**Investigating the Long-term Effects of Prenatal Alcohol Exposure on Inflammatory Responses Mediated by Alveolar Macrophages**

**Background**

Growing evidence in research suggests that alcohol consumption results in impairment of the immune system and increased susceptibility to infectious diseases. In particular, alcohol has been found to have dose-dependent effects that alter the inflammatory response [1], which is a non-specific mode of defence that promotes the recruitment of immune cells to sites of infection through signalling molecules called cytokines [2]. Typical cytokines involved in the inflammatory response include IL-1β, IL-6, and TNF-α [2]. Acute alcohol exposure has been demonstrated to be associated with a decrease in production of IL-1β, IL-6, and TNF-α [1]. In contrast, chronic alcohol exposure has been linked to increased circulating levels IL-1β, IL-6, and TNF-α in the blood [1].

Beyond direct alcohol consumption, in utero alcohol exposure to the developing fetus can also occur when pregnant mothers consume alcohol. Similar to the immunomodulatory effects observed as a result of direct alcohol consumption, children with fetal alcohol syndrome have been observed to have abnormal lymphocytes, impairments in mechanisms that mediate the action of cytokines, and a greater incidence of both life-threatening and minor infectious diseases [3-4].

Compared to children who were never exposed to alcohol in utero, children with fetal alcohol syndrome have been observed to have more hospitalizations as well as longer lengths of hospitalization, with pneumonia being the most common reason for hospitalization [5]. However, it is unknown if prenatal alcohol exposure contributes to increased severity of pneumonia long-term, after children have reached adulthood. As pneumonia is an infection that targets the respiratory tract, resident immune cells such as macrophages in the alveoli act as key players that release pro-inflammatory cytokines to promote the clearance of lung infections [6]. Therefore, this proposed project aims to assess the long-term immunomodulatory effects of prenatal alcohol exposure by investigating if prenatal alcohol exposure contributes to decreased production of inflammatory cytokines by alveolar macrophages during adulthood.

**Research Question**

Is chronic prenatal alcohol exposure sufficient to decrease production of IL-1β, IL-6, and TNF-α by alveolar macrophages in response to infection by *Streptococcus pneumoniae* during adulthood in mouse models?

**Potential Impact**

By increasing our understanding of the long-term consequences of prenatal alcohol exposure, we may gain knowledge that will motivate the population of pregnant alcohol users to reduce alcohol consumption in order to promote healthy children who are less susceptible to infectious diseases. As 1 in 13 pregnant women have reported to consume alcohol during pregnancy [7], this investigation is important because it can promote awareness of immunity-related problems associated with prenatal alcohol exposure that may put children at risk not only during childhood, but potentially throughout life.

Furthermore, unlike many studies that have investigated the immunnomodulatory effects of ethanol by stimulating immune responses using only bacterial structures, such as lipopolysaccharide [8-10], this project proposes to use whole bacteria. By using whole bacteria that are capable of causing infections, the observed immune responses are more representative of true infections because whole bacteria produce additional virulence factors that contribute to pathogenicity and influence immune responses [11]. Therefore, as one of the first studies to use whole *S. pneumoniae* bacteria to investigate alcohol-induced immunomodulation, this proposed project may obtain more relevant information pertaining to alcohol-influenced responses against actual infections compared to previous investigations that only utilized bacterial structures.

**Hypothesis**

The hypothesis of this project is the following: **Chronic prenatal alcohol exposure is sufficient to decrease production of IL-1β, IL-6, and TNF-α by alveolar macrophages in response to infection by *Streptococcus pneumoniae* during adulthood in mouse models.**

The proposed hypothesis is primarily based on evidence presented in a previous study that investigated the effects of acute and chronic alcoholism on lipopolysaccharide-induced activity of the cytokine tumour necrosis factor, where the inflammatory cytokine levels within alveolar macrophages were found to decrease in response to chronic alcoholism [8]. Although this previous investigation examined the effects of direct consumption of alcohol in adult mice, the study did not address whether chronic alcoholism by pregnant mothers produces the same effects in mature offspring. However, the hypothesis of this proposed project infers that mature offspring which are chronically exposed to alcohol in utero will experience the same effects as mice which chronically consume alcohol as adults.

Further evidence that supports the proposed hypothesis is that children with fetal alcohol syndrome have been observed to have abnormal lymphocytes and a greater incidence of both life-threatening and minor infectious diseases, which can be attributed to inadequate inflammatory responses as a result of decreased production of IL-1β, IL-6, and TNF-α [3]. Although this evidence presents findings observed during childhood, the hypothesis infers that the abnormalities observed in lymphocytes as a result of prenatal alcohol exposure will extend to adulthood.

**Predictions**

If chronic prenatal alcohol exposure is sufficient to decrease production of IL-1β, IL-6, and TNF-α by alveolar macrophages in response to infection by *Streptococcus pneumoniae* during adulthood in mouse models, IL-1β, IL-6, and TNF-α production by alveolar macrophages from adult mice that experienced chronic prenatal alcohol exposure should be lower compared to adult mice that did not experience chronic prenatal alcohol exposure.

If chronic prenatal alcohol exposure is not sufficient to decrease production of IL-1β, IL-6, and TNF-α by alveolar macrophages in response to infection by *Streptococcus pneumoniae* during adulthood in mouse models, IL-1β, IL-6, and TNF-α production by alveolar macrophages from adult mice that experienced chronic prenatal alcohol exposure should produce similar levels of IL-1β, IL-6, and TNF-α compared to mice that did not experience chronic prenatal alcohol exposure.

**Experimental Approach**

Mice and breeding

C57BL/6J mice will be inbred to conceive mice that will undergo chronic prenatal alcohol exposure. The parental mice will be housed individually until they have reached sexual maturity, which is at 8 weeks of age [12]. After reaching 8 weeks of age, pairs of male and female mice will be housed together when females are in estrus to allow mating to occur.

There are several reasons why C5BL/6J is a favourable strain of mice to use in this experiment. Firstly, C57BL/6J is a strain which has undergone extensive inbreeding, resulting in genetically identical mice [13]. Therefore, by breeding genetically identical mice to produce offspring for this proposed project, we can attribute observed differences in phenotypes to differences in the environment, rather than differences in genotypes among mice. Secondly, C57BL/6J is a favourable strain of mice to use particularly for experiments involving alcohol because C57BL/6J mice have been observed to show greater voluntary consumption of alcohol compared to other mouse strains [14]. Therefore, by using mice that consume alcohol voluntarily, it can be ensured that consistent alcohol consumption occurs in pregnant mice during the induction of chronic prenatal alcohol exposure.

Inducing chronic prenatal alcohol exposure

After successful insemination of female mice, which is indicated by the presence of a vaginal plug, the diets of females will be replaced with ad libitum access to a fortified liquid diet containing ethanol, with 15% of the total calories derived from ethanol. Fortified liquid diets will be made following the protocol outlined by Zhou et al. [15]. Control mice will receive a liquid diet with sucrose substituted for ethanol, or a solid diet consisting of standard mouse chow and water. The diets will be provided daily until offspring are delivered, at which the diets of all groups will be replaced with standard mouse chow and water in order to prevent the potential for continued alcohol exposure in offspring after birth through breastfeeding.

Liquid diets will be the sole source of food, water, and alcohol for the experimental group, as opposed to incorporating ethanol into the drinking water and feeding solid food, to ensure consistent intake of ethanol relative to the total calories consumed.

Inducing *Streptococcus pneumoniae* infection

After offspring mice reach 8 weeks of age, intubation-mediated intratracheal instillation will be conducted to deliver 50 μL of *S. pneumoniae* suspension containing 1x107 CFU in phosphate-buffered saline solution (PBS) into the lower respiratory tract, following the protocol outlined by Lawrenz et al. [16]. To control for the effect of the procedure, control mice will delivered 50 μL of PBS alone.

Although intranasal infection or standard intratracheal infection can be considered as modes of inducing infection, intubation-mediated intratracheal instillation is advantageous for studying the effects of lower respiratory tract infections, such as pneumonia, in mouse models because it allows direct infection of the lower respiratory tract. Although previous studies have shown that intranasal and intratracheal infection induce similar results after *S. pneumoniae* infection in mice [17], compared to humans, mice have significantly larger upper respiratory tracts, normalized against total lung capacity, which may significantly influence subsequent bacterial presentation and disease progression in the lower respiratory tract [16]. As pneumonia is primarily a lower respiratory tract infection [18], intubation-mediated intratracheal instillation is the preferred mode of infection in order to circumvent the involvement of the upper respiratory tract to allow better extrapolation of results from the mouse model to humans.

Harvesting alveolar macrophages

48 hours after inducing *S*. *pneumoniae* infection, cells from mouse lungs will be extracted by bronchoalveolar lavage, which involves passing a bronchoscope through the mouth and into the lungs, adding fluid to the lungs, and recovering the fluid which now contains components from the respiratory tract. A major advantage of using bronchoalveolar lavage is that the cells extracted most abundantly are alveolar macrophages, which can compose more than 80% of the cells within the extracted fluid [19]. Bronchoalveolar lavage will be performed following the protocol described by Han & Ziegler [20].

To isolate alveolar macrophages from the mixed cell population within the bronchoalveolar lavage fluid, fluorescence activated cell sorting (FACS) will be conducted. Although no single cell surface marker distinguishes alveolar macrophages from other cells, alveolar macrophages are characterized by being Siglec-F+, CD11c+, CD11bint, and F4/80+ [20]. Taking this into consideration, fluorescent antibodies against the aforementioned cell surface proteins will be used in successive rounds of fluorescence activated cell sorting to isolate alveolar macrophages with the stated characteristics.

Measuring cytokine production by alveolar macrophages via intracellular staining and flow cytometry

Intracellular staining of IL-1β, IL-6, and TNF-α, using fluorescent anti-IL-1β, anti-IL-6, and anti-TNF-α antibodies with fluorescence of different wavelengths for each specific antibody, respectively, will be conducted using the protocol by the biotech company, Abcam [21]. Cytokine levels within alveolar macrophages will be quantified by flow cytometry.

Secretion of cytokines by alveolar macrophages will be blocked by treating cells with Brefeldin A to inhibit protein transport from the endoplasmic reticulum to the Golgi apparatus. Cells will be fixed by treatment with formaldehyde to prevent proteins within the cells from being lost during subsequent washing steps. Cell membranes of alveolar macrophages will be permeabilized using detergent to allow fluorescent antibodies to enter the cells and bind to corresponding target cytokines. Cells will be washed to remove any unbound antibodies before analyzing the cells using a flow cytometer.

By performing intracellular staining of alveolar macrophages rather than analyzing the bronchoalveolar lavage fluid to measure levels of IL-1β, IL-6, and TNF-α, we eliminate the possibility of measuring cytokines that were produced by immune cells other than alveolar macrophages, such as neutrophils or T cells, that are present in the mixed population of cells in the bronchoalveolar lavage fluid. As this project focuses specifically on cytokine production by alveolar macrophages, use of intracellular staining is the preferred method to detect cytokines produced specifically by alveolar macrophages.

**Possible Results and Discussion**

Similar expression levels of IL-1β, IL-6, and TNF-α in alveolar macrophages from mice prenatally exposed to alcohol compared to control mice

Despite evidence from previous research that show increased susceptibility to infection among children due to alcohol exposure in utero [3, 4, 9], similar expression levels of IL-1β, IL-6, and TNF-α in alveolar macrophages from mice prenatally exposed to alcohol compared to control mice would suggest that prenatal alcohol exposure is not sufficient to result in immunomodulation during adulthood. Therefore, this observation would demonstrate that the immunomodulatory effects of prenatal alcohol exposure observed during childhood do not extend to adulthood.

Altered expression levels of IL-1β, IL-6, and TNF-α in alveolar macrophages from mice prenatally exposed to alcohol compared to control mice

If the expression levels of IL-1β, IL-6, and TNF-α in alveolar macrophages from mice prenatally exposed to alcohol are different compared to control mice, this suggests that the immunomodulatory effects of prenatal alcohol exposure extend to adulthood. However, the observation of higher or lower expression levels of IL-1β, IL-6, and TNF-α can be interpreted as both beneficial in some respects, yet detrimental in others.

Higher levels of IL-1β, IL-6, and TNF-α would indicate that prenatal alcohol exposure is sufficient to up-regulate IL-1β, IL-6, and TNF-α production in alveolar macrophages. However, two possible implications can stem from up-regulation of inflammatory responses: 1) chronic prenatal alcohol exposure causes the inflammatory response to be more efficient at clearing infection, or 2) the inflammatory response is up-regulated in order to compensate for a different mechanism involved in immunity that has been inhibited, so even if the inflammatory response is up-regulated, these mice may still be more susceptible to infection overall. Furthermore, up-regulation of IL-1β, IL-6, and TNF-α by alveolar macrophages may result in excessive inflammation that can damage the lungs and inhibit efficient gas exchange.

Lower levels of IL-1β, IL-6, and TNF-α would indicate that prenatal alcohol exposure is sufficient to down-regulate IL-1β, IL-6, and TNF-α production in alveolar macrophages. As a result, there is a decrease in inflammatory responses and reduction in collateral damage to the lungs caused by excessive inflammation. However, decreased inflammatory responses may lead to decreased efficiency of pathogen clearance.

Previous studies have shown that regular consumption of beer or red wine in low to moderate amounts can lead to decreased susceptibility to infections [22]. Therefore, it may be possible that chronic prenatal alcohol exposure can also decrease susceptibility to infections. Although this extends beyond the scope of this proposed project, one can determine if altered expression levels of IL-1β, IL-6, and TNF-α in alveolar macrophages due to chronic prenatal alcohol exposure is beneficial in terms of reducing susceptibility to infections by comparing the infectious dose of pathogens in treated mice versus control mice, where a greater required infectious dose would suggest decreased susceptibility to infections.

Sex-specific long-term immunomodulatory effects

It is possible that long-term immunomodulatory effects of prenatal alcohol exposure may be observed in one sex, but not the other. In a previous study that investigated the effects of fetal alcohol exposure on cytokine production mediated by blood macrophages in response to lipopolysaccharide, prenatally exposed male rats demonstrated significantly decreased production of the cytokine TNF-α compared to control males [10]. However, female rats did not show any significant difference in TNF-α production when comparing experimental and control groups [10]. Furthermore, in a different study, unusual maintenance of X-chromosome inactivation has been observed in female lymphocytes, which is speculated to cause increased expression of X-linked immunity related genes in females compared to males. Although none of the cytokines, IL-1β, IL-6, or TNF-α, are encoded by the X chromosome [23-25], it is possible that other proteins encoded by genes on the X chromosome may be involved in regulating the aforementioned cytokines [26].

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