T cell responses.

10.10.2.4 Altered Neuroendocrine–Neuroimmune Networks

Prenatal exposure to alcohol has been shown to have adverse effects on neuroendocrine function, which could in turn alter the bidirectional communication between neuroendocrine and neuroimmune networks. The nervous system, endocrine system, and immune system exist within a complex regulatory network of connections through nerve pathways, hormonal cascades, and cellular interactions [136–138] (Fig. 10.2). The CNS can regulate both the endocrine and immune systems directly, through autonomic innervation of lymphoid organs and tissues and endocrine glands, and indirectly, through release of neurotransmitters. In turn, the nervous system can "read" information from both the endocrine and immune systems. Cytokines produced by immune cells and hormones produced by endocrine glands may be similar or even identical in structure and function. Cytokines not only play an important role in regulating immune responses but also feed back to the CNS to affect neuronal function. Cytokines can also influence or stimulate hormone release from the hypothalamus and pituitary and endocrine glands and in themselves may have neuroendocrine effects. Similarly, hormones produced by the endocrine glands and pituitary not only feed back to the CNS to influence neural and endocrine function but also have immunoregulatory functions. Lymphocytes express receptors for hormones and neurotransmitters, including CRH, ACTH, glucocorticoids, norepinephrine, and epinephrine. The glucocorticoid hormones can exert profound influences on T cell function through their interaction with GRs on T cells, which modulate trafficking, homing, proliferation, activation, and apoptosis [139]. Of note, other endocrine systems besides the HPA axis are involved in mutual regulation of immune function. Thyroid hormones and estradiol are known to have activational effects on the immune response, whereas progesterone, testosterone, and dehydroepiandrosterone (DHEA) reciprocally inhibit immune responses. In addition, the autonomic nervous system can exert regulatory actions on the immune system. Noradrenergic neurotransmitters can target most immune cells including thymocytes, T lymphocytes, macrophages, and plasma cells, resulting in selective suppression of Th1-mediated inflammation and cellular immunity, which, in turn, will favor humoral immunity as well as protecting the organism from the adverse effects of proinflammatory cytokines. Synergistic effects of catecholamines and glucocorticoids have also been described [136]. Indeed, the HPA response is

Fig. 10.2 Bidirectional communication between the nervous, endocrine, and immune systems. (**a**) Neuroendocrine–neuroimmune circuitry under normal conditions. (**b**) Neuroendocrine–neuroimmune circuitry following prenatal alcohol exposure. *RH* releasing hormones act on cells of the anterior pituitary to stimulate synthesis and secretion of appropriate tropic hormones; corticotropin-releasing hormone (*CRH*) stimulates release of adrenocorticotropic hormone (*ACTH*); thyrotropin-releasing hormone (*TRH*) stimulates release of thyroid-stimulating hormone (*TSH*); gonadotropin-releasing hormones (*GnRH*) stimulate release of luteinizing hormone (*LH*) and follicle-stimulating hormone (*FSH*). The pituitary tropic hormones then stimulate peripheral endocrine glands to release their hormones: *CORT* (corticosterone/cortisol), thyroid hormones, triiodothyronine/tetraiodothyronine (*T3*, *T4*), and gonadal hormones (estradiol, E_2, testosterone, T)


a
Stressor
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Thyroid
Gonads
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CORT T3/T4 E2/T
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Prenatal Alcohol Exposure
Stressor
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ACTH TSH LH/FSH
Adrenal Cortex
Thyroid
Gonads
Peripheral cytokines
CORT T3/T4 E2/T
Peripheral Immune Cells

coordinated with that of the autonomic nervous system to enable the organism to respond to stress. The autonomic response is extremely rapid and provides an immediate response to a stressor through initiation of the sympathetic "fight or flight" response (increased heart rate and blood pressure, pupil dilation, bronchodilation, glucose release). The HPA response is slower, and the secretion of the glucocorticoid hormones initiates numerous metabolic and behavioral effects that mediate effective coping with a stressor in the longer term. The HPA and autonomic systems appear to be regulated by similar neurotransmitters (e.g., acetylcholine, serotonin, norepinephrine, GABA). In addition, there is reciprocal stimulation of HPA and autonomic activity by CRH and norepinephrine and reciprocal actions of the glucocorticoids and catecholamines. The glucocorticoids are thought to feed back to restrain activity of both systems. Further, the activity and sensitivity of both systems are modulated by stress and circadian influences [140].

Reciprocal expression of receptors for hormones, neurotransmitters, and neuropeptides and shared hormonal/peptide ligands or products by immune, endocrine, and neural cells underlie the bidirectional communication that allows these systems to "speak a common biochemical language" [137] and thus influence each other to maintain homeostasis. It has been suggested that the immune system acts as a "peripheral receptor organ" able to transmit information to the brain about responses to external or internal antigenic stimuli [136]. Similarly, Blalock and Smith [137] suggest that through the sharing of ligands and receptors, the immune system can serve as a "sixth sense" to detect things the body cannot otherwise hear, see, smell, taste, or touch and to signal and mobilize the body to respond to pathogens, tumors, allergens, and other immune challenges.

Fig 10.2 illustrates the complex bidirectional communication between the nervous system, the endocrine system and the immune system. As shown, reciprocal expression of receptors for hormones, neurotransmitters, and neuropeptides and shared hormonal/peptide ligands or products by immune, endocrine, and neural cells underlie the bidirectional communication that occurs.

Figure 2a shows neuroendocrine–neuroimmune circuitry under normal conditions.

- The CNS can regulate the endocrine and immune systems directly, through autonomic innervation of lymphoid organs and tissues as well as endocrine glands, and indirectly, through release of neurotransmitters (not shown).
- In turn, the nervous system can "read" information from both the endocrine and immune systems.
  - Peripheral cytokines produced by immune cells not only play an important role in regulating immune responses but also provide feedback to the CNS to affect neuronal function.
  - Furthermore, cytokines can influence or stimulate hormone release from the hypothalamus and pituitary and endocrine glands and in themselves may have neuroendocrine effects.
  - Similarly, hormones produced by the endocrine glands (glucocorticoids, thyroid hormones, and the sex hormones) and pituitary (ACTH, TSH, LH, FSH)

not only provide feedback to the CNS to influence neural and endocrine function but also have immunoregulatory functions. Indeed, hormones produced by endocrine glands and cytokines produced by immune cells may be similar or even identical in structure and function.

- The sex hormones as well as many other hormones play a role in the sexually dimorphic nature of immune system function.
- There is evidence for local production of cytokines by microglia in the CNS in response to challenge, including stress.

Together, cytokine expression and activation of receptors in key brain areas such as the cortex, hippocampus, and hypothalamus have the potential to modulate the secretion of releasing hormones (RH) of the endocrine system and thus affect endocrine and immune responses to stress. Finally, mutual regulatory interactions between the autonomic nervous system and the HPA axis (not shown) play an important role in the organism's ability to respond to stressors, and at the same time, the autonomic nervous system can exert important regulatory actions on the immune system (not shown).

Figure 2b shows neuroendocrine–neuroimmune circuitry following prenatal alcohol exposure.

PAE impacts neuroendocrine and neuroimmune function and the interactions between these networks.

- Alterations in hormonal activity under basal conditions and in response to stressors:
  - Increased HPA activity, decreased thyroid function, and decreased levels of gonadal hormones. Altered hormone levels will differentially alter immune function.
  - However, PAE may increase facilitatory effects of estradiol (E_2) and decrease inhibitory effects of testosterone (T) on HPA activity (dotted arrow), further altering immune function.
  - Impaired CORT negative feedback (broken arrow) will further increase HPA activity and responsiveness to stressors.
- Alterations in peripheral cytokine production (in response to challenge) may alter how cytokine signals are transmitted (e.g., elaborated) to the brain (? in figure).
- Alterations in cytokine levels will alter feedback to HPA, thyroid, and gonadal systems (Red arrow).

PAE can link into this neuroimmune–neuro endocrine circuit to exert its teratogenic effects in numerous ways. Alcohol may (1) have direct effects on the ontogeny of the immune and endocrine systems and on CNS development and function; (2) act indirectly through effects on neuroendocrine function; and (3) disrupt the intimate bidirectional link between the neuroendocrine and neuro immune systems.

While several endocrine systems, including the adrenal, thyroid, and gonadal systems, interact with and modulate immune system function, the immune–HPA

axis circuit is of particular interest for our work. The HPA axis mediates the neuroendocrine response to stressors, both systemic stressors that threaten homeostasis and/or survival and perceived threats or psychogenic stressors [141]. Inputs to the HPA axis provided by stressors and the endogenous circadian rhythm [142] act through central neural pathways to the paraventricular nuclei (PVN) of the hypothalamus, where CRH is synthesized. CRH (potentiated by arginine vasopressin [AVP]) [143, 144] stimulates the release of adrenocorticotropin (ACTH) from the anterior pituitary, which in turn stimulates synthesis and secretion of glucocorticoids from the adrenal cortex. The glucocorticoids, cortisol in humans and corticosterone in most rodents, have numerous metabolic and physiological effects and provide negative feedback to inhibit HPA activity at the level of the pituitary, PVN, hippocampus, prefrontal cortex, and other brain areas [142–144]. Glucocorticoids, acting on their receptors (mineralocorticoid receptors, MR; glucocorticoid receptors, GR), initiate metabolic and physiological responses that facilitate response to and coping with the stressor and, ultimately, dampen stress-activated defense reactions, including immune responses, to prevent them from overshooting and themselves causing harm [145, 146]. In the short term, the metabolic and physiological changes induced by the glucocorticoids promote survival (increased gluconeogenesis and blood pressure, suppressed immune and reproductive function). However, prolonged exposure to glucocorticoids can result in metabolic, cognitive, and immune dysfunction [147]. Thus, it is important that the HPA axis be tightly controlled through efficient feedback and efficient termination of the stress response; the ability to turn off the stress response is as important as the ability to respond initially [145]. The HPA axis acts in conjunction with the locus coeruleus–noradrenergic sympathetic adrenal medullary system (hereafter referred to as the LC-NE system) to maintain homeostasis. The LC-NE system is involved in the "fight or flight" response and enables the organism to react rapidly through the secretion of NE from sympathetic nerve terminals and epinephrine from the adrenal medulla. In contrast, through its various physiological and metabolic effects, the HPA axis acts over a longer time frame and helps orchestrate the response and adaptation of the body to the stressor.

10.11 Alcohol Consumption Alters HPA Activity of the Pregnant Female

Data from rodent models indicate that gestational alcohol consumption increases adrenal weights, basal corticosterone concentrations, and the corticosterone stress response in the pregnant dam [148]. This occurs as early as gestation day 11, persists throughout gestation, may increase as gestation progresses, and occurs even with low–moderate concentrations of alcohol (BALs 42–150 mg/dl) in the diet [148, 149]. The stimulatory effects of alcohol on basal hormone concentrations and the corticoid response to stressors can also extend through parturition, even when alcohol administration is discontinued prior to parturition [150]. Thus, regular

consumption of high doses of alcohol during pregnancy not only raises the set point of HPA function in the pregnant female by increasing both basal and stress concentrations of CORT, but it also results in HPA hyperresponsiveness to stressors.

Because the pregnant female and fetus constitute an interrelated functional unit, alcohol-induced alterations in HPA activity in the pregnant female have implications for fetal HPA development. Corticosterone crosses the placenta, at least to some extent [151], resulting in suppression of endogenous fetal HPA activity. At the same time, alcohol can cross the placenta and directly activate the fetal HPA axis.

10.12 Effects of Maternal Alcohol Consumption on Offspring HPA Activity

The complex interaction of the opposing direct and indirect effects of alcohol is apparent in studies using rat models (liquid diets, ~36% EDC), with offspring showing differences from controls in HPA activity over the course of development. Fetuses exposed to alcohol have lower corticosterone concentrations than control fetuses on gestation day 19 [133] but elevated plasma corticosterone and β-endorphin levels at birth [150, 152–154]. Interestingly, throughout the preweaning period, PAE offspring exhibit blunted HPA and β-endorphin responses to a wide range of stressors including ether, novelty, saline injection, and cold stress [150, 154, 155]. In addition, PAE appears to alter the development of expression of CRH and POMC mRNAs in a sexually dimorphic manner, delaying and exaggerating the rise in CRH expression in female (but not male) pups and suppressing POMC mRNA concentrations in male (but not female) pups throughout the preweaning period [156]. Sexually dimorphic effects of PAE on these two important glucocorticoid-regulated genes may contribute to both the immediate and the long-term effects of prenatal alcohol on stress responsiveness of the offspring.

Interestingly, the blunted hormone responsiveness of PAE pups during preweaning life is a transient phenomenon. It appears that maternal alcohol consumption, in the long term, programs the fetal HPA axis such that HPA tone is increased throughout life. From weaning age on, although basal hormone levels are typically normal, PAE offspring are hyperresponsive to stressors and to drugs such as alcohol and morphine, showing increased HPA activation and/or delayed or deficient recovery following stress [157–160]. In addition, central HPA regulation appears to be altered under both basal and stress conditions [31, 124, 161]. Findings in both nonhuman primates and human infants are consistent with those in the rodent model. In rhesus monkeys, prenatal exposure to moderate amounts of alcohol induced higher plasma ACTH and marginally higher plasma cortisol responses to the stress of maternal separation [162]. Similarly, Jacobson and colleagues [163] found that heavy drinking during pregnancy and at conception is associated with higher post-stress (blood draw) cortisol concentrations in 13-month-old infants. Basal cortisol was also shown to be higher in 2-month-old infants exposed *in utero* to alcohol or cigarettes [164]. Moreover, Haley and colleagues [165] found that alcohol consumption during

the early postconception period was related to increases in cortisol reactivity, elevated heart rate, and negative affect in infants in response to a modified "still-face" procedure, with differential effects in boys and girls. It was suggested that the effects of PAE on infant stress systems could underlie problems in cognitive and social–emotional functioning that are common among individuals exposed prenatally to alcohol.

10.12.1 Sexual Dimorphism of the HPA Response in PAE Offspring

Sexually dimorphic effects of PAE on HPA activity may extend into adulthood. Beyond the normal sexual dimorphism of HPA activity, differences in the response of PAE male and female offspring compared to their control counterparts are often observed and may vary depending on the nature of the stressor, and the time course and hormonal endpoint measured [130, 166–168]. For example, PAE males and females (liquid diets, 35–36% EDC) both exhibit increased hormone responses to stressors ranging from repeated restraint to footshock to immune challenges [130, 157, 159, 160, 169], stress-induced increases in expression of mRNAs for immediate early genes and CRH [160], and deficits in habituation to repeated restraint [170]. In contrast, in response to prolonged restraint or cold stress, HPA hyperactivity may be seen primarily in PAE males [167, 169], whereas in response to acute restraint or acute alcohol or morphine challenge, greater corticosterone and ACTH increases occur primarily in PAE females [157, 166, 171].

10.12.2 Role of Maternal Corticosterone in Offspring HPA Responsiveness

Whether the increased plasma corticosterone levels induced in the maternal female by alcohol consumption during pregnancy play a role in mediating HPA hyperresponsiveness in PAE offspring is as yet unresolved. Interestingly, maternal adrenalectomy (ADX) was found to dramatically increase the corticosterone response to restraint stress in offspring of pair-fed mothers but to have no effect on the corticosterone response to stress in PAE offspring [172]. Similarly, corticosterone treatment of ADX dams did not mimic the effect of PAE on HPA activity of offspring [173]. Based on these data, one could conclude that the increased corticosterone concentrations in pregnant females are not the primary mediator of offspring hyperresponsiveness. In contrast, ADX of the pregnant female was shown to reverse the effects of PAE on pituitary POMC mRNA concentrations in PAE offspring [174]. In addition, a compensatory increase in fetal CORT that was seen following maternal ADX of control females was attenuated in fetuses of ethanol-consuming dams, suggesting a direct effect of alcohol on fetal HPA activity [175]. Tritt and colleagues

reported that removal of the adrenal cortex, but not the medulla, in the pregnant female prevents the growth-retarding effects of PAE and may reverse the delay in postpartum weight gain in PAE offspring [176]. Moreover, whereas the febrile response to IL-1β is normally attenuated in PAE offspring, maternal ADX completely eliminated the effect of PAE on this response [177]. These findings suggest that at least some of the adverse effects of alcohol may be mediated fully or partially through the adrenal gland and, possibly, through maternal–fetal HPA interactions. Further studies are needed to resolve the role of corticosteroids derived from the pregnant female on the altered stress responsiveness of their offspring. It is likely, however, that the adverse consequences of alcohol consumption during pregnancy on fetal development and programming of fetal HPA activity arise from a complex interaction between direct and indirect effects of alcohol on both the mother and the fetus.

10.13 PAE Alters the Effects of Stress on Immune Function

Chronic or repeated stress in adulthood provides a challenge to the immune system that differentially affects PAE and control animals. Data suggest that exposure to stressors may unmask specific deficits in the immune system that are not observed under nonstressed conditions. Furthermore, consistent with the findings that PAE may alter HPA responses to stress in a sexually dimorphic manner, sexually dimorphic effects of PAE on immune function may also be observed following stress.

In a rat model (liquid diet, 36% EDC), exposure to chronic intermittent stressors selectively downregulated the numbers of thymic and peripheral blood CD43+ and CD4+ T cells and marginally decreased the number of peripheral blood antigen-presenting cells, whereas CD43 antigen expression on peripheral blood T cells was selectively upregulated in PAE males but not females [178]. The possible role for estrogen as an immunoprotective hormone and testosterone as an immunosuppressive hormone in these sexually dimorphic immune responses remains to be determined.

One of the first demonstrations of fetal alcohol effects on immune function of females is the data from Halasz and colleagues [168] who found that the challenge of chronic alcohol exposure in adulthood selectively increased Con A-induced lymphocyte proliferation in PAE females, but not males. Consistent with these data, work from our laboratory [46] reported an interaction between prenatal alcohol and chronic cold stress. After 1 day of cold stress, PAE females but not males exhibited increased lymphocyte proliferation in response to pokeweed mitogen (a T cell-dependent B cell mitogen) and Con A challenge. In contrast, PAE males exposed to 1 or 3 days of cold stress had increased basal CORT concentrations compared to PAE males not exposed to cold. Altered interactions between T and B cells may underlie the alcohol-induced alterations in immune responsiveness observed in response to stressors.

10.14 PAE, Cytokines, and the HPA Axis

Cytokines secreted by immune cells, including IL-1, IL-2, IL-4, and IL-6, influence the function of hypothalamic neurosecretory and thermoregulatory neurons and pituitary cells [179–182], resulting in activation of the HPA axis and inducing "sickness behavior" [183, 184]. IL-1, IL-6, and TNF-α are also produced in the hypothalamus by microglia and macrophages [185, 186] and, thus, can directly influence neuroendocrine function. For example, IL-1 stimulates the release of CRH and AVP from the hypothalamus [187, 188], and IL-1, IL-6, and TNF-α stimulate ACTH secretion from the anterior pituitary [189–191]. During prolonged stress, cytokines exert effects at the level of the pituitary and adrenal glands [136]. In turn, the glucocorticoids play a major role in the stress-induced suppression of immune/inflammatory reactions.

PAE has been shown to alter neuroendocrine and behavioral responses to cytokines. For example, the LPS-induced febrile response was shown to be blunted in PAE males [192, 193]. It was suggested that a decreased hypothalamic IL-1β response to LPS administration, possibly due to an impaired release of endogenous pyrogens, could underlie the differential responsiveness observed [194–196]. Interestingly, both ADX and sham surgery in the pregnant female abrogated the effect of alcohol on the febrile response of female offspring to IL-1β, but only ADX had an effect on male offspring. This would suggest that maternal adrenal mediators play an important role in the blunted febrile response of PAE males and that non-adrenal mediators participate in modulation of thermoregulatory systems in PAE females [177, 193].

In parallel with the blunted hormonal responses to stressors observed in PAE animals during the preweaning period, preweaning PAE offspring exhibit reduced ACTH, β-EP, and TNF-α responses to the immune challenge of IL-1β and LPS. This reduction was found to persist into adolescence in PAE males, but not in females [197, 198]. An altered ability of IL-1 to stimulate secretion of ACTH and other peptides may underlie this reduced responsiveness [197]. Interestingly, ovariectomy prior to puberty eliminated the difference in the ACTH response between PAE and control females [130], suggesting that alcohol and female sex hormones may regulate HPA activity through a common pathway.

Following weaning and into adulthood, in parallel with the observed HPA hyperresponsiveness to stressors, hormonal responses to immune signals are also increased in PAE animals. Alcohol exposure *in utero* appears to induce a proinflammatory profile bias, suggested by the finding of greater ACTH and/or corticosterone responses to IL-1β and LPS in PAE compared to control offspring [130, 169, 199]. As well, embryos exposed *in vitro* to alcohol had greater tissue levels of TNF-α and IL-6 than control embryos [200]. PAE males also exhibited increased plasma concentrations of proinflammatory cytokines (IL-1β, TNF-α, IL-6) to LPS challenge following repeated restraint stress [31]. Importantly, however, corticosterone responses to LPS were comparable among groups. These data support and extend previous studies suggesting that although PAE animals may not differ in cytokine

responses under basal or nonstressed conditions, they may be more vulnerable to the adverse effects of stress on immune function [31]. Furthermore, these data have important implications for understanding differential vulnerability to inflammatory disorders such as arthritis in PAE offspring, as discussed below (Sect. 10.15).

10.14.1 Effects of Alcohol Intake During Pregnancy on the Maternal Immune System

In order to understand fully how maternal alcohol intake can alter maternal and fetal cytokine profiles and immune status, it is helpful to understand the effects of adult alcohol intake in general. Alcohol use has been shown to impact the immune defense system, altering how the body responds to an infection (reviewed in [201]). These alterations can affect innate and adaptive immune responses, which in turn gives rise to an increased incidence of infections as well as alterations in antigen presentation postinfection [202]. Of note, the effects of alcohol on immune function appear to depend on the frequency of alcohol consumption. Acute alcohol administration has been shown to inhibit proinflammatory cell activation, an essential process in innate immune system activation, whereas chronic alcohol consumption is associated with increased proinflammatory cytokine activation (reviewed in [201]).

Chronic alcohol exposure also affects the maternal immune system, increasing the levels of proinflammatory cytokines including TNF-α, IL-1α, IL-1β, IL-6, IL-8, IL-2, and MCP-1 and giving rise to alcohol-induced physiological alterations and tissue damage (reviewed in [201]). To date, there is an absence of studies examining how an increased proinflammatory cytokine profile in the alcohol-consuming mother will affect the offspring. However, there is a growing body of research examining the effects of prenatal and/or early postnatal infection and the resulting increase in proinflammatory cytokine levels on the developing fetus [203–205]. As both PAE and early immune challenge are associated with increased proinflammatory cytokines, which can have programming effects on the developing fetus, the early inflammation literature has significant implications for understanding the immune effects of early alcohol exposure.

10.14.2 Early-Life Exposure to Maternally Derived Cytokines

It has been proposed that early-life exposure to maternally derived cytokines programs the developing fetus, affecting brain and immune system development and giving rise to altered cognitive and immune function in adulthood (reviewed in [3]). Increased levels of proinflammatory cytokines occur following naturally occurring maternal infections and have also been simulated in experimental models through direct injection of the mother with cytokines such as IL-6 [114] or maternal immune

stimulation with the endotoxin LPS [203] or *Escherichia coli* [115]. An important question, however, remains to be answered—how the maternal cytokine signal is passed on to the fetus. The current literature is contradictory. Cytokines, being small proteins generally less than 50 kDa in size, have the potential to enter the amniotic fluid and affect the fetus [204]. However, while Gayle and colleagues [206] found that maternal inflammation results in elevated levels of IL-1β, TNF-α, and IL-6 in the amniotic fluid and the fetal brain, other studies suggest that there would be minimal transfer of IL-1β and TNF-α and only the potential for modest transfer of IL-6 between mother and fetus [207, 208]. However, a recent study involving radiolabeled IL-6 found that in rodents, this proinflammatory cytokine crosses to the fetus during mid-gestation (GD 11–13) but not late gestation (GD 17–19) [209]. Thus, it appears that cytokine passage to the fetus may depend on the cytokine in question as well as the gestational time point. Importantly, there may be additional, yet currently unknown, mechanisms in place that allow for the maternal cytokine signal to pass to the fetus. Late gestational (GD 18) maternal increases in cytokines, such as TNF-α, IL-1β, and IL-6, that are unable to cross the placenta stimulate a concomitant cytokine increase in the fetal brain [204]. As a result, it is hypothesized that there is a different mechanism in place in the fetus, resulting in local elaboration of the maternal cytokine signal [204]. While it may seem detrimental to increase cytokine levels in the fetal brain in response to maternal infection, this may be a required adaptive strategy, critical to fetal survival in the context of life-threatening infections but perhaps developmentally disadvantageous in the context of less severe infections.

10.14.2.1 Early-Life Exposure to Cytokines May Increase Vulnerability in Later Life

Regardless of the mode by which cytokines gain entry into the fetal system, many lines of evidence link early-life increases in cytokine levels with increased vulnerability to later-life infections and cytokine overproduction [205, 210, 211]. It is hypothesized that immune stimulation during sensitive periods of development may affect cytokine production, glial morphology, brain development, and neuroimmune responsiveness into adulthood (reviewed in [3]). In the case of *in utero* alcohol exposure, maternal increases in cytokines may be transmitted or elaborated on by the fetus, and alcohol may directly activate the fetal immune system resulting in increased proinflammatory cytokines during development. It is important to note that cytokines, in addition to their role in immune system activation, are also involved in normal brain development, including a role in neuronal plasticity, morphogenesis, growth, and differentiation [212, 213]. Cytokines play a role in mediating neuronal migration, synaptogenesis, synaptic pruning, and stem-cell fate during development [214, 215]. Abnormally high cytokine levels, however, may alter many of the important neuronal processes listed above, resulting in abnormal brain connectivity and ultimately increased immune system impairments and vulnerability to neurodevelopmental disorders [3, 205, 210].

10.14.2.2 Possible Role of Microglia in Neuroimmune Changes in Later Life

It is hypothesized that cytokine-induced alterations in microglia may underlie many of the neuroimmune system alterations and increased susceptibilities to cognitive disorders in adulthood (reviewed in [3, 204]). Microglia, resident macrophages of the CNS, are uniquely poised to retain immunological memory of early-life insults. Microglia arise by embryological day 14 in the rodent [216] and exist in an activated, amoeboid state, producing cytokines and contributing to neuronal development [217]. The precise role of microglia in the normal developing brain has yet to be determined; however, they likely play a role in processes such as neuronal pruning and phagocytosis of dying neurons and act as a source of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) [218–220]. Importantly, microglia are very long-lived cells [221]. Thus, early-life exposure to alcohol or immune insult may alter microglial morphology and activation, changes that may persist for long periods of time due to the long-lived nature of these immune cells amid a more ephemeral immune cell background. Importantly, while microglia arise early in development and exist in an increasingly activated state until postnatal day 6, by postnatal day 15, they transition into a quiescent, ramified state and remain this way throughout adulthood and only become activated with immune challenge [216]. The current hypothesis in the field is that early-life immune stimulation, during the developmental window in which microglia are activated, may prime these cells such that they are more easily stimulated in adulthood (reviewed in [3]). As a result, later-life immune challenge may unveil the neuroimmune system deficits, which were engrained in the system during early development. This "two-hit" hypothesis is particularly relevant in the context of PAE, where exposure to alcohol and maternally derived proinflammatory cytokines may have sensitized the fetal system, making it easier for it to be pushed out of the optimal range when further challenged.

10.15 PAE Alters the Development and Course of Adjuvant-Induced Arthritis

Recent studies in our laboratory have utilized an adjuvant-induced arthritis (AA) model to extend our understanding of the adverse effects of PAE on the functional status of the immune system [222]. AA is widely used as a model of human rheumatoid arthritis. Like rheumatoid arthritis, AA in the rodent is an inflammatory disease of the joints, especially the hind paws, shown to be mediated by CD4+ T cells. AA has been used to study disease pathogenesis, chronic pain, and stress and in the context of the involvement of the HPA axis in arthritis, neuroendocrine imbalance, and altered interactions between the neuroendocrine and neuro immune systems [223–226].

In view of the known effects of PAE on HPA and immune function and the interaction between the HPA axis and the immune system, we undertook a study to test the hypothesis that the autoimmune response and the severity of arthritis would be increased in PAE females compared to their control counterparts [222]. At approximately 55 days of age, females from prenatal alcohol, pair-fed, and *ad libitum*-fed control conditions were injected at the base of the tail with complete Freund's adjuvant. The incidence and clinical signs of arthritis, as well as immune markers and HPA hormone levels, were assessed over the course of inflammation. Our data demonstrate, for the first time, an adverse effect of PAE on the course and severity of adjuvant-induced arthritis in adulthood, indicating an important and clinically relevant long-term alteration in functional immune status. PAE females showed a delay in arthritis onset during the induction phase. However, during the resolution phase, PAE females showed a more prolonged course of disease and greater severity of inflammation compared to controls. In addition, PAE females showed a greater increase in basal ACTH levels than controls during the induction phase of arthritis. This increased HPA activation during induction could have played a role in the delayed onset of disease, either directly, by suppressing the inflammatory response, or indirectly, by suppressing the lymphocyte proliferative response to adjuvant. Consistent with this latter possibility and with previous work from our laboratory [49] and others [35, 48], we found an overall reduction in lymph node lymphocyte proliferative responses to Con A in PAE females. Interestingly, the differential elevation in basal HPA activity observed during the induction phase was not sustained over the course of testing. We saw graded corticosterone and ACTH responses at the peak of inflammation, with the greatest activation in adjuvant-injected animals that developed arthritis, lesser activation in adjuvant-injected animals that did not develop arthritis, and low levels of activity in saline-injected controls. During the resolution phase of disease, hormone levels did not differ among the three prenatal groups, and if anything, corticosterone and ACTH levels were somewhat lower in PAE compared to control animals with clinical signs of arthritis. It has been suggested [227] that low levels of glucocorticoid hormones in the context of high levels of inflammation may reflect a disconnect between the immune and endocrine systems. As noted above, we have evidence for such a disconnect in our previous work. That is, following exposure to repeated restraint stress, PAE animals had significantly increased and sustained elevations in plasma levels of IL-1β and TNF-α to LPS injection compared to controls but showed similar corticosterone responses to LPS [31]. These data suggest a possible disruption of the cytokine–HPA axis feedback circuit. It is possible that reduced HPA activity in PAE animals during the resolution phase, in the face of persisting inflammation, may be related to the prolonged course and severity of disease seen in PAE compared to control animals.

As noted above, later-life immune challenges may unveil neuroimmune system deficits, which were engrained in the system during early development. Our PAE model is a prime example of the "two-hit" hypothesis, where prenatal exposure to alcohol sensitizes the offspring neuroendocrine and neuroimmune systems, possibly through increased exposure to maternally derived proinflammatory cytokines, resulting in increased responsiveness to the later-life challenge of adjuvant injection.

10.16 PAE Reprograms Fetal HPA and Immune Function

One conceptual framework for understanding the long-term adverse effects of alcohol on neuroendocrine and neuroimmune function is that of fetal programming. Programming refers to the concept that environmental factors acting during sensitive periods of development can exert organizational effects on physiological/neurobiological systems, resulting in changes that can persist throughout life [228]. Data suggest that the HPA axis is particularly vulnerable to programming by prenatal and early-life events [228, 229]. The overall effect of early HPA programming is altered exposure to endogenous glucocorticoids throughout life [230, 231] which, in turn, can modify behavior, cognition, learning, memory, and emotion and predispose the individual to cardiovascular-, metabolic-, mental health-, and stress-related disorders and disorders of immune function, including the risk for rheumatoid arthritis [227, 232]. Although environmental factors play an important role in fetal programming, it is likely that perinatal environmental and genetic factors mutually influence each other in determining HPA activity and behavior later in life. Moreover, though the effects of programming are often long lasting, postnatal and later environmental events can modulate the effects of prenatal programming.

Fetal programming is generally thought to facilitate the organism's adaptation to the postnatal environment. However, programming can be detrimental if stimuli program systems to function outside their normal physiological range, leading to high allostatic load [233]. In this regard, because HPA dysregulation has been implicated in the pathogenesis of rheumatoid arthritis in humans and adjuvant-induced arthritis in animal models [226, 227], it has been suggested that HPA programming may be one potential mechanism through which early-life factors can predispose the individual to autoimmune diseases [200–203]. As noted, we and others have shown that alcohol exposure *in utero* programs the fetal HPA axis such that HPA tone is increased throughout life, resulting in increased HPA activation, delayed or deficient recovery [157, 160], and altered central HPA regulation [124, 161]. We postulate that imposition of the chronic inflammatory stress of adjuvant-induced arthritis on a system already sensitized by prenatal exposure to alcohol could underlie the increased autoimmune responses and the altered course and severity of disease that we observed in our model. Alcohol-induced disruptions of normal neuroendocrine–neuro immune interactions may provide an indirect route through which early-life experiences can have long-term effects on the immune system.

10.16.1 Possible Role of Epigenetic Mechanisms in Fetal Programming by Alcohol

Over the past decade, it has become apparent that epigenetic processes provide one mechanism for fetal programming of neurobiological systems [234]. Indeed, data suggest that investigation of possible epigenetic mechanisms as mediators of alcohol's

adverse effects on the fetus provides a promising approach for understanding the complex phenotypes associated with FASD and the persistence of these characteristics into adulthood [235, 236].

Epigenetics refers to stable, but potentially reversible, alterations in a cell's genetic information that result from environmental influences. Epigenetic processes cause changes in gene expression without mutations or changes in the underlying DNA structure. Such changes persist through cell divisions for the remainder of the cell's life and are potentially heritable. Epigenetic mechanisms appear to function as mediators connecting the genome to environmental stimuli and exposures, such as *in utero* alcohol exposure [236].

In the cell nucleus, DNA is found spooled around histone proteins. Local changes in this packaging system can influence gene expression. Two major types of epigenetic modifications are methylation—addition of methyl groups to DNA building blocks (cytosine nucleotides) in a regulatory region of the gene called the promoter region—and the addition of various molecules or chemical groups to the histone proteins. Both DNA methylation and histone modifications are dynamic processes. Numerous enzymes can remove and/or replace these chemical modifications, which, in turn, results in increased or decreased expression of the gene. Activity and availability of these enzymes and chemical groups are influenced by environmental factors. Recent data support the possibility that adverse effects of alcohol exposure *in utero* may be mediated, at least partly, by epigenetic mechanisms [204, 205].

10.16.1.1 Alcohol Exposure During Preconception, Preimplantation, and Gastrulation Periods of Development

Some of the strongest evidence for epigenetic programming comes from data suggesting that both *preconception* and *preimplantation* alcohol exposure, when the embryo is not yet implanted in the uterus and thus not yet connected to the maternal system, can cause adverse effects.

Paternal alcohol consumption may be one route through which preconception effects of alcohol can occur. As noted (Sect. 10.8), paternal alcohol exposure has numerous adverse effects of offspring developmental outcome, including immune function. Of relevance to the present discussion, alterations in the expression of DNA methyltransferase 1 (DNMT1), a key enzyme catalyzing DNA methylation, in the sperm of alcohol-consuming paternal rats suggest a potential mechanism mediating paternal alcohol effects [207]. Interestingly, a correlation between chronic alcohol use and altered DNA methylation in sperm DNA from human volunteers has been reported [208].

Preconception effects of maternal alcohol consumption on birth weight and growth have been observed [237]. Moreover, Kaminen-Ahola and colleagues [210] found effects of preconception maternal alcohol consumption (peak BALs ~120 mg/dl) on DNA methylation of a promoter element for a gene called *Agouti viable yellow* (A^{vy}). DNA hypomethylation of a promoter element for A^{vy} is associated with constitutive

expression of the *Agouti* gene, which is reflected phenotypically in a yellow coat color. Conversely, hypermethylation correlates with silencing of the *Agouti* gene and a brownish pseudo-agouti coat color, and intermediate methylation results in mottled coat color. Maternal alcohol consumption increased the probability of transcriptional silencing of A^{vy}, and the percentage of pseudo-agouti offspring more than doubled compared with that in the control group, consistent with a higher probability of hypermethylation and decreased expression of A^{vy}.

Several studies have examined the teratogenic effects of alcohol exposure during the preimplantation period (after the egg cell has been fertilized and before it implants in the uterus), which encompasses the first 4–6 days of mouse and rat development, corresponding roughly to the first 2 weeks of human pregnancy. Interestingly, a recent study by Haycock and Ramsay [238] found that the alleles inherited from the fathers were significantly less methylated in the placentas of alcohol-exposed animals compared with the placentas of control animals, suggesting effects on imprinting (expression of specific genes primarily from the alleles inherited from one rather than from both parents). Similarly, a recent study [212] reported strain-specific vulnerability to PAE via hippocampal parent-of-origin expression of deiodinase-III (*Dio3*), an imprinted thyroid hormone-inactivating gene. Moreover, alcohol effects on paternal and total expression of *Dio3* in the fetus and the adult male were paralleled by hippocampal-dependent behavioral alterations in male but not female offspring. Imprinting may be a novel mechanism underlying certain adverse effects of alcohol.

Global hypomethylation of fetal DNA, likely resulting from a direct inhibitory effect of alcohol on DNMT activity, was reported by Garro and colleagues [239] following alcohol exposure during *gastrulation* (onset of organ development, days 7–14 of gestation in the mouse and gestation weeks 3–8 in humans). Consistent with these findings, Liu and colleagues [214], utilizing whole mouse embryos in culture, observed not only delayed and reduced growth but also a more than tenfold increase in the number of genes with increased promoter DNA methylation on chromosomes 10 and X in alcohol-exposed (dose of ~400 mg/dl for 44 h beginning on gestation day 8.5) embryos with a neural tube defect compared with embryos without a neural tube defect. Importantly, alterations in DNA methylation were associated with significant changes in the expression of 84 genes. Consistent with these data, Zhou and colleagues [215] examined cultured neural stem cells (NSCs) exposed to alcohol in a "binge-like pattern" (6 h, 88 mM alcohol) and found retarded migration, neuronal formation, and growth processes, as well as altered methylation patterns of these cells. Alcohol exposure appeared to prevent the normal DNA methylation programming of key NSC genes, including genes related to neural development, neuronal receptors, and olfaction.

Consistent with the studies above, Guo and colleagues [240], using a rat model of FASD, demonstrated that moderate alcohol exposure (serum ethanol approximately 40–60 mM) during early neonatal life (third trimester equivalent) leads to an almost 50% reduction of CREB-binding protein expression in developing cerebellar neurons, as well as a reduction in histone acetylation. These findings suggest one possible mechanism underlying deficits in motor coordination found in children

exposed to alcohol *in utero* and in animal models of FASD. Together, the studies discussed above suggest that the effects of alcohol exposure during critical developmental periods may be mediated by epigenetic mechanisms.

10.16.1.2 A Possible Role for Epigenetic Mechanisms in Intervention for FASD

Important research led by Dr. Jennifer Thomas and colleagues [217–219] has focused on the effects of *in utero* alcohol exposure on choline, a nutrient important in the methionine–homocysteine cycle. This cycle involves conversion of methionine to SAM with the subsequent generation of homocysteine and the regeneration of methionine through folate-dependent or folate-independent methylation of homocysteine. Of note, the folate-independent pathway involves the contribution of methyl groups from choline. Using well-established animal models involving either PAE or early postnatal exposure, these investigators demonstrated that choline supplementation during the period of alcohol exposure may reduce the severity of a number of fetal alcohol effects, including reductions in birth and brain weights, delays in eye opening and incisor eruption, and impairments in performance on learning and memory tasks. Given the importance of choline in the methionine–homocysteine cycle and the role of this cycle in generating methyl groups for reactions central to the creation of epigenetic marks, it is tempting to speculate that the protective effects of choline are at least in part mediated by epigenetic mechanisms.

10.16.2 Possible Role of Epigenetic Mechanisms in Immune Function and Inflammation

Accumulating evidence suggests that epigenetic mechanisms are likely involved in immune development and some immune system disorders [241]. T cell differentiation, activation, and memory have been shown to be under epigenetic control [242], and epigenetic mechanisms appear to play a role in both Th1 and Th2 differentiation and the development of regulatory T cells (Treg) and proinflammatory Th17 cells [243]. Recent data suggest, for example, that differences in immune responses of allergic and nonallergic children are evident at birth, suggesting that *in utero* environmental exposures can influence epigenetic programming of immune function. It has been suggested that a failure of epigenetic control may underlie an increased risk for allergic disease in infants, with greater immaturity of both Th1 and Treg function, and persistence of Th2 immune responses beyond gestation [243, 244]. Interestingly, dietary folate intake in pregnancy has been implicated in increased allergic risk of offspring through epigenetic mechanisms [245].

Epigenetic processes may play an important role in rheumatoid arthritis (RA). It has become clear that while genetic factors may predispose an individual to RA, the overall contribution of genetic factors is 50% or less, and thus, nongenetic factors, particularly epigenetic mechanisms, are likely important. The presence of hypomethylated cells in the synovial tissue of RA patients suggests that genomic hypomethylation has a role in the pathogenesis of RA. Support for this comes from the finding that mimicking hypomethylation by treating synovial fibroblasts with a DNMT1 inhibitor led to irreversible phenotypic changes such that normal synovial fibroblasts resembled activated rheumatoid arthritis synovial fibroblasts (RASFs) [225, 226]. Hyperacetylation may also be an underlying mechanism in RA. Histone deacetylase activity was found to be about twofold lower in tissue extracts from patients with RA compared to those with osteoarthritis [246, 247]. In addition, studies have suggested that altered expression and function of microRNAs (miRNA), small noncoding RNAs that act as posttranslational regulators, might also be involved in the pathogenesis of RA. Specifically, miR-155 and miR-146 were shown to be constitutively more highly expressed in RASFs than in synovial fibroblasts from patients with osteoarthritis and were upregulated by treating RASFs with TNF-α [247]. These miRNAs might play a role in the modulation of the destructive behavior of RASFs.

In summary, epigenetic processes appear to play a major role in the development of inflammatory and allergic disorders or diseases by directly influencing the expression of genes involved in immune function. Indirect effects on inflammatory diseases may occur through early-life programming of glucocorticoid sensitivity, either through environmental exposure or through adverse early-life events [248]. These findings have important implications for the development of drugs to target histone modifications as well as DNA methylating and demethylating enzymes [249].

10.16.3 Epigenetic Mechanisms May Influence Function Throughout Life

It is important to note that epigenetic mechanisms can act to influence the epigenome and gene expression not only during early developmental periods but throughout life. Although the organism is particularly vulnerable to environmental influences on the genome and on gene expression during periods of rapid growth and development, environmental events may influence the epigenome later in life as well. Thus, positive environmental factors, such as a healthy diet and lifestyle, can positively influence physiological and behavioral function over the life course and may possibly even rescue adverse effects of PAE or other early-life insults. Conversely, diet and other lifestyle factors may adversely influence the epigenome and possibly alter gene expression over time, as indicated by the discussion above on the effects of adult alcohol intake on the epigenome.

10.17 Summary and Conclusions

This chapter discusses the adverse effects of prenatal exposure to alcohol on offspring neuroendocrine and neuro immune function, with particular emphasis on the HPA axis, fetal programming, and interacting neuroendocrine–neuroimmune networks. Data are presented from both clinical studies and animal models to demonstrate the adverse effects of PAE on immune development of offspring. The adverse impact of alcohol at the fetal–placental interface and on lactational transfer of immunity has important clinical implications for understanding alcohol's adverse effects. The discussion of the effects of alcohol exposure *in utero* on the pulmonary immune system and on susceptibility to tumors provides unique examples that highlight specific deficits in immune function in alcohol-exposed offspring. A review of possible mechanisms underlying alcohol's effects on the developing immune system suggests that adverse effects of PAE on the interaction between neuroendocrine and neuroimmune systems play a major role. Maternal alcohol consumption alters HPA activity and regulation in the pregnant female and has long-term effects on her offspring. The fetal HPA axis is reprogrammed by alcohol exposure such that HPA tone is increased throughout life, with increased HPA activation and/or delayed or deficient recovery following stress, as well as altered HPA regulation under both basal and stress conditions, which may manifest in a sexually dimorphic manner. Altered neuroendocrine activity can impact immune regulation as well as the bidirectional communication among the nervous, endocrine, and immune systems. PAE can alter the effects of stress on the immune response as well as neuroendocrine and behavioral responses to cytokines. In addition, maternal alcohol consumption alters the cytokine profile during pregnancy. Increased levels of proinflammatory cytokines in the mother cause physiological changes, including changes in neuroendocrine function. Moreover, exposure to maternally derived cytokines plays a role in programming the developing fetus. While it is not clear how maternal cytokines gain entry into fetal systems, evidence links early-life exposure to increased cytokine levels with increased vulnerability to later-life infections and cytokine overproduction. Thus, fetal programming of HPA function is paralleled by fetal programming of immune function. It is likely that the complex interactions between direct and indirect effects of alcohol on both the mother and the fetus underlie the programming of neuroendocrine and neuroimmune systems and mediate the dysregulation of these systems in alcohol-exposed offspring. Epigenetic mechanisms may, at least in part, mediate the programming effects of alcohol on the fetus. Increasing evidence implicates epigenetic processes in mediating the adverse effects of alcohol exposure on numerous aspects of offspring development, including immune function and inflammation, and promising approaches to intervention have come from consideration of epigenetic mechanisms. Importantly, environmental events influence the epigenome not only during vulnerable periods of development but throughout the life course. This provides hope that the adverse effects of early-life insults such as PAE may be attenuated in the long term. An increased understanding of how PAE impacts the "common biochemical language" of the nervous, endocrine,

and immune systems will guide us in the development of appropriate and targeted interventions and bring us closer to this goal.

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