Understanding more about maternal vaccination and neonatal immunity to improve vaccine induced protection in early life

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Thesis submitted for the degree of Masters of Philosophy

Abstract

Neonates are more susceptible to infectious diseases and often have worse disease outcomes. Influenza virus, Respiratory Syncytial Virus (RSV) and *Streptococcus agalacticae* (Group B Streptococcus: GBS) are three major pathogens associated with neonatal infections. Maternal and neonatal vaccination are two promising strategies to protect this vulnerable population.

Maternal influenza vaccination has been clinically proven to be effective in protecting both the mothers and newborns. However, the limited half-life of antibody response reduces the effectiveness of protection in infants leaving them highly susceptible to infection. More understanding of the complete picture of how the timing of maternal vaccination affects the antibody responses in both the mothers and their newborns are required. One objective of this study was to evaluate influenza specific antibody after vaccination at different stages of pregnancy in the mothers and their newborns. Maternal immunisation led to significantly more antibody in the infant blood, with the highest antibody concentration found in newborns when the time interval between maternal vaccination and delivery falls in 5-24 weeks.

Neonatal vaccination is an alternative option. However, neonates have distinctive immune features compared to adults, including restricted functionality of the innate immune response and limited immune memory. Since these features play an essential role in shaping the subsequent direction of the vaccine induced response, studying the neonatal innate response to infections in comparison with adults may be instrumental to future neonatal vaccine development. Therefore, the other objective of this study was to investigate the neonatal innate immune response to influenza, RSV and GBS. Neonatal innate immune responses to viral and bacterial infection were characterised and compared, following by the study of innate immune cell phenotypes and the corresponding chemokines expression. Viral infection induced a distinctive immune response pattern compared to bacterial infection. Interestingly, neonate displays similar overall immune profile in comparison with adults, with the exception of the chemokine responses.

These studies provide immunological underpinning for the future development of effective maternal and neonatal vaccines.

Acknowledgments

First, I would like to express my sincere gratitude to Dr John Tregoning, for his invaluable support for my study and the project, for his patience and motivation, for being one of the most important mentors in my life. His guidance has helped me throughout the research and writing, he has taught me to become a better scientist, and more importantly, a better person. I have always been and will always be inspired by his passion for science. It is such an honour and pleasure for being part of his group.

I would also like to thank Professor Beate Kampmann, for her insightful comment and encouragement, but also for the hard questions which has helped me look at the project from different perspectives.

I would like to thank Dr Jaqueline McDonald, Dr Ekaterina Kinnear, Dr Hannah Cheeseman, Helen Groves, Dave Busse, Dr Ryan Russell, Dr Simren Gill, Luke Muir, and Chris Pinder for providing technical assistance during the course of the project and for all the fun we had for the past two years. Thank you for not just being the best colleagues to work with, but also close friends. I would also like to thank Dr Beth Holder and Thomas Rice for providing the precious samples to make this study happen. I am grateful for the rest of Shattock group for their help and support throughout the project.

Last but not least, I would like to thank my parents, friends and Hao, for their support throughout the project and in my life, especially for their patience and kindness for taking care of me during the writing stage.

Declarations

Declaration of Originality

The work within this thesis is one's own, and all else is appropriately referenced.

Copyright Declaration

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Abbreviations

ACK – Ammonium Chloride Potassium lysing buffer

APC – Antigen presenting cell

BAL – Bronchoalveolar Lavage

BALB/c – Bagg Albino mouse strain

BCG – Bacille de Calmette et Guerin

BSA – Bovine Serum Albumin

CAL09 – Pandemic Influenza A H1N1/2009 California isolate

CCL/CXCL – Chemokine ligands

CD – Cluster of Differentiation

cDNA – coding DNA

CFU – colony forming units

CPE – Cytopathic Effect

Ct – Threshold Cycle

D10 – DMEM supplemented with 10% FCS and P/S

DAMPs – Damage associated molecular patterns

DC – Dendritic cell

DMEM – Dulbecco's Modified Eagle Medium

ds – Double-stranded

DTP - Diphtheria Tetanus Pertussis triple vaccine

ELISA – Enzyme-linked Immunosorbent assay

ENG195 – Pandemic Influenza A H1N1/2009 England isolate

FCS – Foetal Calf Serum

GM-CSF – Granulocyte macrophage colony stimulating factor

H1N1 – Influenza A strain with type 1 haemagglutinin and neuraminidase envelope proteins

H_2O_2 – Hydrogen Peroxide

HA – Influenza Haemagglutinin envelope protein

HAI – Haemagglutination inhibition assay

HEp-2 – Human lung Epithelial cell line

i.m. – Intramuscular

i.n. - Intranasal

i.p. – Intraperitoneal

ICAM-1 – Intracellular adhesion molecule -1

IFN – Interferon

Ig - Immunoglobulin

IL - Interleukin

LAIV – Live attenuated influenza vaccine

LDH - Lactate Dehydrogenase

LPS – Lipopolysaccharide

LTA – Lipoteichoic Acid

M-CSF – Macrophage colony stimulating factor

MDCK – Marlin Derby Canine Kidney cells

MEM – Minimal Essential Medium

MeOH - Methanol

MHC – Major Histocompatibility Complex

MOI – Multiplicity of Infection

NK – Natural killer cell

OD – Optical Density

P/S – Penicillin and Streptomycin

PAMPs- Pathogen associated molecular patterns

PBMC – Peripheral Blood Mononuclear Cells

PBS – Phosphate Buffered Saline

PCA – Principal component analysis

PFU – Plaque forming units

PGN - Peptidoglycan

PMA – Phorbol Myristate Acetate

Poly (I:C) - polyinosinic: polycytidylic acid

PRR – Pattern recognition receptors

qRT-PCR – Quantitative Real-Time Polymerase Chain Reaction

R10 – RPMI supplemented with 10% FCS and P/S

RIG – Retinoic Acid inducible gene

ROS – Reactive oxygen species

RPM – Revolutions per minute

RPMI – Roswell Park Memorial Institute Medium

RSV – Respiratory Syncytial Virus

RT – Room temperature

SF – Serum Free

ss – Single stranded

Th – T helper cell

TIV – Trivalent inactivated influenza vaccine

TLR – Toll-like receptor

TMB – Tetramethybenzidine solution

TNF-α – Tumour Necrosis Factor alpha

U/ mL – Active units/ millilitre

1. Introduction

1.1. Infectious diseases in neonates

Despite the advance of medicine and technology, infectious diseases still accounted for more than 50% mortality of children under 5 years of age in 2013 (1). Neonates are more prone to infections and severe infections early in life may have a lasting impact on survivors (2). Influenza virus, respiratory syncytial virus (RSV) and *Streptococcus agalacticae* (Group B Streptococcus: GBS) are three major pathogens causing neonatal infections. Whilst these three infections remain mostly asymptomatic or cause mild illness in older children and healthy adults (<65 years old), neonates and young infants are at a higher risk of infection and severe complications (3–6). The neonatal immune system goes through a gradual, age-dependent maturation since birth (7). Investigating how the ontogeny of the immune system is associated with the higher risk of specific infections may provide instrumental information that can be used in protecting and treating infections in this vulnerable population.

1.1.1. RSV

During first year of life, at least 68% of infants experience a RSV infection. By the end of second year of life, most children will have infected by RSV at least once (8). RSV infection is one of the major causes of hospitalisation of children and approximately 2.1 million children under 5 years of age with RSV infections require medical attention each year (9). Notably, very young children (in the first three months of life) suffer the most from severe lower respiratory tract illness caused by RSV infection. Reinfection is common in older children and adults but the symptoms are often absent or confined to upper respiratory tract (10).

1.1.2. Influenza virus

Influenza virus is another significant cause of hospitalisation of young children, particularly children under 2 years of age (11). During the influenza season, the rate of mortality and morbidity is at the

highest among infants younger than 6 months of age (12). Infants less than 6 months of age are 40% more likely to be admitted to ICU due to severe outcomes when compared to older infants (13).

1.1.3. GBS

GBS is a commensal Gram-positive bacterium found in the intestinal and vaginal tracts (14). However, it is also one of the leading causes of infection in the first three months of life, leading to significant morbidity and even mortality. Approximately 50% of neonates born from GBS colonised mothers acquire GBS during or shortly after labour and 1-2% of this population develops invasive disease including sepsis, pneumonia and meningitis during the first 3 months of life (15). The limited ability of newborns to trigger sufficient immune response has been associated with the high susceptibility of GBS infection (7).

Altogether, age is an important risk factor for RSV, influenza and GBS infections. Neonates are particularly at high risk and more likely to suffer from worse disease outcomes compared to older children and adults.

1.1.4. Factors for elevated risk of infections in neonates

There are a number of factors that may contribute to the high susceptibility of infections in neonates, including physical differences, restricted functionality of the innate immune response and limited immune memory.

1. Physical differences

Neonates have much smaller airways than adults and hence a greater reduction in airflow as a result of increased pulmonary secretions such as mucus, inflammation, cell infiltration or infection. Therefore, neonatal airways are more likely to be blocked by during respiratory disease caused by RSV and influenza infections. This may contribute to the worse disease outcomes. Neonates also have lower reserves of energy to support the increased effort of respiration when airways are blocked.

2. Limited immune memory in neonates

There is no immunological memory in the neonatal immune systems because it is the first time the system encounters the pathogens, which directly contributes to disease severity. Also, neonates have a higher proportion of naïve regulatory T cells (16) and B cells which may dampen the immune response to infection. After exposure to antigen, the neonatal antibody response is delayed and less persistent compared to adults (17).

3. Neonates have restricted functionality of the innate immune response

Neonatal innate immunity undergoes age-dependent development (18). For example differential patterns of innate cytokine production throughout the first few years of life drive the development of cellular immune responses. These patterns of cytokine responses are also associated with age-dependent windows of pathogen susceptibility (19).

Importantly, innate immunity plays a crucial part in the initiation and subsequent direction of adaptive immune response. Given the limited exposure to antigens and the well-studied defects in neonatal adaptive immune responses (16,17), newborns rely heavily on the innate immunity for protection. Studying the neonatal innate immune response to infections in comparison with adults may be instrumental to understanding the reasons behind the elevated risk of specific infections in post-natal period. Therefore, one focus of this project is on the neonatal innate immune response to selected infections**.**

1.2. Functional differences of the neonatal innate immune system

One widely circulated hypothesis is that neonatal innate immune responses are deficient compared to adults. In early postnatal life, the neonatal cytokine response is distinct compared to adults, both qualitatively and quantitatively (17). This pattern of cytokine responses is associated with the pattern of neonatal susceptibility to common serious infections (21). Compared to pre-term infants, term newborns have much higher inflammatory Th17 supporting cytokines, including IL-6 and IL-23 (19). Th17 cells are responsible for defending against bacterial and fungal infection at mucosal surfaces. The deficiency of Th17 responses in pre-term infants makes them more vulnerable to these infections. Th1 supporting cytokines (e.g. IL-12p70 and IFNγ) reach similar levels as adults later in

infancy. Since Th1 is important for defence against intracellular pathogens, this might be associated with the high susceptibility of intracellular pathogens in early post-natal life (Figure 1). Altogether, the cytokine profile influences the clinical susceptibility to specific pathogens in neonates and this functional connection may be valuable in developing treatment and vaccines for neonates (14,15).

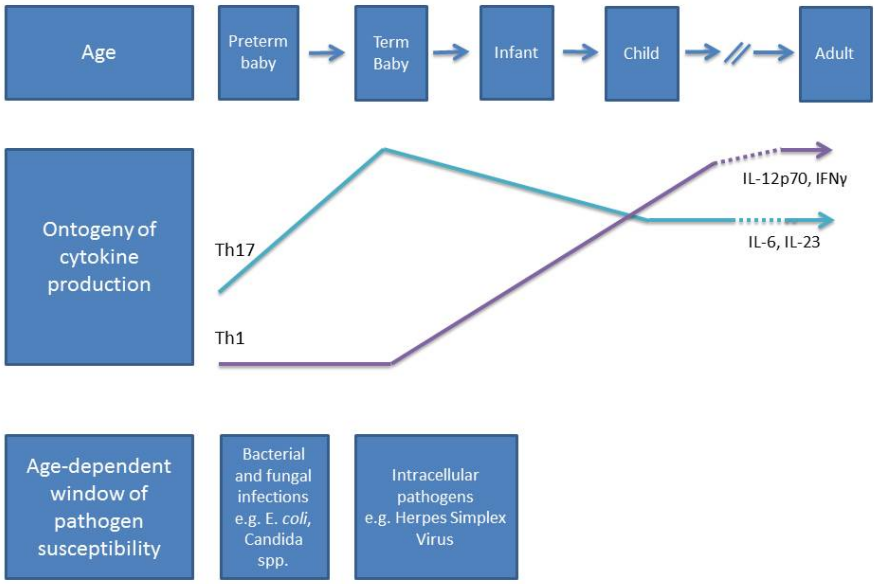

Figure 1.1. Age-dependent changes in cytokine response and the associated susceptibility to pathogens.
Adapted from (19).

1.3.Approaches to protect neonates from infection: Maternal vaccination

However, the innate immune response is not the only mechanism by which the infant is protected against infection. Adaptive immunity plays a role, particularly antibody, however as described above the neonatal adaptive immune system is also deficient compared to the adult response. Therefore an alternate strategy is maternal immunisation. This has the added benefit of protecting the mother and the infant. This is important because pregnant women are at higher risk of severe illness caused by influenza virus infection (22–26). During the 2009 H1N1 pandemic, pregnant women were 7.2 times more likely to be admitted to hospital due to serious influenza associated diseases compared

to non-pregnant women (27). Furthermore, maternal influenza infection has been associated with serious complications, such as fetal malformation, still birth and pre-term delivery (28–30).

1.3.1. Maternal influenza vaccination

Vaccination remains as the most efficacious way to protect both pregnant women and their offspring (31). Trivalent inactivated influenza vaccine (TIV) is licensed for pregnant women and children over 2 years of age. For infants younger than 6 months of age, which is one of the highest risk population for influenza infection, current influenza vaccine is not available because of its poor immunogenicity in this population. An alternative strategy to protect young infants from influenza infection is through passive protection from maternal influenza specific antibody. During pregnancy, maternal derived antibodies are actively transported through placenta from mother to foetus, which offers passive immunity for infants up to 6 months against infection (32). Immunisation of pregnant women with TIV has been recommended by the World Health Organisation since 2005, to protect both the mothers and their infants. The aim of maternal immunisation is to enhance the antibody-mediated immunity against influenza infection in mothers and results in transfer of protective level of antibodies to newborns.

1.3.2. Efficacy of maternal influenza vaccination

Multiple randomised controlled trials and observational studies have shown that vaccinating pregnant women with influenza vaccine was safe throughout pregnancy (33–36). For example, Zaman et al. showed that immunisation with influenza vaccine during pregnancy was not associated with fetal, perinatal or maternal serious adverse events (37). Maternal vaccination is not only safe, but also immunogenic and effective, several studies showed that pregnancy did not compromise the efficacy and immunogenicity of influenza vaccine in terms of inducing antibody response, with a 35.8% reduction in respiratory illness in vaccinated pregnant women (38,39). Jackson et al. showed that with only a single dose of monovalent influenza vaccine, both pregnant and non-pregnant women had high rate of seroprotection (40). Zaman et al. demonstrated that trivalent influenza vaccine during pregnancy was effective in achieving seroconversion rate to H1N1 (83.6%), to H3N2

(69.2%) and influenza B (39.7%) (37). This was similar to the result from immunisation of healthy adults with seasonal influenza vaccine (41). Furthermore, Madhi et al. showed that in South African HIV-uninfected pregnant women, immunisation of influenza vaccine during pregnancy induced high seroprotection rate to all three vaccine strains (42). Along similar lines, Schlaudecker et al. showed that TIV achieved similar seroprotection and seroconversion rate in pregnant women compared to non-pregnant women (43). Kay et al. also confirmed that there was no defect in the quantity and quality of inducing influenza specific antibody response in pregnant women after receiving influenza vaccine (44). Maternal immunisation with influenza vaccine is clinically efficacious in protecting infants, with 45-63% reduction in laboratory-confirmed influenza among newborns and infants depending on models (38,45). Steinhoff et al. confirmed that the maternal derived antibody induced by influenza vaccine was protective for both the mother and the infant against influenza infection (33). Omer et al. investigated the infant outcomes from maternal influenza vaccine and found that during influenza season, infants with vaccinated mother were 70% less likely to suffer from prematurity and 69% less likely to be small for gestational age (30). Eick et al. found that infants with vaccinated mother had significantly higher HAI antibody at birth and up to 2-3months of age (46). Immunoglobulin G (IgG) is the only class of antibody that is transferred across placenta during pregnancy (47). It is suggested that the active transport of influenza specific IgG to foetus during pregnancy was associated with the high HAI titre in infants with mothers who received influenza vaccine during pregnancy (38).

1.3.3. Timing of influenza vaccination in pregnancy

It is important to determine the effect of vaccination timing during pregnancy on the induction of optimal immune response in both the mother and the newborn, in order to confer better protection of infants younger than 6 months of age. Nunes et al. investigated the kinetics of maternal influenza specific antibody in infants from transplacental transfer and revealed that the half-life of these antibody in infants was 42-45 days (48). They also showed that the efficacy of post-pregnancy maternal influenza vaccine for infants younger than 8 weeks was 85.6%. However, the efficacy decreased as the age of infant increased, reaching only 30.3% by 16-24 weeks of age. The rate of

seroprotection in infants decreased significantly over time, from 56% at first week of age to less than 41 % by 16 weeks of life (49). These suggested that the protection from maternal derived antibody against influenza infection in infants only lasts for a limited period of time and lack of protection after 16 weeks of life may be associated with the reduced maternal antibody in infants.

Interestingly, recent studies by Eberhardt et al. suggested that with maternal Diphtheria, Tetanus, Pertussis vaccine (DTap), early second trimester maternal immunisation increased the antibody titre in the newborns (50,51). It remains unclear whether maternal influenza vaccine has a similar effect. So far, only 5 studies have investigated the effect of vaccination timing on antibody response in either the mother or the infant. Sperling et al. revealed that there was no significant difference in the rate of seroprotection (HAI titre) in mothers at postpartum when compared by the timing of influenza vaccination during pregnancy (52). Eick et al. and Blanchard-Rohner et al. showed that the timing of maternal vaccination during pregnancy had no effect in the percentage of seroprotection (HAI titre) in newborns (46,53). Tsatsaris et al. conducted a vaccine trial using monovalent H1N1 influenza vaccine and found that newborns had lower HAI antibody titre when the mother received vaccinated at second trimester compared to third trimester (54). Along the similar line, Ohufuji et al. showed that mothers had lower HAI antibody titre at postpartum if they were vaccinated at first trimester of pregnancy with monovalent H1N1 influenza vaccine (55). However, all the studies have failed to offer a complete picture of how the timing of maternal vaccination affects the antibody response in both the mother and the newborns.

1.4. Thesis Aims and Hypotheses

The aim of this project is to understand more about factors affecting maternal and neonatal vaccination in order to improve the level of immune protection these approaches offer to the new-born child.

Part 1: Timing of maternal vaccination

Recent studies demonstrated that DTaP immunisation in the second trimester leads to greater antibody level than immunisation in the third trimester. The initial hypothesis to be tested was influenza specific antibody titres in cord blood would be higher after maternal immunisation in trimester three than other trimesters. The aim of the first part of the project was:

1. Determine the optimum timing of maternal influenza vaccination to get the highest level of antibody in the infant by measuring influenza specific antibody response in both neonates and mothers with different timing of maternal vaccination.

Part 2: Neonatal innate responses to infection

Neonatal responses to vaccination are not always as effective as in adults. One consideration is that the neonatal innate immune response to infection is dampened. This has previously been observed using TLR ligands but not well characterised after infections. The hypothesis for this part of the project was that the innate response would be less in the cord blood derived cells after live pathogen infections. The aims of part 2 were:

1. Characterise neonatal cytokine responses to three major pathogens for neonatal infectious diseases (influenza virus, RSV and GBS) compared to adult responses through immunoassay on both human in vitro experiment and animal studies.
2. Determine the mechanism that shapes neonatal innate immune responses to selected pathogens compared to adults through flow cytometry on human in vitro experiment.

2. Materials and Methods

2.1.Solutions

Complete media (D10): 500 mL Dulbecco's Minimal Essential Medium (DMEM) was supplemented with 100 units of penicillin and 100 µg streptomycin, 5 mL of 200 mM L-glutamine and 50 mL of 10% heat inactivated foetal calf serum (FCS).

Complete media (R10): 500 mL RPMI 1640 medium was supplemented with 100 units of penicillin and 100 µg streptomycin, 5 mL of 200 mM L-glutamine and 50 mL of 10% FCS.

Madin-Darby Canine Kidney Epithelial cells media (MDCK media): 500 mL DMEM was supplemented with 5 mL 100x MEM Non-Essential Amino Acid solutions, 1 mL MycoZap™ Antibiotics and 50 mL of 10% FCS.

Serum free (SF) media: 500 mL DMEM or RPMI 1640 medium was supplemented with 100 units of penicillin and 100 µg streptomycin, 5 mL of 200 mM L-glutamine.

Serum free MDCK media (SF MDCK media): 500 mL DMEM was supplemented with 5 mL 100x MEM Non-Essential Amino Acid solutions and 1 mL MycoZap™ Antibiotics.

ELISA blocking buffer/FACS buffer: 500 mL was supplemented with 5 g Bovine Serum Albumin (BSA) (1%).

Washing buffer: 500 µL Tween-20 (0.05%) was added to 1 litre phosphate-buffered saline (PBS).

Columbia Horse blood Agar: Per litre: Special peptone 25 g, Starch 1 g, Sodium chloride 5 g, Agar 10.25 g, Defibrinated horse blood 50 mL. Pre-poured plates were purchased from Oxoid.

2.1.1. Materials used in study

Material	Supplier	Catalogue Number
15 mL centrifuge tube	Corning	734-0452
50 mL centrifuge tube	Corning	734-0453
ACK lysing buffer	Lonza	10-548E
Agarose, low gelling temperature	Sigma Aldrich	A9414
Anti-Mouse Ig, κ Compensation Particles Set	Becton Dickinson Ltd	552843
Anti-Rat and Anti-Hamster Ig κ Compensation Particles Set	Becton Dickinson Ltd	552845
ArC Amine Reactive Compensation Bead Kit	Thermo Fisher Scientific	A10346
Bovine Serum Albumin	Sigma Aldrich	A7906
Columbia Blood Agar with Sheep Blood Medium	Thermo Fisher Scientific	PB5039A
Cytofix/Cytoperm fixation/permeabilsation kit	Becton Dickinson Ltd	555028
Dulbecco's Modified Eagles Medium (DMEM)	Sigma Aldrich	D6546
Fast DAB	Sigma Aldrich	D0426
Glycerol	Sigma Aldrich	G5516
GolgiStop	Becton Dickinson Ltd	554724
Heat Inactivated Foetal Bovine Serum (FBS)	Gibco	10500
Histopaque – 1077	Sigma Aldrich	10771
Human Fc Block	Becton Dickinson Ltd	564219
Human IFN-α pan ELISA development kit (HRP)	Mabtech	3425-1H-20
Human TNF-alpha DuoSet ELISA	R&D Systems	DY210
IgG from human serum	Sigma Aldrich	I2511-10MG
Ionomycin	Sigma Aldrich	I0634
MEM Non-Essential Amino Acids	Thermo Fisher Scientific	11140050
MycoZap™ Antibiotics	Lonza	VZA-2011
NUNC Maxisorp 96-well plates	Thermo Scientific	439454
Pen/strep [100 units penicillin 100 µgstrep]	Gibco	15140-122
Phosphate Buffered Saline (PBS)	Gibco	14190-094
PMA	Sigma Aldrich	P8139
Recombinant Human CCL2	R&D Systems	279-MC
Recombinant Human CCL4	R&D Systems	271-BME
Recombinant Human CCL5	R&D Systems	278-RN
Recombinant Human CCL8	R&D Systems	281-CP
Recombinant Human CXCL10	R&D Systems	266-IP
Recombinant Human CXCL8	R&D Systems	208-IL
Recombinant Human CXCL9	R&D Systems	392-MG
Recombinant Human GM-CSF	R&D Systems	215-GM
Recombinant Human IFN-β	PBL Biomedical Laboratories	11410-2
Recombinant Human IFN-γ	R&D Systems	285-IF
Recombinant Human IL-12	R&D Systems	219-IL
Recombinant Human IL-1α	R&D Systems	200-LA
Recombinant Human IL-1β	R&D Systems	201-LB
Recombinant Human IL-2	R&D Systems	202-IL
Recombinant Human IL-4	R&D Systems	204-IL
Recombinant Human IL-6	R&D Systems	206-IL
Recombinant Human TGF-β1	R&D Systems	240-B
Recombinant Human TNFα	R&D Systems	210-TA

Material	Supplier	Catalogue Number
RPMI-1640 medium	Sigma Aldrich	R0883
Streptavidin-HRP	R&D Systems	DY998
TMB substrate	Thermo Fisher Scientific	2023
Todd-Hewitt Broth	Oxoid	CM0189
TPCK Treated trypsin	Lorne Labs	LS003740
Trypan Blue	Thermo Fisher Scientific	15250061
Trypsin EDTA	Thermo Fisher Scientific	25200056
Tween-20	Fisher Scientific	337-500

Methods for part 1: Timing of maternal vaccination

2.2.Recruitment and blood sample processing

2.2.1. Recruitment

The study was conducted during three influenza seasons from November 2013 to October 2016 . This study was nested within a larger project investigating maternal pertussis vaccination. It was an opportunistic study using samples from the same individuals and not specifically powered to look at the questions. Healthy pregnant women were recruited antenatally. Exclusion criteria included; pregnancy with twins, maternal infection, chronic maternal pathology, pregnancy pathology, and babies with chromosomal or structural abnormalities. The study was approved by the National Research Ethics Service (NRES), NHS, UK (REC 13/LO/1712) and stored under the Imperial College Healthcare Tissue Bank (ICHTB). Written informed consent was obtained.

2.2.2. Blood sample Collection

Cord blood and maternal blood were collected in sodium heparin-anti-coagulated Vacutainer tubes (BD Biosciences). Cord blood was obtained from the placenta of healthy neonates at the Maternity Unit of St Mary's Hospital, London. Through collaboration with Dr Beth Holder and Professor Beate Kampmann (Department of Paediatrics, Imperial College London), all participants had completed written informed consent and the study was approved by the ethics committee of the faculty of Medicine, Imperial College London. Blood samples from non-pregnant women were also collected as a comparator, these blood samples were obtained from volunteers in the Department of Mucosal Immunity and the Department of Paediatrics.

2.2.3. Blood sample processing

Maternal blood samples were collected from mother at or within 2 days of delivery. Umbilical cord blood samples were collected at delivery. Prior to the processing of blood samples, 50 µL of whole blood was centrifuged at 4929 xg for 10 minutes. The plasma was then collected and stored in – 80 °C for further analysis.

2.3. Immunoassay

2.3.1. Semi-quantitative influenza specific IgG ELISA

A standardised ELISA assay was adapted from Donnelly et al. (56) and used to measure antibodies (IgG) specific to Influenza virus H1N1, H3N2, Influenza B virus. Since the virus strains included in the seasonal influenza vaccine change in the three influenza seasons (2013-2016), corresponding virus antigens were used in the ELISA based upon the year of participant received the influenza vaccine (Table 2.1).

Flu Season	Influenza Virus Vaccines Composition
2013-2014	A/California/7/2009 (H1N1) pdm09-like virus
	A/Texas/50/2012 (H3N2)-like virus
	B/Massachusetts/2/2012-like virus
2014-2015	A/California/7/2009 (H1N1) pdm09-like virus
	A/Texas/50/2012 (H3N2)-like virus
	B/Massachusetts/2/2012-like virus
2015-2016	A/California/7/2009 (H1N1) pdm09-like virus
	A/Switzerland/9715293/2013 (H3N2)-like virus
	B/Phuket/3073/2013-like virus

Table 2.1 Viruses in vaccine in study period

Purified influenza virus antigen was diluted 1 µg/mL in PBS and used to coat 96-well plates overnight at 4 °C. Anti-human IgG Kappa/Lambda (Southern Biotech) was diluted 1:3,200 in PBS to coat the wells for a standard curve. After washing with washing buffer, the plate was blocked by ELISA blocking buffer for 1 hour. A serial dilution of human IgG from serum (Sigma-Aldrich) was used for the standard curve in order to quantify the influenza virus specific IgG in the sample. Plasma from

samples was diluted 1:50, 1:500 and 1:5,000 before adding to the plate. This is to ensure that the absorbance reading is within the linear range of the standard curve for an accurate quantification. The plate was incubated at 37 °C for 2 hours and the bound IgG was then detected by HRP-conjugated goat anti-human IgG antibodies (Sigma-Aldrich) by 2 hour incubation at 37 °C. Following the washing step, tetramethybenzidine solution (TMB) and 1M H_2SO_4 stop solution were used to detect the response and the optical density was read at 450nm.

2.3.2. Haemagglutination inhibition (HAI) assay

Samples were tested for haemagglutination inhibition against A/California/7/2009 (H1N1) virus strain. A 96-well V bottom plate was used to perform the assay. Cord and maternal serum samples were pre-treated with Receptor Destroying Enzyme (RDE) for 18 hours at 37 °C before inactivating the enzyme at 56 °C for one hour. All the treated sera were tested for non-specific agglutination before analysing with HAI assay. RDE-treated serum was two-fold serial diluted across the plate with PBS and incubated with pre-diluted 4HA unit virus per well for 15 minutes at room temperature. 100 µL of 0.5% turkey erythrocytes (Enivgo, Cambridge) diluted in PBS was then added to each well of the test and the plate was incubated for 30 minutes at room temperature. The plate was then tipped to 45 to interpret the result in comparison with positive and negative controlled well. Titre was defined as the reciprocal dilution of antibody where agglutination first seen.

Methods for Part 2: Neonatal innate responses to infection

2.4.In vitro study

2.4.1. Peripheral blood mononuclear cell (PBMC) isolation

Blood samples were processed no more than 8 hours after birth. Blood samples were diluted with PBS in 1:2 ratio. This mixture was then layered with equal volume of Histopaque (density: 1.077 g/mL, Sigma-Aldrich) and centrifuged at 352 xg for 20 minutes with the brake off. Th`e cloudy interphase layer, which contains the PBMC, was collected with a Pasteur pipette into a new Falcon tube. PBMC were then washed with 15 mL PBS and centrifuged at 530 xg for 10 minutes with the

brake on. Supernatant was discarded and washing was repeated with 15 mL PBS to remove platelets. After the second washing, supernatant was discarded and 2 mL ACK buffer was added to resuspend the pellet and remove any contaminating red blood cells. After incubating in room temperature for no more than 5 minutes, 10 mL R10 media were added and centrifuged for 5 minutes at 530 xg. The pellet was resuspended with R10, ready for counting. PBMC were infected with live RSV, influenza virus or GBS in the stimulation experiment immediately after isolation and the rest of PBMC were frozen at -80 °C for flow cytometry experiment.

2.4.2. Whole blood assay

Whole blood samples were diluted in 1:1 ratio with pre-warmed R10 media.

2.4.3. Culture of GBS

A primary clinical isolate of GBS isolated from a patient in Gambia, was kindly provided by Dr Kirsty Le-Doare. 500 µL frozen aliquot of sub-master stock of GBS was removed from -80 °C and allowed to thaw at room temperature. Stock was then added to 20 mL Todd Hewitt Broth (THB) in an Erlenmeyer non-baffled shake-flask and incubated at 37 °C with shaking at 180 RPM until a final optical density (OD) at 600 nm of 1 was reached. The aim was to achieve the concentration of 1.0 x 10^7 CFU/mL of the culture. The total viable count of GBS was confirmed by the following steps. 100 µL of culture was diluted 1:10 to 10^{-7}. 10 µL of each dilution was spread onto horse blood Colombia agar plates. Plates were incubated at 37 °C in 5% CO_2. Colonies were counted and averaged for each plate and colony-forming unit (CFU)/mL was calculated using dilution factor.

2.4.4. Optimisation of GBS concentration for stimulating PBMC

Following growth, GBS was transferred to a 50 mL Falcon tube and centrifuged at 3,060 xg for 5 minutes. Supernatant was discarded and pellet was resuspended in 10 mL of PBS. The suspension was centrifuged again at 3,060 g for 5 minutes and supernatant was discarded. The remaining pellet was resuspended with 5 mL PBS and used for PBMC stimulation. To optimise the concentration of GBS assay, PBMCs were isolated from blood cones (NHS Blood and Transplant Centre, Colindale, UK). A series of multiplicity of infection (MOI) of GBS were added to 2 x 10^5 cells PBMC for 24 hours and

supernatant was collected afterwards. The TNFα response was measured by TNFα ELISA and used to determine the optimal MOI for subsequent stimulation of PBMC from cord, maternal and non-pregnant women blood samples. The optimal MOI was 20 CFU/cell.

2.4.5. GBS stimulation of PBMC

PBMC were isolated from cord, maternal and non-pregnant women blood samples. 2 x 10^5 PBMC were incubated with GBS at a MOI 20. The samples were incubated for 24 hours at 37 °C (5% CO_2) before harvesting 200 µL supernatant in Eppendorf tubes and stored at -80 °C for further analysis.

2.4.6. Influenza growth

Sub-confluent monolayer of Madin-Darby Canine Kidney Epithelial (MDCK) cells were used to grow H1N1/Influenza A/California/7/2009 and H1N1/Influenza A/England 195. Cells were washed with PBS prior to infection. 1 mL of virus was thawed and diluted in 1:3000 with 3 mL SF MDCK media. Diluted virus were added to MDCK and incubated at 37 °C (5% CO_2) for 1 hour with rocking occasionally to prevent cells drying out. Cells were then washed twice with SF MDCK media. 10 mL SF MDCK media supplemented with 1 µg/mL trypsin were added to the cells which were then incubated at 37 °C (5% CO_2) for two days. Viruses were harvested by centrifuging the cell supernatant at 528 xg for 5 minutes and aliquotted for storage at -80 °C.

2.4.7. Influenza virus plaque assay

1X10^6 MDCK cells were seeded into 12-well cell culture plate until it reached confluent monolayer (>90% confluency). Virus was thawed and diluted in SF MDCK media with 10-fold serial dilutions. MDCK cells were washed with PBS twice and 200 µL of each virus dilution was added to the well, incubating for 1 hour at 37 °C (5% CO_2). Sterile water was used to make up 2% low melting plaque agarose. Overlay medium was prepared, composed of 15% 10x MEM, 15 mM HEPES, 0.3% BSA, 3 nM L-glutamine, 30 mM $NAHCO_2$, 0.01% dextran, 1.5% 10x P/S and 0.001% Trypsin. An assay overlay consisted of 1/3 of 2% low melting agarose and 2/3 overlay media. After 1 hour incubation, cells were washed with PBS and 1 mL of assay overlay was added. Assay overlay was allowed to dry and

the plates were inverted, followed by 2-3 days incubation at 37 °C (5% CO_2). 1% Crystal Violet (diluted in water) was used to stain the area of cell clearance and allow counting of plaques. The viral titre was calculated as plaque forming unit (PFU)/mL according to the dilution of the virus.

2.4.8. RSV growth

HEp2 cells were grown to 75% confluency in T-175 flasks before infecting with RSV with MOI 0.1 in SF DMEM media. Flasks were rotated horizontally in the incubator every 15 minutes to achieve aneven distribution. After 2 hours incubation at 37 °C (5% CO_2), 27 mL D10 media was added. The concentration of FCS was reduced to 2% after 24 hours incubation. Cells were incubated for another 24 hours before harvesting the virus by scraping the flask contents, sonicating and aliquotted into 1.5 mL cryovials. Virus was snapped frozen and stored in liquid nitrogen.

2.4.9. RSV plaque assay

RSV plaque assays were performed to assess the viral titre. 2 x 10^4 cells were seeded per well in 96-well flat bottom plate and allowed to grow to 80-90% confluent monolayer. Virus was diluted 1:500 in SF D10 media and 50 µL of diluted virus were added to first row. Dilution was double for each row across the plate. 150 µL D10 media was added 2 hour post infection and incubated for 24 hours. Virus was then removed and cells were washed, followed by fixing with MeOH containing 2% H_2O_2 for 20 minutes at room temperature. Cells were then incubated at rest, with biotinylated polyclonal goat anti-RSV antibody for 1 hour at room temperature. After washing with PBS, 100 µL extravidin peroxidase was added and incubated for 30 minutes. The washing step was repeated and 50 µL amino-ethylcarbazole substrate was added and incubated in the dark. Colour change was monitored until it reached the desired colour. Cells were then washed with PBS and plaques were counted to calculate the viral titre using the dilution factors.

2.4.10. Optimisation of RSV concentration for PBMC stimulation

Adapted from the protocol in the lab (Myriam Haltalli's Protocol), the optimal MOI of RSV A2 (stock concentration 7 x 10^6 PFU/mL) for PBMC stimulation was determined by IFNα assay. PBMC were

isolated from blood cones (NHS Blood and Transplant Centre, Colindale, UK). 2 x 10^5 cells PBMC was incubated with a series of MOI of RSV A2 for 24 and 48 hours. Supernatant was collected and IFNα ELISA was used to measure IFNα response to determine the optimal MOI of RSV A2. The optimal MOI is 3 PFU/cell. The same MOI was used in influenza virus strain A/England/195/2009 (stock concentration 7.6 x 10^6 PFU/mL, H1N1) stimulation of PBMC.

2.4.11. Influenza virus/RSV stimulation of PBMC (cord, maternal and non-pregnant women blood samples)

PBMC from cord, maternal and non-pregnant women blood samples were isolated. 2 x 10^5 cells PBMC was incubated with either influenza virus or RSV at MOI 3 on 96-well U-bottom plate. The samples were incubated for 24 hours at 37 °C (5% CO_2) before harvesting 200 µL supernatant in Eppendorf tubes and stored at -80 °C for further analysis.

2.4.12. Semi-quantitative RSV specific IgG ELISA

A standardised ELISA assay was adapted from Donnelly et al. (56) and used to measure antibodies (IgG) specific to RSV. Purified RSV antigen was diluted 1 µg/mL in PBS and used to coat 96-well plates overnight at 4 °C. Anti-human IgG Kappa/Lambda (Southern Biotech) was diluted 1:3,200 in PBS to coat the wells for a standard curve. After washing with washing buffer, the plate was blocked by ELISA blocking buffer for 1 hour. A serial dilution of human IgG from serum (Sigma-Aldrich) was used for the standard curve in order to quantify the RSV specific IgG in the sample. Plasma from samples was diluted 1:50, 1:500 and 1:5,000 before adding to the plate. This is to ensure that the absorbance reading is within the linear range of the standard curve for an accurate quantification. The plate was incubated at 37 °C for 2 hours and the bound IgG was then detected by HRP-conjugated goat anti-human IgG antibodies (Sigma-Aldrich) by 2 hour incubation at 37 °C. Following the washing step, tetramethybenzidine solution (TMB) and 1M H_2SO_4 stop solution were used to detect the response and the optical density was read at 450nm.

2.4.13. *Cytokine ELISA*

A pan IFNα ELISA kit (Mabtech) and TNFα ELISA kit (Mabtech) were used to measure IFNα and TNFα, respectively in supernatant collected from GBS, influenza virus and RSV stimulated PBMC. Capture antibody was diluted to 4 µg/mL with PBS and high protein binding ELISA plate was coated with 50 µL of these antibody per well for overnight at 4 °C. The plate was then washed with washing buffer for 5 times before blocking with blocking buffer (PBS + 0.01% BSA) for one hour at room temperature. 100 µL of samples and standards were added to the well after the plate was washed with washing buffer for 5 times. Samples and standards were incubated at room temperature for 2 hours. Washing was repeated. 50 µL of biotinylated detection antibody was then added to each well and incubated for 1 hour at room temperature. Streptavidin-HRP was diluted 1:1,000 in blocking buffer and added to each well for 1 hour following the washing with washing buffer. After the final wash, 50 µL of TMB substrate was added and plate was left in dark to develop for 5 to 10 minutes before stopping the reaction with 50 µL/well 1M Sulphuric acid (H_2SO_4). The absorbance was measured at 450nm. The IFNα concentration in the sample was determined by the standard curve.

2.4.14. *Luminex assay*

An in-house Luminex kit was used (57–59). The standards were purchased from R&D systems with corresponding antibody pairs. Individual Luminex bead sets (Luminex, Riverside, CA) were coupled to cytokine-specific capture antibodies according to manufacturer's recommendations. 19 analytes were measured in this assay (Table 2.2). Each analyte binds to magnetic beads via specific capture antibodies. Each bead is filled with different ratio of dyes, resulting in up to 500 unique fluorescent profiles/bead regions. The measurement of multiple analytes is possible because each capture molecule with the specific analyte is attached to a specific bead region (60).

Inflammatory cytokines	**Antiviral and adaptive cytokines**	**Chemokines**	**Growth factors**
IL-1α	IFNβ	CCL2/MCP-1	GM-CSF
IL-1β	IFNγ	CCL4/MIP-1β	TGFβ
IL-6	IL-2	CCL5/RANTES	
IL-12	IL-4	CCL8/MCP-2	
TNFα		CXCL8/IL-9	
		CXCL10/IP-10	

Table 2.2 Human Luminex panel

Magnetic beads conjugated with capture antibody were diluted in Luminex assay buffer (PBS supplemented with 1% goat serum, 1% mouse serum, 0.05% Tween 20 and 20 mM Tris-HCL). 50 µL of beads mix was put in 96-well flat-bottomed plate with 50 µL undiluted samples or pre-diluted standards. Plates were incubated for 1.5 hours on a plate shaker. Plates were then washed with washing buffer on a magnetic platform for three times before adding 50 µL pre-mixed detection antibody cocktail. Plates were incubated for 1 hour on a plate shaker as per the previous incubation step. The washing step was repeated, followed by the addition of 50 µL streptavidin-PE and incubation for 30 minutes on a plate shaker. The washing step was repeated and 100 µL washing buffer was added to each well. The plate was shaken for another 10 minutes to thoroughly disperse the beads before reading the plate on Bio-Plex®100 Luminex machine (BIO-RAD).

2.5. Flow cytometry

2.5.1. Viability stain

PBMC isolated from cord, maternal and non-pregnant adult samples were stimulated with influenza virus. Cells were harvested 24 hours post-infection and stained to define the innate cell subsets. Once harvested, cells were stained for viability with 1:400 Live/Dead Aqua stain in PBS for 20 minutes at room temperature. Cells were then washed twice with PBS and centrifuged at 679xg for 5 minutes. Cells were then stained with surface stain markers.

2.5.2. Surface stain

In order to block any non-specific binding, cells were incubated with 1:10 human FC block (BD) for Fc blocking for 10 minutes at room temperature. Cells were centrifuged and washed twice with PBS before incubating with antibodies for surface staining diluted in FACS buffer for 1 hour at 4 °C. Antibodies used in surface staining panel are listed in Table 2.3. Cells were washed twice with FACS buffer and centrifuged, prior to intracellular cytokine staining.

2.5.3. Intracellular cytokine stain (ICS)

After surface staining, cells were incubated with 1X BD Cytofix/Cytoperm solution for 20 minutes at 4 °C, followed by washing with 1:10 BD Perm/Wash buffer diluted in distilled water twice and centrifugation at 679xg for 5 minutes. Antibodies for ICS were diluted in BD Perm/Wash buffer and added the cells for 1 hour at 4 °C. Antibodies used in ICS were listed in Table 2.3 and innate cell populations investigated in this project and the associated cell markers were listed in table 2.4.Post staining, washing and centrifugation step were repeated twice. Cells were then resuspended in 2% Formaldehyde solution and kept in dark at 4 °C until acquisition.

Antibody	Flurochrome	Clone/Cat. number	Company	Volume per sample
CD3	V500	UCHT1	Becton Dickinson Ltd	2.5µL
CD56	BV711	NCAM16.2	Becton Dickinson Ltd	2.5µL
CD14	Qdot605	TüK4	Thermo Fisher Scientific	1.25µL
CD16	PECY7	3G8	Biolegend	2.5µL
CD123	PECY5	9F5	Becton Dickinson Ltd	5µL
CD11c	FITC	3.9	Biolegend	1µL
HLA-DR	APC-Cy7/H7	L243	Becton Dickinson Ltd	5µL
GM-CSF	BV421	BVD2-21C11	Becton Dickinson Ltd	5µL
CXCL10	PE	6D4/D6/G2	Becton Dickinson Ltd	1.25µL
Viability	Live/Dead Fixable Aqua	L34965	Thermo Fisher Scientific	N/A

Table 2.3 Antibody used in flow panel

Cell types	Cell makers
NK cells	CD3-, CD56+, CD16+/-
Dendritic cells	CD3-, HLA-DR+, CD11c+/CD123+
Monocytes	CD3-, CD14+, CD16+/-

Table 2.4 Cell types and associated cell markers

2.5.4. Flow cytometry Acquisition and Data Analysis

Cells were acquired on BD Fortessa flow cytometer and data analysis was performed using FlowJo (TreeStar, Ashland, OR, USA).

2.6.In vivo assay

2.6.1. Animals

All procedures were approved by the Animal Welfare and Ethical Review Board (AWERB) of Imperial College. Studies were carried out under the UK Animals (Scientific Procedures) Act Project Licence (PPL 70/7457). Animals were kept in the Central Biomedical Services (CBS) facility at St Mary's Hospital under Specific Pathogen Free conditions (SPF). BALB/c mice were obtained from Charles River UK Ltd (Margate, UK).

Neonatal mice were 7-8 days old and adult mice were 6-8 weeks old at the start of experiments. Animals were culled by intraperitoneal injection (IP) with pentobarbitone (20 mg dose, Pentoject, Animalcare Ltd. UK).

2.6.2. Intranasal infections

Prior to infection, mice were anesthetized by inhaled isoflurane. Virus was pre-diluted to desired concentration in PBS and kept on ice prior to infection. For influenza virus infection, $2X10^2$ PFU influenza virus was delivered intranasally to neonatal mice in 20µL and $5X10^5$ PFU influenza virus was delivered intranasally to adult mice in 100µL. For RSV Adult mice were infected with $2.5x10^6$ PFU in 100µL, neonatal mice were infected with $0.5x10^6$ PFU in 20µL. Concentrations of virus used in the experiments were based on previous studies in Dr John Tregoning's group.

2.6.3. Lung harvest

Lungs were removed from sacrificed animals and processed through 100µm cell strainer passage for homogenisation. After centrifugation at 200x g for 5 minutes, supernatant was collected and stored at -80 °C for Luminex assay.

2.6.4. Luminex assay for lung supernatant

A custom made Magnetic Luminex assay (R&D Systems) was used according to the manufacturer's instruction. 13 analytes were measured in the assay (Table 2.5). Plate was read on Bio-Plex® 100 Luminex machine.

Inflammatory Cytokines	Chemokines	Growth Factors
IL-1α	CCL2	GM-CSF
IL-1β	CCL3	
IL-5	CCL5/RANTES	
IL-6	CXCL1	
IL-12	CXCL2/MCP-2	
TNFα	CXCL10/IP-10	

Table 2.5 Cytokine panel in mouse Luminex panel

2.7.Statistics

2.7.1. Principal component analysis

Principal component analysis was performed in R version 3.3.3 using basic command function. PCA graphs were plotted with packages ggfortify (61) and ggplot2 (62). The script used for PCA is shown in appendix 1.

2.7.2. Statistical analysis

One-way ANOVA, Student's t-test, Chi-square test and Fisher's exact test were used in statistical analysis and performed using in GraphPad Prism 7 (California, USA). MANOVA was performed in analysing variables in PCA using R version 3.3.3. $P \leq 0.05$ was considered significant (* $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$ and **** $p \leq 0.0001$). The standard error of mean or the standard deviation within an experiment was calculated and presented.

Descriptive statistics were carried out to investigate any differences in baseline characteristics between the vaccinated and un-vaccinated groups using the Student's t-test. Univariate analyses were then conducted to assess associations of mean H1N1 maternal and cord antibody concentration with

a range of socio-demographic and clinical risk-factor variables. Crude associations between vaccination status, trimester of vaccination, and other variables, with mean antibody concentration were obtained using the Student's t-test. Variables that showed evidence of association with mean antibody concentration at the $p \leq 0.1$ level were analysed further in multivariable logistic regression models. Maternal antibody transfer was quantified by the mother to cord H1N1 antibody ratio and reported by trimester of vaccination. The correlation between H1N1 IgG and HAI titres in mother and cord blood samples were analysed using linear regression. Bonferroni correction was used to adjust the bias from multiple comparisons.

In multivariable analyses the relationship between vaccination status; trimester of vaccination; and season of birth; and H1N1 cord blood titre were quantified using a logistic regression model. The outcome was defined as the upper 50^{th} centile of H1N1 antibody titre in cord blood.

H1N1 IgG concentrations in cord blood were used to predict infant antibody levels up to 6 months assuming an antibody half-life of 45 days. The predicted decay of H1N1 IgG concentrations were stratified by vaccination status and trimester of vaccination.

3. Effect of maternal influenza immunisation timing on antibody transfer to child

The aim of this part of the study was to describe and evaluate the influenza specific antibody after trivalent influenza vaccination administered at different stages of pregnancy in both the mother and the newborns in United Kingdom, with two different assays (haemagglutination inhibition test and semi-quantitative influenza antigen specific IgG ELISA).

3.1. Result

3.1.1. Study population

In total, 96 pregnant women were enrolled into this sub-study from the main study during three influenza seasons between 2013 and 2016 (Table 3.1). 63.5% of the study population received seasonal influenza vaccine during pregnancy. There were no significant differences in age, body mass index (BMI) or parity between vaccinated and unvaccinated pregnant women enrolled in the study. Of the vaccinated mothers, 56% of study population received the vaccine in the 2015-16 influenza season, 31% in the 2014-15 season and 13% in 2013-14. For the newborns, there were no significant differences in terms of gestational week, birth weight and ratio of female gender between those born to vaccinated mother compared to newborns with unvaccinated mothers. When comparing the volunteers by trimester in which they were immunised, 26% of participants were vaccinated in trimester one, 26% in trimester two and 48% in trimester three. There were no significant differences in terms of gestational age, birth weight and ratio of female sex in newborns when they were grouped by the maternal vaccination trimester (Table 3.2).

Characteristic	Vaccinated (n=59)	Unvaccinated (n=36)	p Value
Women			
Age, mean (SD)	32.4 (5.1)	32.3 (4.5)	0.879
BMI, mean (SD)	24.2 (4.5)	25.9 (3.6)	0.079
Parity, mean (SD)	0.58 (0.8)	0.89 (1.0)	0.101
Newborns			
Gestational age, weeks (SD)	39.9 (1.3)	39.9 (1.1)	0.912
Mean birth weight, g (SD)	3.5 (0.4)	3.5 (0.4)	0.944
Female sex, n (%)	27 (44%)	16 (46%)	0.900
Mode of delivery: Vaginal delivery, n (%)	33 (56%)	24 (71%)	0.162
Season of birth			
Month of birth (Median)	6.5	6	0.303
Born in flu season (Sept-Mar), n (%)	33 (56%)	18 (50%)	0.574

Table 3.1 Characteristics of the Study Populations

Characteristics	Maternal flu immunisation in First trimester (n=16)	Maternal flu immunisation in Second trimester (n=18)	Maternal flu immunisation in Third trimester (n=27)	p value
Mothers				
Age, year, mean (SD)	31.6 (4.8)	30.8 (5.9)	33.6 (4.7)	0.18
Parity, mean (SD)	0.6 (0.8)	0.5 (0.8)	0.6 (0.9)	0.92
Influenza season at immunisation:				
2013-2014	3 (19%)	2 (13%)	3 (11%)	
2014-2015	9 (56%)	6 (37%)	18 (64%)	
2015-2016	4 (25%)	8 (50%)	7 (25%)	
Newborns				
Gestational age, weeks (SD)	40.2 (1.1)	39.6 (1.5)	40.2 (0.9)	0.27
Season of birth:				
Birth month (median)	6 (June)	4 (April)	10 (October)	**0.0183**
Born in flu season, n (%)	2 (13%)	8 (44%)	23 (85%)	**<0.0001**
Month of vaccination (median)	10	11	10	0.8
Mean birth weight, g (SD)	3.5 (0.3)	3.6 (0.5)	3.5 (0.5)	0.78
Female sex	8 (50%)	9 (56%)	10 (36%)	0.38
Mode of delivery: Vaginal delivery, n (%)	11 (69%)	10 (56%)	13 (48%)	0.42

Table 3.2 Characteristics of vaccinated pregnant women in the study by the stage of pregnancy at immunisation

3.1.2. Influenza vaccination during pregnancy significantly elevates cord anti-H1N1 IgG level

Blood samples were collected from the mother and the cord at the time of birth. Antigen specific ELISA was used to quantify the influenza specific IgG response in both the cord and maternal serum samples. The H3N2 and B strain components of the inactivated influenza vaccine changed in 2015-16, whilst the H1N1 component remained constant (Table 2.1). To enable comparative analysis for these two viruses, H3N2 and B specific IgG responses were measured only in the infants and their mothers who were vaccinated or gave birth during 2013-2015 influenza seasons.

H1N1 specific IgG concentration were significantly higher in the cord blood of babies born to vaccinated rather than unvaccinated mothers ($p<0.05$, Figure3.1A). The cord samples also had a significantly higher concentration than the maternal responses. The transfer ratio for H1N1 concentration in matched vaccinated pairs (3.019 ± 0.4388) was significantly greater than in unvaccinated pairs (1.539 ± 0.1926). There was no significant difference between vaccinated and unvaccinated mothers. No significant differences were seen in the H3N2 and influenza B specific IgG concentration of either cord or mother regardless of the vaccination status (Figure 3.1B, 1C).

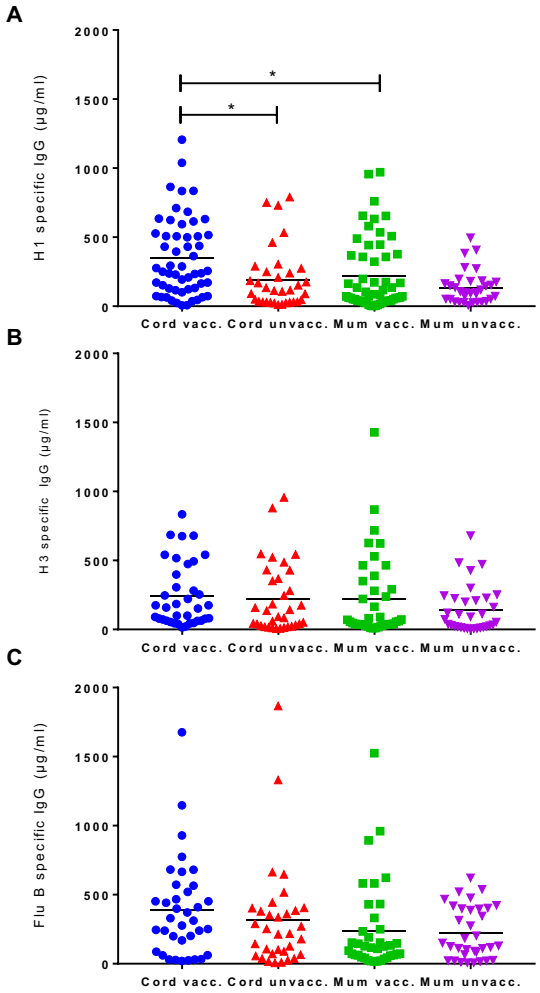

Figure 3.1. Immunisation in pregnancy increases the anti-influenza antibody concentration in the infant.

Blood was collected from mothers and cord at the time of delivery. Individuals were grouped by vaccination status of the mother. IgG responses to H1N1 (A), H3N2 (B) and influenza B (C) antigens were measured by ELISA. One way ANOVA and post test was performed to compare the means of IgG concentration in each group. Line represents mean, points represent individuals, n=59 vaccinated and 36 unvaccinated. *p≤0.05

3.1.3. Vaccination in the second trimester significantly elevates H1N1 specific IgG at birth in mothers and newborns

The cord blood samples were further subdivided by the stage of pregnancy at the time of immunisation (Table 3.2). Cord blood samples from infants born to mothers vaccinated at the second or third trimesters had significantly higher H1N1 specific IgG concentration at birth compared to infants from unvaccinated mothers ($p<0.05$, Figure 3.2A). Babies born to mothers vaccinated in the first trimester had no increase in concentration compared to those born to non-vaccinated mothers. In the maternal samples at the time of delivery, H1N1 specific IgG concentration in women vaccinated in the second trimester were significantly higher than either unvaccinated mother or mothers that received vaccination at first trimester (Figure 3.2B). There were no significant differences in the H3N2 and B antigen concentration (Figure 3.2C-F).

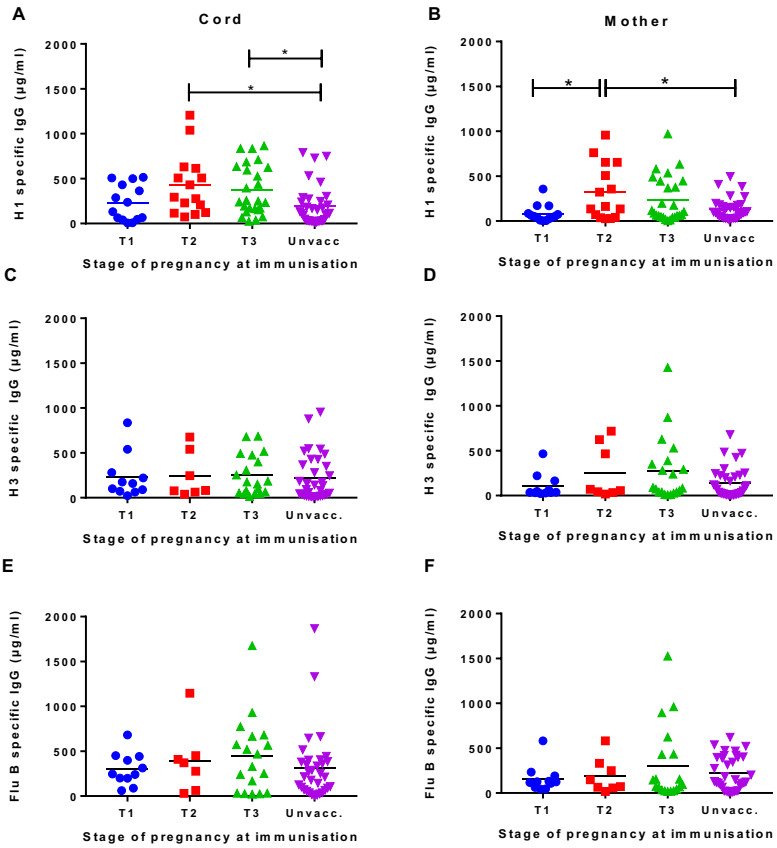

Figure 3.2. Immunisation in the second trimester significantly increases the anti-influenza antibody concentration.

Individuals were grouped by the trimester of maternal vaccination, into first (n=16) second (n=18) or third (n=27) trimesters or unvaccinated (n=36). Antigen specific IgG concentration in cord (A, C, E) and maternal (B, D, F) serum samples was measured by ELISA against H1N1 (A, B), H3N2 (C, D) and influenza B antigen (E, F). One way ANOVA was performed to compare the means of IgG concentration in each group. Line represents mean. *p≤0.05

3.1.4. Maternal immunisation induced a high, seroprotective HAI titre in newborns

To further dissect the degree of protection provided by maternal vaccination, we measured the haemagglutination inhibition (HAI) titre in the samples. The HAI titre that is currently used as a correlate of influenza protection was defined in the 1970's in a series of human influenza challenge studies (63). Since the H1N1 vaccine strain was unchanged through the course of the study, we focussed the HAI studies on a representative H1N1 virus (A/California/7/2009). The HAI titre in the cord blood of babies born to vaccinated mothers was significantly greater than the titre in infants born to unvaccinated mothers (Figure 3.3A). The seroprotection rate (HAI≥40) of infants with vaccinated mother was 54%, which was significantly higher than those infants born to unvaccinated mothers ($p<0.001$). The HAI titre was also greater in cord blood from vaccinated mothers than the maternal blood, indicating active transfer of functional influenza specific antibodies across the placenta. Whilst there was no significant difference in HAI titre between vaccinated and unvaccinated mothers, significantly more vaccinated mothers (21%, $p<0.02$) had an HAI titre ≥ 40. The HAI titre in cord blood samples of infants and maternal blood samples were moderately correlated ($r^2=0.4356$, $p<0.001$, Figure 3.3B), though the maternal levels were lower. The HAI titre in cord blood of babies born to mothers vaccinated in the second or trimester of pregnancy had significantly higher HAI antibody titres than those born to unvaccinated mothers (Figure 3.3C). When results were compared by trimester at immunisation, there was no difference in the proportion of infants with an HAI titre ≥ 40. Comparing the functional (HAI) and binding (ELISA) assays for measuring antibody there was a weak correlation for both the cord (Figure 3.4A) and maternal (Figure 3.4B) samples.

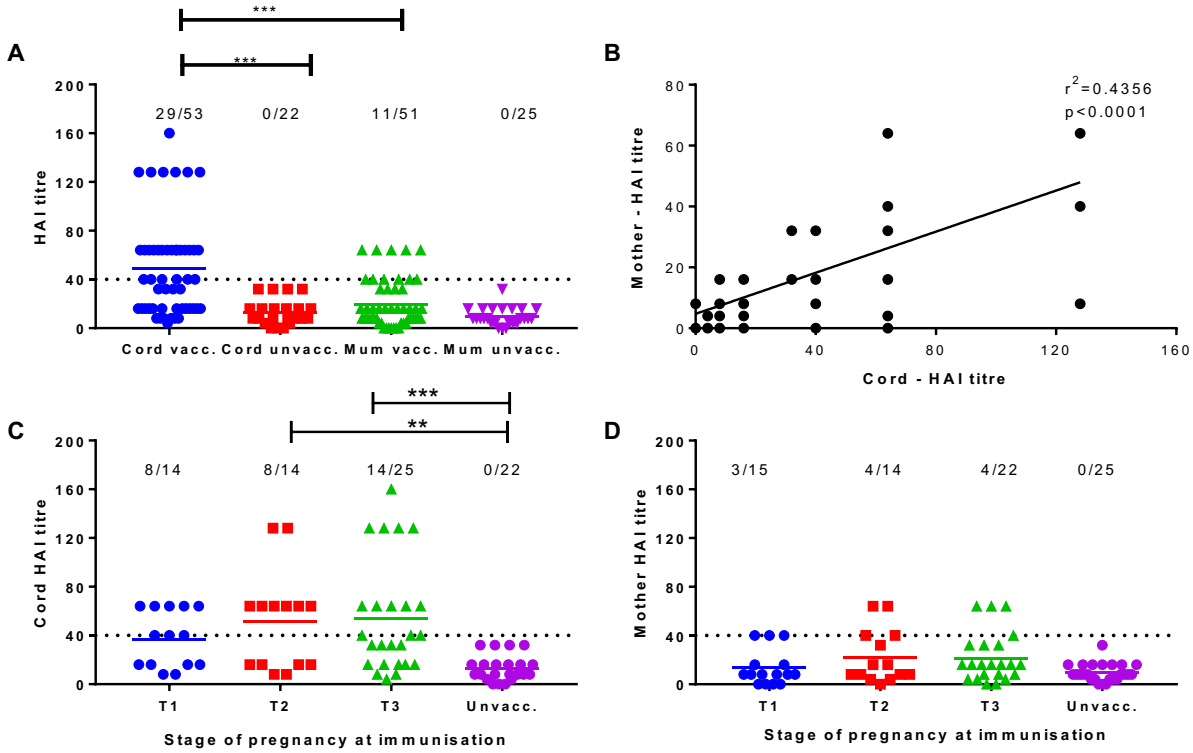

Figure 3.3. Effect of maternal immunisation on maternal and cord HAI titres.
Blood was collected from mothers and cord at the time of delivery. Donors were grouped by vaccination status of the mother. HAI titres to H1N1 influenza (A). The dotted line represents the level of seroprotection (HAI titre ≥1:40): numbers indicate the proportion with a titre over the seroprotective level. Correlation between HAI titre in cord and maternal serum samples (B). HAI titres by stage of pregnancy at immunisation in cord (C) and maternal samples (D). One way ANOVA was performed to compare the means of HAI titre in each group. Line represents mean. **p≤0.01, *** p≤0.001.

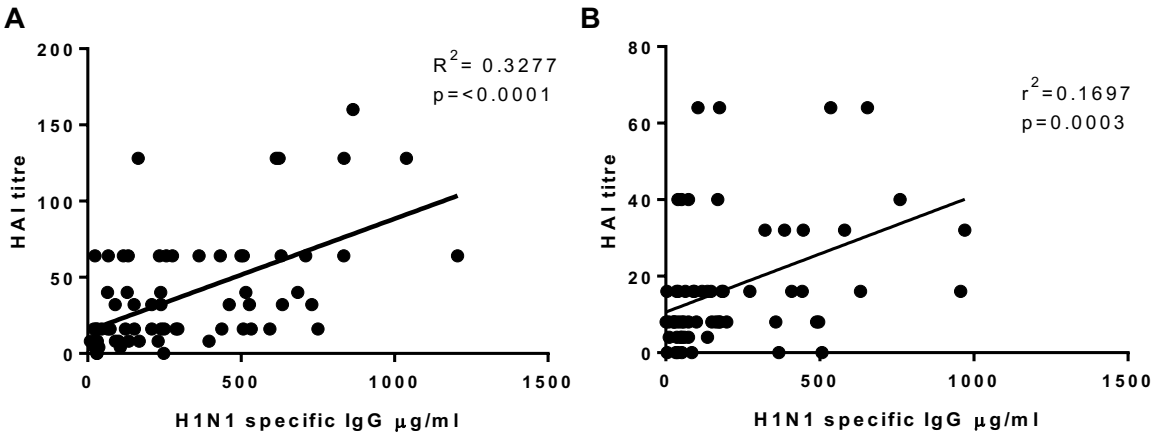

Figure 3.4. HAI and ELISA titre correlate in both cord and maternal samples
HAI measured in cord blood was compared to HAI titre in the same sample from cord (A) and mothers (B).

3.1.5. Projected decay in antibody in infant

The antibody transferred to the infant decays over time after birth, the half-life of HAI antibody in infants is between 43-45 days (53). We applied a half-life of 45 days to our data to investigate the rate of decay after immunisation. Based on this projection, the antibody titre in infants born to vaccinated mothers will still be significantly higher than children born to unvaccinated mothers 1.5 months after birth. This was seen for both IgG concentration by ELISA (Figure 3.5A) and HAI titre (Figure 3.5B). Based on this projection there is a rapid decline in transferred immunity, the infants from immunised mothers fall below the seroprotective level within the first month of life, in line with a previous study that showed immunisation conferred protection less than eight weeks (64). When subdivided by trimester of birth, infants born to mothers immunised in trimester 2 and 3 had a higher starting titres and remained higher than children of unvaccinated mothers or trimester 1 vaccinated mothers till 3 months after birth (Figure 3.5C and D).

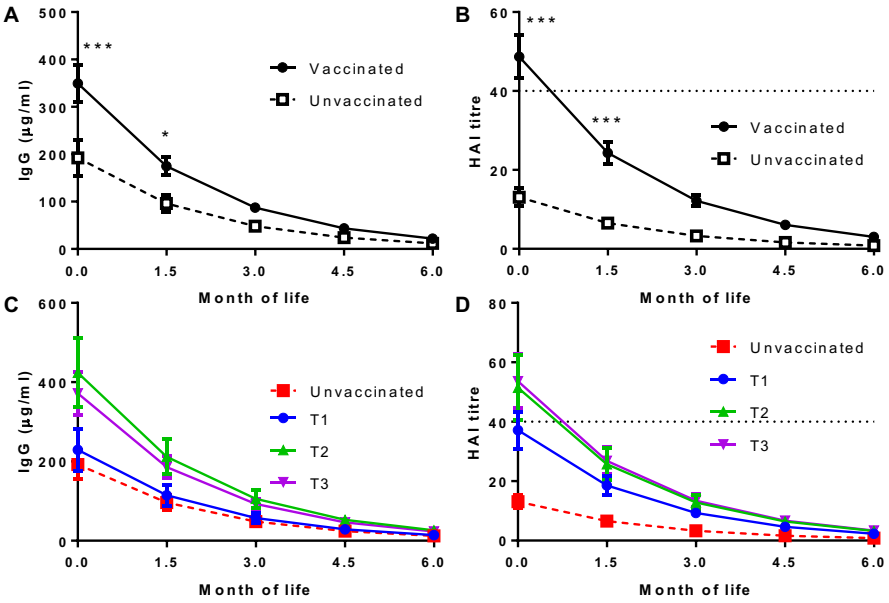

Figure 3.5. Projected decay kinetics of infant antibody titres.
IgG (A, C) and HAI (B, D) antibody titres in the infant were projected based on the predicted half-life of 45 days. * p<0.05, *** p<0.01 by two way ANOVA.

3.1.6. Immunisation between 24 and 5 weeks before birth led to the highest antibody titre in the cord samples

When thinking about the best time for maternal immunisation, an alternative consideration is the interval between immunisation and delivery. Infants from mothers vaccinated either 5-12 or 13-24 weeks before delivery had significantly higher H1N1 specific IgG concentration at birth compared to those from unvaccinated mothers (Figure 3.6A). Infants whose mother received vaccination either less than 4 weeks or more than 24 weeks before birth had lower IgG concentration than those whose mothers were vaccinated between 5-24 weeks. Mothers vaccinated 13-24 weeks before delivery had the highest IgG concentration among all vaccination groups at birth and the concentration was significantly higher than those vaccinated more than 24 weeks before delivery or unvaccinated mothers (Figure 3.6B).

HAI titres were also compared by interval to delivery. Newborns from mothers vaccinated 5-12 weeks before delivery had the highest HAI titre (Figure 3.6C). Infants born to mothers vaccinated 13-24 weeks before delivery also had significantly higher HAI titre at birth than infants from unvaccinated mothers. Interestingly, administration of influenza vaccine less than 4 weeks before delivery achieved the lowest HAI titre in newborns when compared with other timing of vaccination. Maternal HAI titres at birth were similar regardless of the timing of vaccination during pregnancy (Figure 3.6D). In terms of seroprotection, administration of maternal influenza vaccine 5-12 weeks before delivery conferred the highest percentage of infants with HAI titre ≥ 40 (69.7%). This data suggests that immunising too soon before birth leads to less antibody transferred to the infant.

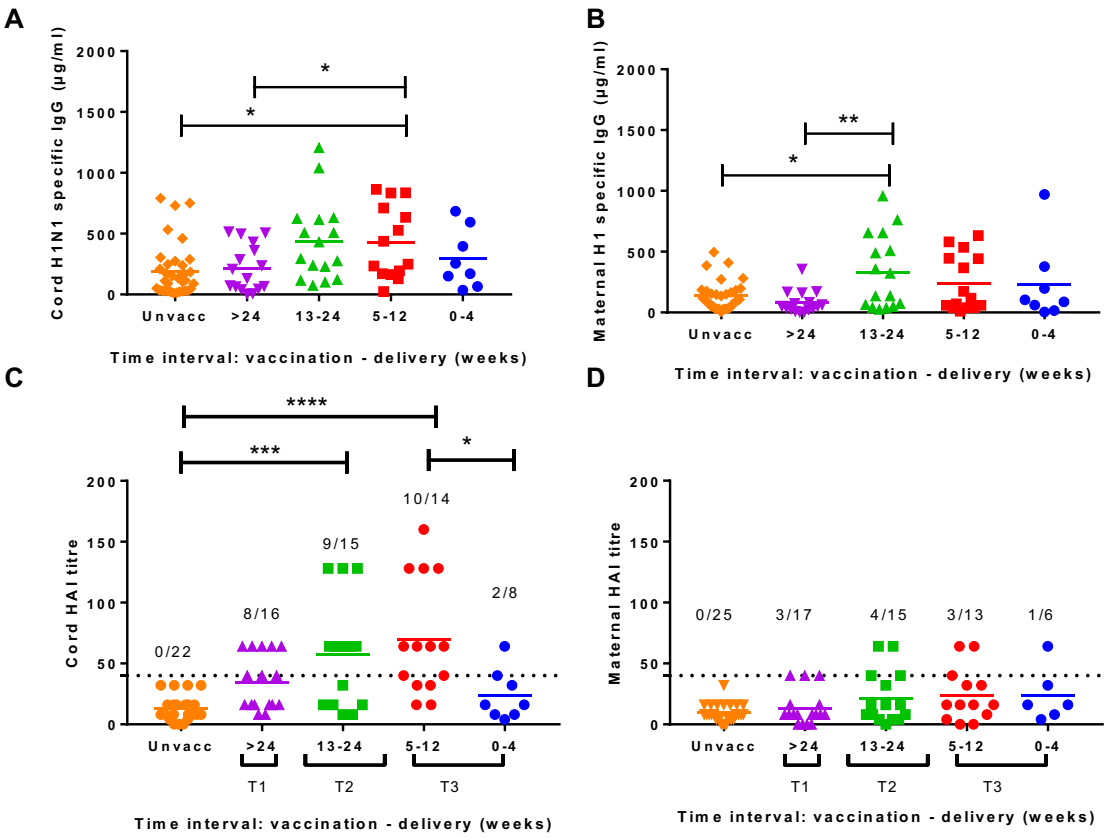

Figure 3.6. The effect of time interval between maternal vaccination and delivery on anti-influenza titres.

Individuals were grouped by the time interval between vaccination and delivery. H1N1 specific IgG concentration were measured by ELISA in cord (A) and maternal serum (B). H1N1 specific HAI titre was measured by HAI assay in cord (C) and maternal (D) serum samples. The dotted line represents the level of seroprotection (HAI titre ≥1:40). One way ANOVA was performed to compare the means of HAI titre in each group. Line represents mean. *p≤0.05, ***p≤0.001, ****p≤0.0001.

3.1.7. Season of birth

Unlike other vaccines, the influenza vaccine changes annually to reflect changes in the circulating virus (Table 2.1). Furthermore, the peak of influenza virus infection is seasonal – in the Northern Hemisphere the season runs from September to March. The combination of the seasonality of influenza and the changes in the strains included in the vaccine mean that the licensed vaccine is available in the UK each year from September to March. This has an impact on when mothers can be vaccinated. We wanted to determine how season affected antibody concentration in both mothers and their infants. There was no significant difference in the median date of birth between vaccinated and unvaccinated groups (Table 3.1). Pooling both vaccinated and unvaccinated mothers and their children, the anti-H1N1 IgG concentration was significantly greater at birth in infants born in the influenza season ($p<0.05$, Figure 3.7A). This seasonal difference was also observed in the cord antibody concentration of children born to unvaccinated (Figure 3.7B), but not vaccinated mothers (Figure 3.7C). We then looked more closely at the relationship between month of birth and antibody concentration. When both groups were combined, there was a decline in antibody concentration (Figure 3.7D) from children born at the start of the influenza season (September) to those born before the beginning of the next season (August). This relationship between birth month and antibody concentration was significant in the unvaccinated (Figure 3.7E) but not the vaccinated (Figure 3.7F) group. Since season had an effect on antibody concentration we then compared month of birth with trimester of vaccination. There was a significant difference with the median month of birth for the trimester 3 immunised group earlier in the influenza season than the trimester 1 group (Figure 3.7G, Table 3.2). This was a reflection of the month of vaccination, which for all three groups had a median of October – the start of the influenza season (Figure 3.7H).

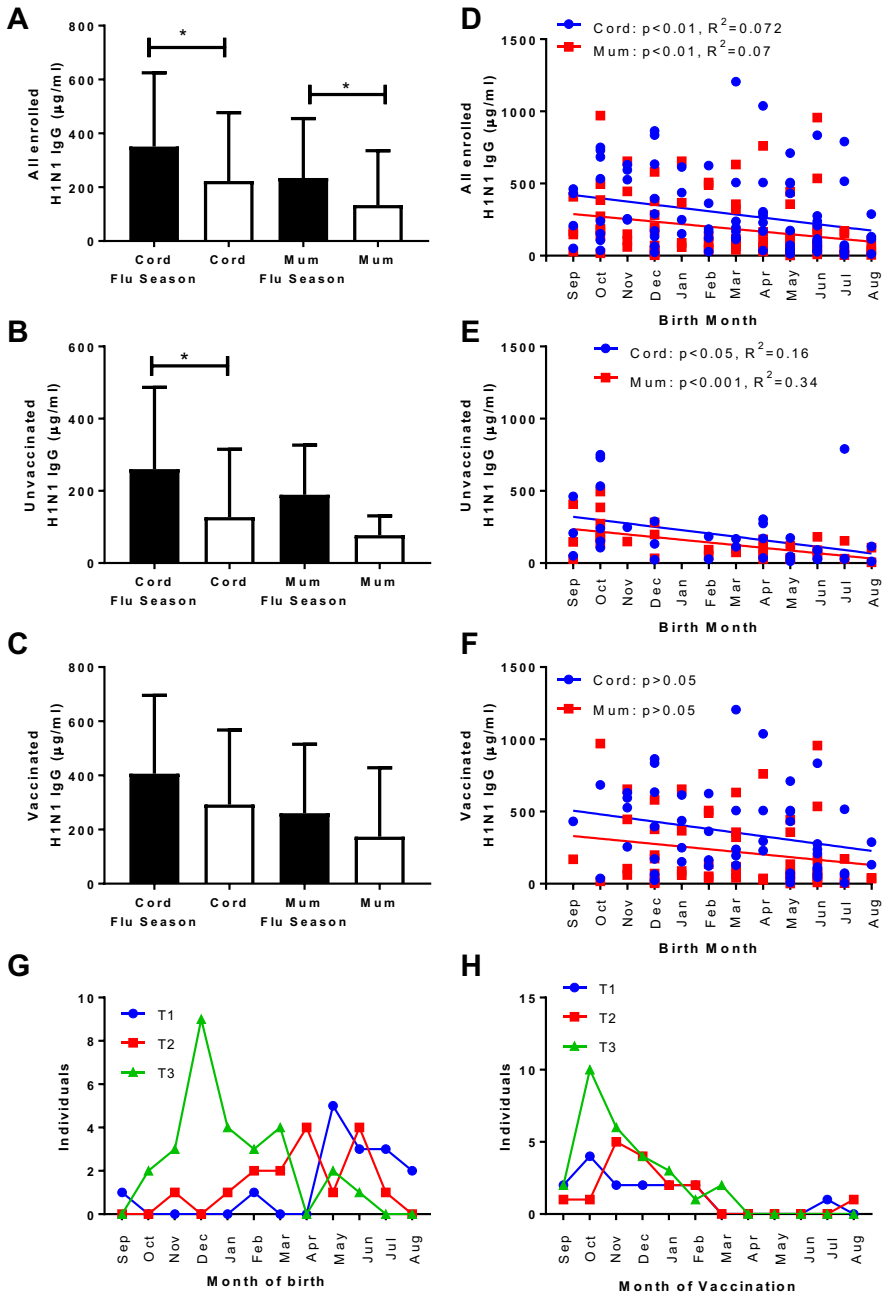

Figure 3.7. Effect of season of birth and immunisation on antibody concentration in mother and cord.

Maternal and cord samples were grouped by the season of birth comparing those in flu season (born Sept-March) to those outside it (Apr-Aug). Antibody concentration were compared by birth season in all donors (A), unvaccinated (B) and unvaccinated donors (C). Concentration in all (D), unvaccinated (E) and vaccinated (F) groups were compared against month of birth, ordered from the start of the influenza season (Sept). Month of birth (G) and month of vaccination (H) for children born to mothers vaccinated in different trimesters.

3.1.8. Determinants of influenza specific antibody titre in cord and maternal blood samples

Having observed that a number of factors contributed to antibody levels in pregnant women and their infants at birth, we used univariate (Table 3.3) analysis to determine their relative contributions. Vaccination status significantly impacted cord IgG concentration (Table 3.3) and both cord and maternal HAI titre (Table 3.4). Vaccination trimester and birth season also had a significant impact on cord and maternal antibody titres. Parity, maternal age, maternal BMI and mode of delivery did not significantly impact vaccine-specific antibody levels in mothers or their infants. The gestational week at birth and birth weight were not included in the univariate analysis because all the infants included in the study were delivered at term pregnancy (mean gestational age 39.9 weeks) and no infants were categories as low birth weight (defined as less than 2.9kg male or 2.8 kg female at 40 weeks).

			H1N1 Cord IgG Concentration			H1N1 Maternal IgG Concentration	
		N	*Mean (95%CI)*	p *value*	*N*	*Mean (95%CI)*	p *value*
Vaccination Status	Vaccinated	53	352.6 (273.9-431.4)	**0.006**	54	221.7 (151.8-291.5)	0.062
	Un-vaccinated	35	191.7 (117.78-265.7)		34	133.3 (92.3-174.2)	
Season of Birth	April-Aug	43	223.1 (145.0-301.3)	**0.026**	41	133.7 (70.0-197.4)	**0.029**
	Sept-Mar	45	351.2 (268.9-433.5)		47	234.5 (169.7-299.2)	
Trimester of vaccination	Un-vaccinated	35	191.7 (117.8-265.7)	-	34	133.3 (92.3-174.2)	-
	Trim 1	14	229.0 (112.9-345.1)	0.580	14	81.4 (26.2-136.5)	0.150
	Trim 2	15	423.7 (235.5-611.9)	**0.005**	15	323.6 (152.1-495.1)	**0.003**
	Trim 3	24	380.4 (262.3-498.4)	**0.005**	25	239.1 (133.4-344.8)	0.038
Parity	Primiparous	49	266.7 (188.2-345.2)	0.398	48	152.5 (93.5-211.6)	0.098
	Multiparous	39	316.2 (229.3-403.1)		40	229.5 (156.7-302.3)	
Mother's age	20-34	57	296.8 (223.0-370.6)	0.704	57	117.3 (122.7-231.9)	0.552
	35-42	31	273.7 (178.3-369.1)		31	206.3 (119.0-293.6)	
Mode of Delivery	Vaginal	52	254.9 (175.0-334.7)	0.120	53	154.4 (100.1-208.6)	0.054
	C-Section	34	348.4 (264.1-423.7)		33	247.5 (161.7-333.3)	
Maternal BMI	<25	47	294.6 (226.2-362.9)	0.902	48	173.4 (115.3-231.5)	0.362
	≥25	38	301.9 (198.6-405.4)		37	217.4 (136.7-298.0)	

Table 3.3 Univariate analysis of factors determining the H1N1 antibody concentration rate in cord and maternal blood samples.

*p values are results of paired sample t-test relative to the un-vaccinated group.

			HAI Cord Titre			HAI Maternal Titre	
		N	*Seropositive (%)*	p *value*	*N*	*Seropositive (%)*	p *value*
Vaccination Status	Vaccinated	53	29 (54.7%)	**<0.001**	50	11 (22.0%)	**0.011**
	Un-vaccinated	22	0 (0.0%)		25	0 (0.0%)	
Parity	Primiparous	38	17 (44.7%)	0.274	38	4 (10.5%)	0.304
	Multiparous	37	12 (32.4%)		37	7 (18.9%)	
Mother's age	20-34	47	18 (38.3%)	0.932	49	7 (14.3%)	0.898
	35-42	28	11 (39.3%)		26	4 (15.4%)	
Mode of Delivery	Vaginal	43	17 (39.5%)	0.943	46	8 (17.4%)	0.434
	C-Section	31	12 (38.7%)		28	3 (10.7%)	
Gestation	35-39 weeks	30	10 (33.3%)	0.439	26	4 (15.4%)	0.898
	≥40 weeks	45	19 (42.2%)		49	7 (14.3%)	
Season of Birth	April-Aug	34	13 (38.2%)	0.944	33	4 (12.1%)	0.581
	Sept-Mar	41	16 (39.0%)		42	7 (16.7%)	
Maternal BMI	<25	62	23 (37.1%)	0.171	64	7 (10.9%)	**0.004**
	≥25	10	6 (60.0%)		8	4 (50.0%)	
Trimester of vaccination	Trim 1	14	8 (57.1%)	0.932	14	3 (21.4%)	0.763
	Trim 2	14	8 (57.1%)		14	4 (28.6%)	
	Trim 3	25	13 (52.0%)		22	4 (18.2%)	

Table 3.4 Univariate analysis of factors determining the HAI seroprotection rate in cord and maternal blood samples

3.1.9. Multivariate analysis of factors associated with cord blood antibody level

For multivariable analyses I defined positive outcome as being in the upper 50th percentile of H1N1 IgG cord blood concentration, this percentile ranged from 194.0 – 1205.6 µg/mL (Table 3.5). 74.1% of cord blood samples with a protective HAI titre >40 fell within this group reflecting a correlation between binding ELISA binding and functional antibody. Vaccinated mother-infant pairs were 3.7 (1.5-9.3) times more likely to be in the upper half of H1N1 IgG concentration relative to unvaccinated pairs, controlling for season of birth. Mother-infant pairs vaccinated in trimesters one, two and three had were 3.4, 8.1 and 2.5 times more likely to be in the upper H1N1 cord blood concentration group respectively, relative to unvaccinated pairs, after adjustment for season of birth. This was significant only for trimester two (p=0.005). Mother/infant pairs that gave birth/were born in the flu season had significantly increased odds of 3.1 (1.1-9.3) of being in the upper H1N1 IgG cord concentration group, relative to those in the non-flu season, after controlling for vaccination status and trimester of vaccination.

		Odds Ratio (95%CI)	**p Value**	**Adjusted Odds Ratio (95%CI)**	**p Value**
Vaccination status^{+}	Unvaccinated	1	-	1	-
	Vaccinated	3.6 (1.5-8.9)	**0.005**	3.7 (1.5-9.3)	**0.006**
Trimester of vaccination*	Unvaccinated	1	-	1	-
	Trimester 1	2.2 (0.6-7.8)	0.228	3.4 (0.9-13.9)	0.084
	Trimester 2	5.9 (1.6-23.1)	**0.009**	8.1 (1.9-34.3)	**0.005**
	Trimester 3	3.6 (1.2-10.8)	**0.020**	2.5 (0.8-8.0)	0.120
Birth Season ^{x}	April-August	1	-	1	-
	September-March	2.29 (0.9-5.4)	0.057	3.1 (1.1-9.3)	**0.046**

Table 3.5 Crude and adjusted odds ratios for being in the upper 50th percentile of H1N1 IgG cord blood concentration.

3.2. Discussion

3.2.1. Conclusion

The aim of the study was to assess the effect of seasonal influenza vaccination at different stages of pregnancy on the antibody concentration in both infants and mothers. The key question was to use influenza vaccination as a model to determine the ideal timing of vaccination during pregnancy, which leads to the maximal protection of the infants. A previous study has reported a significant increase in HAI antibody titre in the cord blood of infants born to mothers vaccinated at second and third trimester (53). However, there were no data on either the infant or the mother antibody concentration when the influenza vaccination took place at first trimester of pregnancy. In this study, I reported for the first time the impact of maternal influenza immunisation at three different trimesters on paired mother and infant antibodyconcentration. I found that infants from mothers who received influenza vaccine at 5-24 weeks before delivery (second trimester to early third trimester) had significantly higher HAI antibody titres against influenza A (H1N1) and H1N1 specific IgG concentration than infants with unvaccinated mothers. Interestingly, when maternal vaccination took place later than 4 weeks before delivery, the HAI and IgG concentration were much lower at the point of birth in both the mother and the child.

3.2.2. Timing of immunisation

The goal of maternal influenza immunisation is twofold to protect the mother from influenza infection throughout pregnancy and also to protect the infant during first months of life. There is a fine balance between protecting the mother by inducing an early antibody response and maintaining high maternal antibody throughout high risk period during pregnancy verses conferring strong and prolonged protection for the infants by inducing high antibody concentration at birth. The timing of vaccination may be critical in achieving this balance.

Pregnant women at all stages are at higher risk of respiratory illness associated with both seasonal and pandemic influenza infections (65). The risk of influenza associated commodities increases as pregnancy proceeds to later stage (66). Dodds et al. showed that pregnant women in the second and

third trimester of pregnancy were at the highest risk of respiratory illness during influenza season compared to those at first trimester (67). Furthermore, women in the second and third trimester of pregnancy suffered the most from influenza associated comorbidities with higher proportion of ICU admission and mortality rate compared to those at first trimester during 2009 pandemics (68). The current regime of influenza vaccination in the United Kingdom is to provide the seasonal influenza vaccine to women at all stages of pregnancy as soon as the vaccine is available (usually later September, early October). In the current study administration of influenza vaccine 5-24 weeks (second trimester and third trimester) before delivery conferred both high HAI titre and seroprotection rate in both the mother and infants at birth. In contrast, vaccination either late in the third trimester or earlier than 24 weeks before delivery did not seem to confer prolonged protection for the infants with low HAI titre at birth. This suggests that a more refined opportunity window of maternal influenza vaccination (5-24 weeks before delivery) may be a more effective strategy to reduce risk of influenza infections for the infants during first months of life.

3.2.3. Mechanisms of maternal-fetal antibody transfer

The high antibody concentration in newborns induced by immunisation at the second or third trimester during pregnancy is the result of cumulative effect of antibody transfer across placenta. Transfer of IgG antibody across placenta is facilitated by fetal Fc receptor, which begins at gestation week 13 (second trimester). The efficiency of transport increases overtime (69). Administration of influenza vaccine to mother 5-24 weeks before delivery matches with the time period of maximal antibody transfer. This gives enough time for the mother to develop a high level antibody concentration and enabling prolonged fetal maternal antibody transfer. The antibody transfer reaches its peak efficiency at Gestational Week 37. However, the shorter time period of exposure of antibody resulted from immunisation at later stage of pregnancy (4 weeks before delivery) may not be compensated by the peak transfer efficacy.

3.2.4. Elevated neonatal antibody concentration

In this study, I also found a consistent trend of higher HAI antibody and influenza specific IgG antibody concentration in cord blood samples, when compared to the maternal blood samples at delivery. A similar pattern of concentration was also observed in the study immunising pregnant women with inactivated H1N1 monovalent vaccine (40). The increased blood volume in pregnant women may contribute to the lower antibody concentration as the result of dilution effect. Kat et al. showed that the total IgG antibody concentration reduces as pregnancy proceeds to later stages (39). The active transfer of antibody across the placenta may be another factor for the lower antibody concentration in pregnant women. Other studies have also found that there were higher levels of antibody specific for pertussis antigen and pneumococcal antigens in cord blood samples than maternal samples as a result of transplacental transfer (70–72).

3.2.5. Comparison of ELISA and HAI

Another interesting aspect of this study is the comparison of two methods analysis (HAI assay and antigen-specific IgG ELISA) in antibody concentration in cord blood samples from maternal immunisation of influenza, which was performed for the first time. Zhu et al. showed that the concentration of IgG specific to H1N1 antigen was correlated to HAI titre in the serum of patients infected with Influenza A(H1N1)pdm 09 in United State (73). Consistent with the previous study, I found that the HAI titre (H1N1) in cord blood sample was correlated with H1N1 specific IgG concentration measured by ELISA regardless of the maternal vaccination status. Infants with significantly high H1N1 IgG concentration resulted from maternal immunisation 5-24 weeks before delivery also showed high HAI titre. In contrast, infants with mother vaccinated less than 4 weeks before delivery had significantly lower HAI titre. Interestingly, the IgG concentration in these populations was lower but not significantly different from those with high HAI titre. This suggests that there may be differences in the subtypes of antibody transferred in infants with mother vaccinated at different stage of pregnancy, in which infants with mother vaccinated 5-24 weeks before delivery received more functional neutralising antibody contributing to the protection.

There are several limitations of this study. First, the previous immunisation record of pregnant women was not available. Therefore, the effect of prior vaccination history on the induction of antibodies in mother and their infants at birth was not evaluated. There may be cross-reactivity of antibody from previous influenza immunisation or infection, which may explain the detectable HAI titre and antigen specific IgG concentration in unvaccinated women and their infants in this study (53). However, Kay et al. showed that the pregnancy status had greater effect on inducing antibody concentration from influenza vaccine during pregnancy than the previous immunisation before pregnancy (39). Second, as a result of being a nested study within a larger study, the sample size of this study was relatively small and overlapping with 3 influenza seasons, with different influenza vaccine administered (H3N2 and influenza B antigen). This may explain why the H3N2 and influenza B specific antibody concentration did not show similar pattern as the H1N1 antibody concentration observed in this study. Further studies with larger numbers of participants in the same influenza season is required to determine if there is a consistent pattern of differences in infant antibody concentration according to the timing of vaccination during pregnancy. Third, it is not possible to draw a final conclusion of the timing of vaccination during pregnancy on true vaccine efficacy since no follow up was conducted for the participants after delivery. Therefore, it is not possible to monitor the antibody level and its correlation with laboratory-confirmed influenza infection outcomes in infants of this study. However, previous studies showed that at least 63% of reduction of influenza infection in infants born to mother vaccinated during pregnancy and the protection was correlated with the increased antibody concentration (38,46). Overall, the literature confirmed the administration of influenza vaccine was clinically efficacious in preventing neonatal morbidity.

There are a number of considerations that are specific to maternal influenza vaccination. Unlike maternal RSV vaccination, the goal of maternal influenza immunisation is to protect both the mother from influenza infection throughout pregnancy and the infant during first months of life. However, it is of note that the peak of susceptibility to infection increases as pregnancy proceeds to later stage (74) with pregnant women at second and third trimester of pregnancy at the highest risk of respiratory illness during influenza season (67). Secondly the vaccine for influenza, unlike other

pathogens changes annually to reflect changes in the circulating strains of virus; this restricts the availability of the vaccine to certain times of the year. Changes in vaccine strain also affects vaccine efficacy against subsequent seasons: vaccination later in the influenza season to achieve optimum antibody transfer to the child may lead to the transfer of antibodies against the wrong strains.

4. Response of neonatal cells to infection

4.1. Introduction

Whilst it is an attractive strategy, maternal immunisation may not be sufficient to completely protect infants against all infections. The first hurdle is producing immunogenic vaccines, and whilst progress is being made with GBS and RSV vaccines, a candidate RSV antigen currently that is being evaluated in maternal vaccination studies recently failed in a phase III efficacy trial in the elderly (75). The second issue is that levels of transferred maternal antibody wane rapidly in the infant with an approximate half-life of 35 days, leading to concentration falling below protective levels within the first 5 months of life (76). So even if the vaccine elevates maternal antibody concentration (77), there is still a window of susceptibility in the infant; this is of particular concern in premature infants who may only receive a smaller amount of maternal antibody and will still be developmentally immature when antibody falls below the protective level. Therefore we need to provide additional immune protection to the child to close this window of susceptibility. In order to develop better, neonatal specific vaccines, we need to understand more about the neonatal immune response. Many studies have focused on studying the difference between neonatal and adult cytokine response with the stimulation of immune receptor ligands (78–81). Very few studies have used the live whole virus or bacterial stimulation to study the differences in cytokine response.

4.2. Result

4.2.1. GBS assay optimisation and stimulation of PBMC

Prior to running the assays we needed to optimise the growth and PBMC stimulation conditions for GBS. Cord blood and maternal blood samples must be processed within 8 hours of delivery, and the timing of receiving the samples was very unpredictable. GBS normally requires overnight culture before using it for infection, which is not feasible in this context. Therefore, the protocol for the GBS assay was optimised so that overnight culture was not required, whilst ensuring the same CFU of GBS was used for PBMC samples each time. To standardise GBS growth, a frozen aliquot of 500 µL GBS stock was added to 20 mL of THB and incubated at 37 °C, whilst shaking at 180 RPM. The optical density of the culture was measured every 30 minutes for 3 hours. It took 130 minutes culture time to reach OD 1.0 (Figure 4.1A). The concentration of the GBS liquid culture was approximately 1.0 x 10^7 CFU/mL at OD 1.0 (Figure 4.1B). Previous studies have shown GBS infection induces a strong TNFα response (82,83), therefore, the TNFα response was used to determine the optimal concentration of GBS for PBMC stimulation. PBMC were isolated from blood cones purchased from NHS Blood and Transplant centre and a series of concentrations of GBS from culture were added to 2 x 10^5 PBMC/well on 96-well plate for 24 hours. TNFα ELISA was used to measure the TNFα response in the supernatant (Figure 4.1C). The TNFα response was the highest when PBMC were stimulated with 1.0 x 10^7 CFU/mL of GBS (equivalent to MOI 20), this concentration was used for subsequent GBS stimulations of PBMC from cord blood, maternal and non-pregnant women control blood samples.

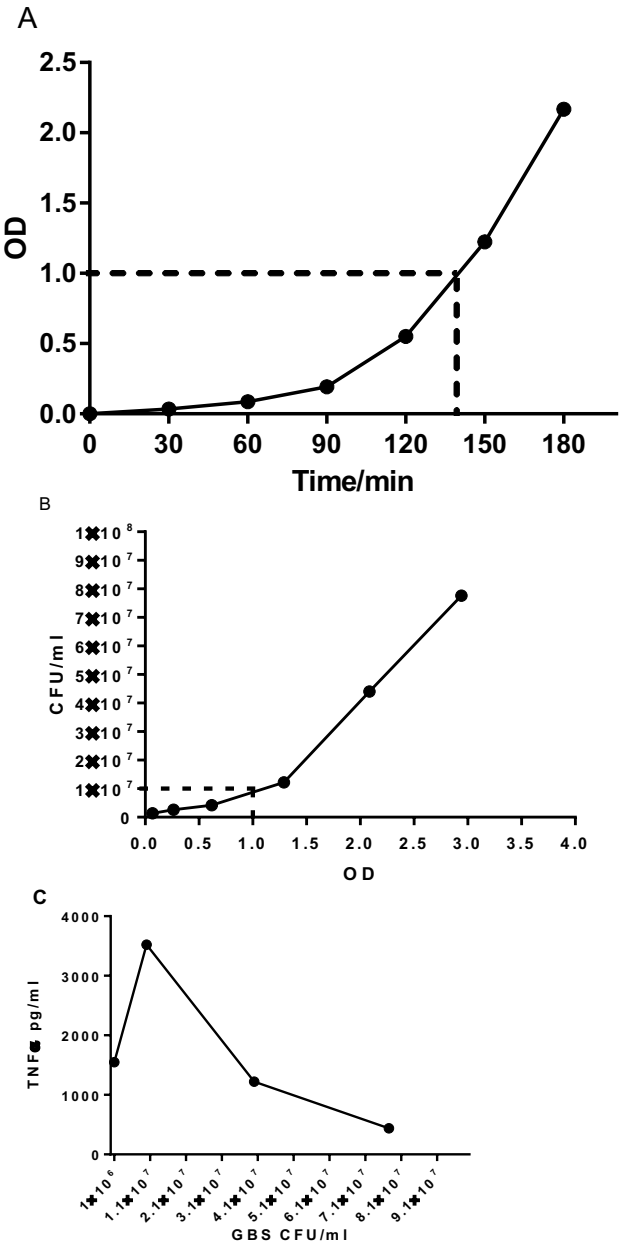

Figure 4.1. Optimisation of GBS stimulation on PBMC

A) Growth curve of GBS over three hours in liquid culture of THB. **B)** Measurement of GBS CFU in different OD. **C)** PBMCs were isolated from blood cones (NHS blood and transplant centre, UK). TNFα response was used as a positive control for determining the optimised concentration of GBS for subsequent assay for cord, maternal and non-pregnant women blood samples. TNFα response was measured by ELISA.

4.2.2. Optimisation of protocol of stimulating cord and maternal blood samples with viruses

IFNα plays an essential role in the defence against viral infections. Adapted from the protocol in the lab (Myriam Haltalli's protocol), the IFNα response was used to determine the optimal concentration of viral stimulation of PBMC. PBMC isolated from blood cones (NHS blood and transplant centre, UK) were used. 2 x 10^5 PBMC were incubated with a series of MOI of RSV for 24 and 48 hours. Supernatant was collected and IFNα response was measured by pan-IFNα ELISA (Figure 4.2A). PBMC stimulated with RSV at MOI 3 for 24 hours had the highest IFNα response. I then used this concentration for the subsequent experiments.

4.2.3. Comparison of PBMC and whole blood responses

Previous studies show that 1:10 diluted whole blood cultures can be an alternative assay to measure cytokine production when compared with a conventional PBMC assay (84,85). I used four cord blood samples to determine the optimal protocol for this study. Whole blood and PBMCs from the corresponding cord blood samples were incubated at 37 °C with either influenza virus (Figure 4.2B) or RSV (Figure 4.2C) at MOI 3 and the IFNα response was quantified by ELISA. As shown in the figure, the IFNα response from whole blood samples when stimulated with influenza virus was very low compared to PBMC from the same cord blood samples. A similar IFNα response was also observed in samples stimulated with RSV. I determined from this that isolating PBMC from cord and maternal blood samples for viral stimulation was the best method for measuring the immune responses in these samples.

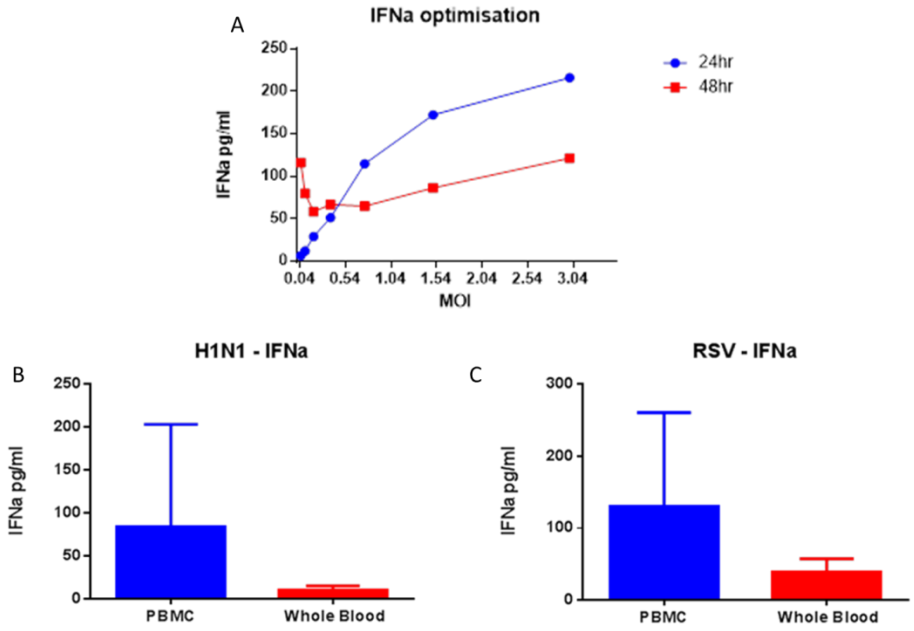

Figure 4.2. Optimisation of influenza virus and RSV stimulation of PBMC$_L$ and whole blood samples by measuring the IFNα response.

(A) PBMC was isolated from blood cones (NHS blood and transplant centre, UK) and a series of MOI of RSV A2 were used to stimulate the PBMC for 24 hours at 37 °C. Supernatant was used to measure IFNα response by pan-IFNα ELISA kit. PBMC and whole blood were obtained from four cord blood samples and incubated with A/England/195/2009 H1N1 influenza (B) and RSV A2 (C) at MOI 3 for 24 hours at 37 °C. Supernatant was collected after 24 hours and used to measure IFNα response with pan-IFNα ELISA kit. n=4.

4.2.4. Distinct immune profile of neonatal, maternal and adult PBMC after stimulating with different pathogens

PBMC isolated from cord blood (n=21) and blood from mothers post-partum (n=13) or non-pregnant women (n=9) were incubated with either influenza virus or RSV for 24 hours. PBMC from 4 pairs of matched cord and maternal blood and an additional 7 cord blood samples were stimulated with GBS for 24 hours. Supernatants from these stimulations were collected 24 hours post infection and analysed by Luminex and pan-IFNα ELISA to investigate the immune response profile. The Luminex platform allows quantification of multiple inflammatory mediators at the same time. In this project, 19 analytes (4 categories) were measured (Table 2.2).

To look at the overall patterns of response, Principal Component Analysis (PCA) was used to compress and transform the multivariate Luminex data. PCA was used to determine whether the different pathogens were a main contributing factor to the variability in terms of cytokine profile in the three populations, newborns, mothers and non-pregnant women. Two clusters were observed associated with differences in stimulation (Figure 4.3A); the cytokine profiles after viral infection were significantly different from those after bacterial infection (p<0.01). When comparing RSV and H1N1 exposure, two clusters were observed and the cytokine profiles from H1N1 infections were significantly different from those from RSV infections (Figure 4.3B, p<0.01). There were no significant differences in the global cytokine profiles between newborns, mothers, and non-pregnant women (Figure 4.3C and D). However, one of the limitations of PCA is it presents the majority of the variance in the data set but may miss out the differences in some data. Therefore, I explored the Luminex data by each individual cytokine measured. For the individual cytokine analysis, I have grouped the cytokines by function into inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-12 and TNF), antiviral and adaptive cytokines (IFNα, IFNβ, IFNγ and IL-2), chemokines and growth factors (CCL2, CCL4, CCL5, CCL8, CXCL8, CXCL9, CXCL10, TGFβ and GMCSF).

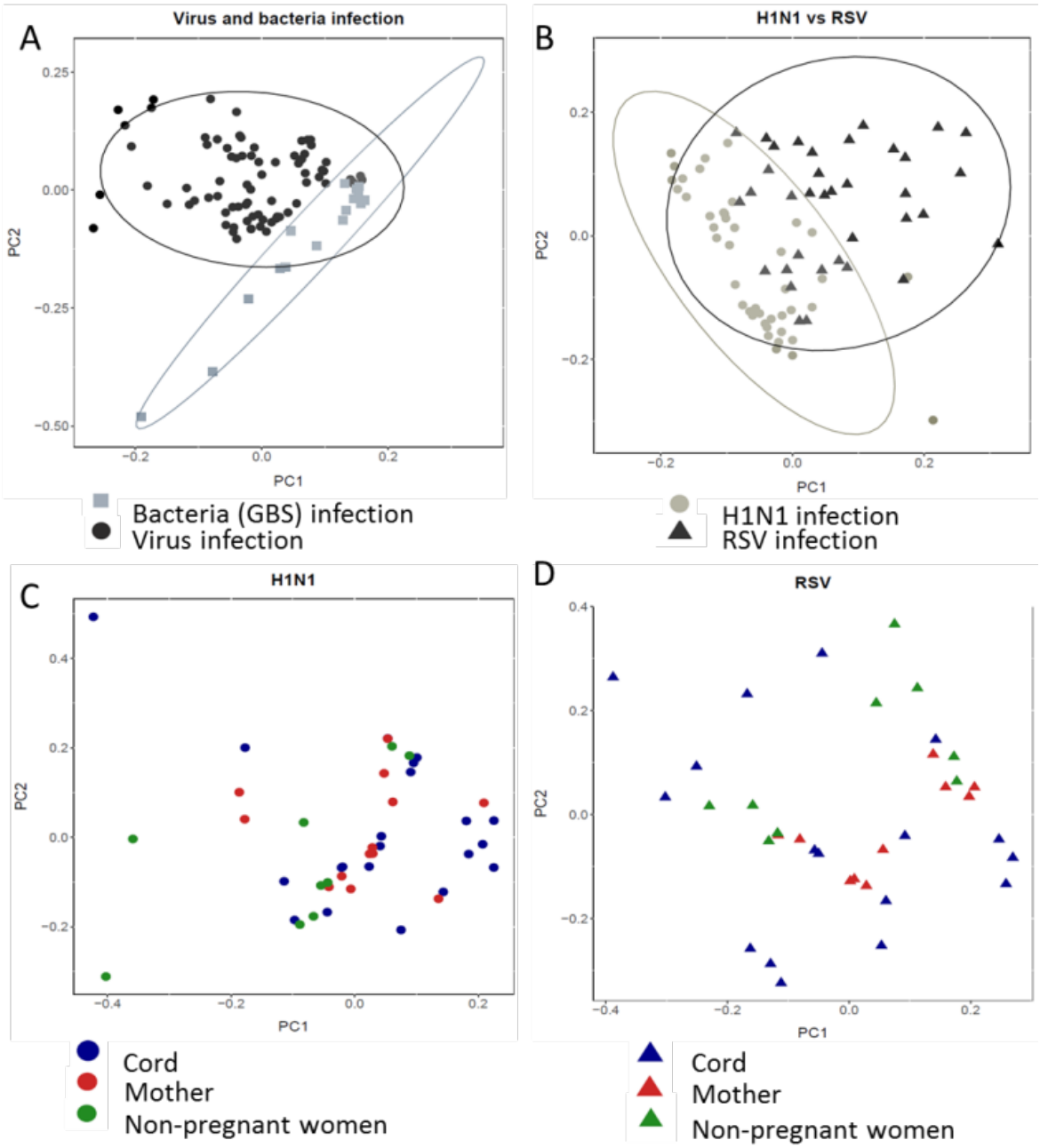

Figure 4.3. Principal component analysis of cytokine response to infection.
PBMC isolated from cord blood, maternal blood post-partum and non-pregnant mothers were stimulated with influenza, RSV or GBS for 24 hours. Cytokines were measured in the supernatant by Luminex. A custom Principal component analysis (PCA) script was developed to analyse the results. Differences in global cytokine response between bacterial and viral (A) and Influenza and RSV (B) infections were compared by PCA. Differences between global cytokine response for different donor types (cord, mother, non-pregnant) were compared for influenza (C) and RSV (D) by PCA. Number of donors, cord blood n=21, maternal n=13 and non-pregnant n=9.

4.2.5. Differential cytokine response after Influenza virus infection in cord or adult blood

After influenza virus stimulation, the majority of the inflammatory cytokines measured except for IL-6, had similarly low level of responses among the three populations (Figure 4.4). There was no significant difference in inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-12 and TNF) in PBMC derived from cord, maternal and non-pregnant women blood samples (Figure 4.5). Likewise antiviral, adaptive cytokines, chemokines and growth factors were expressed by cells from all three populations (Figure 4.5). Non-pregnant women had significantly higher IFNβ and IL-2 responses compared to cord blood samples, whilst other antiviral and adaptive cytokine responses were similar across the three populations (Figure 4.5). The CCL5 response to influenza virus in non-pregnant women was also significantly higher than newborns (Figure 4.5). Notably, the CXCL10 and GM-CSF responses after influenza virus stimulation in non-pregnant women were significantly higher than PBMC derived from both cord and maternal blood samples (Figure 4.5).

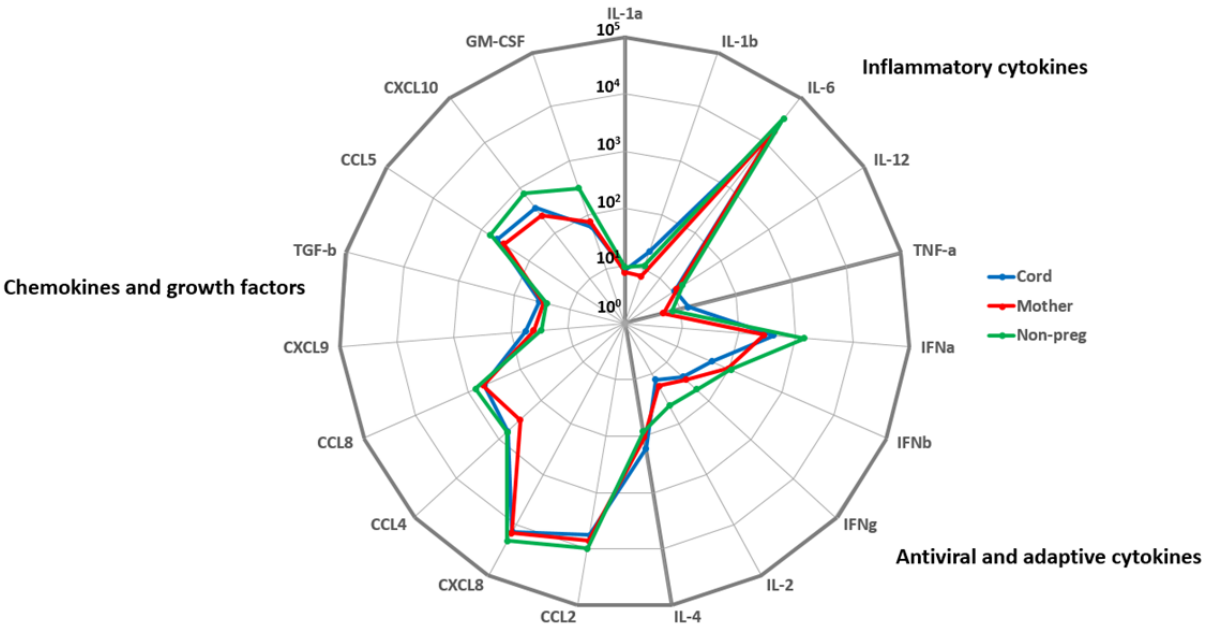

Figure 4.4. Overall profile of cytokine response to influenza infection.
Spider plot showing cytokine responses to infection by PBMC derived from cord blood, maternal blood post-partum and non-pregnant mothers.

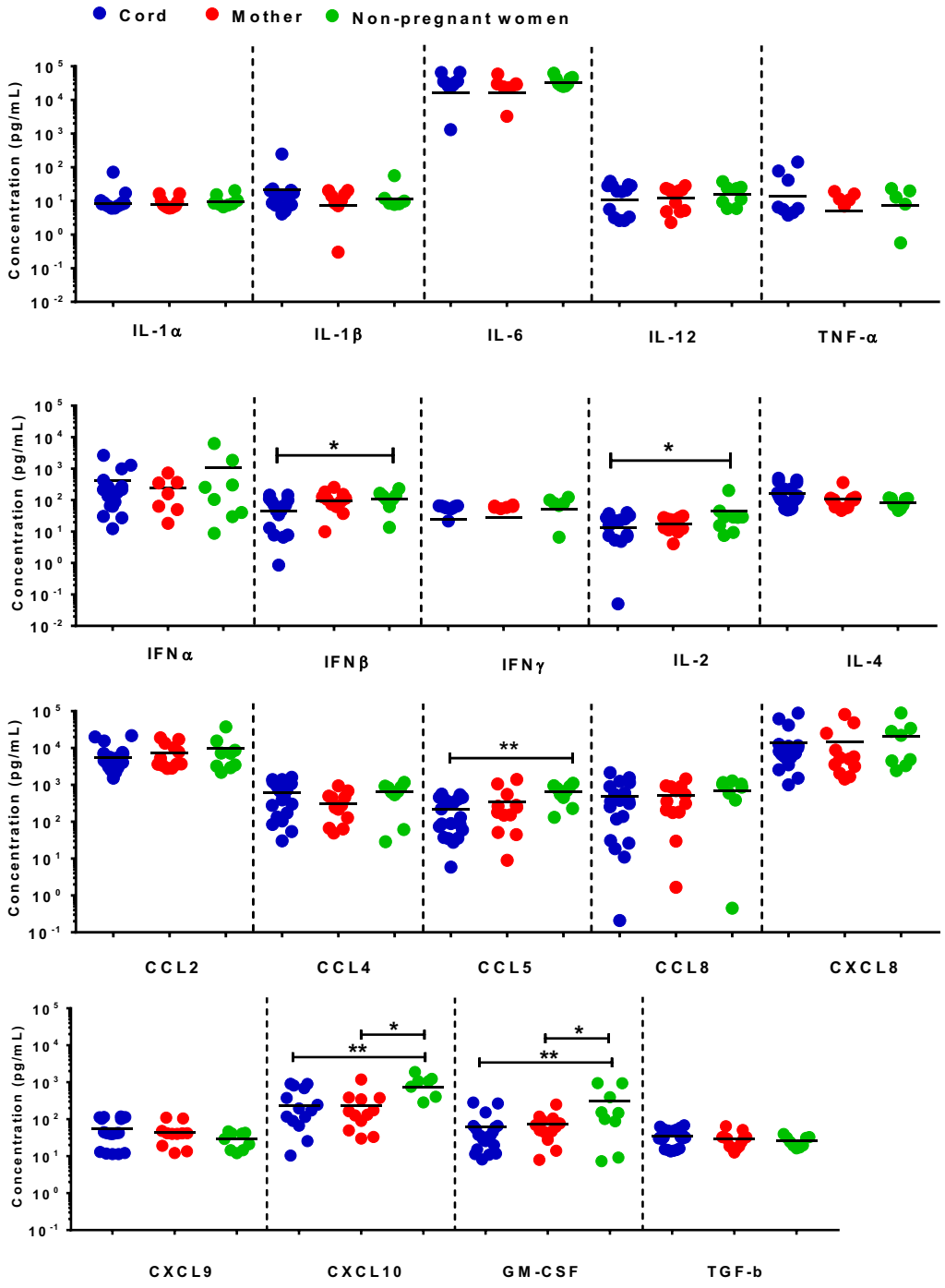

Figure 4.5. Individual cytokine responses to influenza infection

Individual cytokine responses in supernatant after PBMC derived from cord blood, maternal blood post-partum and non-pregnant mothers infected with influenza. Points represent individual donors, lines represent means. Cord blood n=21, maternal n=13 and non-pregnant n=9. Comparisons by ANOVA with Tukey's post test, * $p<0.05$, ** $p<0.01$.

4.2.6. RSV infection

Unlike H1N1 stimulation, all three populations had moderate to high levels of inflammatory cytokines expression after RSV infection (Figure 4.6). There was no significant difference between the three groups of donors in individual inflammatory cytokines (Figure 4.7). The pattern of antiviral and adaptive cytokine expression after RSV infection among the three populations was similar to those after H1N1 stimulation. PBMC derived from the blood samples of all three populations had similar levels of antiviral and adaptive cytokine responses after RSV infection (Figure 4.7). All three populations had high levels of chemokine and growth factor expression except for CXCL9, TGFβ and GM-CSF (Figure 4.7). Interestingly, the TGFβ response in cord blood samples was significantly higher than in maternal blood samples after RSV stimulation.

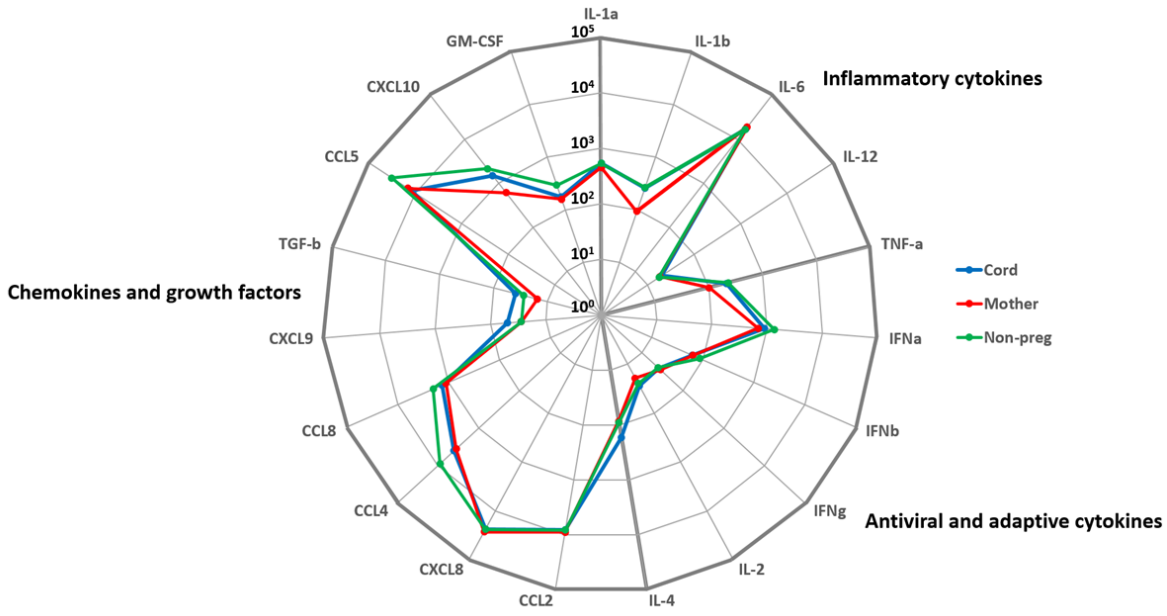

Figure 4.6. Overall profile of cytokine response to RSV infection.
Spider plot showing cytokine responses to infection by PBMC derived from cord blood, maternal blood post-partum and non-pregnant mothers.

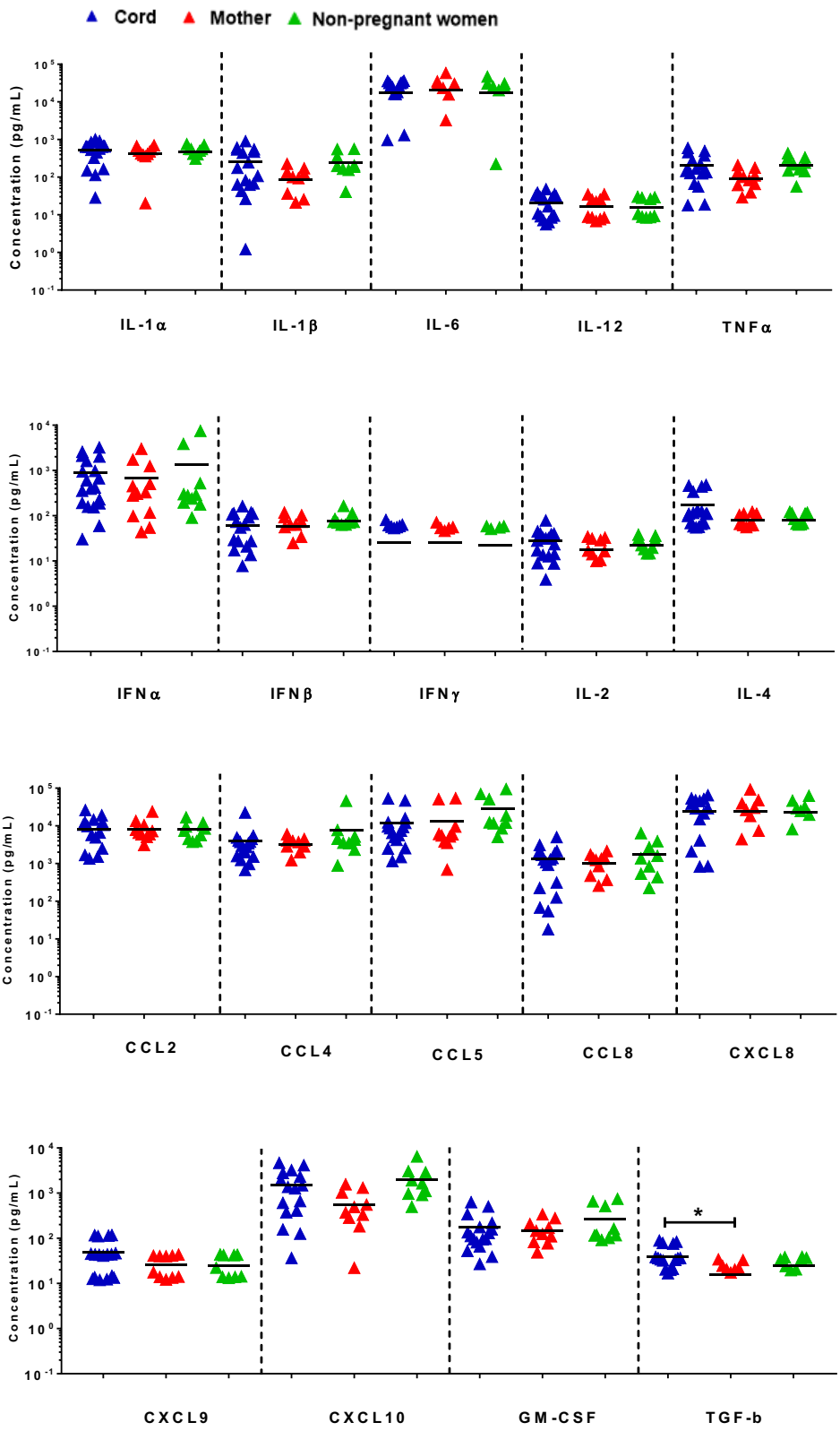

Figure 4.7. Individual cytokine responses to RSV

Individual cytokine responses in supernatant after PBMC derived from cord blood, maternal blood post-partum and non-pregnant mothers infected with RSV. Points represent individual donors, lines represent means. Cord blood n=21, maternal n=13 and non-pregnant n=9. Comparisons by ANOVA with Tukey's post test, * $p<0.05$, ** $p<0.01$.

4.2.7. GBS infection

A smaller number of samples were available for infection with GBS. After GBS stimulation, both cord and maternal samples show a distinct pattern of cytokine responses compared to viral stimulation. For inflammatory cytokines, both populations had high levels of IL-1β and IL-6 to GBS infection (Figure 4.8). Both populations also had high levels of TNFα and IL-4 among the other antiviral and adaptive immune responses. In terms of chemokines and growth factors, GBS stimulation induced a high level of CXCL8 in both populations. However, there were no significant differences in any of the immune parameters measured when compared between cord and maternal blood samples (Figure 4.9).

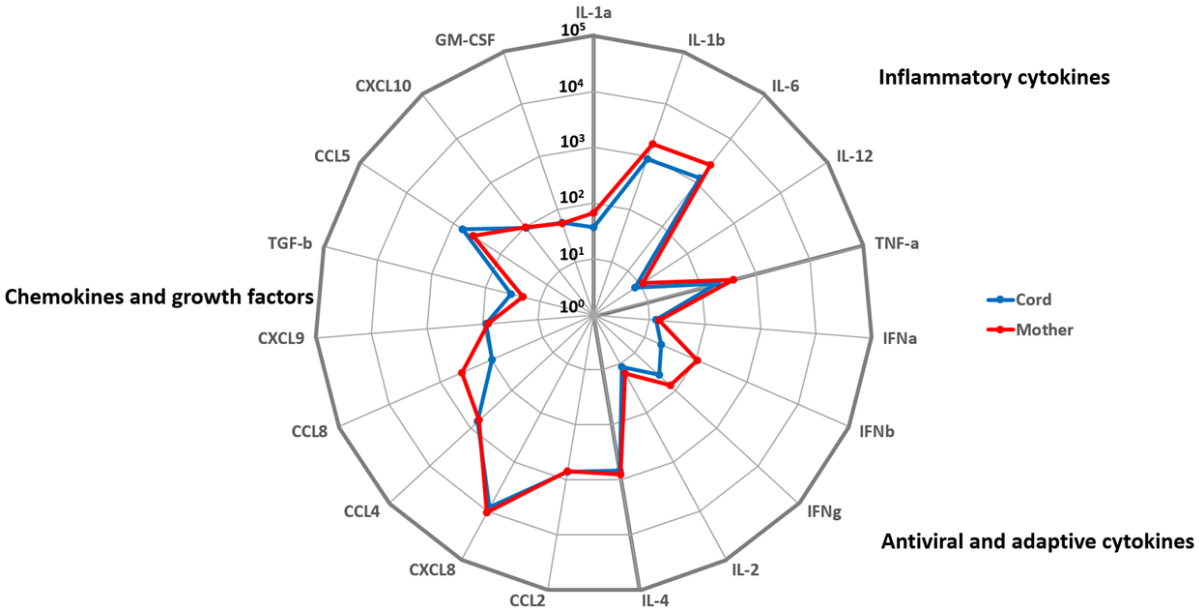

Figure 4.8. Overall profile of cytokine response to GBS infection.
Spider plot showing cytokine responses to infection by PBMC derived from cord blood and maternal blood post-partum.

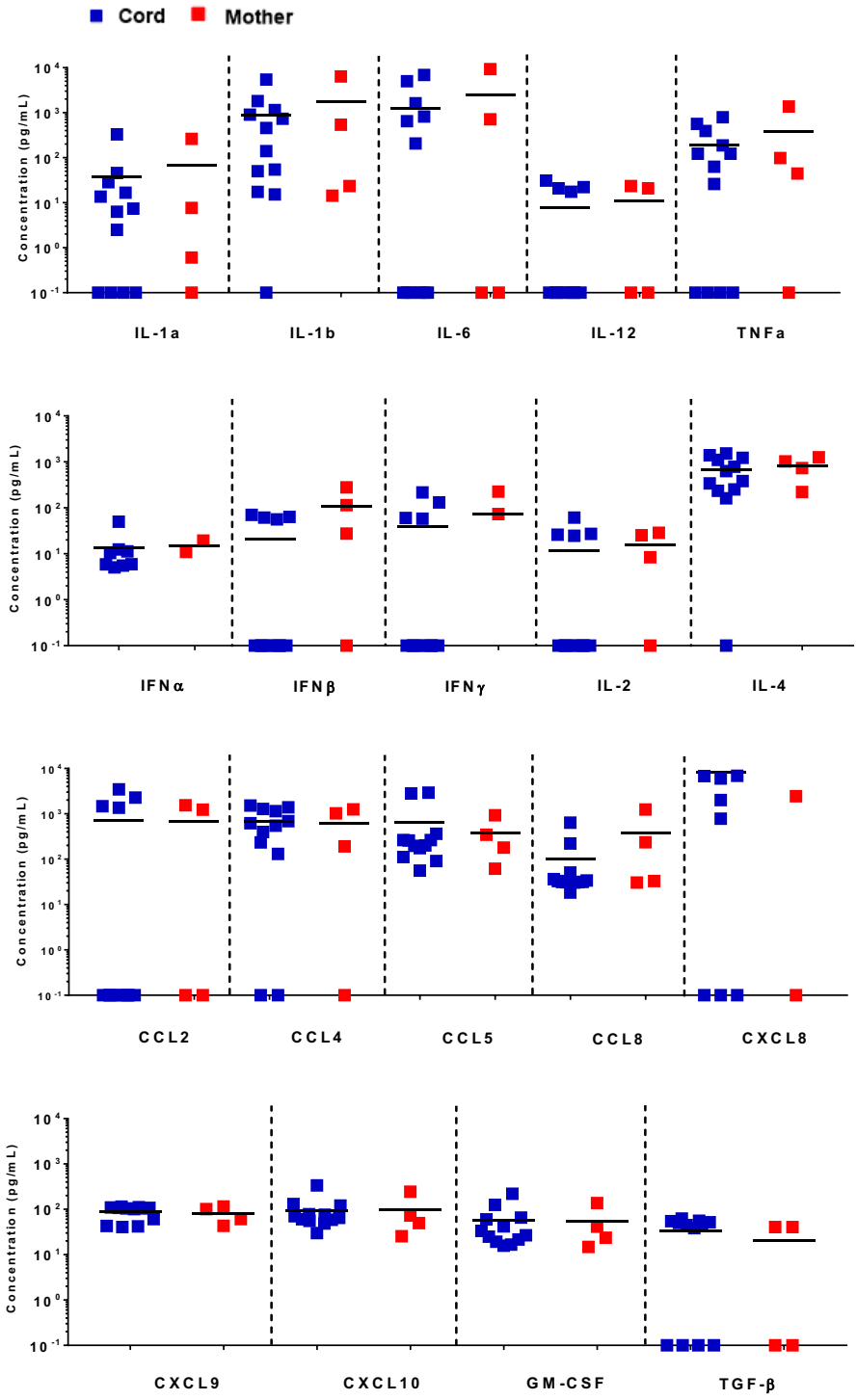

Figure 4.9. Individual responses to GBS infection.
Individual cytokine responses in supernatant after PBMC derived from cord blood and maternal blood post-partum were infected with GBS. Points represent individual donors, lines represent means. Cord blood n=12, maternal n=4.

4.2.8. Pre-existing antibody response to influenza virus or RSV did not significantly affect the innate cytokine responses among three populations.

One of the confounding factors for studying the cytokine response to viral infection might be pre-existing antibody. The majority of adults have encountered RSV and influenza infection at some point. This could also affect neonates, because neonatal antibody is mostly of maternal origin for the first 6 months of life due to the transplacental transfer (32). Therefore, I wanted to find out whether pre-existing antibodies have a significant impact on cytokine responses to influenza virus and RSV in this experimental setup. H1N1 and RSV specific IgG were measured in plasma samples by antigen-specific ELISA. The six cytokine/chemokine responses that were significantly different among the three populations in the Luminex assay were compared with the antigen-specific IgG responses. No correlation was found between IFNβ or IL-2 responses and H1N1 specific IgG in either cord or mothers (IFNβ: r^2_{cord}=0.221, r^2_{mother}=0.258; IL-2: r^2_{cord}<0.01, r^2_{mother}=0.202) (Figure 4.10A and B). There was no correlation between either CCL5 or CXCL10 response to the influenza virus, and pre-existing H1N1 specific IgG in both cord and maternal blood samples (CCL5: r^2_{cord}=0.132, r^2_{mother}=0.068; CXCL10: r^2_{cord}=0.117, r^2_{mother}=0.296) (Figure 4.10C and D). The GM-CSF response to influenza virus was not correlated with pre-existing H1N1 specific IgG titre in neonates and their mother (r^2_{cord}=0.097, r^2_{mother}=0.011) (Figure 4.10E). For RSV infection, TGF-β responses in cord and maternal blood samples had no correlation with the pre-existing RSV specific-IgG response (r^2_{cord}<0.001, r^2_{mother}=0.051) (Figure 4.10F). These results suggest that the pre-existing pathogen specific antibody does not have a significant impact on the innate cytokine response to influenza virus and RSV in the experimental setup.

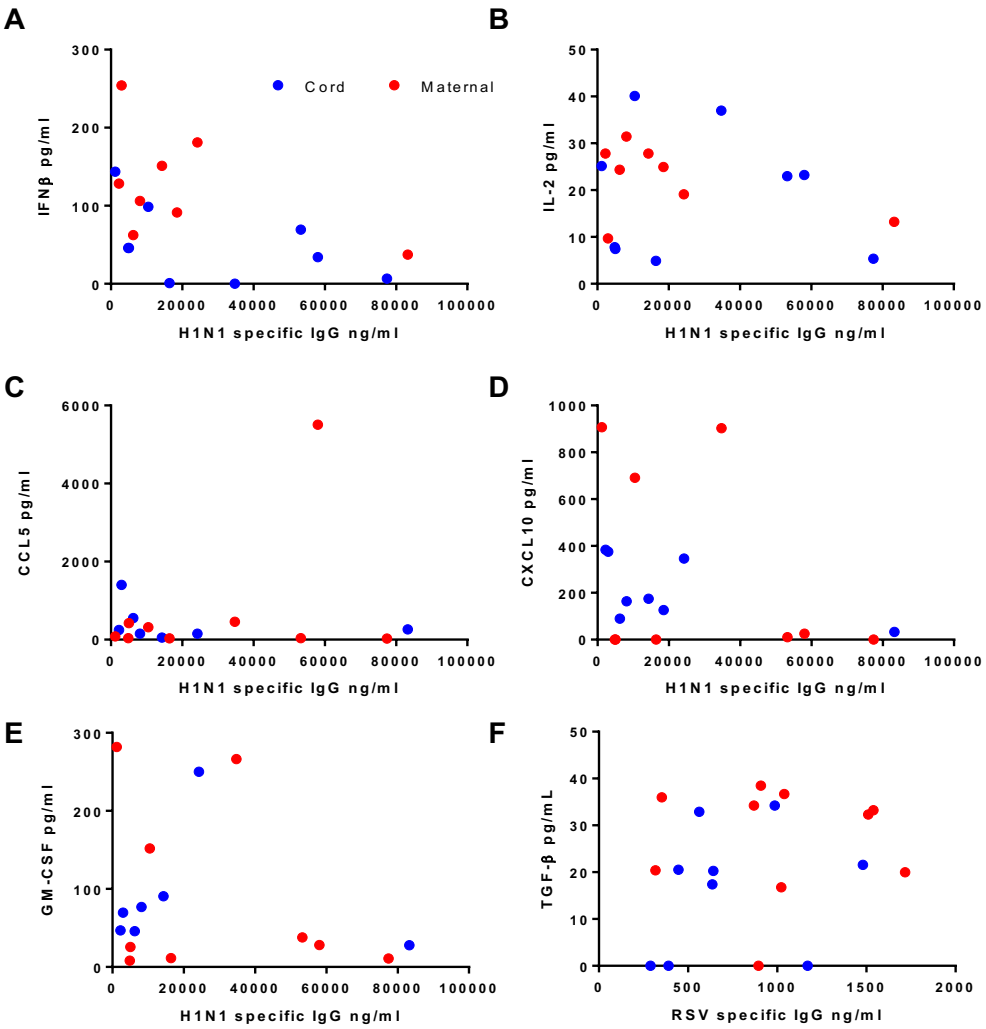

Figure 4.10. H1N1 specific IgG, RSV specific IgG and flu vaccine did not have a significant impact on innate immune response among three populations in the experimental context.
Plasma was collected by centrifugation from cord, maternal and non-pregnant women blood samples upon arrival. H1N1 specific IgG **(A, B, C, D, E)** and RSV specific IgG **(F)** in plasma were measured by antigen-specific ELISA. IFNα response was measured by pan-IFNα ELISA. n = 10 - 15

4.2.9. Effect of cellular composition on responses to viral infection

One possible explanation for the differences in innate cytokine responses between neonates and healthy adults is distinct immune cell compositions of these two populations. This led me to investigate the mechanism of the differential cytokine responses in neonates by flow cytometry.

4.2.10. Optimisation of FACS experimental protocol

4.2.10.1. Flow Cytometry Panel Development

To enable the characterisation of immune cell composition, a flow cytometry panel was designed (Table 2.3). The design of the flow panel allowed characterisation of immune cell populations in PBMC derived from cord and healthy adult blood samples after H1N1 stimulation, focusing on the identification of NK cell, monocyte, and dendritic cell (DC) populations. Intracellular cytokine staining of CXCL10 and GM-CSF were also included in the panel to identify the cell populations responsible for different cytokine production. The selection of antibodies and fluorochromes was based on the surface markers of cell populations of interest and fluorochrome brightness. Cell surface markers that were at low-level of density were paired with brighter fluorochromes and vice versa.

4.2.10.2. FACS antibody Selection and Titration

FACS antibody titration was performed using PBMC isolated from blood cones (NHS blood and transplant centre, UK) to determine the optimal concentration of staining. For CD14, HLA-DR, CD16 and CD123 antibodies, titration was started from 2 fold of the recommended concentration, making up total volume of 50 µL and adding to the cells. A 2-fold serial dilution was performed across the plate. For the rest of the antibodies, titration was started from the recommended concentration and a 2-fold serial dilution was performed across the plate. To avoid the bleeding over between different staining channels, only the channel of one fluorochrome was opened each time during acquisition of the stained cells. The optimal concentration of staining was determined by the greatest difference in MFI between positive and negative populations (Figure 4.11). The concentrations were defined for use in subsequent studies based on the acquired data.

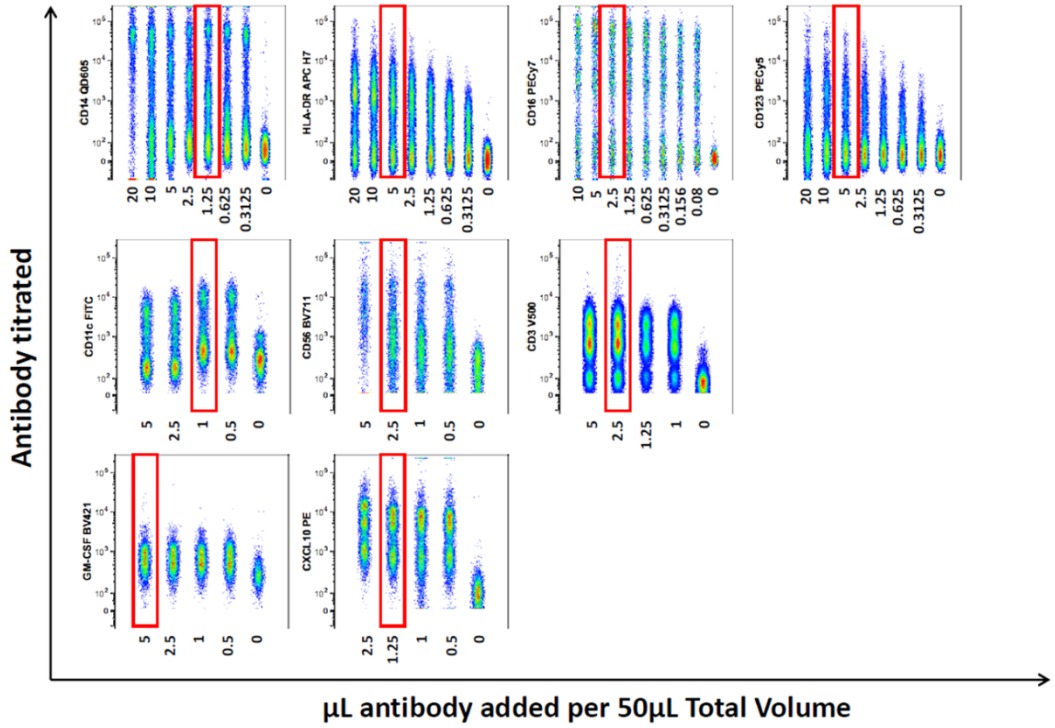

Figure 4.11. Antibody titration for flow cytometry
PBMC from blood cones were incubated with increasing concentrations of antibody prior to acquisition on BD Fortessa flow cytometer. Only the channel of one fluorochrome was opened each time during acquisition of the stained cells. CD14, HLA-DR, CD16 and CD123 titration were performed by Dr Hannah Cheeseman.

4.2.10.3. Fluorescence Minus One (FMO)

FMO was used to allow the proper interpretation of flow cytometry data in the context of spectral overlap due to multicolour flow cytometry panel. It was performed using PBMC isolated from blood cones (NHS blood and transplant centre, UK), and was used to identify and gate the cell populations of interest. Staining the PBMCs using a full panel with one fluorochrome removed sequentially enables the identification of the spread of fluorescence into the channel measured and determination of where the gate should be placed (Figure 4.12). The final gates and voltages were based upon this data.

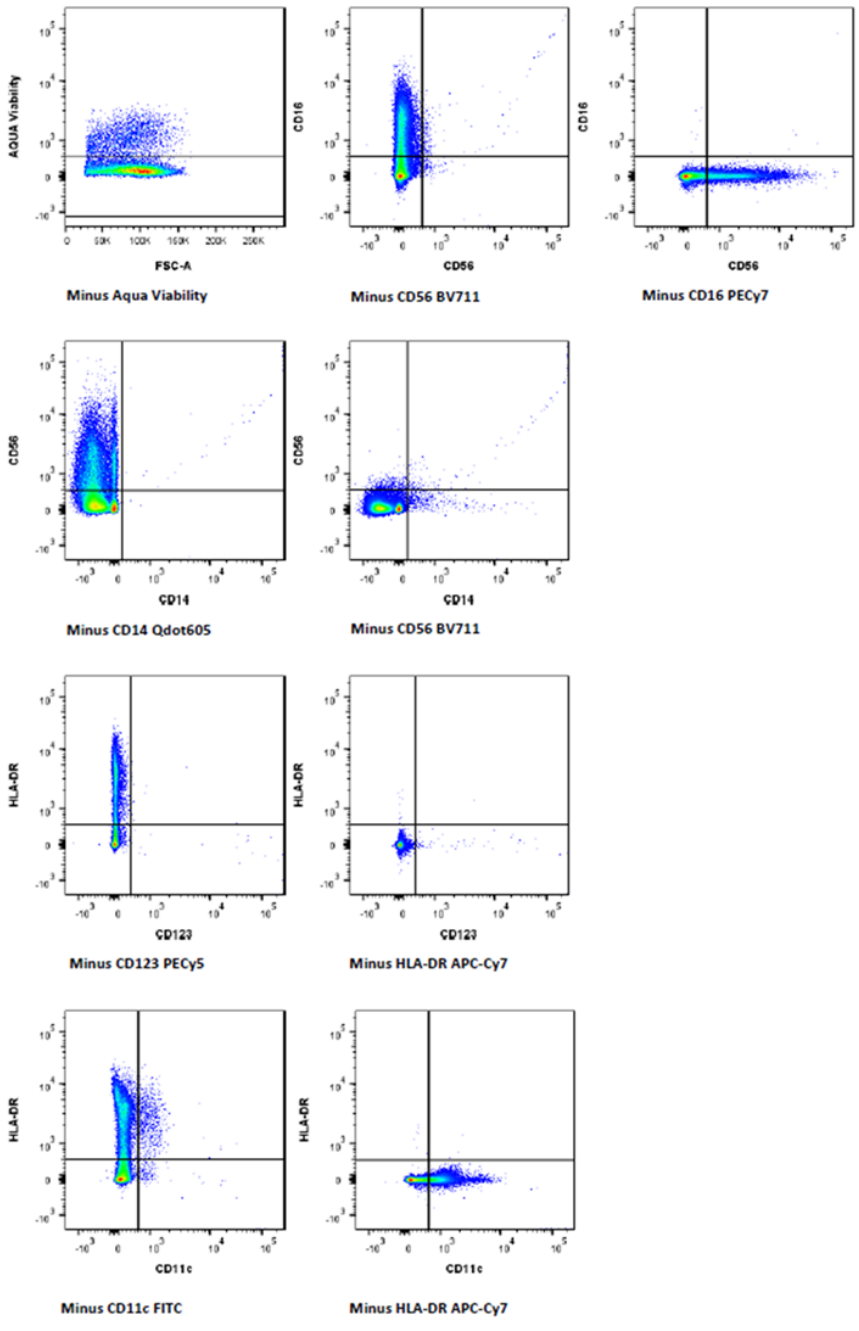

Figure 4.12. FMO analysis Fluorescence Minus One analysis was performed on PBMC isolated from blood cones. Sample data from an individual donor presented.

4.2.10.4. Flow Gating strategy

The data generated in the FMO experiment enabled the development of a gating strategy of cell populations of interest (Figure 4.13). This gating strategy was applied in all subsequent experiments.

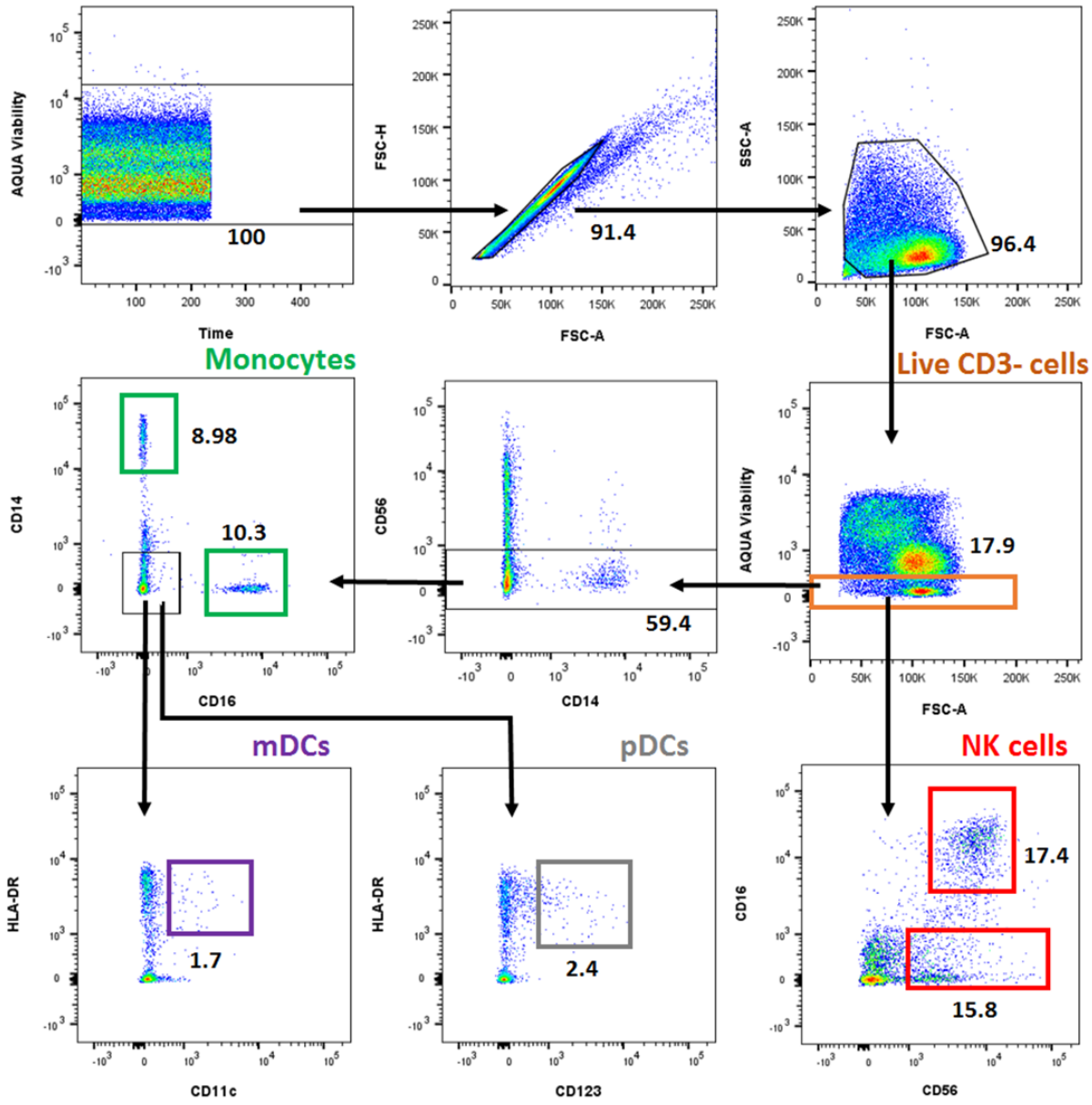

Figure 4.13. Gating strategy used for flow cytometry
Figure demonstrates gates and antibody staining used to define cell subsets in the subsequent studies. Live, singlet cells were first selected as CD3- to exclude T cells, before subdividing into NK cells (CD16/ CD56+), monocytes (CD14+/CD16-), myeloid dendritic cells (mDC: CD11c+/ HLA-DR+) or plasmacytoid dendritic cells (pDC: CD123+/ HLA-DR+). Data from an individual donor.

4.2.10.5. Cell stimulation optimisation

Having established the surface staining FACS panel to determine different cell types, it was also necessary to set up the intracellular staining. Various conditions were tested to obtain the optimal condition for CXCL10 expression using intracellular staining (ICS). This included the dose of virus, two different MOIs of H1N1 virus, the length of stimulation and the defrosting process used unrested vs rested defrosting. Unrested PBMC were defrosted by washing with pre-warmed R10 media twice, followed by centrifugation at 528 xg for 5 minutes, prior to H1N1 infection for 7, 15, or 24 hours (Figure 4.14B,D,F). Rested PBMC were defrosted in pre-warmed R10 media and rested at 37 °C overnight prior to stimulation with influenza virus for 7, 15, or 24 hours (Figure 4.14C, E, G). Optimal conditions were determined by generating the largest difference in MFI of CXCL10-PE signal compared to negative control (Figure 4.14A). The background signal of CXCL10-PE was similar when comparing the two defrosting conditions. Unrested PBMC stimulated with MOI 0.5 H1N1 for 24 hours generated the peak signal of CXCL10-PE (Figure 4.14F). The subsequent experiments were carried out using this protocol.

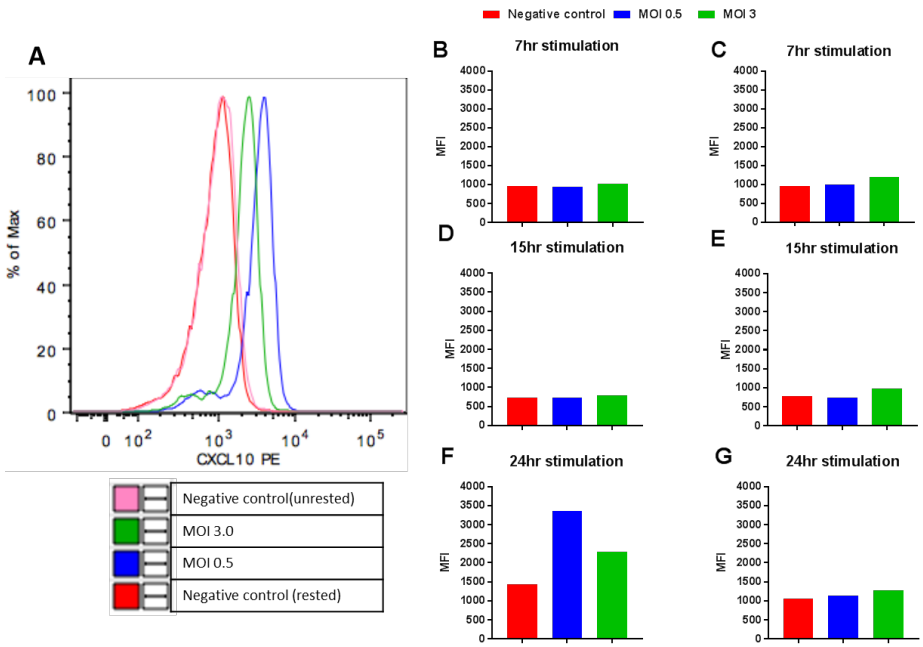

Figure 4.14. Optimisation of cell stimulation for detection of CXCL10
Example acquisition plot for CXCL10 (A). Unrested (B, D, F) or rested (C, E, G) PBMC were stimulated with influenza for 7 (B, C), 15 (D, E) or 24 hours (F, G) before fixing, permeabilising and staining.

4.2.11. Characterisation of immune cell composition and CXCL10 expression in PBMC isolated from cord and adult blood samples

The Luminex data showed a significant difference in the CXCL10 response to H1N1 infection of cells from cord and non-pregnant adults. To investigate whether this was driven by a difference in CXCL10 expression level in cells, stimulation with H1N1 was performed. Basic characterisation of immune cell composition and the associated CXCL10 production for each cell type was performed using frozen PBMC isolated from cord and adult blood samples. Frozen cell aliquots were thawed and stimulated with influenza virus for 24 hours before harvesting for FACS analysis. CD3+ cells were gated out to exclude any adaptive cell populations.

A comparison of NK cells, Monocytes, and dendritic cells (DCs) between the two populations was made. There were no significant differences in the percentage of NK cells (either CD56+CD16- or CD56+CD16+ cells) isolated from cord and adult blood samples (Figure 4.15A). The level of CXCL10 expression was similar for both NK cells subtypes between neonates and adults (Figure 4.15B).

A trend towards higher percentage of CD14+CD16- monocytes was observed in cells isolated from cord blood samples, though it did not reach statistical significance (Figure 4.15C). Both populations had similar percentages of CD14-CD16+ monocytes after H1N1 simulation. Interestingly, cells isolated from adult blood samples had significantly greater CXCL10 expression among CD14+CD16- monocytes, when compared with cells from cord blood samples after H1N1 stimulation (Figure 4.15D).

There was a greater percentage of CD11c+HLA-DR+ DCs (Myeloid DC, mDC in cells isolated from adult blood samples) after H1N1 stimulation, when compared with cells isolated from cord blood samples (Figure 4.15E). Due to the small percentage of mDCs in cells isolated from cord and adult blood samples, it was not possible to analyse the CXCL10 expression among these populations. Although there was no statistical significance, a trend for increased expression of CD123+HLA-DR+ DCs (Plasmacytoid DC, pDC) in cells isolated from adult blood samples was also observed, when compared with those from cord blood samples (Figure 4.15E).

Altogether, the phenotypic analysis of the three cell subsets of interest (NK cells, monocytes and DCs) and the corresponding CXCL10 expression showed that, there was a distinct immune composition and different pattern of CXCL10 expression in cells isolated from cord blood compared to those from adult blood samples after H1N1 stimulation.

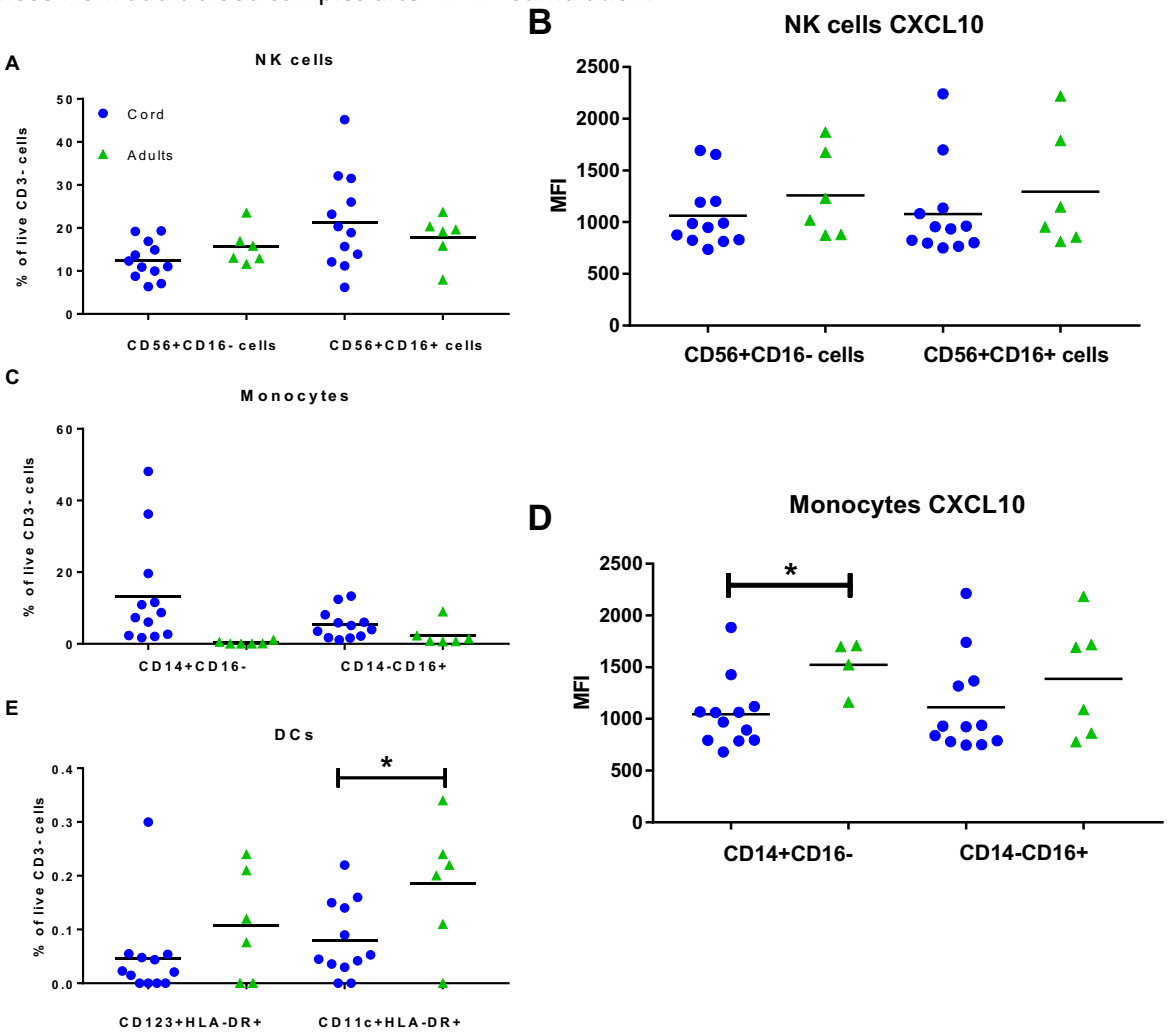

Figure 4.15. Differences in Cell populations and CXCL10 production after stimulation of cord and non-pregnant donor PBMC

PBMC derived from cord (n=12) and non-pregnant (n=6) adult blood were infected with influenza for 24 hours before harvesting for FACS analysis. CD3+ cells were gated out to exclude any adaptive cell populations. Cells were gated as described in figure 4.13 and grouped into NK cells (A), monocytes (C) and DCs (E). Expression of CXCL10 was measured by intracellular staining in NK cells (B) and monocytes (D). Comparisons by ANOVA with Tukey's post test, * $p<0.05$.

4.2.12. Innate immune response profile in lungs of neonatal and adult mice after Influenza virus or RSV infection

One of the limitations of using PBMC to study the immune response of infections is that immune cells in the blood may have distinct responses to immune cells in tissues (86). Since access to human neonatal tissue is not practical, *in vivo* studies using neonatal mice are an alternative way to measure the immune response in the respiratory tract after influenza virus or RSV infections.

Neonatal (7-8 days) and adult (6-8 weeks) mice were infected intranasally with influenza virus or RSV. Mice were culled 24 hour post infection. Cytokine and chemokine levels in the lungs were measured by Luminex.

4.2.13. *Influenza infection in neonatal mice*

The lungs of influenza infected adult mice had significantly higher levels of IL-1α and IL-1β compared to infected neonatal mice (Figure 4.16A). Similar level of IL-6, IL-12 and TNFα were found in the lungs of infected neonates and adult mice (Figure 4.16B). For chemokine responses, adult mice after influenza infection showed higher levels of CCL3, CCL5, CXCL1, CXCL2 and CXCL10 in the lungs compared to neonatal mice (Figure 4.16C, D). Compared to the naïve control group, influenza infection induced an elevated level of CXCL1 and CXCL10 response in the infected adult mice (Figure 4.16D).

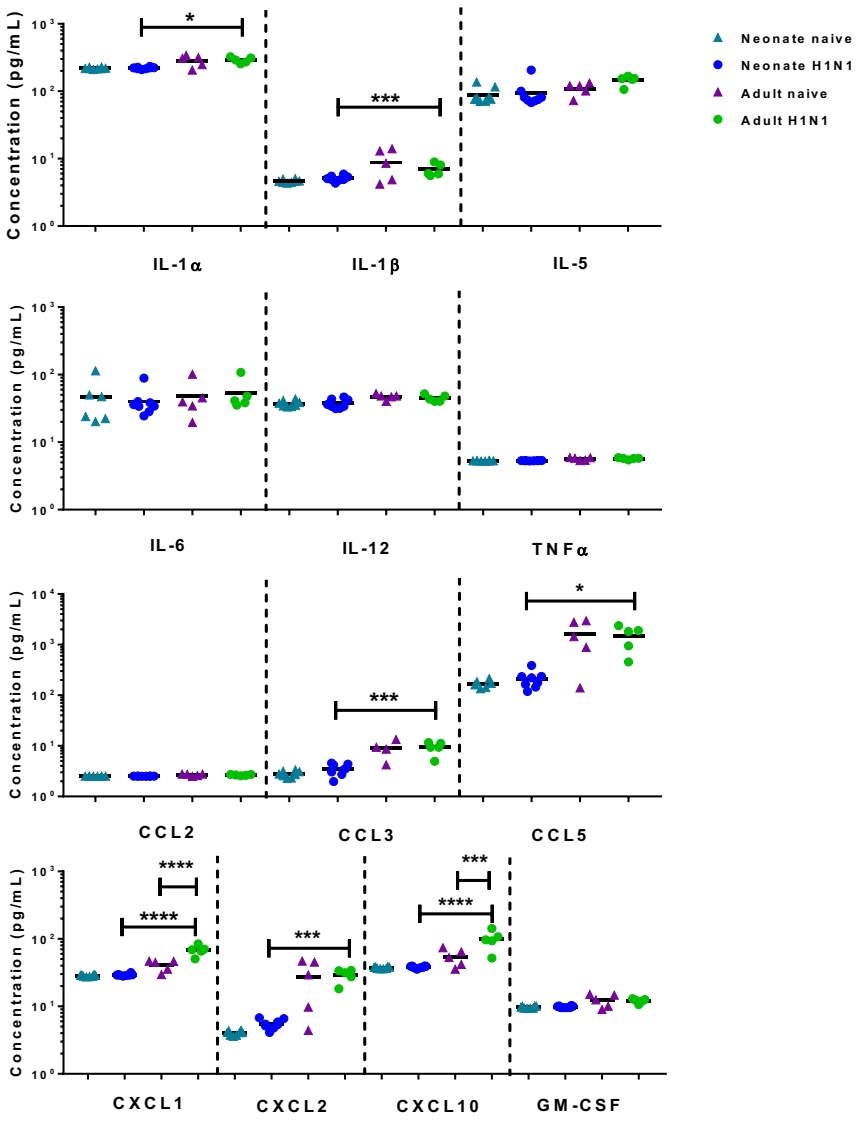

Figure 4.16. Cytokine response in mice to influenza infection
Adult Mice (n=5) were infected intranasally with $5x10^5$ PFU and neonatal (n=8) mice were infected with 10^5 PFU A/England/195/2009 H1N1 influenza, responses were compared to age matched adult (n=5) and neonatal (n=6) naïve animals. Lungs were collected at 24 hours after infection. Lung cytokine levels were measured by Luminex. Comparisons by ANOVA with Tukey's post test, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Points represent individual animals, lines represent mean.

4.2.14. *RSV infection in neonatal mice*

As seen in the cord blood data, the immune profile of RSV infected mice was different from influenza infected mice. RSV infected adult mice had a significantly increased IL-1α, IL-6, and TNFα response in the lung (Figure 4.17A, B). There was significantly less IL-1α, IL-6, and TNFα in the lungs of neonatal mice after infection when compared to infected adults. The age of mice also affected the chemokine response to RSV infection: neonatal mice had significantly elevated CCL5 responses, and adult mice showed increased responses in CCL2, CCL3, CXCL1, and CXCL2 (Figure 4.17C, D). Compared to neonatal mice, there were significantly higher levels of CCL2, CCL3, CXCL1, CXCL2, CXCL10 and GM-CSF in the lungs of RSV infected adult mice (Figure 4.17 C, D).

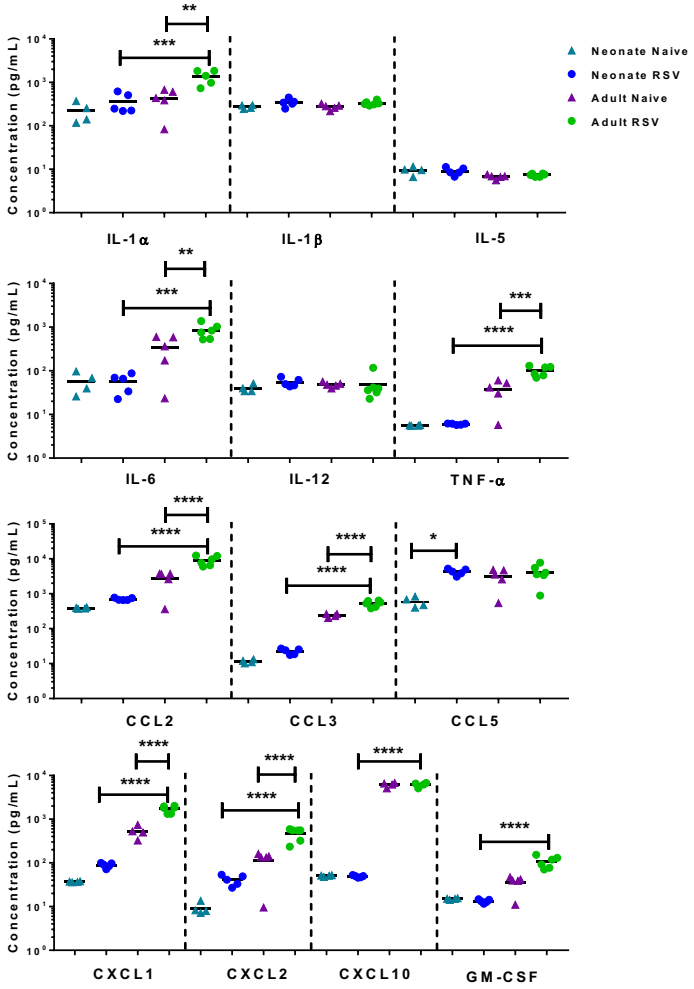

Figure 4.17. Cytokine response in mice to RSV infection
Adult Mice (n=5) were infected intranasally with $5x10^5$ PFU and neonatal (n=5) mice were infected with 10^5 RSV, responses were compared to age matched adult (n=5) and neonatal (n=4) naïve animals. Lungs were collected at 24 hours after infection. Lung cytokine levels were measured by Luminex. Comparisons by ANOVA with Tukey's post test, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Points represent individual animals, lines represent mean.

4.3. Discussion

Previous studies have focused on the differences between neonatal and adult cytokines in response to the stimulation of immune receptor, particularly TLR, ligands (78–81). Very few studies have used live whole virus or bacterial stimulation to study the differences in cytokine response. Likewise previous studies have mainly focussed on cytokines such as TNFα, IL-6, and IL-1β, few studies have investigated the complete innate immune profile, which provides a complete picture of the host's defence against invading pathogens.

4.3.1. Bacterial stimulation induced a distinctive immune profile compared to viral stimulation

The pattern of immune profile with viral stimulation was different from those resulted from bacterial infection. This may be caused by the differences in the mechanisms of pathogen recognition by the innate immune system and the associated signalling pathways.

4.3.2. The sensing of bacterial and viral infections

Infection is detected by the innate immune system through pattern recognition receptors (PRR), which are located at both the cell surface and in the intracellular compartment. PRRs bind to microbial components called pathogen-associated molecular patterns (PAMPs), which subsequently induce the activation of downstream signalling pathways and the transcription of anti-microbial effectors (87). The Toll-Like Receptor (TLR) family is one of the most important PRRs, recognising a range of viral and bacterial PAMPs.

GBS has peptidoglycan (PGN) and lipoteichoic acid (LTA) on the bacterial cell wall. LTA is recognised at the extracellular compartment by the cell surface receptors TLR1, TLR2 and TLR6, which activates the signalling pathway via MYD88 and induce the release of cytokines and chemokines (88). LPS is recognised by TLR4, PGN is recognised by TLR2. On the other hand, viral infection is detected at the intracellular compartment by the nucleic acid sensors, including endosomal TLRs RIG-I like receptor (RIR) and NOD-like receptor (NLR) (87). Viral DNA/RNA at the endosome is recognised by TLR3, 7, 8 and 9, resulting in the activation of downstream signalling pathway through TRAF3 or MYD88 and

the release of antiviral cytokines and type I interferon (89). RIR and NLR are specific for the detection of viral nucleic acid at the cytoplasm, which induces the activation of inflammasome through mitochondrial antiviral signalling protein (MAVS) or Caspase-1 pathway. This then results in the expression of pro-inflammatory cytokines (90,91). Overall, the two distinctive mechanisms of detecting virus and bacterial infection may contribute to the differential cytokine profile observed in this study.

4.3.3. Stimulation with different viruses resulted in distinctive immunological profiles

Of the two virus tested, influenza infection induced significantly different cytokine responses compared to RSV infection in neonates, mothers and non-pregnant women. Interestingly, both influenza virus and RSV are negative sense single-stranded RNA (ssRNA) viruses. The differences in cytokines responses between influenza and RSV infection may be associated with the differences in the innate sensing for these two viruses. For influenza infection, there are three main PRRs that are responsible for pathogen recognition: TLR, RIG-I and NLRP3 (92). TLR3 at the endosome senses the virus infected cells through the recognition the double-stranded RNA (dsRNA) released from phagocytised cells, inducing the antiviral state through the release of type I interferon and pro-inflammatory cytokines (93,94). TLR7 is activated by the interaction with ssRNA, resulting the expression of type I interferon and cytokine through IRF7 and MyD88 respectively (95–97). RIG-I recognises 5' triphosphate on viral RNA. RIG-I signalling is facilitated by MAVS through NF-kB and IRF3 to induce the release of pro-inflammatory cytokines and activation of ISG (92). NLRP3 mediates the host tolerance to influenza infection through the sensing of cellular damages and promotes the recruitment of the leukocytes to the respiratory tract. The activation of NLRP3 induces the release of IL-1β and IL-18 but does not affect the replication of influenza virus (98,99).

The innate sensing of RSV infection is less well characterised. Similar to influenza infection, multiple PRRs are involved. RIG-I recognises the ssRNA of RSV at early time point of infection and induces the release of type I interferon through MAVS, activating numerous ISG (100). TLR3, on the other hand, recognises the viral ssRNA at later time point of the infection (101). TLR7 is also involved in the

sensing of the dsRNA in the endosome (102). Interestingly, cell surface TLR4 and TLR2/6 heterodimer are shown to be active in the recognition of RSV infection (103,104). NLR is capable of recognising cytosolic RSV ssRNA and protein kinase R (PKR) binds to the viral dsRNA in the cytoplasm (105,106).

Overall, various PRRs have been shown to sense both influenza and RSV infection. The potential cross-talk between different PRRs in sensing different viruses may be responsible for the distinctive immunological profiles observed in this study.

4.3.4. The effect of age on chemokine responses against infections and the associated impact

Interestingly, the overall pattern of cytokine profiles against influenza/RSV infection was similar among neonates, mothers and non-pregnant women.

There were significant differences in terms of the magnitude of cytokine/chemokine responses between neonates and adults. It is possible that the lower neonatal innate cytokine and chemokine responses observed in this study are associated with the inadequate activation of adaptive immunity.

4.3.5. Type I Interferons

Type I interferon (key members: IFNα and IFNβ) play an essential role in the defence against viral and microbial infections. Mice with deficiency in type I IFN signalling have increased susceptibility to many viral infections, including influenza (107). Upon infection the pathogen is detected by pattern recognition receptors (PRR) triggering the downstream signalling pathway, leading to the production of type I IFN. Type I IFN then activate numerous interferon stimulated genes (ISGs), including myxovirus resistance gene (Mx), the protein kinase stimulated by dsRNA (PKR) and the 2'-5' oligoadenylate synthetases (OAS). With strong antiviral activity, these ISG target every step of viral

life cycle. Early production of type I IFN is required to limit initial viral replication before the adaptive immune becomes effective (108).

As well as controlling intracellular pathogen infections, types I IFN have a profound impact on defence against extracellular bacteria, including GBS (109). Mice with deficiency in type I IFN receptor showed shorten survival and/or more bacterial growth than wild type (110,111). The immune response against GBS infections by type I IFN might be through the priming of macrophages and induces the production of pro-inflammatory cytokines against the pathogen (112). Infants less than 3 months of age are at the peak of susceptibility to GBS infections and this may be correlated with the period of low Type I IFN (18). It is possible that the diminished IFN response against pathogens in neonates contribute to the elevated risk of infectious diseases.

Type I interferon also affects adaptive immune response against infection, including enhancing the actions of DCs and monocytes (113–115), promoting T cell activation (116,117) and having a positive effect on the NK cell functions and survival (118,119).

4.3.6. Inflammatory cytokines

Inflammatory cytokines are essential in the defence against viral and microbial infections. Differentiation of T cells is pathogen specific, and dependent on the surrounding cytokine signal, particularly IL-2 which plays an important role in T cell survival and expansion (120). The reduced level of IFNβ and IL-2 in neonates may contribute to poor clearance of the viral replication.

4.3.7. Chemokines

In the post-natal period, chemokines are essential for localising lymphoid progenitors to the mucosal-associated lymphoid tissues and promoting their development. Foetal-derived monocytes stimulated by GM-CSF in the lung proliferate into alveolar macrophages (121). In the case of infections, chemokines are important for recruiting the circulating neutrophils, monocytes and lymphocytes to the site of infections. Production of CCL5 in RSV infected mice within 48 hours post infection was important in recruiting T cells (both $CD4^+$ and $CD8^+$ T cells) to the respiratory tract and controlling the viral replication. Administration of a CCL5 inhibitor (Met-RANTES) throughout the

first 7 days of infection resulted in a reduction in cell infiltration, and increased virus concentration in the lungs, compromising the immune memories for a secondary challenge (122).

CXCL10 also plays an important role in the generation and recruitment of effector T cells to the site of infections. $CXCL10^{-/-}$ mice had a significant reduction in the trafficking of virus-specific T cells, accompanied by decrease in cell infiltration at the site of infection (123). In previous studies, neonatal mice stimulated with LPS via the intrapharyngeal route had lower CXCL10 and CCL5 responses and a prolonged inflammation in the lung in comparison to juvenile mice (124). CXCL10 was also found to be associated with the function of macrophages and neutrophils. The blockade of CXCL10 response had a detrimental impact on neonatal mice with polymicrobial sepsis by reducing the survival and recruitment of macrophages and granulocytes (125).

Therefore, understanding the mechanism behind the differential chemokine response between neonates and adults will be instrumental in finding the potential treatment and vaccine strategy for the leading pathogens of neonatal infections (such as influenza, RSV and GBS).

4.3.8. Mechanism of the differential chemokine response to infection in neonates

There are two possible mechanisms that contribute to the differential chemokine response to infection, differences in cell composition and differences in signalling pathways in the cells that are present. In this study I focussed on the contribution of the distinct immune cell composition to the differential cytokine/chemokine response between neonates and adults. In this study, 3 different immune cell populations and the corresponding expression level of CXCL10 in each population were quantified.

4.3.9. Dendritic cells

In the case of infection, dendritic cells (mDC and pDC) play a critical role in both innate and adaptive immunity. As antigen-presenting cells, both mDC and pDC activate and promote T cells response by presenting antigen together with co-stimulatory molecules. By rapid production of IFN, pDC create a

local anti-viral state and activates NK cells. Moreover, pDC regulate adaptive immunity and recruits effector to the cells to the site of infection through the release of cytokines and chemokines, such as CXCL10 (126).

I found that neonates had significantly lower percentage of pDCs after influenza infection compared to adults. Studies have shown that pDCs isolated from cord blood samples were fully functioning and capable of inducing potent IFN and TNF response to HIV, influenza and HSV infection (127–129). The deficit in the number of pDC in neonates may contribute to the differential chemokine response to infections. Administration of Fms-related tyrosine kinase 3 ligand (FLT3L) promotes the production of pDC and mDC and restored protection to viral infections in neonatal mice (130–132). However, the function of neonatal pDC was selectively impaired when stimulated with adjuvants. Neonatal pDCs had limited response to CpG-containing DNA (CpG-DNA) which targeted TLR9 (127,128,133). In contrast, the stimulation of R848 targeting TLR7/8 induced a robust activation of neonatal pDCs (81). Fragiadakis suggested that neonatal pDCs had potential defect in the signalling pathway involved in cell growth, proliferation and differentiation after LPS stimulation (targeting TLR4) of fetal cord whole blood samples (134). It is possible that the function of neonatal pDCs can be influenced by the presence of immune cells and the adequate stimulants. Further studies are required to draw the conclusion.

4.3.10. Monocytes

During infection or inflammation, monocytes at non-lymphoid tissues differentiate into inflammatory, pro-resolving or tissue macrophages depending on the environment. In this study, there was a trend of higher percentage of both classical $CD14^{+}CD16^{-}$ and non-classical $CD14^{low}CD16^{+}$ monocytes subsets in neonates than adults. Interestingly, the expression level of CXCL10 in classical monocytes to influenza infection was significantly lower in neonates than those in adults. This may be due to the diminished functionality and distinctive basal gene expression in neonatal monocytes compared to adults. Monocytes in neonates showed impaired responses to the stimulation of agonists which targeted on the TLR and inflammasome pathways (135). A decrease was found in the proportion of neonatal $CD14^{+}CD16^{+}$ subsets with high TLR4 expression, resulting in a reduction in

TLR2 and TLR4-associated response of neonatal monocytes (136). Moreover, a greater level of adenosine was found in neonates than adults, which was associated with the impaired TNF response to adjuvants in neonatal monocytes (137). High levels of alarmins were found in the blood samples of newborns and contributed to the reduction in TLR4-induced TNF response of neonatal monocytes through the modulation of TRIF/MyD88 pathway (138). Intriguingly, similar levels of CXCL10 expression were found in both neonatal and adult monocytes after IFNγ stimulation (139). However, the expression of CXCL10 may be different when cells were infected with live pathogens. Further studies are required to determine whether the function of CXCL10 induction in neonatal monocytes is impaired and whether this has contributed to the inadequate immunity against infection.

4.3.11. Effect of differences in cell signalling on the chemokine response

Alternatively, differential signalling activities in neonatal immune cells may explain the differential chemokine responses. A previous study has shown that the differential cytokine responses between neonates and adults was not associated with basal expression level of TLRs (78). However, MYD88, an important adaptor protein in TLR signalling pathway, has diminished expression in neonates, which may contribute to the reduced TNFα response to LPS stimulation (140). Furthermore, neonates had blunted signalling response to LPS stimulation, including p-CREB, pERK1/2, p-P38 and p-NF-κB, which are important in cell activation, differentiation and growth (134). As described before, I saw decreased signalling responses in multiple immune cell subsets. It is likely that the difference in cell type and function are connected and both contribute to the differential chemokine response to infection in neonates.

4.3.12. Limitations

There are some limitations to the study. Cord blood samples were used to isolate PBMC of neonates. Whilst *in vitro* studies on human neonatal immune response often use cord blood samples (141), it remains unclear whether the blood taken from umbilical cord fully represents the peripheral blood of neonates. Likewise, PBMC may have distinct responses to tissue resident immune cell (86). Since the access to human neonatal tissue is not practical, *in vivo* studies using neonatal mice are an

alternative way to measure the immune response at the level of the respiratory tract in the case of influenza virus and RSV infections. However, there are some issues in extrapolating the results from animal study in the particular experimental setting to the case of human exposure in real life. A combination approach as used here, may offer the best compromise, looking for overlaps between the two systems.

In order to compare the age-dependent maturation of immune response to infection, blood samples from older infants and young children are the ideal patient samples for this study. However, it is very difficult to obtain these blood samples. Thanks to collaboration in the Department of Paediatrics, the matched maternal blood samples with cord samples were obtained and were used in this study to compare with neonatal response. However, antibiotics, neuraxial anaesthesia and surgical intervention during labour can induce inflammatory and hormonal response in mothers. Non-pregnant healthy women were introduced as a control to minimise the environmental influences on the immune response.

The multiplex measurement of analytes may bring in the 'Multiple Comparisons Problem', which increases the probability of Type I error (142). Several statistical tests with a higher significance threshold can be used to prevent this problem, including Bonferroni's correction and Hochberg procedure (143).

5. Conclusion

Maternal immunisation is one of safest and most useful tool to address the immunity of infants during first months of life, covering the gap in infant protection until the primary immunisation regime is completed at 6 months of age. The administration of influenza and DTaP vaccines during pregnancy have shown to be clinically efficacious in protecting infants from infections (31). Developing maternal vaccination for other major pathogens that cause infections in early life, including RSV and GBS is a research priority. The timing of vaccine administration is critical in achieving prolonged protection. The present study, consistent with other studies in maternal immunisation against pertussis, suggests that the second trimester and early third trimester may be the optimal time for maternal immunisation when the goal is to maximise protection in the infant (50,51).

Both maternal and neonatal vaccines have focused on inducing strong antibody responses for protection. However, there is a limitation for this approach: inhibitory effect of maternal antibody on infant antibody response. Infants who were immunised in the presence of maternal antibody were found to have lower seroconversion rate and lower antibody concentration compared to those were seronegative at the time of vaccination (144–149). Although the inhibitory effects were reported in different vaccines, the outcomes of immunisation varied depending on the specific vaccines and diseases. The suppressions of infant antibody by maternal antibody were more pronounced in the immunisation of tetanus and pneumococcus, while the effect against *Haemophilus influenzae* B and pertussis vaccines were less significant (150). Edwards suggested that the priming of infant immunisation was not largely affected by the presence of maternal antibody but the level of booster response was affected variably (151).

The maternal antibody may inhibit the infant antibody production through the removal of vaccine antigen by phagocytosis of macrophages through the Fc receptor (32,152). However, there was no significant evidence suggesting that antigen removal had an impact on the infant antibody response. Live attenuated live vaccines may be neutralised by the maternal antibody in newborns and infants. The replication of live-attenuated RSV vaccine was inhibited at the respiratory tract by the maternal

antibodies (153). However, the neutralisation effect may largely depend on the replicating capacity of the vaccine. Epitope masking may be another mechanism of the suppression effect of maternal antibody. The epitope from immunisation can be bound by the maternal antibody, which in turns inhibit the interaction between epitope with infant B cells (154–156). The proliferation and antibody secretion of infant B cells can be inhibited by the crosslink between the FcγIIB mediated signalling and the vaccine-maternal antibody complex (157). The level of inhibition was associated with the concentration of maternal antibodies and its associated interaction with Fc receptor.

Therefore, neonatal vaccination can be an alternative to overcome the above limitations. However, neonatal vaccines against RSV or GBS are not available despite years of development. This may be associated with the lack of knowledge of the underlying mechanism of neonatal immune system, which is different from the adults. The current study characterised the neonatal innate immune profile against live viral infection and bacterial infections. Consistent with the existing theory that neonates have a suppressive immune response, I have found key differences in the chemokine response to infection. These changes in chemokine responses may play a critical role in recruitment of effector immune cells. The similar patterns of immune profile were also observed in neonatal mice *in vivo* infection model. Furthermore, I found that neonates fewer dendritic cell populations and whilst the number of monocytes were similar, the neonatal monocytes had lower expression of CXCL10.

Similar patterns of immune profile were observed in previous study which I participated in. McDonald et al. investigated the impact of age on the immune response of influenza vaccination and found that neonates had distinctive immune profile after vaccination when compared to adults and aged mice (158). The published study also suggested that age had an important impact on the efficacy of influenza vaccine.

One possible approach is the induction of a protective T-cell mediated response. Use of adjuvant is one of the most effective ways to enhance the efficacy of the vaccines. Adjuvants may also be used to shape the direction of immune response and in particular, increase the immune response in

populations with distinct immunity. PRRs are found to be expressed in antigen-presenting cells and leucocytes. The expression of PRR is age-dependent and neonates are found to have significantly different PRR pattern compared to adults. PRR agonist/inhibitors are used extensively as adjuvant. As part of a study I was involved in with Fischetti et al., we found that combination of TLR adjuvants exhibited a synergistic effect on the cytokine response (159). It is possible that with the particular combination of TLR adjuvants, the neonatal immune response to the vaccine can be shaped towards the T-cell mediated response. Adjuvants can be an alternative solution to overcome the drawbacks including limited half-life and maternal antibody interference (160). An alternative is to use live attenuated viruses to induce local immunity. I also contributed to a study that showed an immunomodulatory role for the SH protein of RSV, which could help in the design of better live attenuated vaccines (161).

Overall, infections cause a significant burden on young children during first months of their life. An effective immunisation strategy would be of great public benefit. Maternal immunisation is a safe and clinically effective method in protecting neonates. However, the efficacy and length of protection varies depending on the pathogen. A complete picture of the effect of maternal immunisation on neonates may provide insight to develop a more flexible and efficacious regime. Neonatal vaccination is a more direct and potentially more potent strategy to protect newborns. The distinctive neonatal immune profile is one of the main hurdles for the vaccine development. A better understanding of the neonatal immune response may close the knowledge gap and enlighten the progress into the development of a vaccine with a more targeted, effective and long-lasting immune protection.

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Appendix 1: Script for Principle Component Analysis

This a brief description of the code used to perform the Principle Component Analysis (PCA). It uses as an example the Luminex data comparing the cytokine responses of donors to viral and bacterial stimulation.

The following packages were installed and loaded in R Version 3.3.1

```
library(ggplot2)
library(ggfortify)
```

1. Loading data into R

The data was loaded into R as a comma separated value file. The cytokine data was then subsetted to leave only numerical variables.

```
cytokine_data = read.csv("Virus and bacteria.csv", header = T, row.names = 1)
cytokines = (cytokine_data[,2:21])
```

2. PCA

PCA was performed using the base R function **prcomp**, which includes scaling the cytokine data variables to have unit varience before the analysis took place. The results of the PCA were viewed using the **summary** function, which gives the standard deviation and proportion of varience asociated with each principle component.

```
cytokine_pca = prcomp(cytokines, scale. = TRUE)
summary(cytokine_pca)
```

3. Plotting

The results of the PCA were visulised using the **ggplot2** and **ggfortify** packages.

```
autoplot(cytokine_pca, data = cytokine_data, colour = "Stimulation", shape="Stimulation", size=4,
asp=1, frame=TRUE, frame.type="norm") +
 scale_colour_manual(values = c("tan4", "lightslategray")) +
 scale_fill_manual(values = c("white", "white")) +
 scale_shape_manual(values = c(15,18)) +
 labs(x="PC1", y="PC2") +
 ggtitle("Virus VS bacteria infection") +
 theme(axis.text = element_text(size=14),
   panel.background = element_rect(fill="white", colour="black"),
   axis.title.x = element_text(size=16, margin=margin(15,0,0,0)), title
   axis.title.y = element_text(size=16, margin=margin(0,15,0,0)),
   plot.title = element_text(size=18, face="bold", hjust = 0.5, margin=margin(0,0,15,0)))
```

4. Statistical analysis

In order to evaluate whether the cytokine profiles were different between groups, a multivariate analysis of varience (MANOVA) was performed.

First the independent variable of interest, in this example *Stimulation*, was turned into a factor

```
stim = as.factor(cytokine_data[,1])
```

Then a matrix of the dependent variables, *the cytokine profile*, was created

```
variables = as.matrix(cytokine_data[,2:21])
```

Then a MANOVA to determine whether the overall *cytokine profile* was significantly different between *Stimulation* groups was performed. The **summary.aov** function provides the individual

statistical results for each cytokine while the **summary.manova** function gives the overall result for the MANOVA.

Stim.Manova = **manova**(variables~stim)
summary.aov(Stim.Manova)
summary.manova(Stim.Manova)