Mechanisms of Maternal and Fetal Resistance to Influenza A Virus Infection

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Father, this is the beginning of my contribution to humanity's ether. This one's for you.

The question, O me! So sad, recurring – What good amid these, O me, O life?

Answer.

That you are here – that life exists and identity,

That the powerful play goes on, and you may contribute a verse.

-Walt Whitman

List of Abbreviations

AEC - airway epithelial cell

AHR - airway hyperresponsiveness

AM - alveolar macrophage

AMP - antimicrobial peptide

ARDS - acute respiratory distress syndrome

Arg1 - arginase I

BAL - bronchoalveolar lavage

CCL2 - C-C motif chemokine ligand 2

CCL4 - C-C motif chemokine ligand 4

CCR2 - C-C chemokine receptor type 2

COVID-19 - coronavirus 19

cRNP - complementary ribonucleoprotein complex

CXCL1 - CXC-chemokine ligand 1

DAMP - damage-associated molecular pattern

DC - dendritic cells

dNK - decidual natural killer cell

DPI - day post infection

E# - embryonic day

ELISA - enzyme-linked immunosorbent assay

eIF - eukaryotic translation initiation factor

GD# - gestation day

γδ - gamma delta

HA - hemagglutinin

HBD - human beta defensin

H&E - hematoxylin and eosin

HNP - human alpha defensin

IAV - influenza A virus

IFITM - interferon induced transmembrane protein

IFN - interferon

IFNAR - IFN-I receptor

Ig - immunoglobulin

IL - interleukin

ILC - innate-like lymphoid cells

i.n. - intra-nasal

iNOS - inducible nitric oxide synthase

i.p. - intra-peritoneal

IRF - interferon regulatory factor

ISG - interferon stimulating gene

ISG56 - interferon stimulating gene 56

ISRE -interferon-sensitive response elements

i.t. - intra-tracheal

IUGR - intrauterine growth restriction

JAK - Janus family kinases

KC - keratinocyte chemoattractant

LDH - lactate dehydrogenase

LTB4 - leukotriene b4

M1 - matrix protein 1

M2 - matrix protein 2

MAVS - mitochondrial antiviral-signaling protein

mBD - mouse beta defensin

M-CSF - macrophage colony-stimulating factor

MDA5 - melanoma differentiation-associated gene 5

MDCK - Madin-Darby canine kidney

MOI - multiplicity of infection

MUC - mucin

Mx - myxovirus resistance protein

NA - neuraminidase

NEP - influenza nuclear export protein

NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells

NK - natural killer cells

NLR - NOD-like receptor

NP - nucleoprotein

NS1 - non-structural protein 1

OAS - 2'5'- oligoadenylate synthase

PAMP - pathogen associated molecular pattern

PB1 - polymerase basic protein 1

PB1-F2 - PB1 frame 2

PBS - phosphate buffered saline

PFU - plaque forming units

PKR - protein kinase R

PRR - pattern recognition receptor

RdRp - RNA-dependent RNA polymerase

RT-PCR - reverse transcription polymerase chain reaction

RIG-I - retinoic acid-inducible gene I

RLR - retinoic acid-inducible gene I-like receptor

SARS-CoV2 - severe acute respiratory syndrome coronavirus 2

SP1 - specificity protein 1

SP - surfactant protein

STAT - signal transducer and activator of transcription

STBs - syncytiotrophoblasts

TCR - T-cell receptor

TGF-β - transforming growth factor β

Th - T helper

TLR - toll-like receptor

TNF - tumor necrosis factor

TSLP - thymic stromal lymphopoietin

uDC - uterine dendritic cell

Vγ - γ-chain variable regions

vRNP - viral ribonucleoprotein

Abstract

Host survival during infection requires a delicate balance between host resistance, which is essential for eliminating pathogens, and disease tolerance, which is critical in minimizing tissue damage. Pregnant women represent a high-risk group during influenza A (IAV) outbreaks, in part due to type-2 immune responses which favor reproductive fitness. Such changes were thought to impair host resistance; however, human, and murine studies demonstrate that pregnancy induces a more robust inflammatory response following IAV infection [16, 17]. Thus, it is still unclear how pregnancy regulates immunity to IAV and whether impairment in pulmonary antiviral immunity or an exorbitant inflammatory state contributes to maternal morbidity. The overall aim of this thesis was to characterize both host resistance and disease tolerance in mid- gestation C57BL/6 pregnant mice following IAV infection and the subsequent consequences on offspring immunity.

The first part of this thesis is dedicated to understanding the maternal immune response to IAV infection. Contrary to the accepted dogma surrounding pregnancy and susceptibility to viral infection, we found that pregnancy in fact confers protection against maternal IAV infection depicted by a lower pulmonary viral burden, reduced inflammatory responses, pathology, and airway hyperresponsiveness in a mid-gestation (E10) IAV C57BL/6 mouse model. This protection was ultimately conferred by enhanced upper airway antimicrobial immunity driven by IL-17^{+} γδ^{+} T cells. This approach to assessing protection of the respiratory system during pregnancy remains unexplored yet is evolutionarily favorable, as it suggests that the mother may have conditioned upper airway defense mechanisms to restrict IAV replication and propagation to support maternal fitness.

The second part of the thesis focuses on the sequelae of maternal IAV infection on offspring immunity. We show that prolonged protection against IAV infection in offspring is

dependent on both the transplacental and breast milk transfer of IgG1 maternal antibodies. In addition, the number of antibodies transferred is conditional upon the initial maternal concentration and the antibody profile generated is influenced by pregnancy. Thus, maternal recovery from infection or vaccination during pregnancy and child-bearing years demonstrably improves offspring immunity.

In totality, this project provides insight into the significance of upper airway antimicrobial immunity and may shed light into cellular and molecular players that distinguish severe versus mild cases of IAV infections in pregnant women. The mechanisms described in this enhanced upper airway host resistance during pregnancy may also be extrapolated and harnessed as potential novel therapeutics or vaccine strategies for the general population. Moreover, it will bring to attention the fine balance of maintaining maternal and fetal fitness during pregnancy and IAV infection and the beneficial but also detrimental costs to offspring health.

Résumé

La survie de l'hôte pendant l'infection nécessite un équilibre délicat entre la résistance de l'hôte, requise pour éliminer les agents pathogènes, et la tolérance à la maladie, essentielle pour minimiser les dommages tissulaires. Les femmes enceintes représentent un groupe à haut risque lors des épidémies par le virus de l'influenza, en partie à cause des réponses immunitaires de type-2 qui favorisent la croissance fœtale. Ces changements ont souvent été considérés comme inapproprié pour la clairance virale; cependant, des études humaines et murines démontrent que la grossesse est associée à une réponse inflammatoire très robuste après une infection par le virus de l'influenza [16, 17]. Ainsi, il n'est toujours pas connu comment la grossesse régule l'immunité contre le virus de l'influenza et si une déficience de l'immunité antivirale pulmonaire ou un état inflammatoire exacerbé contribue à la morbidité maternelle. L'objectif général de cette thèse est de caractériser à la fois la résistance de l'hôte et la tolérance à la maladie chez des souris C57BL/6 gestantes lors d'une infection par le virus de l'influenza, et les conséquences ultérieures sur l'immunité de leur progéniture.

La première partie de cette thèse est consacrée à la compréhension de la réponse immunitaire maternelle à l'infection par le virus de l'influenza. Contrairement au dogme accepté associant la gestation et susceptibilité aux infections virales, nous avons constaté que la gestation confère en réalité une protection contre l'infection par le virus de l'influenza, représentée par une charge virale pulmonaire plus faible, une réduction des réponses inflammatoires et de la pathologie pulmonaire ainsi qu'une diminution du dysfonctionnement des voies respiratoires à mi-gestation (E10) dans un modèle de souris C57BL/6. Cette protection est conférée par une immunité antimicrobienne des voies respiratoires supérieures renforcée grâce aux cellules T IL-17$^{+}\gamma\delta^{+}$. Cette approche pour évaluer la protection du système respiratoire pendant la grossesse reste inexplorée mais est favorable à l'évolution humaine, car elle suggère que la mère pourrait avoir conditionné

ses mécanismes de défense des voies respiratoires supérieures pour restreindre la réplication et la propagation du virus de l'influenza afin de soutenir la santé maternelle.

La deuxième partie de la thèse se concentre sur les séquelles de l'infection maternelle par le virus de l'influenza sur l'immunité de leur progéniture. Nous montrons qu'une protection prolongée contre l'infection par le virus de l'influenza chez la progéniture dépend à la fois du transfert des anticorps maternels IgG1 transplacentaire et par le lait maternel. De plus, le nombre d'anticorps transférés dépend de la concentration maternelle initiale et le profil d'anticorps généré est influencé par la grossesse. Ainsi, la guérison maternelle d'une infection ou la vaccination pendant la grossesse et pendant les années de procréation supporte l'immunité de la progéniture.

En résumé, ce projet donne un aperçu de l'importance de l'immunité antimicrobienne des voies respiratoires supérieures pendant la grossesse et peut éclairer sur les mécanismes cellulaires et moléculaires qui peuvent distinguer les cas graves des cas légers d'infections par le virus de l'influenza chez les femmes enceintes. Les mécanismes décrits dans cette résistance pendant la grossesse peuvent également être extrapolés et exploités en tant que nouvelles thérapies potentielles ou stratégies vaccinales pour la population générale. De plus, cela attirera l'attention sur le compromis évolutif entre la santé maternelle et fœtale et les coûts bénéfiques mais également désavantageux pour la santé de la progéniture.

Contribution to Original Scientific Knowledge

Chapters 2 and 3 represent the results section of this thesis and comprise novel contributions to the greater scientific community. The thesis is submitted in accordance with the standards set by McGill University and the Division of Experimental Medicine and is presented here in a "manuscript based" format.

Chapter 2: In this manuscript submitted as a brief definitive report to the *Journal of Experimental Medicine,* we outline the importance of IL-17 predominantly produced by $\gamma\delta^+$ T cells in upper airway immunity against Influenza A infection during pregnancy.

I. Pregnancy confers resistance to IAV infection.
II. Pregnancy reduces downstream inflammatory responses and promotes disease tolerance to limit IAV-induced pulmonary pathology.
III. Maternal resistance is independent of pulmonary antiviral immunity and alveolar macrophage function.
IV. IL-17 producing $\gamma\delta^+$ T cells mediate enhanced antimicrobial immunity in the upper airways restricts viral replication in pregnant mice.
V. Upper airway antimicrobial immunity protects pregnant mice against IAV-induced airway dysfunction.

Chapter 3: In this published manuscript (Chronopoulos, J., Martin, J.G., Divangahi, M., *Frontiers Immunology*, 2022), we reveal the importance of both maternal transplacental and breast milk transfer of IgG1 for prolonged protection of offspring against IAV infection.

I. Maternal IgG1 is detected in neonatal and adult offspring sera at the time of weaning and 8 weeks of age.
II. The concentration of maternal IgG1 is correlated with degree of protection in offspring.

III. Antibody profiles differ between pre-conception and mid-gestation IAV infection, however only IgG1 persists in offspring circulation in adulthood.

IV. IgG1 from breast milk and *in utero* are both necessary to confer prolonged protection against IAV infection.

Co-author Contributions

1. Tran, K.A. *et al.,* BCG immunization induces CX3CR1^{hi} effector memory T cells to provide cross-protection via IFN-γ-mediated trained immunity. Accepted in *Nature Immunology* (2024).
2. Pernet, E., Sun, S., Sarden, N. *et al.* Neonatal imprinting of alveolar macrophages via neutrophil-derived 12-HETE. *Nature* **614**, 530–538 (2023). https://doi.org/10.1038/s41586-022-05660-7
3. Kaufmann E, Landekic M, Downey J, Chronopoulos J, Teimouri Nezhad S, Tran K, Vinh DC, Barreiro LB, Divangahi M. Lack of evidence for intergenerational inheritance of immune resistance to infections. *Nat Immunol*. 2022 Feb;23(2):203-207. doi: 10.1038/s41590-021-01102-0. Epub 2022 Jan 20. PMID: 35058614.
4. Farahnak S, Chronopoulos J, Martin JG. Nucleic Acid Sensing in Allergic Disorders. *Int Rev Cell Mol Biol.* 2019; 345:1-33. doi: 10.1016/bs.ircmb.2018.11.001. Epub 2018 Dec 27. PMID: 30904191.

Contribution of Authors

The work outlined in this thesis was completed under the direction of Dr. James G. Martin and Dr. Maziar Divangahi. Authors are depicted by their initials (*e.g.,* Julia Chronopoulos, JC) and their respective contributions to each chapter are presented below:

Chapter 1: The literature review was written by JC.

Chapter 2: JC wrote the manuscript with edits from MD and JM. JC and EP designed experiments. JC performed all experimentation with technical assistance from EP, KT, AM, TMcG, OT, SW, and KM. Study was supervised by MD and JM.

Chapter 3: JC performed all experiments and wrote the manuscript under the direction of MD and JM.

Chapter 4: JC wrote the discussion.

Chapter 1: Introduction

1.1 A Brief History of Influenza

Influenza has scourged humanity since ancient times and left its footprint in history as the culprit of the most lethal pandemic to strike the modern world in 1918 with the emergence of the H1N1 Spanish flu [18, 19]. *"Influenza killed more people in a year than the Black Death of the Middle Ages killed in a century; it killed more people in twenty-four weeks than AIDS has killed in twenty-four years"[18].* With an estimate of 50-100 million deaths and 500 million cases worldwide, influenza attacked around one fifth of the world's population, threatening our very existence [18].*"Civilization could have disappeared within a few more weeks*," warned American physician and dean of the University of Michigan Medical School during that time, Victor Vaughn [18]. Since the 1918 depopulation, influenza A virus (IAV) has been extensively studied for over a century as one of the biggest biological threats to humanity, with pandemic strains emerging every few years [19]. Yet, despite the implementation of vaccinations and antiviral therapies worldwide, IAV still accounts for up to 5 million severe cases and claims the lives of 500 000 people annually [1, 19, 20]. Unmistakably, there is an urgent need to expand our repository of immune strategies to combat this impending threat.

In order to understand why the 1918 IAV pandemic is viewed as the *"greatest failure of medical science in the 20th century [21],"* we must recount the events that lead to mankind's approaching quietus and dissect the pathogen responsible. One could argue that the preface of the health disaster of 1918 was a sign of the times. Human transmission is a result of human activity as was the case with the Spanish flu. World War I generals unknowingly mapped the virus' trajectory when planning military troop deployments [18, 19, 22, 23]. Summated with poor sanitation, overcrowding, and a lack of medical facilities, equipment, and personnel, a vista of

plague stretched across the planet [18, 19, 22, 23]. In fact, it wasn't until the 1930s when Richard Shope discovered the causative agent of influenza was indeed a virus instead of the well-accepted bacteria known as Pfeiffer's *Bacillus influenzae* [18-20, 23]. Limited staff and supplies, rudimentary knowledge on the pathogen and understanding of the disease coupled with the raging World War slowed significant advancements in the development of a cure [23]. Although some researchers questioned the origin of the pathogen, the vacillation between the potential causative agent of the disease was quickly redirected as the number of bodies laying in disarray increased. The consternation towards this upheaval that threatened human survival resulted in the vitiation of scientific rigour across research labs in a desperate attempt to save lives [18, 23].

Surely, the 1918 influenza commonly presented as viral pneumonia which often preceded or was concomitant with secondary bacterial pneumonia unless treated with antibiotics [18, 24]. Indeed, bacterial pneumonia was the major cause of death during the 1918 Spanish flu [24]. Autopsy reports showed denudation of the alveolar epithelium, fibrosis, intra-alveolar hemorrhage, edema, cellular inflammatory infiltrates associated with necrosis, formation of hyaline membranes, and bacterial invasion [20, 22, 24, 25]. A similar observation would be noted in pathology reports from patients from the 1957 and 1968 influenza pandemics [22]. The best immune strategy developed at that time was a vaccine containing streptococcal, pneumococcal, staphylococcal, and Pfeiffer's *Bacillus influenzae.* Even so*,* this intervention was largely ineffective as it only prevented the development of pneumonia caused by a fraction of bacterial serotypes and certainly did not protect against cases of early viral pneumonia [18]. In fact, there was much diversity in the clinical manifestations and course of disease following IAV infection in

1918. In a number of patients who died rapidly after contracting the virus, consolidation characteristic of bacterial pneumonia was rarely observed, and the alveolar compartment was essentially damaged beyond repair, suffused with necrotic immune cells and blood [13, 18, 25]. Certain clinical features were redolent of the bubonic plague, dysentery, cholera, dengue, typhoid or occasionally unlike anything pathologists had previously encountered, often leading to misdiagnoses [18, 25]. What was even more puzzling and distinguished the 1918 IAV strain was its high case fatality rate amongst an unusual target population, creating this characteristic "W" shaped mortality curve **(Figure 1.1)** [18, 20, 22, 25]. It victimized healthy young adults between the ages of 20-40 years old, largely sparing the middle-aged who had likely been exposed to a similar strain of H1N1 in their lives, which conferred some degree of protection, known as *original antigenic sin* [19, 20, 22]. Additionally, the influenza of 1918 did not leave any organs unscathed [23]. With regards to respiratory symptoms, mechanical pressures from violent coughing tore muscles of the rib cage and expectoration was saturated with blood [18]. Apart from the initial pulmonary symptoms, complications spread systemically and included extreme cyanosis, liver damage, pericarditis, renal failure, extrapulmonary mucosal hemorrhaging, and neurological impairments including hysteria and delirium [18, 20, 22]. Acute infection psychosis and systemic spread would not be unique to the this influenza, as the involvement of the central nervous and distal organ systems would resurface in the 1997 H5N1 Hong Kong outbreak [18]. This was fulminating viral pneumonia with a distinctive "cytokine storm" progressing to acute respiratory distress syndrome (ARDS). Patients' lungs were essentially battlegrounds for the

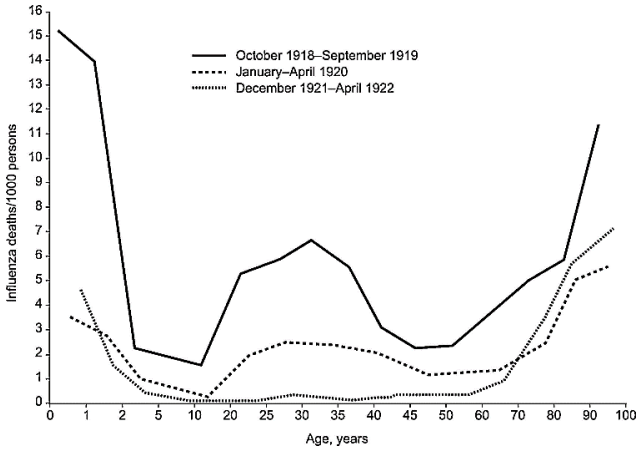

Figure 1.1 Characteristic W-shaped mortality curve depicting age-related influenza deaths in Breslau, Silesia from July 1918 to April 1922. Graph taken from [13].

invading virus and tissue became collateral damage. The immune system deployed every tool in its arsenal to clear the infection and scorched the very ground that supported it.

A common feature of highly pathogenic IAV strains such as the 1918 H1N1 and the 1997 H5N1 is the exuberant pro-inflammatory gene signature in airways of infected hosts which results from a combination of viral replication but more importantly, inadequate resolution of inflammation [10]. The differences in the pathology of infection from autopsy reports of those who died quickly during the 1918 pandemic compared to those in later years eventually lead to the appreciation of two important arms of immunity: **host resistance and disease tolerance** [10]. These concepts outline the necessity of both adequate antiviral responses to clear the pathogen as well as host repair mechanisms to resolve the inflammatory response [10]. If either of these defenses are compromised, the host inevitably succumbs to infection **(Figure 1.2).**

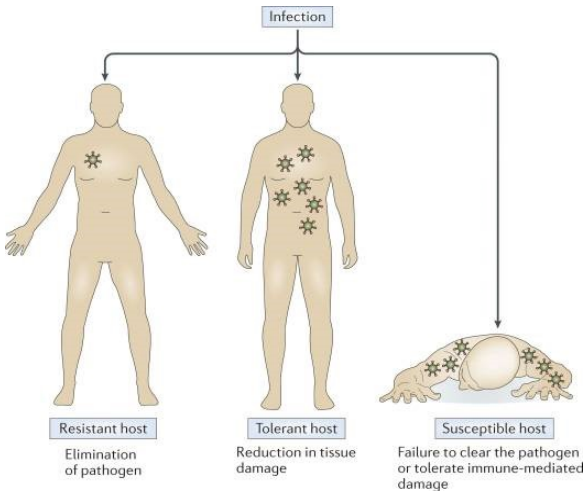

Figure 1.2. Arms of host defense: host resistance which encompasses clearing pathogen burden and disease tolerance which is required for inflammatory resolution. Inability to control pathogen replication or inflammatory responses leads to mortality of the host. Image taken from [10].

1.2 Biology and Pathophysiology of Influenza Viruses

Three types of influenza viruses have been identified, A, B, and C with IAV being the most pathogenic in humans and the one with the highest pandemic potential [1, 10, 20]. IAV infects and estivates in a number of species including pigs, birds, horses, and humans, with its natural host being of avian origin [1, 10, 20]. IAV can infect both upper and lower airways depending on the characteristics of its main surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) [1, 10, 20]. Of the 16 HA and 9 NA subtypes identified in avian strains, only H1, H2, H3 and N1, N2

have established successful infections in the human population that are readily transmissible [1]. There have been some reports of human infection with highly pathogenic avian IAV strains (H5N*x*, H7N*x*) however, interhuman transmission is infrequently observed in these cases [1]. In this regard, IAV of avian origin rarely infects humans [1, 10, 20]. Notwithstanding, the segmented nature of IAV allows it to undergo *antigenic drift* through the introduction of point mutations in its highly error-prone replicative machinery [1, 10, 20]. Previous exposure to IAV can still provide some protection against minor mutations within its structure. The real danger arises when two IAV strains co-infect an intermediate host. When a human and avian strain infect the same animal, such as a pig (which can be readily infected by human, avian, and swine IAV strains) genetic reassortment occurs between IAV strains, typically in the HA and NA genes, and results in the emergence of a novel zoonotic virus capable of interspecies transmission with a proclivity for human sialic acids [1, 10, 20]. This process is known as *antigenic shift* and yields antigenically unique viruses to which humans are immunologically naïve [1, 10, 20]. Antigenic shift resulted in the emergence of the five major influenza virus strains that caused the 1918 (H1N1), 1957 (H2N2), 1968 (H3N2), 1977 (H1N1), and the 2009 (H1N1pdm09) pandemics.

Infection occurs through inhalation of aerosolized droplets and is usually confined to the upper airways resulting in an acute infection with characteristic symptoms of fatigue, fever, sore throat, cough, and body aches [20]. Once inside the airways, the HA protein recognizes either α-2,3- or α-2,6-N-acetylneuraminic (sialic) acids on the surface of host epithelial cells. In the human upper respiratory tract, the dominant sialic acids are α-2,6-linked whereas α-2,3-linkages are more prevalent in the duck gut epithelium and in the distal human respiratory tract, mainly the bronchioles and the alveoli [1, 7, 10, 20]. However, both α-2,3 and α-2,6 residues can be found along the entire human respiratory tract. Avian IAV strains have higher affinity for sialic acid residues with α-2,3 sialic linkages whereas human strains favor α-2,6 sialic acid residues [26]. This

receptor preference gives IAV significant cell tropism and may account for the difference in viral distribution in the airways of those infected with seasonal or pandemic-causing strains of IAV [26].

The distal airways are not usually accessible to respiratory viruses that are mainly cleared by the mucociliary system in the upper respiratory tract [1, 10, 20]. Therefore, the success of many highly pathogenic respiratory viruses, such as the 1918 IAV strain, is directly linked to their ability to escape upper airway barriers and to cause lower respiratory tract infections [10]. The lower respiratory tract is home to sentinel cells known as alveolar macrophages (AM) whose function is to sense the microenvironment and clear debris, dust, bacteria, and other inhaled microparticles [27, 28]. Virulent strains of IAV bypass the upper airways and activate AMs often leading to a "cytokine storm" characterized by massive inflammatory cellular responses leading to pulmonary edema and hypoxemic respiratory failure [19, 22]. Hence, it can be argued that the virus is not the only threat, but rather, the host's own inflammatory response is jeopardizing survival. The 1918 pandemic primarily affecting young adults with strong immune systems was not only a result of an IAV strain capable of replicating within the alveolar compartment of a host with essentially no prior immunity. Rather, it was a perfect combination of viral virulence factors and host mechanisms driving lung pathology and respiratory failure [19, 22].

1.3 Influenza A Viral Life Cycle

The origin of viruses is largely debated. Some virologists consider that viruses evolved from simple organisms or were once an organelle [29]. Others argue that viruses devolved from complex organisms to become primitive *"quasi-species"* with only one main function: **replication** [30]. Of the viruses known to man, influenza is a paragon of this sole function. Belonging to the *Orthomyxoviridae* family of respiratory viruses, IAV is a single stranded anti-sense RNA virus

containing eight segments that code for at least ten essential proteins [1]. Upon attachment of HA to sialic acids on the surface of epithelial cells, IAV is endocytosed in a clathrin-dependent manner or through macropinocytosis [1, 26]. Successful replication requires an optimal pH to mediate viral exit of the endosome [1]. Acidification of the endosomal vesicle opens the floodgates for hydrogen ions to flow through the matrix-2 protein (M2) ion channel and induce a conformational change in HA which stimulates membrane fusion [1, 26]. Fusion of HA with the endosomal membrane triggers the uncoating of the viral core and the release of viral particles decorated by nucleoprotein (NP) copies known as viral ribonucleoproteins (vRNPs) from matrix-1 protein (M1) scaffolds into the host cytoplasm. Nuclear localization signals in the viral polymerase then direct the vRNPs to the nucleus via host α/β-importins to initiate replication and transcription [1, 26].

Cleavage of HA is essential in mediating the protein's function and fusion to the endosomal membrane. Depending on the cleavage site, HA will be processed by different host proteases within the cell. Highly pathogenic IAV strains have multi-basic cleavage sites that are processed by the ubiquitously expressed furin proteases [26]. In comparison, strains of lower pathogenicity and most human strains have monobasic cleavage sites and are processed by transmembrane-protease serine S-1 members and human airway trypsin-like proteases [26]. The differential distribution and expression of these proteases along the respiratory tree adds another layer of viral tropism which could explain the preferential infection sites and confinement of human versus avian IAV strains [26].

Replication of influenza occurs in the nucleus as opposed to most viruses which replicate in the cytoplasm [1, 10, 26]. Due to its antisense nature, replication begins with the formation of complementary RNA through a positive-sense intermediate known as complementary ribonucleoproteins (cRNPs). This process is executed by the viral RNA-dependent RNA polymerase (RdRp) complex composed of three subunits (PB1, PB2, PA) which is associated with each of the eight vRNPs. Viral mRNAs are transcribed from the vRNP templates by a stealth mechanism known as *"cap snatching"*. In this process, the viral RdRp complex cleaves off the 5' caps of nascent host mRNAs which serve as primers to promote positive sense transcription from the vRNP templates and conceal the existence of viral mRNA from the host [1, 26]. The resulting viral mRNAs are polyadenylated and exported into the cytoplasm for translation. Enveloped proteins, HA, M2, and NA, are transcribed on the surface of the endoplasmic reticulum, trafficked to the Golgi apparatus, and inserted into the host plasma membrane. Newly synthesized components of the RdRp complex and NP proteins return to the nucleus via nuclear localization to increase transcription efficiency of new anti-sense vRNPs [26]. Guided by M1 and influenza nuclear export protein (NEP), newly synthesized vRNPs and associated proteins are then exported into the cytoplasm, packaged into new viral particles coated by HA, M2, NA and host-derived plasma membrane, and bud off to restart their life cycle [1, 26]. This process is facilitated by NA which catalyzes the hydrolysis of sialic residues on the surface of epithelial cells so that newly formed virions can exit and infect neighbouring cells [1] **(Figure**

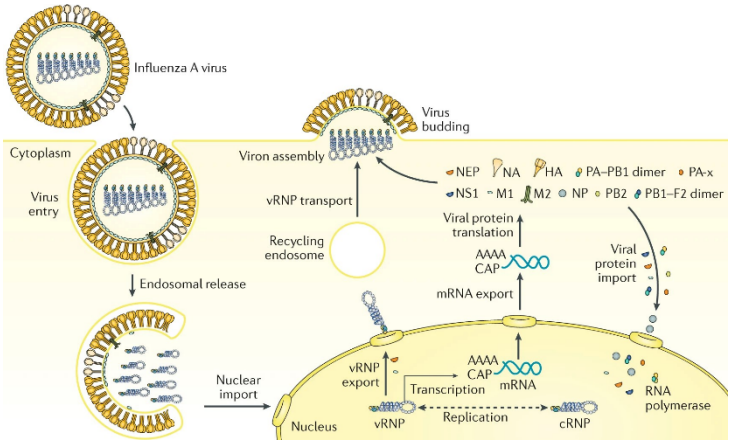

Figure 1.3 Influenza viral life cycle. Image taken from [1].

1.3). By hijacking host machinery to complete its life cycle, IAV shrouds itself from host immune responses to drive its pathogenesis.

Studies characterizing some of these viral factors in the subsequent years following the 1918 pandemic concluded that PB1, HA, and NA genes were associated with the high pathogenicity of that IAV strain [22, 31-33]. Importantly, multiple sites and motifs within IAV including non-structural protein-1 (NS1) have also been identified in pandemic strains as key virulence factors determining IAV pathogenicity. Although not packaged into newly formed virions, NS1 is highly upregulated in infected cells and serves to suppress host antiviral defenses (specifically the type I interferon (IFN) axis) and modulate cellular apoptosis to maximize replication efficiency [34]. Other IAV non-structural proteins with immune evasion and suppression functions include PB1 frame 2 (PB1-F2) and PA-X. The process of IAV replication is notorious for inducing epithelial cell death and pathology reports from the 1918 pandemic revealed that disease severity was correlated with high viral virulence and apoptosis of epithelial cells in the large conducting airways but more importantly, the alveolar compartment [24].

The peregrination of influenza research has been long-lasting. Information gathered from previous outbreaks allowed us to gain insight into the immune response and its interaction with IAV which lead to the emergence of novel vaccine technologies and the development of antiviral therapies [35]. Vaccination has proven mankind's best weapon serving as a foresighted strategy to intercept the pathogen's royal flush: **its novelty**. In addition to vaccination, two antiviral drug classes were manufactured: ion channel blockers (*e.g.,* amantadine) which prevent acidification of the endosomal compartment by the M2 protein and NA inhibitors (*e.g*. oseltamivir and zanamivir) which prevent viral exit [36]. However, epidemiological data following the 2009 H1N1 pandemic highlighted the inadequacy of current vaccination and antiviral therapies whose efficacy have become mediocre due to IAV's high mutation rate conferring resistance to treatment and rendering

its structure and behaviour highly unpredictable [36]. Therefore, despite a century of research, revolutionary scientific achievements, medical milestones, and documented historic notes of disease propagation and progression, we are still burdened by annual seasonal influenza outbreaks and threatened by looming pandemic strains.

1.4 Immune Response to Influenza A Virus (IAV)

1.4.1 Mechanical and Chemical Barriers in the Upper and Lower Airways

Epithelial cells of the respiratory tract serve as the front-line soldiers as they are the primary site of host-pathogen interactions. Epithelial cells are well-equipped to sense and resist invading threat and signal the infantry. Mice deficient in signal transducer and activator of transcription 1 (STAT1), which is essential in the induction of antiviral responses, exclusively in the epithelium, have severely impaired immune responses to respiratory viruses [37]. Collectively, epithelial cells supply the upper airways with a physiochemical barrier to prevent the dissemination of pathogens into the lower airways with the help of the mucociliary escalator. In fact, it was in 1922, when Alexander Fleming first discovered that human nasal secretions had potent antimicrobial properties [38, 39]. Although at the time he attributed these properties to the action of lysozymes, over the years, this cadre expanded to include other antimicrobial peptides (AMPs) such as lactoferrin, defensins, and cathelicidins, as well as collectins, mucus glycoproteins, and immunoglobulins (Ig) to name a few [38, 40, 41]. Together with the production of pro-inflammatory and antiviral cytokines and chemokines, they orchestrate

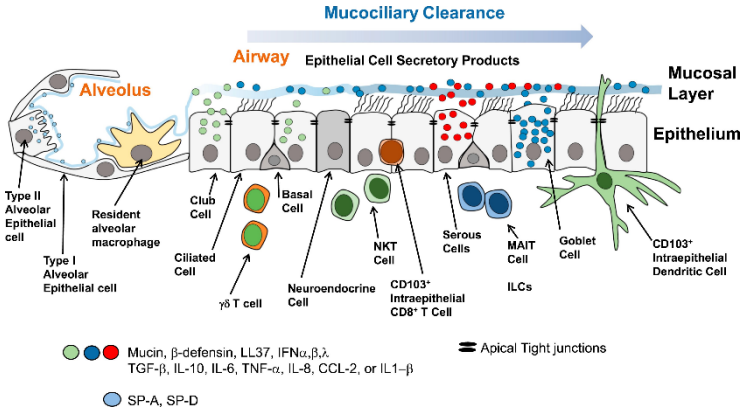

Figure 1.4. Structure and components of the respiratory epithelium with associated cellular and soluble factors involved in host defense against IAV infection. Image taken from [7]. Copyright https://creativecommons.org/licenses/by-nc-nd/4.0/

the downstream immune responses necessary to clear infection **(Figure 1.4)**. *The following section will describe in detail the antimicrobial properties of the respiratory tract and some of its constituents.*

The respiratory tract can be divided into two compartments: the upper and lower airways. The lower airways can be further sub-divided into large conducting (bronchial) and peripheral (bronchiolar) airways. The respiratory tract for both humans and mice begins with the nasal cavity which contains three regions starting from the most cephalic and moving distally down the respiratory tree: (1) the nasal vestibule, (2) the respiratory epithelium, and (3) the olfactory epithelium [2] **(Figure 1.5)**. Each region has characteristic epithelial structures and associated functions. The nasal vestibule is lined with a stratified, squamous, non-ciliated epithelium and is located at the entrance of the nostrils/nares [2, 42]. The respiratory region is a columnar pseudostratified epithelium containing ciliated and non-ciliated cells, basal cells, and goblet cell [2]. This region regulates airflow and is a rich source of mucins and AMPs that trap inhaled particles and prevent their progression to the lower airways [42]. The olfactory region is a thick pseudostratified columnar epithelium, distinguished by the presence of the Bowman's gland, and contains olfactory sensory neurons, and basal cells [2, 42]. The conducting airways contain the mucociliary escalator and are lined by a pseudostratified columnar epithelial layer which branches out into smaller bronchioles of a simple cuboidal structure [41, 43]. The terminal end of the respiratory tree is lined by alveolar type I and II cells which constitute a major part of the surface of the alveolar compartment [41, 43].

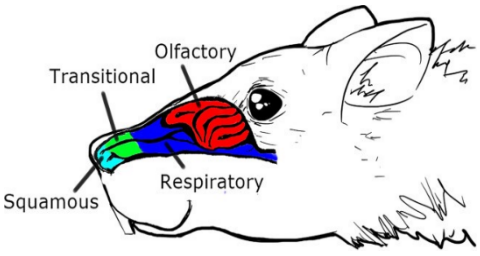

Figure 1.5. Anatomy of the rodent nasal cavity. Image taken from [2].

The primary protective barrier in the upper airways includes the mobile mucous or gel layer containing soluble mucins, a separating surfactant layer, and the periciliary layer composed of tethered mucins, cilia, and microvilli on the surface of nasal and bronchial epithelial cells [44] **(Figure 1.6).** Upper airway secretory cells and ciliated cells function interdependently to produce mucus and AMPs and drive the mucociliary escalator to hem in and impel foreign particles in a sort of conveyer belt-like fashion to be coughed out or swallowed [41, 43].

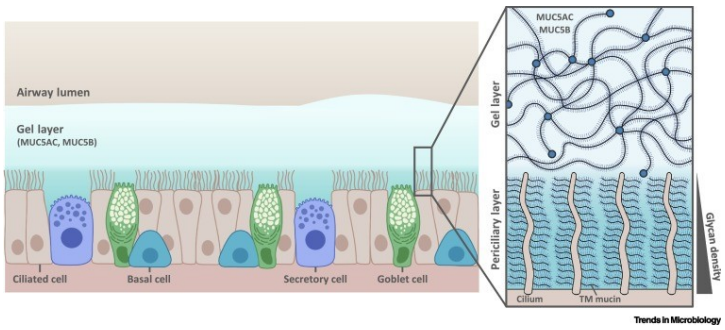

Figure 1.6. Schematic of the mucous and periciliary layer with associated mucins. Image taken from [3].

Mucin (MUC) glycoproteins are the main structural components of mucus which not only confer viscoelasticity to the mucous gel, but also constitute a matrix to contain secreted AMPs [41, 43, 45]. The most prominent mucins, MUC5AC and MUC5B, are secreted by goblet cells and submucosal glands found in secretory cells, respectively, and are upregulated during infection with respiratory pathogens such as IAV to help with trapping and clearance [3, 41, 43]. MUC5B is the chief mucin in healthy airways, the predominant mucin in the distal airways, and the main driver of the mucociliary escalator for the initial protection against respiratory pathogens [43, 46]. Mucin expression is induced by the interaction of pathogen associated molecular patterns (PAMPs) with pattern recognition receptors (PRR) and through the production of cytokines such as interleukin (IL-)1β and IL-17 following nuclear factor κB (NFκB) activation [41, 43]. MUC5AC production is induced after viral infection, and in the case of IAV, as military deception, acting as a decoy receptor preventing viral binding to sialic acid receptors on epithelial cells [47-49]. Mucins slow the movement of IAV throughout the respiratory tract and reduce transmission of IAV strains with low NA activity [26]. Not surprisingly, the NA portion of IAV can desialylate mucins to reduce

their viscosity and facilitate the binding of HA to neighbouring epithelial cells to flank this defense mechanism [26, 50]. However, the proportion of MUC5AC and MUC5B within the airways, with a tendency towards MUC5AC hypersecretion, varies with individual health, such as in cases of asthma, allergic inflammation, infection, and even pregnancy [3, 51-54].

AMPs are present in nearly all human mucosal secretions and are important contributors to host defense at barrier sites [55-58]. The two major classes of AMPs are defensins and cathelicidins. They have dual roles being both potent immunomodulators and direct inhibitors of infection, synchronously ensuring optimal pathogen clearance and inflammatory resolution. First identified in 1985, human defensins were categorized into two major families (α and β) of which human α -defensin (HNP) subtypes HNP-1, -2, -3, and -4 are stored in neutrophil granules while -5 and -6 are produced by Paneth cells and by respiratory epithelial cells [39]. *It is important to note that mouse neutrophils do not express α-defensins [59] and therefore may not be ideal surrogates to investigate their contribution to host defense against infectious pathogens that require neutrophilic responses for clearance.* In contrast, human β-defensins (HBD) are expressed predominantly by epithelial cells of mucosal surfaces such as the respiratory, intestinal, and female reproductive tract and are secreted rather than released by degranulation [55, 56, 60]. Epithelial expression of β-defensins may be constitutive, such as HBD-1, or induced upon infection or inflammatory stimuli [39, 55, 56]. Mouse β-defensins, mBD-3 (HBD-2 ortholog) and -4, have been shown to be upregulated in the upper and large conducting airways following infection with the mouse-adapted A/Puerto Rico/8/34 H1N1 strain (PR8) [61] while mBD-4 reduced pulmonary viral titers following infection with A/CA/04/2009 H1N1 [62].

Cathelicidins are first synthesized and stored as prepropeptides that require proteolytic cleavage by proteinase-3 to become active [63]. The most abundant cathelicidin variant and the only one identified in humans is LL-37 (mouse ortholog, CRAMP). LL37 and HBD-2 are

regulated in response to vitamin D as well as infection and inflammation [64]. LL37 is predominantly secreted by neutrophils but can also be produced by epithelial cells, natural killer (NK) cells, and macrophages and has been shown to neutralize viruses, including IAV, respiratory syncytial virus (RSV), and dengue virus *in vitro* and *in vivo* [56, 65-68]. Administration of LL37 to mice lowered viral titers, inflammation, and morbidity following IAV infection [67, 69]. LL37 and HNP-1 have also been shown to induce the expression of MUC5AC in airway epithelial cells, highlighting the important interplay and coaction of antimicrobial defenses [70, 71].

Defensins and cathelicidins have synergistic functions and exert their antimicrobial and antiviral activities by directly disrupting pathogen membranes through the formation of amphipathic secondary structures, destabilizing viral envelopes, inducing viral aggregation, and inhibiting replication and protein synthesis of non-enveloped viruses to name a few [55, 56, 72]. Additionally, AMPs possess immunomodulatory properties by regulating cytokine, chemokine, and adhesion molecule production, and promoting chemotaxis of leukocytes, predominantly of neutrophils and monocytes to effectively clear viral and bacterial pathogens [73-76]. Activation of the NF-κB pathway is a major inducer of AMP production akin to mucin [77]. Of the cytokines that induce this pathway, the IL-17 family of cytokines (IL-17A/F, IL-22) have been shown to increase AMP production to clear viral and bacterial infections and support tissue repair [76, 78-81]. Importantly, AMPs also exert potent anti-inflammatory effects by suppressing Toll-like receptor (TLR)-induced production of inflammatory cytokines, driving macrophage pro-resolving phenotypes, and supporting epithelial barrier integrity and tissue regeneration by stimulating angiogenesis, cell migration and proliferation [82-87]. Thus, AMPs can promote both host resistance and disease tolerance.

In some cases, such as in 1918, the HA portion of IAV had a single glycosylation site and was therefore small enough to bypass mucociliary clearance and travel deeper into the airways

causing severe disease in an otherwise impenetrable system [88]. The lower airways are lined with type I and II alveolar epithelial cells (AEC1/2) and provide a chemical barrier by regulating surfactant production, gas exchange, and inflammation through the secretion of cytokines and enzymes [41, 89, 90]. AEC1s, although numerically in the minority, cover the majority of the alveolar surface at the main site of gas exchange [7, 91, 92]. AEC2s (and Club cells on a smaller scale) are the primary replicative niche of IAV in the alveolar compartment, the main producers of surfactant proteins (SP) necessary for proper respiration, and can regenerate and repopulate both AEC1 and AEC2s [7, 91, 92]. Belonging to the collectin family of AMPs, SP-A and SP-D are important C-type lectins in the clearance of pathogens, especially heavily glycosylated strains of IAV [7, 88, 91, 92]. Examples of such protection were observed through the addition of glycans to the otherwise devoid HA component of PR8 and poorly glycosylated HA portion of H3N2 and A/Brazil/11/78 H1N1 which increased their sensitivity to SP-D resulting in viral clearance [88, 93-95]. Both SP-A and SP-D have antiviral effects but differ slightly in their mechanism of action. SP-A neutralizes IAV and prevents its entry into host cells by binding to HA and SP-D best serves to opsonize and aggregate viral particles [7, 88, 91, 92]. Both surfactant proteins have also been shown to promote macrophage phagocytosis [96]. Polymorphisms in these proteins are linearly correlated with worse disease outcomes [97, 98] and deficiencies have been shown to increase susceptibility to many pulmonary infections including IAV, RSV, and bacterial infections [96]. Studies using SP-A$^{-/-}$ and SP-D$^{-/-}$ mice showed impaired pulmonary viral clearance, enhanced pulmonary inflammation, and T-helper 1 (Th1) immune responses [7, 91, 92]. Surfactant proteins are therefore vital in modulating innate immune responses and may also be responsible for reducing inflammation and tissue damage in the lung during viral infections [7, 91, 92]. Although their expression and function in the lungs is well characterized, SP-A and SP-D have also been

identified in the human nasal mucosa [99-101]. Considering their role in host defense in the lower airways, they are likely also contributing to upper airway immunity through similar mechanisms.

In addition, a plethora of other antimicrobial molecules such as nitric oxide, lysozyme, lactoferrin, and complement C3 protein can also serve as primary defenses to opsonize pathogens and trigger phagocytosis, block viral replication, and activate the immune system [90, 102]. Antibodies, such as secretory IgA, synergistically contribute to mucosal immunity against IAV by hindering initial infection of epithelial cells along the respiratory tract [40, 43]. Natural IgM antibodies produced predominantly by B-1 cells collectively comprise the innate humoral immune system of mice and men. B-1 responses are induced early following influenza infection via stimulation by pro-inflammatory and antiviral cytokines to differentiate into potent IgM-producing cells [102-105]. Early production of IgM contributes to innate responses by directly inducing viral clearance but also bridges the adaptive immune system by ensuring optimal influenza-specific IgG responses [102, 103, 106]. Together, the stratum of upper airway antimicrobial defenses ensures the rapid cession of replication and exclusion of respiratory pathogens from the alveolar compartment.

1.4.2 Immune Response to Acute Infection

The immune system is a very elaborate network, like a matrix. Unlike the adaptive immune response which relies on memory, the innate immune branch is a primitive collection of warriors born for battle. If a virus manages to escape this first line of defense and head towards the more vulnerable lower airways, epithelial cells will heed a call to arms. Epithelial cells signal the infantry with the help of AMs, the resident cells of the alveolar compartment and the first immune cells exposed to virus. This early response includes the production of cytokines, chemokines, and cytotoxic factors, and the recruitment and activation of inflammatory monocytes, dendritic cells (DC) neutrophils, NK cells, innate-like lymphoid cells (ILC), and gamma delta (γδ) T cells [107].

In the later stages of infection, DCs traffic viral antigen to the lymph nodes to call for back-up and engage the adaptive immune system through antigen presentation to yield antigen-specific effector T cells, antibody production, and ultimately immune memory.

The immune response is enabled by sensing invading microbes through PRRs including the first PRRs discovered in the late 1990s known as TLRs, as well as NOD-like receptors (NLRs) and retinoic acid inducible gene-1 (RIG-I)-like receptor (RLRs) [108]. Epithelial cells and AMs recognize IAV through TLR3, TLR7, and RIG-I together with melanoma differentiation-associated protein 5 (MDA5) [7, 41, 43, 89, 90]. There is much convergence on the downstream signalling pathways following receptor activation of all these PRRs. This is not a glitch in the matrix. This redundancy is necessary if one considers biological context. Infection is a powerful driver of evolution, and so over time, humans have developed multiple classes of receptors to survey different parts of our cells to establish a barricade to prevent entry of an array of microbes. In this way, regardless of where a pathogen tries to invade, there are checkpoints on each front with TLRs positioned extracellularly on the plasma membrane and intracellularly (*e.g.,* on endosomes and lysosomes), while NRLs and RLRs are found intracellularly within the cytosol [10].

In both epithelial cells and AMs, initial recognition of IAV occurs through cytosolic RIG-1 (recently found to be sequestered in stress granules) which senses free viral double-stranded or single-stranded RNA with 5'triphosphates in IAV infected cells following replication [7, 41, 43, 89, 90]. Viral RNA bound to RIG-I leads to the interaction with mitochondrial antiviral signaling proteins (MAVS) and the subsequent phosphorylation and translocation of interferon inducible factor 3 (IRF3) to the nucleus [7, 41, 43, 89, 90]. This pathway is critical in the initial antiviral response through the production of IFN-β [109]. In addition to RIG-I signalling, TLR3 and TLR7 recognize double-stranded RNA from dying infected cells and single-stranded RNA through viral

endocytosis, respectively [10]. The downstream signalling cascade of TLR3 and TLR7 leads to the translocation of NF-κB to the nucleus to induce the transcription of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and IL-6 [10]. The other branch downstream of pathogen sensing through TLR3 and TLR7 leads to the production of type I IFNs through the translocation of IRF3 and IRF7, respectively, to the nucleus [10]. Signalling of this pathway results in the activation of the Janus Kinase (JAK)/STAT pathway to upregulate hundreds of genes collectively known as interferon-stimulated genes (ISGs) which inhibit viral replication, degrade viral nucleic acids, and promote host resistance [10]. Both pathways downstream of TLR3 and TLR7 ensure the establishment of a proper antiviral and inflammatory environment. Interestingly, studies using TLR3 knockout mice have demonstrated a higher survival rate than wild-type mice despite a high viral burden. Thus, TLR3 recognition, although necessary for reducing viral replication, may lead to exacerbated inflammatory responses [110]. Studies to underline the role of TLR7 in IAV have shown that TLR7 antagonism promotes disease tolerance by reducing late type I IFN-dependent inflammatory responses [111]. Other studies found a requirement for TLR7 in host resistance by inducing B cell antibody responses [112, 113]. Thus, the exact role of TLR7 in immune defense against IAV infection is still not completely understood.

Epithelial cells and AMs are important sources of antimicrobial and antiviral proteins which regulate innate immune responses and inflammation [41, 43, 90]. Of these, type I (IFN-α and IFN-β) and III (IFN-λ1, IFN-λ2, and IFN-λ3) IFNs are key mediators in the orchestration of antiviral and subsequent immune-inflammatory responses following IAV infection. [10, 114]. Both IFN-α and IFN-β act in an autocrine and paracrine fashion to establish antiviral immunity [109, 114]. The initial production of IFN-β through RIG-I signalling binds to the heterodimeric IFN-α/β receptor (IFNAR1/2) which yields a second wave of type I IFNs, this time also producing IFN-α through the additional induction of IRF7 expression [115]. Work in the Divangahi lab has

elegantly demonstrated the importance of this biphasic induction of type I IFNs during IAV infection for the procurement of both early antiviral immunity (through IFN-β, promoting host resistance) [116-118] and late host immunomodulation (through IFN-α, promoting disease tolerance) [109]. Indeed, intranasal administration of IFN-α has been shown to reduce IAV and RSV viral titers in animal models [119, 120]. However, during the late phase of IAV infection, Pernet *et al.* demonstrated that IFN-α mediated suppression of inflammatory monocyte proliferation via the engagement of the leukotriene b4 (LTB4)–BLT1R axis and promoted disease tolerance, providing, in fact, a dual role for IFN-α in both arms of host defense [109].

Type III IFNs were discovered in 2003 and are produced during the very early stages of viral infections [14]. IFN-λ binds to its own independent receptor complex composed of two subunits: IL-10R2 and IFNλR1 [14]. Type I IFN responses can be generated in almost all nucleated cells due to the ubiquitous expression of IFNAR, whereas type III IFN responses are restricted to mucosal sites such as the airways or gut epithelium [14, 121]. For this reason, unregulated type I IFN responses can contribute to severe immunopathology however, the restriction of type III IFN responses at barrier surfaces establishes an adequate antiviral environment whilst limiting inflammatory processes which could be damaging to the host [122]. Evidence of IFN compartmentalization has also been documented with type III IFNs being more prominently produced in the upper airways to restrict viral propagation to the lower airways. In contrast, type I IFN production will assume dominant viral control if dissemination to the lower airways occurs, albeit at the potential expense of host fitness [123]. Therefore, although there is significant functional redundancy, the kinetics and localization of selective IFN production are apportioned along the respiratory tract to synergistically ensure appropriate antiviral responses with minimal damage to host tissues [124].

Despite their distinct induction differences and usage of receptor complexes, once type I and III IFNs bind to their respective receptors, the downstream signaling process is virtually identical [114]. Binding of IFNs to their receptors leads to the transcription of IFN-stimulated gene factor 3 (ISGF3), a complex composed of STAT1, STAT2, and IRF9, and induces transcription of hundreds of ISGs via interaction with interferon-sensitive response elements (ISRE) in their promoters to interfere with viral replication, degrade viral RNAs, and induce apoptosis of the infected cells **(Figure 1.7)** [114].

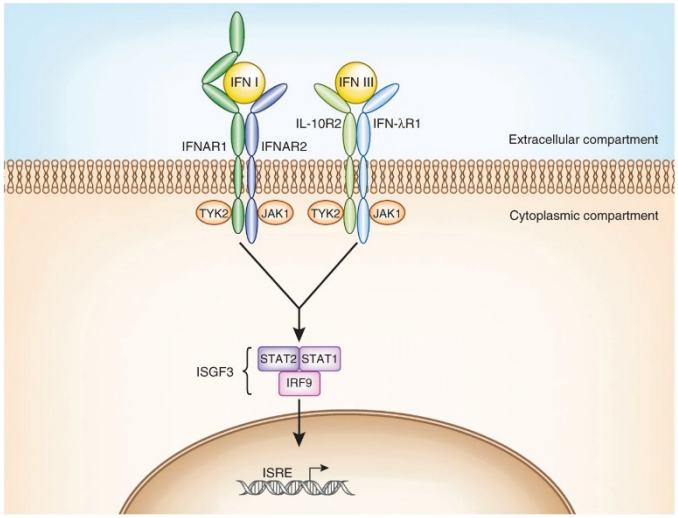

Figure 1.7. Type I and III IFN receptor signalling cascade through different receptor complexes leading to the transcription of ISGs to induce an antiviral state. Image taken from [14].

Some of the important and well-studied ISGs include myxovirus resistance (Mx) proteins, interferon induced transmembrane proteins (IFITMs), 2-5'oligoadenylate synthetase (OAS), and protein kinase R (PKR) [114]. Cytosolic Mx proteins in humans prevent nuclear import of viral nucleocapsids and viral transcription [10]. *However, since almost all inbred mice lack functional Mx proteins, one must pay heed to the interpretation of results from mouse studies studying type I IFN-mediated responses* [10]. IFITMs directly block viral entry by preventing viral fusion with host membranes [10]. IFITM3, which is constitutively expressed in macrophages and epithelial cells, is also essential in limiting tissue damage, as IFITM3 deficient mice succumb quickly to infection due to pulmonary edema and alveolar hemorrhaging despite controlling initial viral burden [10]. OAS activates RNase L to destroy cytosolic viral RNA and disrupt the protein synthesis process making it an important ISG in controlling viral replication [10]. PKR inhibits IAV replication through Fas expression and FADD-dependent apoptosis [125] and by directly

binding RNA to inhibit both host and viral protein translation through the phosphorylation of the α-subunit of eukaryotic translation initiation factor (eIF) 2α [10, 126]. PKR also stabilizes type I IFN mRNA to uphold IFN-mediated antiviral responses [10]. Due to the importance of ISGs in antiviral immunity, surely IAV has evolved mechanisms to block the IFN axis and directly run interference on the mechanisms of action of these ISGs. Mutations in the NP of the 1918 pandemic influenza granted immune evasion from Mx restriction [127, 128]. NS1 blocks interferon signaling by blocking PKR and increases translation efficiency through the recruitment of eIF-4G to the 5'region of viral mRNA [1]. Recent studies show that NS1 also disrupts the stress granules in which RIG-I and PKR co-localize to reduce IFN-responses [10]. Evidence of IAV evolving to block RIG-I signaling further demonstrates its indispensable role in viral recognition following infection. Another potent inhibitor of antiviral responses is viral PBI-F2 which binds MAVS to inhibit IFN production and induces apoptosis of infected cells [117]. Of note, host mitochondrial protein nucleotide-binding oligomerization domain-like receptor (NLRX1) has been shown to prevent PB1-F2-mediated macrophage apoptosis to maintain type I IFN responses [117]. As demonstrated, this dance between influenza and our immune system is a perpetual internal chess match where one tries to outsmart, outwit, and outmaneuver the other.

Although epithelial cells are the first line of defense, they are succored by the innate immune system. AMs are the first cells to encounter a pathogen in the alveolar compartment. They are pivotal in innate and adaptive immune responses and in preventing respiratory failure as depleting this cell type leads to increased viral titers, immunopathology, and mortality [116, 129, 130]. Due to their importance in tissue homeostasis and driving initial host defenses, IAV evolved to quickly deplete AMs through cell death pathways, paving the way for respiratory failure and secondary bacterial infection [131]. Recognition of IAV by epithelial cells and AMs stimulates an increase in the production of cytokines such as TNF-α and IL-1 and chemokines which in turn

upregulate the expression of intracellular and vascular adhesion molecules to recruit neutrophils, monocytes, NK cells, and DCs to kill infected cells and initiate subsequent adaptive immune responses [132, 133]. Neutrophils and monocytes are recruited primarily through keratinocyte chemoattractant (KC or IL-8 in humans) and chemoattractant protein 1 (CCL2), respectively [134, 135]. Although studies using C-C chemokine receptor type 2 (CCR2) knockout mice show reduced survival following IAV infection, $CCR2^{+}$ monocytes become the main source of inflammatory cytokine production as disease progresses and become major drivers of immunopathology [134]. Neutrophils produce α-defensins, reactive oxygen species, and extracellular traps to help clear infection, however, continuous production of cytotoxins can cause considerable tissue damage [136]. Therefore, the clearance of inflammatory monocytes and neutrophils from the lungs following infection is essential as their persistence and accruing production of cytotoxic molecules and pro-apoptotic signals could exacerbate tissue damage and jeopardize epithelial integrity. Instead, their timely migration to the site of infection should be succeeded by inflammatory resolution to limit pathology [90].

The first wave of cytokine secretion can also induce type-2 immune responses and exacerbate asthma symptoms. Chang *et al.* showed that IAV is implicated in the induction of pulmonary type-2 immunopathology through the release of damage-associated molecular patterns (DAMPs), such as IL-33 and thymic stromal lymphopoietin (TSLP), followed by the recruitment of Th2 cells and ILC2s, and the secretion of IL-5 and IL-13

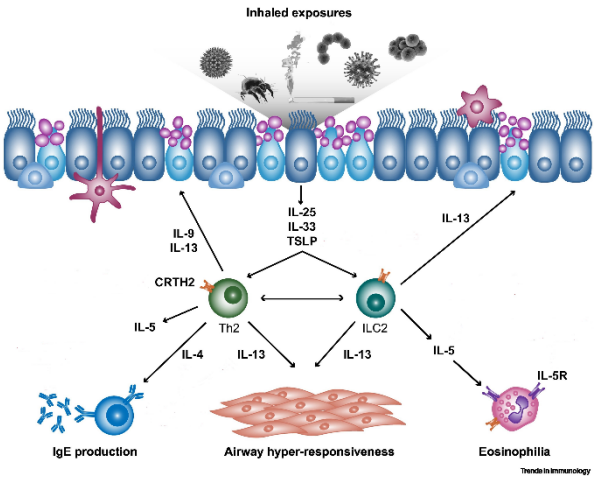

Figure 1.8. Adaptation on type-2-induced airway hyperresponsiveness following inhaled exposures. Image taken from[12].

causing eosinophilia and airway hyperresponsiveness (AHR) **(Figure 1.8)** [137, 138]. Indeed, ILC2s can promote tissue repair through the production of amphiregulin [139]. However, under certain conditions, type-2 cytokine-producing ILCs can exacerbate lung pathology through excess mucus production and airway smooth muscle constriction [10]. IL-13 has been associated with the disruption of a healthy MUC5AC/MUC5B ratio, ramping MUC5AC production through the epidermal growth factor receptor, STAT6 activation, and specificity protein 1 (Sp1) resulting in AHR [48, 140-142]. This type-2 mediated inflammation can occur independently of adaptive immunity although Th2 cells will induce the same cytokine profile [12, 43]. In the case of severe infection where unresolved type-1 inflammation is already damaging delicate epithelial tissue, it is evident that layering a type-2 based immune response which leads to constriction of the airways renders the host exceedingly susceptible to respiratory dysfunction [90].

Upon phagocytosing infected cells, conventional DCs migrate to the draining lymph nodes to present antigen to naïve T cells and pass the torch to the adaptive immune system to assume charge. In response to IAV infection, newly activated T cells proliferate, differentiate, and acquire effector functions of the classical Th1 profile which predominantly secrete IFN- γ [133]. However, $\gamma\delta^+$ T-cells, a unique subset of T cells identified in the mid-1980s that express γδ subunits of the TCR rather than αβ, can also contribute to host defense even without TCR engagement, through the production of IL-17A/F, IL-22, and IFN-γ [143]. $\gamma\delta^+$ T cells arise from $CD3^-CD4^-CD8^-$ double negative thymocytes similar to conventional T cells and represent around 1-5% of circulating lymphocytes. However, unlike αβ T cells, which are naïve upon exiting the thymus until they acquire appropriate effector functions in the periphery, $\gamma\delta^+$ T cells are bestowed with effector fates during thymic development [144]. Depending on their γ-chain variable regions (Vγ), pre-programmed subsets of $\gamma\delta^+$ T cells exit the thymus in sequential waves during embryogenesis to populate peripheral organs, primarily mucosal tissues [145, 146]. However, evidence of

extrathymic maturation has been reported for certain Vγ subsets that reside in intestinal [147, 148], hepatic [149], and decidual [150-152] tissues. Importantly, $\gamma\delta^+$ T cells possess bona fide innate functions and have been most extensively studied for their protective roles against an array of pathogens at mucosal sites [107, 153]. IL-17-producing $\gamma\delta^+$ T cells during early IAV infection have been shown to promote viral clearance through the recruitment of NK cells and neutrophils in the trachea [75] and B-1a differentiation and IgM production in the lungs [154, 155]. Moreover, Thomas and colleagues reported that $\gamma\delta^+$ T cells protected neonatal mice against IAV-mediated pulmonary immunopathology through the IL-33-amphiregulin axis which mirrored elevated levels of IL-17 in nasal secretions of children with mild disease [156]. Pulmonary IL-17-producing $\gamma\delta^+$ T cells have also been shown to induce pathogen clearance via AMP production [157] and increased neutrophilia [158]. Nonetheless, the relevance of IL-17 in innate immunity has been mainly studied in the context of bacterial and fungal infections, and most IAV literature in adult mice outlines its detrimental role in perpetuating positive pulmonary inflammatory feedback loops [159-162]. Recently, IL-17 has been gaining consideration for its role in healthy responses to injury and infection [163]. **As we investigated in our study in Chapter 2, there is a beneficial role for IL-17 primarily produced by $\gamma\delta^+$ T cells in the upper airways in adult mice driving antimicrobial immunity against IAV infection in the context of pregnancy.**

1.5 Pregnancy

1.5.1 Immunological Changes in Pregnancy

1.5.1.1 Systemic Immune Changes

Many anatomical, physiological, and immunological changes occur during pregnancy and are of relevant importance for disease outcomes. Of the most important changes concerning infection are the immunological adaptations that are necessary to sustain a growing fetus. Changes

of the maternal immune system as gestation progresses are postulated to favor reproductive fitness, partly due to increasing estrogen and progesterone concentrations which drive type-2 immune responses [164]. It was thought for a very long time that the maternal immune system was in constant antagonism with the semi-allogeneic fetus and that fetal development was devoted to maternal immunosuppression. Although there is evidence of such a switch in immune profiles with Th1/Th17-type disorders such as psoriasis, multiple sclerosis, and arthritis, ameliorating or remitting in a subset of women during gestation, this paradigm originates from the immune adaptations occurring at the maternal-fetal interface [4, 164-166]. Even so, Th2 cytokine-deficient mice experience normal allogeneic pregnancies [167]. And although excessive pro-inflammatory signals can lead to abortion, pre-term labour and preeclampsia, cytokines such as IFN-γ are vital in the first trimester of pregnancy for vascular remodelling and in the final trimester to induce labour [168, 169]. Therefore, the complex spatiotemporal expression of the cytokine network and its interaction with immune cells make the immunology of pregnancy extremely challenging to generalize.

This shift in immune profiles driven by female reproductive hormones has been long thought as compromising the maternal immune response, rendering the woman susceptible to infections that require type-1 immune responses essential for viral clearance [170]. At high concentrations, estrogen [171-177] and progesterone [178, 179] inhibit NF-κB signaling and reduce the expression of pro-inflammatory and antiviral cytokines (*i.e.,* type I IFNs) in favor of an anti-inflammatory environment. Based on this, a pregnant woman is faced with contradictory demands to sustain immune tolerance required for fetal survival versus mounting an appropriate immune response to fight infection. Although there is a limited number of studies reporting influenza incidence in pregnant women, studies report a complex environment during pregnancy, in which the concentration of both serum pro- and anti-inflammatory cytokines are increased [175,

180, 181]. Serum cytokine profiles in women vary greatly from patient to patient, and the profiles change dramatically particularly during the second and third trimesters of pregnancy [181]. As women in these two gestational periods are more prone to severe infection, these changes may influence their immune response against invading pathogens.

1.5.1.2 Local Immune Response at the Fetal-Maternal Interface

Immunological changes throughout pregnancy are dynamic and adopt specific profiles to permit embryo implantation, fetal growth, and parturition **(Figure 1.9)** [4]. Pregnancy begins as a pro-inflammatory state in which the maternal decidua (a specialized layer of endometrial tissue lining the uterus) undergoes extensive remodeling characterized by immune cell infiltration and upregulation of adhesion molecules in order to establish an environment capable of receiving the implanting blastocyst [4, 169]. NK cells are the most abundant cell type, accounting for 70% of immune cells in the uterus, while macrophages make up 20-25%, DCs tally to 1.7%, and T cells (including $CD4^+$, $CD8^+$, $\gamma\delta^+$ T cells, regulatory T cells (Tregs)) account for 3-10% [182-185]. Analogous to tissue injury, the first stage of pregnancy is rich in inflammatory cytokines including IL-6, IL-8, IFN-γ, CXC-chemokine ligand 1 (CXCL1), CC-chemokine ligand 4 (CCL4), and TNF-α and requires the infiltration of innate cells, mainly DCs, M1-like macrophages, neutrophils, and NK cells [168, 182]. Maternal leukocytes home to the decidua via chemokine gradients secreted by decidual and fetal tissues and are phenotypically and functionally different from their peripheral counterparts which are generally more cytotoxic [182]. Decidual NK (dNK) cells, M1-like macrophages, and uterine DCs are crucial in vascular

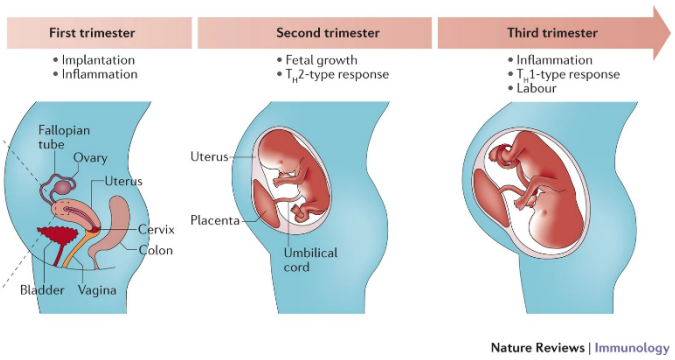

Figure 1.9. Adaptation of the current views of the immunology of pregnancy defined by trimester. Image taken from [4].

remodeling to allow the flow of nutrients and gases to the fetus, embryo implantation, apoptosis and cellular clearance, and decidual angiogenesis [182]. Murine studies show that absence of dNK cells leads to reduced fetal viability and abnormal decidual architecture [186, 187]. Moreover, transgenic CD11b-Dtr mice post diphtheria toxin administration show complete implantation failure [188], highlighting the importance of dNK cells and macrophages in the initial establishment of a successful pregnancy.

Following embryo attachment, the microenvironment at the maternal-fetal interface shifts to an anti-inflammatory stage that allows for fetal growth [4]. At this stage, the mother and fetus could be viewed as symbionts. This milieu is rich in M2-like macrophages and Tregs which synergistically function to maintain a tolerogenic environment. They do so by secreting anti-inflammatory cytokines, such as TGF-β and IL-10, and restricting immune recognition of paternal antigens [4]. Polarized by macrophage colony-stimulating factor (M-CSF) and IL-10, M2-like macrophages in this stage are critical in the initiation of tissue repair and the clearance of remnant cellular debris and dying cells from the first pro-inflammatory stage [4, 182, 189]. Decidual macrophages also produce indoleamine 2,3-dioxygenase to suppress maternal effector T cells to maintain immune tolerance [190]. To compensate for the Th2 immune profile within the uterus at this time, Th17 and $\gamma\delta^+$ T cells are also present and protect against invading pathogens through the production of IL-17 [4, 9, 191-193].

Throughout pregnancy, the maternal immune system balances Th1/Th2/Treg/Th17 immune responses to maintain maternal tolerance to the fetus [190, 194]. Tipping the scale in favor of Th1 and Th17 responses is associated with several adverse obstetric outcomes such as preeclampsia and pre-term birth [194, 195]. However, decidual Th17 cells are detected in normal pregnancies, specifically in the first and third trimester [196]. Increasing Th1/Th17 and decreasing Treg profiles in the final stages of pregnancy is, in fact, necessary to induce a pro-inflammatory

environment and initiate parturition [195, 197]. TLR4 activation by fetal ligands and elevated levels of endogenous DAMPs activates the NF-κB signaling pathway, which is essential to initiate uterine contractions and induce labor [4, 169]. Despite our growing understanding of the complex maternal-fetal dialogue, the impact of such drastic immune changes during pregnancy on outcomes of maternal infection outside of this local environment is still unclear.

1.6 Pregnancy and Infectious Disease

1.6.1 Placental Defenses against Invading Pathogens

Pregnant women represent a unique group who have high morbidity to infectious diseases. Both congenital and non-congenital viruses including influenza, Zika, cytomegalovirus, and human immunodeficiency virus (HIV) continue as a profoundly global clinical threat to mothers and their offspring. These viral infections not only lead to severe maternal disease but also increase the risk of adverse pregnancy outcomes specifically when infection occurs in later stages of pregnancy [198]. Infections at the maternal-fetal interface can be localized in different areas of the uterus with the most common routes of entry being by vertical transmission through the placenta and ascension through the vagina **(Figure 1.10)** [5]. In the early stages of pregnancy, the embryo develops two extraembryonic tissues that provide nutrition and immune defenses necessary for proper fetal development: the chorioamniotic membranes (including the chorion and amnion) and the placenta [199]. The human placenta acts as a primary

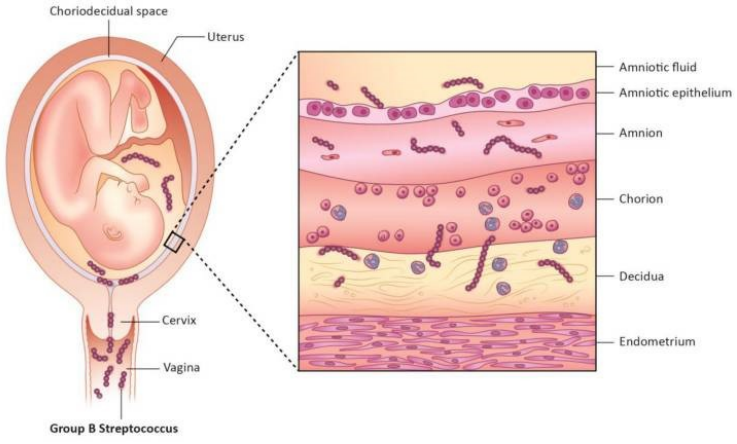

Figure 1.10 Ascending Group B Streptococcus infection form the vaginal cavity, through the chorioamniotic membranes to the fetus. Image taken from [5].

barrier to infectious agents and has evolved an abundance of non-redundant mechanisms of antimicrobial defenses [4, 182, 200]. The placenta is delimited by multinucleated syncytiotrophoblasts (STBs) which serve as a primary defense against the external environment and function to resist invading pathogens [4]. STBs recognize pathogens via PRRs, which trigger the induction of inflammatory and antimicrobial signaling pathways and the production of pro-inflammatory cytokines, antiviral molecules (IFN-λ), microRNAs, chemokines, and AMPs [4, 9, 182, 201].

At the forefront of this mucosal barrier are antimicrobial defenses conferred at large by AMPs and mucus [202]. Female reproductive hormones, specifically estrogen, differentially influence AMP concentrations and mucus viscosity along the reproductive tract and levels fluctuate during pregnancy and throughout the menstrual cycle [9, 203]. During pregnancy, AMPs protect the fetus from uterine infections by providing a chemical barrier to bacterial (*e.g. Chlamydia trachomatis*), fungal (*e.g. Candida albicans)*, and viral (*e.g.*, HIV-1 and herpes simplex virus type-2) infections [9]. Epithelial cells and phagocytic cells secrete AMPs either in the extracellular environment or within intracellular compartments, respectively [202]. Epithelial cells are the main producers of basal AMP expression and serve as a pre-emptive defense strategy in case of incoming danger. Some of the predominant AMPs expressed in the female reproductive tract are similar to those discussed in **Section 1.4.1** and include defensins, cathelicidins, lysozymes, collectins, and lactoferrin and could be constitutively expressed or induced in response to inflammatory stimuli and infection [202].

HNPs and HBDs are found throughout both maternal and fetal tissues. HBD-1 is constitutively expressed along the entire reproductive tract whereas HBD-2, -3, -4 predominate in the lower female reproductive tract, the endometrium, and cervico-vaginal fluid [204, 205]. HNP 1-4 are mainly stored in neutrophil granules alongside LL-37, which is also highly expressed in amniotic fluid, fetal membranes, and cervico-vaginal fluid [206-208]. Cervico-vaginal fluid is also rich in mucins (*e.g.,* MUC5AC and MUC5B) which provide another protective layer against ascending infection [57, 209]. AMPs and mucus that are strategically secreted in the lower female reproductive tract serve as guardians and protectors of the uterine cavity from ascending pathogens that could potentially compromise pregnancy [204] **(Figure 1.11)**.

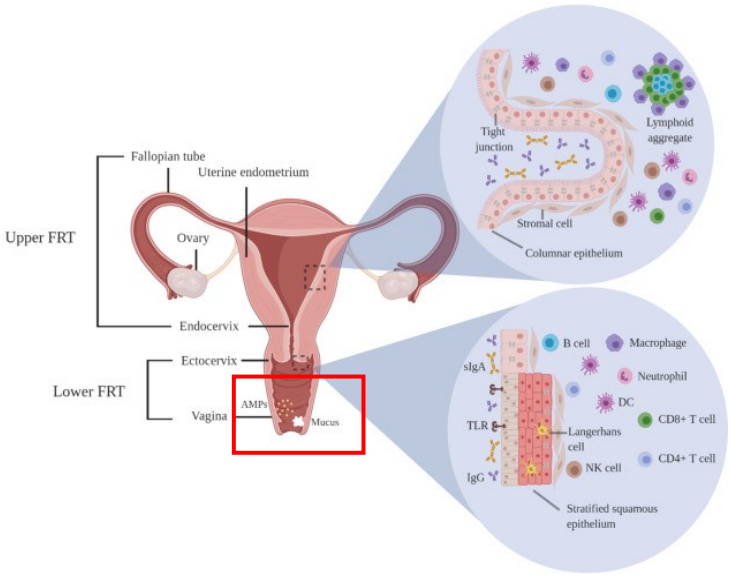

Figure 1.11 Cellular and molecular composition of the upper and lower female reproductive tract (FRT) with important visual depiction of AMPs and mucus in the lower FRT. Image taken from [9] .

The paradox of pregnancy is the need for immunosuppression of specific responses to prevent fetal rejection whilst not compromising maternal immune defenses to infection. From this point of view, $\gamma\delta^+$ T cells are of particular interest. Reportedly, $\gamma\delta^+$ T cells constitute 34% of uterine T cells and are located predominantly within the endometrium [210]. This preferential tissue localization underlines their importance in host defense and fetal tolerance as they combine both unique effector functions with high cytotoxic potency through the release of cytolytic molecules and immunoregulation through the secretion of IL-10 and TGF-β [183, 211]. Additionally, uterine $\gamma\delta^+$ T cells represent the main source of IL-17 in the female reproductive tract [191, 210, 212, 213]

and several investigators have reported on their protective role against an array of pathogens [191, 193, 214-216]. Given their role in potentiating antimicrobial immunity in the lungs, arguably, these cells may also be inducing IL-17-mediated AMP production in the female reproductive tract for additional protection.

1.6.2 Pregnancy Outcomes and Consequences of Maternal Infection on Offspring Immunity

Although the placental barrier is a privileged environment not easily accessible, depending on the nature of the pathogen, intrauterine infection may occur resulting in spontaneous abortion, stillbirth, intrauterine growth restriction, and preterm labor [199, 217]. In the absence of vertical transmission, maternal infection may still result in inflammation and long-term consequences on offspring health due to the engagement of maternal and placental immune responses [199]. In spite of this, a positive prospect of mild maternal infection is the ability to provide passive immunity to the growing fetus. Antibody responses following natural infection are longer-lasting compared those induced via vaccination [218]. Thus, preventing severe disease in the mother will not only assure maternal survival but also grant immunity to the newborn.

During the first year of life, a newborn's underdeveloped immune system renders it extremely susceptible to infection. Therefore, protection during this critical period comes from the transfer of maternal antibodies *in utero* and through breastfeeding [6, 219]. IgG is the most prevalent circulating antibody, having the longest half-life of around 3 weeks in the newborn and is the primary antibody generated following current vaccination strategies against IAV alongside IgA [218]. Importantly, IgG1 and IgG2 subtypes aid in viral clearance through antibody-mediated complement activation, cytotoxicity, phagocytosis, and direct viral neutralization to provide protection against severe influenza infection [220-224]. Therefore, the transport of IgG to the offspring is crucial during that window of vulnerability in early life.

Antibody transport occurs predominantly via the neonatal Fc-receptor (FcRn) on the surface of the STBs which bind to the Fc portion of maternal IgG [6, 219, 225] **(Figure 1.12)**. IgG antibodies will continue streaming to the fetus until maternal serum levels reach the zenith at 15 g/L, at which point all FcRn are saturated and excess IgG are degraded in lysosomes [226]. Thus, the magnitude of IgG transferred depends on maternal serum antibody concentrations and the amount of FcRn expressed, which increases as of the second trimester and peaks at around 36 weeks of pregnancy [182] **(Figure 1.13)**. Among the different subsets of IgG classes, IgG1 is at the top of the hierarchical pyramid being the most efficiently transferred due to its abundance and high FcRn affinity [218, 220, 226-228]. However, the prevalence of IgG subclasses and glycovariants influenced by antigen exposure, disease, inflammation, and pregnancy regulate IgG-Fc receptor interactions thereby modulating antibody effector functions and transfer efficiencies [229-234].

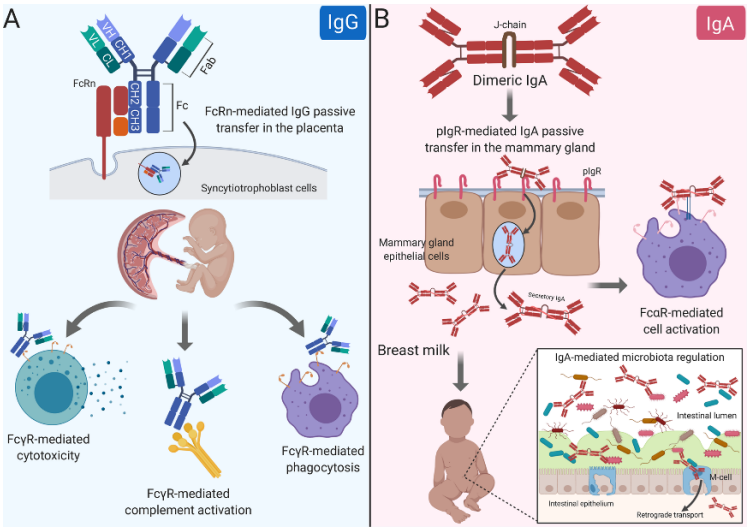

Figure 1.12. (A) Transplacental transfer of maternal IgG to the fetus. (B) Transfer of maternal IgA to neonate through breast milk. Image taken from [6]. Copyright https://creativecommons.org/licenses/by/4.0/

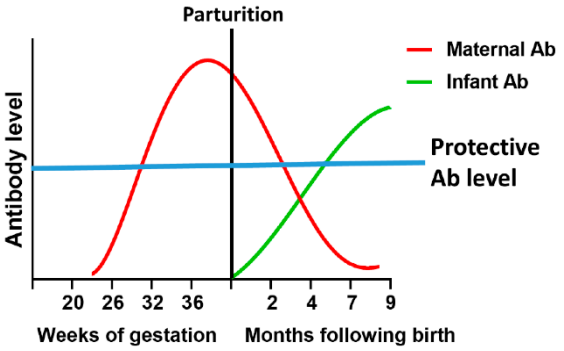

Figure 1.13. Pre-and post-natal kinetics of maternal vs. infant antibody levels. Graph taken from [11]. Copyright http://creativecommons.org/licenses/by/4.0/

After birth, mothers continue passing immunity through breast milk primarily through the transfer of IgA [6, 219]. The polymeric Ig receptor on the surface of mammary glands binds IgA and transfers it across epithelial cells to the breast milk via transcytosis [6, 219] **(Figure 1.12)**.

IgA provides protection against respiratory pathogens through similar mechanisms to that of its IgG counterpart and prevents viral attachment to epithelial cells [235, 236]. Together, both maternal IgA and IgG provide protection in both the upper and lower airways, respectively, until the infant starts developing its own antibody responses. However, despite evidence of maternal antibodies providing protection to infants from IAV infection [219], whether protection is primarily mediated through placental transfer or breastfeeding, predominantly by IgG and/or IgA, and the duration of protection remains elusive. **The contribution of maternal antibodies transferred *in utero* and through breast milk in protective immunity against IAV infection in offspring is the focus of Chapter 3.**

1.6.3 Pregnancy and Influenza

Pregnant women specifically seem much more prone to severe cases of seasonal and pandemic IAV infections. Accounts of case fatality rates in the United States during the 1918 pandemic reported the death rates for hospitalized pregnant women ranged anywhere from 23% to 71% depending on the cohort and 26% of the surviving women miscarried [18, 237]. In addition, severe maternal illness was correlated with preterm labor and low birth weights, despite the exclusion of the virus from the fetal-placental unit [4, 18, 238, 239]. In the case of the pandemic of 1957, pregnant women accounted for half of the influenza-related deaths among women in childbearing years [240]. Epidemiological data from the H1N1 2009 pandemic have also shown that pregnant women experienced disproportionately higher morbidity and constituted around 5% of all hospital and subsequent intensive care unit admissions [241-245]. This susceptibility linearly increased with gestational age and women in their third trimester were 3-4 times more likely to require hospitalization [241]. However, for perspective, the number of total hospitalized pregnant women is a small fraction of the total pregnant population. In fact, a Canadian study on the 2009-2010 H1N1 pandemic found that pregnant women essentially had proportionally fewer severe

outcomes when compared to all H1N1 admitted hospital cases, concluding only 1.5% of pregnant women died compared to accounts reporting death rates at around 6% in the United States [245, 246]. However, due to ethical reasons, pregnant women remain an understudied population [247]. Thus, to date, the clinical or biological parameters linking maternal morbidity to IAV infection have been poorly defined. Additionally, few studies have focused on the effect of maternal IAV infection on the neonatal immune system and the subsequent susceptibility or predisposition to infection or other disorders [248, 249]. Altogether, there is a discernable urgency in the continued research of maternal immunity and the maternal-fetal dialogue.

1.7 Murine Models of IAV Infection in Pregnancy

Murine models have served as indispensable tools for the understanding of pregnancy and infectious disease. Murine gestational lengths range from 19 to 21 days long and can be demarcated analogously to human trimester equivalents whereby days 1-10 refer to trimester 1, 10-16 trimester 2, and 16-21 representing trimester 3 **(Figure 1.14)** [15, 250]. However, there are limitations to these models and the conclusions as will be discussed below are often contradictory. Susceptibility in pregnancy has been modeled in BALB/c mice and allogeneic pregnancies, whereby a C57BL/6 female is mated with a BALB/c male. Some pregnancy studies using BALB/c pregnant females reported greater viral replication than non-pregnant females [251, 252], but others suggest that susceptibility is independent of viral burden [253]. The Th2 bias in pregnant BALB/c mice was shown to be associated with a more M2-like macrophage phenotype and was proposed as the basis for adverse maternal disease outcomes [254]. Allogeneic breeding also yields susceptible mothers with an inability to clear infection, a

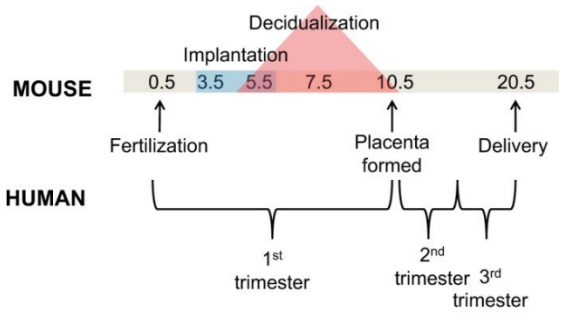

Figure 1.14. Human and analogous murine pregnancy timelines. Image taken from [15].

reduced Th1 response with lower IFN responses, and increased inflammation, [17] with profound long term effects on offspring immunity [249]. In contrast, when infected with PR8 during pregnancy, C57BL/6 mice showed less lung dysfunction [255]. The relationship between female reproductive hormones and immune responses also influences birth and disease outcomes. Although estrogen and progesterone in high concentrations inhibit the development of Th1 and promote Th2 immune responses, exogenous 17-β-estradiol provided protection against infection with PR8 whereas progesterone either worsened [256] or ameliorated [257] disease outcomes. Such contrasting conclusions are not only the result of different mouse strains used in the studies (C57BL/6 vs. BALB/c) but also the mating strategies (syngeneic vs. allogeneic breeding), the timing of IAV infection (*i.e.,* first, second, third trimester mouse equivalents), and the strain of IAV used in the study (seasonal vs. pandemic).

1.8 Rationale, Hypothesis, and Objectives

Throughout human history, pregnant women have fallen victim to seasonal and pandemic outbreaks of influenza. Although our understanding of the immunology of pregnancy is improving, the local maternal-fetal microenvironment may not represent the immune status of distal sites. Evidently, there is an urgent need to expand the repertoire of studies assessing maternal immune responses to IAV infection to gain a better understanding of how pregnancy-related immunological changes impact pulmonary immune responses and outcomes of disease. Although most studies have used BALB/c mice and allogeneic mating strategies to uncover mechanisms of susceptibility during pregnancy, the approach taken in our study is one of addressing resistance. Contrary to dogma, the fundamental reason for our study was to carefully dissect early host defense strategies in a mid-gestation mouse model of maternal resistance to IAV in C57BL/6 mice, which may expound the key immune players as determinants of mild vs. severe maternal disease.

The overarching hypothesis of the current thesis is that the exploration of mechanisms of resistance to IAV infection in the C57BL/6 mouse will reveal not only the mechanisms of resistance but as a corollary, the sources of susceptibility. Such an approach has proven to be informative in HIV infection, in identifying the chemokine receptor CCR5 as a mechanism of viral entry [258]. The two main objectives were to (1) characterize host resistance and disease tolerance in a mid-gestation model of IAV in C57BL/6 mice (2) delineate the importance of mild maternal disease and passive immunity to offspring against IAV infection.

Preface to Chapter 2

Pregnancy is a complex immunological state balancing maternal host defense to infectious pathogens and tolerance to a growing fetus. Epidemiologically, pregnant women are at risk of developing severe disease to both seasonal and pandemic strains of IAV. However, they do not represent a substantial majority of this population. Recently, there is also evidence of maternal resistance in response to SARS-CoV-2 infection [259-263]. Therefore, despite our understanding of the local maternal-fetal environment, immune-related adaptations during pregnancy and their role in preventing severe disease in vital organs, such as the lungs, is incompletely understood. In our study, we modeled maternal resistance using mid-gestation (E10) C57BL/6 mice. We showed that resistance to IAV was conferred by IL-17^{+} γδ^{+} T cells boosting antimicrobial immunity in the upper airways which prevented lower airway pathology and respiratory failure. This approach to assessing protection of the respiratory system during pregnancy challenges the current dogma yet is evolutionarily favorable, as it suggests that the pregnant female may be primed with defense mechanisms to withstand IAV infection to support the conservation of the species.

Pregnancy enhances antiviral immunity via recruiting IL-17-producing γδ^{+} T cells into the nasal mucosa.

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2.1 Highlights

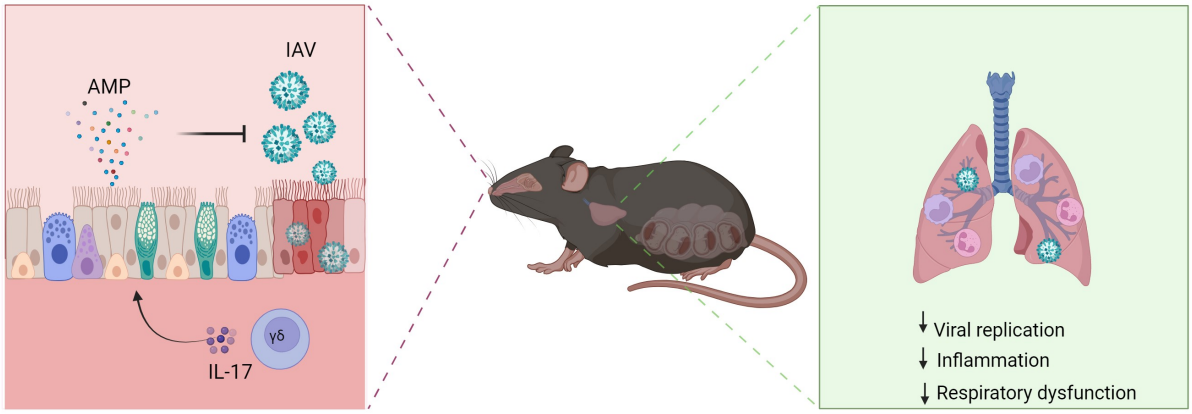
- Pregnancy enhances host defense against IAV infection.
- Pregnancy induces IL-17 producing $\gamma\delta^+$ T cells in the upper airway promoting antimicrobial immunity and restricting influenza propagation into the lung.
- Pregnancy-induced upper airway immunity promotes the maintenance of optimal lung function during influenza infection.

2.2 Summary

Conventionally, pregnant women are considered at high risk of developing serious respiratory illness during seasonal or influenza pandemics. However, pregnancy-related immune adaptions outside of the local maternal-fetal environment and their impact on disease severity remains unclear. Surprisingly, here we found that mid-gestation pregnancy confers enhanced host defense to Influenza A virus (IAV) infection in C57BL/6 mice that limits the viral replication and reduces the magnitude of the intrapulmonary immune response. Consequently, the pregnant mice show reduced pulmonary pathology and augmented lung function after IAV infection. Interestingly, the restriction of the viral replication is dependent on the upper airways immunity via increased antimicrobial peptides driven by IL-17^+ $\gamma\delta^+$ T cells. This novel pathway of protection against IAV infection during pregnancy is evolutionarily favorable as it suggests that the pregnant female upregulates upper airway barrier defenses to restrict early viral infection and prevent virus dissemination to the lung, supporting maternal fitness.

Key words: pregnancy, influenza, host resistance, upper airways, antimicrobial immunity

2.3 Graphical Abstract



2.4 Introduction

Influenza virus is one of the biggest biological threats to humanity, with pandemic strains emerging every few years [1]. Pregnant women are considered as a group at high risk of developing severe disease during influenza outbreaks, with a higher incidence of pulmonary immunopathology and respiratory failure than the general population, specifically in their 2nd and 3rd trimesters [2]. Although the main causes of maternal morbidity to influenza virus are unclear, much of the notion of susceptibility comes from a dogma whereby pregnancy was viewed as having dichotomous Th1/Th2 immunity, leading to an immunocompromised state required to tolerate a semi-allogeneic fetus [3]. However, it is now well-established that pregnancy comprises transitional periods of distinct immunological states engaged at each trimester [4, 5]. The immune response during the major part of pregnancy is actively skewed towards type-2 immunity but is type-1 biased and pro-inflammatory in early pregnancy to allow for placentation and implantation and reverts to a pro-inflammatory state that promotes labor and placental rejection in the later stages of pregnancy [3]. While much of the focus of the immunology of pregnancy pertains to the maternal-fetal interface, our understanding of immune responses driving maternal morbidity with infectious pathogens outside of this local environment is still incomplete.

Host defenses against IAV are balanced between *host resistance*, which is essential for reducing pathogen burden, and *disease tolerance*, which is crucial in limiting the extent of inflammation and subsequently tissue damage [6]. Both arms of host defense are required for effective immunity against pathogens [7]. Most studies have focused on susceptibility to IAV infection during pregnancy using BALB/c mice, with a highly Th2 skewed immunity, which have shown that maternal morbidity in these mice is driven either by a lack of antiviral immunity [8] or exuberant inflammatory responses [9]. In contrast, IAV infection in syngeneic pregnancy C57BL/6J mice has revealed protection against IAV-induced pulmonary dysfunction [10]. This strain-dependent

protection is reportedly attenuated in allogeneic pregnancy when C57BL/6J females are crossed with BALB/c males [5, 11]. These discrepancies may be attributed to strain-specific differences of either the mouse or influenza used, the mating strategy, and the gestational period at the time of infection. For this reason, it is still unclear how pregnancy regulates immunity to IAV infection. In this study, we characterized maternal immune responses in a mid-gestation model of IAV during pregnancy in C57BL/6J mice to investigate the potential protective mechanisms of maternal immunity. We have identified a novel mechanism of resistance that is dependent on enhanced antimicrobial immunity within the upper airways conferred by IL-17^{+} γδ^{+} T cells. These upper airway antimicrobial defenses ultimately reduced pulmonary viral titers and promoted disease tolerance.

2.5 Results

Pregnancy in C57BL/6 mice enhances host resistance and limits IAV-induced immunopathology

First, we investigated disease severity by infecting non-pregnant and pregnant (E10) C57BL/6 mice with a sublethal dose of IAV (PR8; 50pfu) intra-nasally (*i.n.)* (Figure 1A) and monitored their morbidity (weight loss). The IAV-infected pregnant mice lost significantly less weight than the non-pregnant females compared to their respective uninfected controls (Figure 1B). Interestingly, the pulmonary viral burden at 10h and 24h post-IAV infection was significantly lower in pregnant IAV-infected mice compared to control groups (Figure 1C). Using the reporter Ruby-NS1 PR8 strain [12] (500pfu), we examined the dissemination of IAV within the lung and observed an overall reduction in infected cells in the airways (Figure 1D) and parenchyma (Figure S1A) of the pregnant mice. Since epidemiologically maternal morbidity worsens with gestational age[2], we asked whether maternal resistance would persist in the third trimester. Indeed, IAV infection in the 3rd trimester (E16) also resulted in reduced viral titers in the pregnant mice (Figure S1B), suggesting that pregnancy afforded enhanced host resistance to IAV infection.

In characterizing the intrapulmonary inflammatory response, we found that the pregnant IAV-infected mice had fewer neutrophils and Ly6C$^+$ monocytes, both expressed in terms of frequency (Figure S1C and D) and as absolute cell numbers (Figure 1E and F). Additionally, the pregnant IAV-infected mice had lower levels of inflammatory cytokines and chemokines, including pulmonary CCL2 (Figure 1G), KC (Figure 1H), IL-6 (Figure 1I), IL-1β (Figure 1J). BALF lactate dehydrogenase (LDH) levels were also lower (Figure S1S) and pulmonary IL-10 levels (Figure 1K) were higher in the pregnant IAV-infected mice. Consequently, histology exhibited reduced areas of inflammation and hemorrhage by 9 days post IAV infection in the pregnant mice (Figure 1L)**.** Therefore, enhanced pulmonary pro-inflammatory responses were not associated with increased host resistance in the pregnant IAV-infected mice.

One of the hallmarks of IAV replication is the induction of epithelial cell death [13]. In agreement with the reduction in total viral burden (Fig. 1C-D), there were fewer infected epithelial cells (Fig. 1M) and cleaved/activated caspase-3$^+$ cells in the pregnant IAV-infected mice (Fig. 1N and S1T). Therefore, early control of viral replication in the pregnant mice was associated with reduced morbidity, pulmonary inflammation, cell death, and immunopathology, consistent with increased host resistance to disease.

Enhanced maternal resistance during pregnancy protects against IAV-induced airway dysfunction.

IAV infection is associated with asthma attacks [14] and increases respiratory responses to inhaled aerosols of methacholine in mice [15]. Thus, we examined the consequences of IAV infection for airway dysfunction, comparing pregnant and non-pregnant mice at days 0, 1, and 3 post infection. In accordance with lower viral titers, less epithelial cell death, and reduced inflammatory responses, pregnant IAV-infected mice showed lower airways resistance and elastance at days 1

and 3 post infection (Figure 2). Collectively, maternal resistance reduced pulmonary immune and inflammatory responses and minimized airway dysfunction.

Maternal resistance to IAV infection is independent of pulmonary antiviral mechanisms

We next sought to identify the cellular and molecular mechanisms enhancing host resistance in IAV-infected pregnant mice. Type I and III interferons (IFN) are essential components of host resistance as they orchestrate antiviral and inflammatory responses to IAV infection. To assess whether the increase in host resistance was a consequence of enhanced antiviral immunity, we quantified type I (IFN-α/β) and III (IFN-λ) IFNs in lung homogenates and observed a reduction in the concentrations of all three IFNs in the pregnant IAV-infected mice (Figure 3A, 3B, S3A). To further support the type I IFN independency in protection against IAV in the pregnant mice, we utilized *Ifnar1*$^{-/-}$ mice. At day 3 post-IAV infection, although the total viral burden in both pregnant and non-pregnant *Ifnar1*$^{-/-}$ mice exceeded that of WT mice, pregnant mice showed an early resistance against IAV infection (at day 1 post-infection), despite their inability to signal in response to type I IFNs (Figure 1C, Figure 3C). These data further confirmed that resistance attributable to pregnancy during very early stages of infection is independent of type I IFN-mediated antiviral immunity.

Alveolar macrophages do not promote pregnancy-mediated enhanced antiviral immunity

AMs are the first immune cells to encounter IAV within the lung and have indispensable roles in host defense against IAV [16]. The protective role of AM in IAV infection has been well documented by our group and others and their depletion quickly accelerates viral replication and renders the host highly susceptible to secondary bacterial infection and severe disease [16-20]. Following IAV infection, although there were no differences in the frequency of AMs (Figure 4A), the total number of AMs in the pregnant IAV-infected mice was significantly higher than control

groups at day 3 post-infection (Figure 4B). However, there were no differences in the frequency of Ki67$^+$ AMs (Figure S3B), but the frequency of caspase-3$^+$ AMs was lower in pregnant IAV-infected mice (Figure 4C). Thus, this absolute increase in total AMs was a result of reduced cell death rather than increased *in situ* proliferation.

AMs adopt specific functional phenotypes from inflammatory (M1) to regulatory (M2) spectrum by undergoing changes in surface marker expression, cytokine secretion, and metabolism in response to environmental stimuli and pulmonary pathogens [21, 22]. The Th2 bias in pregnant BALB/c mice is associated with a more alternatively activated macrophage phenotype which has been proposed as the basis for unfavourable disease outcomes [9, 23]. Since our study modelled resistance, we investigated the possibility of having a more "classical" functional AM phenotype in C57BL/6 pregnant mice. Flow cytometry was performed on lung homogenates to assess the functional capacity of AMs based on the expression of iNOS and Arg1. However, there was no differences in the frequency or in mean fluorescence intensity of Arg1$^+$ or iNOS$^+$ AMs between pregnant and non-pregnant mice (Figure S3C). A glycolytic shift in macrophage metabolism promotes pro-inflammatory phenotypes and effector functions [24]. To assess the cellular metabolism, AMs were isolated from the BALF of non-pregnant and pregnant mice and then infected with IAV (MOI 1) and subjected to seahorse. Interestingly, AMs from pregnant mice had lower basal respiration prior to and following IAV infection (Figure 4D), and lower maximal respiratory capacity at baseline (Figure S3D). The data suggest that AMs from pregnant mice do not adopt a specific functional program tailored towards antiviral immunity and pro-inflammatory effector functions.

We next investigated whether the intrinsic properties of AMs from pregnant mice were increasing host resistance to IAV infection. AMs were isolated from the BALF of naïve non-pregnant and pregnant mice and infected *in vitro* with IAV (MOI 1) and viral *Ns1* expression was quantified by

RT-PCR. The data revealed that AMs from non-pregnant and pregnant mice had comparable *Ns1* levels 24h post infection (Figure 4F). This data was further supported by adoptively transferring AMs derived from non-pregnant and pregnant mice intra-tracheally into non-pregnant *Rag1*$^{-/-}$ mice. Two hours post transfer, *Rag1*$^{-/-}$ recipient mice were infected with IAV (50pfu) (Figure 4G). Since viral replication with PR8 peaks 3 days post infection, we assessed the capacity of AM to control pulmonary viral load at the time of maximal viral yield. However, there was no differences in the control of viral replication among AM transferred groups (Figure 4H). To further confirm that AMs did not account for the protection against IAV during pregnancy, AMs were depleted via intranasal administration of clodronate liposomes as previously described [20] two days prior to infection with IAV, which resulted in ~80% depletion of AMs in lungs (Figure S3G). Pulmonary viral titers were then quantified one day post IAV infection at the onset of observable differences in host resistance between non-pregnant and pregnant mice (Figure 4I). Although total viral burden in both non-pregnant and pregnant mice increased following clodronate administration, confirming the importance of AMs in restricting viral replication [19, 25], the AM depletion did not abrogate the relative protection in the pregnant mice (Figure 4J). Therefore, although canonically known for their indispensable role in antiviral immunity during IAV infection, AMs did not confer enhanced resistance during pregnancy.

Given that pulmonary antiviral mechanisms were not responsible for maternal resistance against IAV, we hypothesized that the protection of pregnant mice can be due to antiviral immunity of the upper airways. To test this possibility, we initially bypassed the upper airways by infecting the pregnant mice via intra-tracheal route (Figure 4K). Bypassing the upper airways via intra-tracheal infection completely ablated protection in the pregnant IAV-infected mice (Figure 4L). These data indicate that the ability of pregnant mice to restrict viral replication is mainly mediated by upper respiratory tract antiviral immunity.

IL-17-producing γδ^{+} T cells enhance nasal airway antimicrobial immunity to restrict IAV replication during pregnancy

To address the upper airway antimicrobial immunity, nasal cavities from non-pregnant and pregnant mice were harvested after IAV infection and assessed for viral burden (Figure 5A). Viral replication was significantly reduced in the pregnant IAV-infected mice at days 1 and 3 post infection (Figure 5B). To identify the location of viral restriction in the upper airways, we used the NS1-PR8 Ruby reporter strain and observed a marked reduction in the intensity and number of infected nasal epithelial cells, specifically in the olfactory region of the nasal mucosa in the pregnant mice (Figure 5C). It has been previously reported in pregnant rats that maternal morbidity is due to an increase in sialoglycan receptors, facilitating viral entry [26]. To investigate whether the reduction in nasal viral burden was a consequence of reduced viral attachment, we stained the nasal cavity of naïve mice for the IAV receptors, α2,3- and α 2,6-sialic acids using *Maackia amurensis* and *Sambucus nigra agglutinin* lectins, respectively. However, there were no significant differences in the frequency (Figure S4B), nor mean fluorescence intensity (Figure S4C), of sialic acid receptors between pregnant and non-pregnant mice, suggesting reduced viral replication was not a consequence of alteration in the sialic acid receptor expression. Surprisingly, this resistance in the nasal cavity was also independent of type I IFN-mediated antiviral immunity as pregnant *Ifnar1*$^{-/-}$ mice had lower viral titers at day 1 post infection (Fig. 5D).

Considering type I IFNs did not account for the protection at the early stage of infection, we shifted our focus to IL-17, which is an important cytokine produced at mucosal surfaces reported to provide host resistance to IAV infection[27]. The expression level of *il-17* by RT-PCR was significantly upregulated in the pregnant mice at days 1 and 3 post IAV-infection compared to non-pregnant mice (Figure 5E). IL-17 is primarily produced by γδ^{+} T cells and Th17 cells, therefore, to identify the cellular source of IL-17, we performed flow cytometry on cells isolated

from the nasal cavities of naïve and 1-day post IAV infected mice stimulated with murine recombinant IL-1β and IL-23 [28]. Importantly, naïve pregnant mice had a higher proportion of $\gamma\delta^+$ T cells (Figure 5F) and a higher absolute cell count 1 day post IAV infection in the nasal cavity (Figure 5G). During pregnancy, $\gamma\delta^+$ T cells accounted for ~65% of all IL-17-producing cells (Figure 5H) and following IAV infection, the pregnant mice had higher IL-$17^+\gamma\delta^+$ T cell counts compared to non-pregnant mice (Figure 5I). Collectively, these results indicate that nasal $\gamma\delta^+$ T cells are the major source of IL-17 and are increased during pregnancy.

The IL-17 family of cytokines is known to regulate antimicrobial peptides (AMP) production at mucosal surfaces to provide protection against invading pathogens [29-32]. AMPs such as cathelicidins (LL37; mouse ortholog CRAMP), defensins (mouse β defensin (mBD)), and mucins (MUC5AC) have been shown to interfere with viral pathogenesis by direct inhibition of virus-host interactions, viral replication and membrane integrity, and host immunomodulation [33-38]. Therefore, we assessed the expression of AMPs by RT-PCR of nasal cavity tissues from pregnant and non-pregnant naïve and IAV-infected mice (1- and 3-days post-infection). We observed a broad upregulation of multiple AMPs including *Muc5ac*, *Cramp*, *Mbd-3*, *-4*, and *-14* (Figure 5J-N) post IAV- infection in the pregnant mice that mimicked the kinetics of *Il-17* expression. Although all AMPs were negatively correlated with *Ns1* expression to some degree, *Mbd-3* and *Cramp* were the most significantly inversely correlated at peak viral replication (Figure 5O).

To consolidate the link between IL-17 and AMPs production, an anti-IL-17 neutralizing antibody was administered intraperitoneally, and AMP expression was quantified by RT-PCR in the nasal cavity of pregnant mice. Administration of anti-IL-17 decreased AMPs expression in the pregnant IAV-infected mice, with the most pronounced reduction observed in *Mbd-3* and *Cramp* (Figure 5P-S). This reduction in AMPs production consequently resulted in increased nasal viral titers in

the pregnant mice (Figure 5T). Taken together, the data suggests that IL-17, predominantly produced by $\gamma\delta^+$ T cells, mediates antimicrobial upper airway resistance to IAV during pregnancy.

2.6 Discussion

Pregnant women are faced with contradictory demands to maintain immune tolerance required for fetal survival versus mounting an appropriate immune response to invading pathogens [39]. However, the consequences of pregnancy-associated immune changes on outcomes of infection beyond the maternal-fetal interface are still unclear. Many of the immune changes occurring during pregnancy and their impact on host defense have often been explored in BALB/c mice and allogeneic pregnancies [8, 9, 23] which model well the subset of women who develop severe disease following IAV infection. Susceptibility to severe infection with influenza is not uniform across the population of pregnant women but rather dependent on the individual [40]. Emerging evidence from the recent COVID-19 pandemic also suggests that pregnant women experienced mild disease compared to the general population [41-45]. In the current study, pregnant (mid-gestation) mice infected with IAV showed enhanced host defense against infection, specifically via augmented antiviral immunity in the upper airways. The reduced viral replication in the nasal cavities of the pregnant mice resulted in significant reduction in viral propagation into the lungs with subsequent decreased pulmonary inflammation and immunopathology. Thus, the results conceptually highlight the interplay between host resistance in upper versus lower airways and subsequent disease tolerance whereby the initial pathogen load reduced the magnitude of the immune response and consequently its associated collateral tissue damage.

The resistance of pregnant C57BL/6 mice to IAV infection is in sharp contrast to the observed susceptibility manifested by BALB/c mice, which is in part conferred by a more alternatively activated macrophage phenotype [9, 23]. However, in our study, AMs from pregnant mice did not adapt their metabolism nor their phenotype to be more conducive to viral clearance. In fact, neither

their depletion nor did their adoptive transfer abrogate the relative differences in viral load between non-pregnant and pregnant mice. Whereas IAV quickly depletes AMs and significantly compromises host defenses and increases morbidity and mortality [17, 19, 46], in our study, the AM population was conserved during pregnancy as a consequence of reduced cell death, presumably due to a reduction in viral burden in the lower airways. Considering their role in tissue homeostasis [47] and maintaining lung function during IAV infection [16], we postulate that AMs may be contributing to disease tolerance rather than host resistance in our model.

The relevance of IL-17 in innate immunity has been mainly studied in the context of bacterial infections [29-31], however, the precise role of IL-17 in the upper airways during the early response to IAV infection remains largely unknown. It has been previously shown that pulmonary $\gamma\delta^+$ T cells are the dominant IL-17-producing cells following influenza infection [48] and IL-$17^+\gamma\delta^+$ T cells have been implicated in restricting IAV replication in the trachea during early infection [27] and in the lungs of mice infected with *Bordetella pertussis* [49] through AMPs secretion. In our study, we observed that $\gamma\delta^+$ T cells were the major source of IL-17 in the nasal mucosa of pregnant mice. Using the reporter NS1-Ruby strain, we observed a reduction in the number and magnitude of infected nasal epithelial of pregnant mice following viral passage through the respiratory zone of the nasal mucosa that synthesizes AMPs and mucins [50]. Of the AMPs measured in our study, *Cramp* and *Mbd-3* were the strongest negatively correlated with viral *Ns1*. Neutralizing IL-17 significantly decreased AMP expression and concomitantly increased viral titers in the nasal cavities of pregnant IAV-infected mice. Therefore, the immune protection during pregnancy is mediated via two distinguished programs: the early phase, which leads to increase induction of IL-$17^+\gamma\delta^+$ T cells and the production of AMPs which limits the initial viral replication in the upper airways, and the late phase which subsequently modulates innate immune responses to maintain viral restriction through type I IFN signaling in the lower airways.

AMPs are known to be modulated by steroid hormones [51-53] and clinically, pregnancy-induced adaptations of the upper respiratory tract include increased mucopolysaccharides and glandular secretions in the naso- and oropharyngeal mucosa which in ~18-30% of patients results in nasal congestion [54-57]. The etiology of this gestational rhinitis is unknown; however, it could arguably serve as a host defense strategy against respiratory infections. Notably, AMPs and mucus secreted in cervico-vaginal fluid and uterine tissues serve as essential primary barriers to invading pathogens [58-62]. Furthermore, uterine $\gamma\delta^+$ T cells are the major source of IL-17 within the female reproductive tract [62-65] and provide protection against viral [62, 66, 67], fungal [68], and bacterial infections [69], suggesting a similar mechanism of host protection in this site.

Apart from their roles in reducing viral replication, both defensins and cathelicidins promote chemotaxis of leukocytes, predominantly of neutrophils and monocytes, to aid in viral and bacterial clearance [27, 30, 70, 71]. Although epithelial cells are the predominant producers of β-defensins, macrophages and neutrophils have also been shown to secrete defensins and LL37/CRAMP [70, 72-74]. Therefore, future studies are necessary to elucidate the source of AMPs within the nasal passages and whether they are directly interacting with IAV and restricting replication or are promoting chemotaxis of inflammatory cells to clear the infection.

Several limitations of the study warrant consideration. We have observed reduced pulmonary pathology and airway dysfunction in pregnant mice. However, the characterization of pulmonary immune responses in pregnant and non-pregnant mice was performed with unequal pulmonary viral burdens. The reduced pathobiology may have been a consequence of reduced viral burden in pregnant mice and so it is still unclear whether lower airway immunity would manifest greater resistance if faced with an equal viral inoculum. Arguably, enhanced upper airway immunity during pregnancy compensates for an inability of the lower airways to clear large viral inocula and resolve subsequent inflammatory responses as a result of a Th2 environment. Greater

investigation into upper versus lower airway immunity using dose-response experiments or intra-tracheal administration of IAV is required to better understand how each compartment responds to inhaled pathogens.

In summary, the data presented herein are a description of a pregnant murine model resistant to IAV infection. Evolutionarily, pregnancy may alter the mode of host defense against pulmonary pathogens by augmenting upper airway antimicrobial immunity, limiting lung tissue damage, and maintaining optimal lung function to support maternal fitness. Our study suggests that strategies to prevent severe influenza infection in both pregnant women and the general population need to consider the induction of antimicrobial immunity in the nasal mucosa as a determining factor of disease severity.

2.7 Materials and Methods

Mice

8-10-week-old female wild-type and *Rag1*$^{-/-}$ mice (B6.129S7-*Rag1*$^{tm1Mom/J}$) C57BL/6 mice were purchased from Jackson Laboratories, housed, and bred with male C57BL/6 at the animal facility of the Research Institute of the McGill University Health Centre. Interferon alpha/beta receptor deficient (*Ifnar1*$^{-/-}$) mice purchased from Jackson Laboratories. All protocols were approved by the Animal Care Committee of McGill University.

Infection

Pregnant mice were infected on day 10 post mating (E10) or E16 with the mouse-adapted influenza A/Puerto Rico/8/34 (H1N1) virus provided by J.A. McCullers (St. Jude Children's Research Hospital). The Ruby-NS1 PR8 strain kindly provided by N. Heaton (Duke University) was used for fluorescent imaging of infected cells in the lungs and nasal cavities. Virus was propagated in hen eggs and titrated in Madin-Darby Canine Kidney (MDCK) cells using standard plaque assays

[75]. Mice were intra-nasally or intra-tracheally infected with sublethal doses of 25 or 50 pfu (PR8) and 500pfu (Ruby-NS1) in 25µl PBS for lung studies and 10µl for nasal cavity studies.

Isolation of nasal tissues

Following euthanasia, the skin was removed to expose the skull. The head was dissected from the body and the lower jaw and tongue were excised. The eyes, surrounding tissues, and zygomatic bones were then removed, and the skull was cut down a sagittal plane to expose the nasal cavities. Both halves of the nasal cavities were cut away from the rest of the skull and all associated tissues were scraped from the inner surfaces of the cavities.

Quantification of viral load

Viral titres were determined in lung homogenates and nasal cavities (in 500µl DMEM) using MDCK plaque assays or by RT-PCR assessing the viral gene, *non-structural protein 1 (ns1)* using GENEzol^{TM} TriRNA Pure extraction kit (Geneaid). MDCK cells (ATCC) were plated in 6-well plates at a density of 750 000 cells/well in complete DMEM (Gibco) (10% FBS (Heat-inactivated; Wisent), 1% L-glutamine (Gibco), 1% antibiotic-antimycotic (Gibco)), at 5% CO_2, 37ºC. After 48hr, the cell layer was washed 2x with PBS and incubated with 100µl of serial dilutions of lung or nasal homogenates for 1hr. The inoculum was washed 1x with PBS and wells were topped with modified 2X MEM (Thermofisher) and 1% agarose (Sigma-Aldrich) in a 1:1 ratio supplemented with 2µl/ml trypsin treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) (Fisher) and incubated (5% CO_2, 37ºC) for 48hr. Cells were then fixed with methanol (Glacial; Fisher) and acetic acid at a ratio of 3:1. Agarose plugs were removed, and plates were dried for 24h under the fume hood.

Assessment of morbidity

Non-pregnant and pregnant mice were administered PBS or 50pfu IAV intra-nasally. Change in body weight was measured daily until pregnant mice gave birth. To account for the impact of pregnancy on morbidity, the change in body mass relative to gestational-age matched uninfected animals was analyzed as previously described [76].

Fluorescence microscopy

Lungs were inflated with 1ml of 4% PFA by intra-tracheal cannulation, excised from the thoracic cavity, and placed in 4% PFA overnight. Lungs were washed in PBS twice over 48 hr and transferred into a sucrose/OCT (Tissue-Tek) solution overnight. Lungs were then placed in OCT-filled blocks and flash-frozen in liquid nitrogen. Blocks were cut in 5 μm sections using a cryostat (Leica Biosystems). For nasal cavities, skin was removed from the skull, heads were placed in 4% PFA overnight, and transferred to PBS for another 24 hr. Skulls were then serially transferred into 15% and 30% sucrose + 0.1% sodium azide over two days. Tissues were sectioned and mounted with anti-fade mounting medium with DAPI (Vector Laboratories). Images were quantified with ImageJ.

Assessment of lung pathology

Bronchoalveolar lavage fluid (BALF) was collected with a 26-gauge needle though the tracheal cannula using 3 x 1 mL cold, sterile PBS. The total volume recovered after lavage was ~0.7 mL. Samples were centrifuged at 1,500 rpm for 10 minutes. Lactate dehydrogenase in BALF supernatants of IAV-infected mice was quantified using a commercial assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega), following the manufacturer's recommendations. Additionally, lungs were perfused through the right ventricle with 10ml PBS and subsequently fixed in inflation with 1ml formalin instilled intra-tracheally. Lungs were immersed in 5ml of 10%

formalin for 48 hours, then placed in 10ml of 70% ethanol before paraffin-embedding. Sections were stained with hematoxylin-eosin. The extent of inflammation present in H&E lung tissue was quantified using the Aperio ImageScope (Leica Microsystems, USA). The percentage of inflammation expresses the ratio of the total inflamed area to the total lung area determined by manual digitization of areas infiltrated with inflammatory cells and the outer perimeter of the lung, respectively. Quantification was analyzed by an observer blinded to the group status. For the quantification of apoptotic cells, sections 4μm-thick sections were deparaffinized, rehydrated, and incubated for 24 min at 37ºC with anti-cleaved caspase-3 (1:300; #96615, Cell signaling) then incubated with secondary antibody OmniMap anti-rabbit HRP (#760-4311, Roche) for 20 min at room temperature followed by ChromoMap DAB detection kit (#760-4304, Roche). Slides were then counterstained with hematoxylin, dehydrated, cleared, and cover slipped. Slides were digitally scanned with Aperio Turbo 2T scanner for morphometric analysis with Imagescope software.

Cytokine and chemokine production

Interferon (IFN)-β and IFN-α concentrations in lung homogenates were quantified using the Verikine Mouse IFN-β ELISA kit (PBL Assay 746 Science) or Mouse IFN-β ELISA kit (Abcam) and Verikine Mouse IFNα ELISA kit (PBL Assay). Interleukin (IL)-33, IL-13, IL-5 were quantified in the BALF and IFN-λ, IL-6, IL-1β, chemokine (C-C motif) ligand 2 (CCL2), keratinocyte chemokine (KC) were quantified by ELISA in lung homogenates according to the manufacturer's instructions (R&D Systems).

Flow cytometry – immunophenotyping

Lung tissues were perfused through the vasculature with 10ml of PBS, harvested, minced, and digested with collagenase type I (Worthington CLS-1; 150 U ml^{-1}), DNase (Worthington D 1000

U ml^{-1}), and elastase (Worthington ESL 80 U ml^{-1}) for 30min at 37 °C. Nasal cavities were harvested as previously described [77] and subjected to the same enzymatic digestion. Enzymes were neutralized with 300 µl HI-FBS, lung and nasal digests were sieved through a 70 µm nylon mesh, and red blood cells were lysed. Lung and nasal samples were re-suspended, and cell counts were determined with a hemacytometer. 500 000-1 000 000 cells were used for staining. Cells were initially stained with the viability dye e506 or ef780 (Invitrogen; 30 min; 4 °C) in PBS and anti-CD16/32 (BD Biosciences) in 1% BSA/PBS solution to block non-specific interaction with Fc receptors (10 min; 4 °C). To identify innate immune cells, cells were surface stained (30 min; 4 °C) with BV786-conjugated anti-SiglecF, BUV737-conjugated anti-CD45.2, BUV395-conjugated anti-CD11b, PerCP-eFluor710-conjugated anti-Ly6G, allophycocyanin (APC)-or FITC conjugated anti-Ly6C, BV421-conjugated anti-CD11c, PeCy7-conjugated anti-MerTK, PE-conjugated anti-CD64, APC conjugated anti-F4/80, and BV650-conjugated anti-NK1.1. For intracellular staining, cells were fixed and permeabilized using BD CytoFix/CytoPerm (BD #554714) and stained with APC-conjugated anti-Ki67 (Biolegend), APC-conjugated anti-nitric oxide synthase (iNOS), PeCy7-conjugated anti-arginase-1 (Arg1), and PE-conjugated anti-caspase-3. To identify adaptive immune cells in the lungs, cells were surface stained with FITC-conjugated CD45.2, BUV395-conjugated anti-CD19, PerCP-eFluor710-conjugated anti-gamma delta TCR, PE-conjugated anti-CD3, V450-conjugated anti-CD4, AlexaFluor700-conjugated anti-CD8.

To assess the expression of NeuAc(α2-3)Gal and NeuAc(α2-6)Gal receptors in the nasal cavity, cells were stained with BUV737-conjugated anti-CD45.2, PE-conjugated anti-CD326, biotinylated *Maackia amurensis* (MAA) (Vector Laboratories, Burlingame, CA), Cy5-conjugated anti-*Sambucus nigra agglutinin* (SNA) (Vector Laboratories, Burlingame, CA), and PeCy7-conjugated streptavidin (30 min; 4 °C).

For the characterization of IL-17 synthesis by $\gamma\delta^+$ T cells and $\alpha\beta^+$ T cells within the nasal passages, nasal cells were stimulated with rIL-23 (10ng/ml) and rIL-1β (10ng/ml) and incubated with GolgiStop™ (1:1000) for 4 hr at 5% CO_2, 37ºC. Cells were then surface stained with V500 for viability, BUV737-conjugated anti-CD45.2, BV650-conjugated anti-CD3, BUV395-conjugated anti-CD11b, APC-Cy7-conjugated anti-BTCR, and FITC-conjugated anti-gamma delta TCR (Biolegend) (30 min; 4 °C). Cells were then permeabilized using the Foxp3 transcription factor fixation/permeabilization kit (Thermo Fisher) and intracellularly stained with APC-conjugated anti-IL-17A (50 min; 4 °C). Flow cytometry was performed on the BD LSRFortessa X-20 (BD Biosciences) using FACSDiva Software version 8.0.1 (BD Biosciences). Analysis was performed using FlowJo software version 10.9.1. All antibodies were purchased from BD Biosciences unless otherwise specified.

RNA isolation and reverse transcription PCR

RNA was extracted from nasal cavities using the GENEzol™ TriRNA Pure extraction kit (Geneaid). RNA was reverse transcribed using the LunaScript RT SuperMix kit (New England BioLabs), as directed by the manufacturer and complementary DNA was quantified using BrightGreen SYBR Green (Applied Biological Materials) by qPCR. Cq values of all target genes including *gapdh* (F,5'-GGTCCTCAGTGTAGCCCAAG-3'; R,5'-AATGTGTCCGTCGTGGATCT-3)*, viral ns1 (*F, 5'-AGAAAGTGGVAGGCCCTCTTTGTA-3'; R, 5'-GGGCACGGTGAGCGTGAACA-3'), *mucin 5ac (muc5ac) (*F, 5'-CCATGCAGAGTCCTCAGAACAA-3'; R, 5' TTACTGGAAAGGCCCAAGCA-3')*, mouse β-defensin-1 (mbd1) (*F, 5′-CCAGATGGAGCCAGGTGTTG-3′*;* R, 5′-AGCTGGAGCGGAGACAGAATCC-3′), *β-defensin-2 (mbd2)* (F*,* 5′-AAGTATTGGATACGAAGCAG-3′; R, 5′-TGGCAGAAGGAGGACAAATG-3'), *β-defensin-3 (mbd3)* (F, 5′-GCATTGGCAACACTCGTCAGA-3′; R, 5′-CGGGATCTTGGTCTTCTCTA-3′*),*

β-defensin-4, (mbd4) (F, 5′-GCAGCCTTTACCCAAATTATC-3′; R, 5′-ACAATTGCCAATCTGTCGAA-3′), *β-defensin-14 (mbd14)* (F, 5′-GTATTCCTCATCTTGTTCTTGG-3′; R, 5′-AAGTACAGCACACCGGCCAC-3′) , *surfactant protein d (sp-d)* (F, 5′-TGTGATGGTGGGAATGGGTCAGAA-3′; R, 5′-TGTGGTGCCAGATCTTCTCCATGT-3′), *cramp* (F, 5′-CGAGCTGTGGATGACTTCAA-3′; R, 5′-CAGGCTCGTTACAGCTGATG-3′) , *il-17A* (F, 5'-CACCTCACACGAGGCACAAG-3'; R, 5'-GCAGCAACAGCATCAGAGACA-3'), *il-22* (F, 5'-TTCCAGCAGCCATACATCGTC-3'; R, 5'-TCGGAACAGTTTCTCCCCG-3'), *ifn-γ* (F, 5'-ATGAACGCTACACACTGCATC-3'; R, 5'-CCATCCTTTTGCCACTTCCTC-3') were obtained on a CFX96 PCR System (Bio-Rad) and analyzed using the formula either $2^{-\Delta Ct}$ normalizing target gene expression to GAPDH or $2^{-\Delta\Delta Ct}$ where applicable.

Alveolar macrophage depletion

Non-pregnant and pregnant (E8) WT mice were treated with control or clodronate liposomes (70 µl, intra-nasally) (Liposoma BV). Two days post depletion (E10), mice were intra-nasally infected with 50pfu IAV PR8 and lungs harvested one day post infection for quantification of viral load. All instillations were performed under light isoflurane anesthesia.

Isolation and infection of alveolar macrophages

Murine alveolar macrophages were isolated from non-pregnant and pregnant mice (E10). BALF was collected following 5 washes with 1mL cold PBS via a tracheal cannula. The total volume recovered after each lavage was ~0.8 ml. Samples were spun down at a speed of 1,500 rpm for 10 min, re-suspended in RPMI (Gibco) (10% FBS (Heat-inactivated; Wisent), 1% L-glutamine (Gibco), 1% antibiotic-antimycotic (Gibco)), and plated at a density of 100 000 cells/well in a 96-well plate. Cells were incubated for 1hr, washed 3x with PBS and infected with IAV (MOI 1) for

24hr. RNA from alveolar macrophages was extracted using the RNeasy Kit (Qiagen) according to the manufacturer's instructions.

Assessment of metabolism in alveolar macrophages

Alveolar macrophages were seeded at a density of 100 000 cells/well and infected with IAV (MOI 1) overnight. After 24hr incubation, cells were washed and re-suspended in serum-free medium supplemented with 4.5 g/L D-glucose and 2 mM glutamine in DMEM. Following a 1hr incubation without CO_2 at 37 °C, basal oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were assessed using a Seahorse XF extracellular flux analyzer (Seahorse Bioscience, Inc, North Billerica, MA, USA). Mito Stress assay was performed using 1 μg/ml oligomycin, 0.7 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and 1 μM rotenone/antimycin A which inhibits ATP synthesis, uncouples the electron transport chain, and inhibits complex I and III of the respiratory chain, respectively.

Alveolar macrophage adoptive transfer model

AMs from non-pregnant or pregnant mice (E10) were harvested, re-suspended at a density of 5 x 10^4 cells per 50 μL, and intra-tracheally transferred into Rag-$1^{-/-}$ mice. Two hours post transfer, mice were infected with 50pfu IAV PR8 intranasally. Lungs were harvested 3 days post infection for the quantification of viral load.

PCA analysis

Pearson correlation coefficients between viral load and antimicrobial peptide concentration, as well as Principal Component Analysis (PCA) on centered and scaled features, were computed using the R "stats" library [78].

Administration of IL-17 blocking antibody

Non-pregnant and pregnant (E10) WT mice were administered 200 μg/mouse anti-IL-17 (Cat#: BE0173, BioXCell Lebanon, New Hampshire) or anti-IgG1 (Cat #:BE0083, BioXCell Lebanon, New Hampshire) in 200 μl PBS intraperitoneally two days prior and the day of IAV infection. One day post intranasal infection with 50pfu IAV PR8, nasal cavities were harvested for *ns1* and antimicrobial peptide expression.

Assessment of respiratory mechanics and airway responsiveness to methacholine

Mice were administered anaesthetic agents xylazine (8-12 mg/kg) and sodium pentobarbital (30-70 mg/kg) and the paralytic agent pancuronium (0.8-1.2 mg/kg) intraperitoneally, cannulated via tracheotomy and connected to a mechanical ventilator (flexiVent™, Scireq, Montreal, Qc, Canada)). Mice were challenged with increasing concentrations of aerosolized methacholine (6.25, 12.5, 25, 50 mg/m of MCh) using the flexiVent^{TM} on days 0, 1, and 3 post infection while assessing respiratory mechanics [79]. Commercial software was used to estimate the respiratory parameters, resistance and elastance.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9 (GraphPad Software, San Diego, California, USA). All data are presented as mean ± SEM. Differences were considered significant if $p<0.05$ using Two-way ANOVA followed by Sidak's multiple comparison, One-way ANOVA followed by Holm- Sidak's multiple comparison, or unpaired Student's T-test, as appropriate.

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2.9 Author Contributions

JM, MD, and JC conceptualized the study and designed the experiments. JC with the assistance of EP, KAT, AM, OT, and KM performed *in vivo* and *in vitro* experiments. JC and EP analyzed and graphed the data. TMcG, AM, and SW performed microscopy image analysis. JC, JM, and MD wrote the manuscript.

2.10 Figures and Figure Legends

Figure 1.

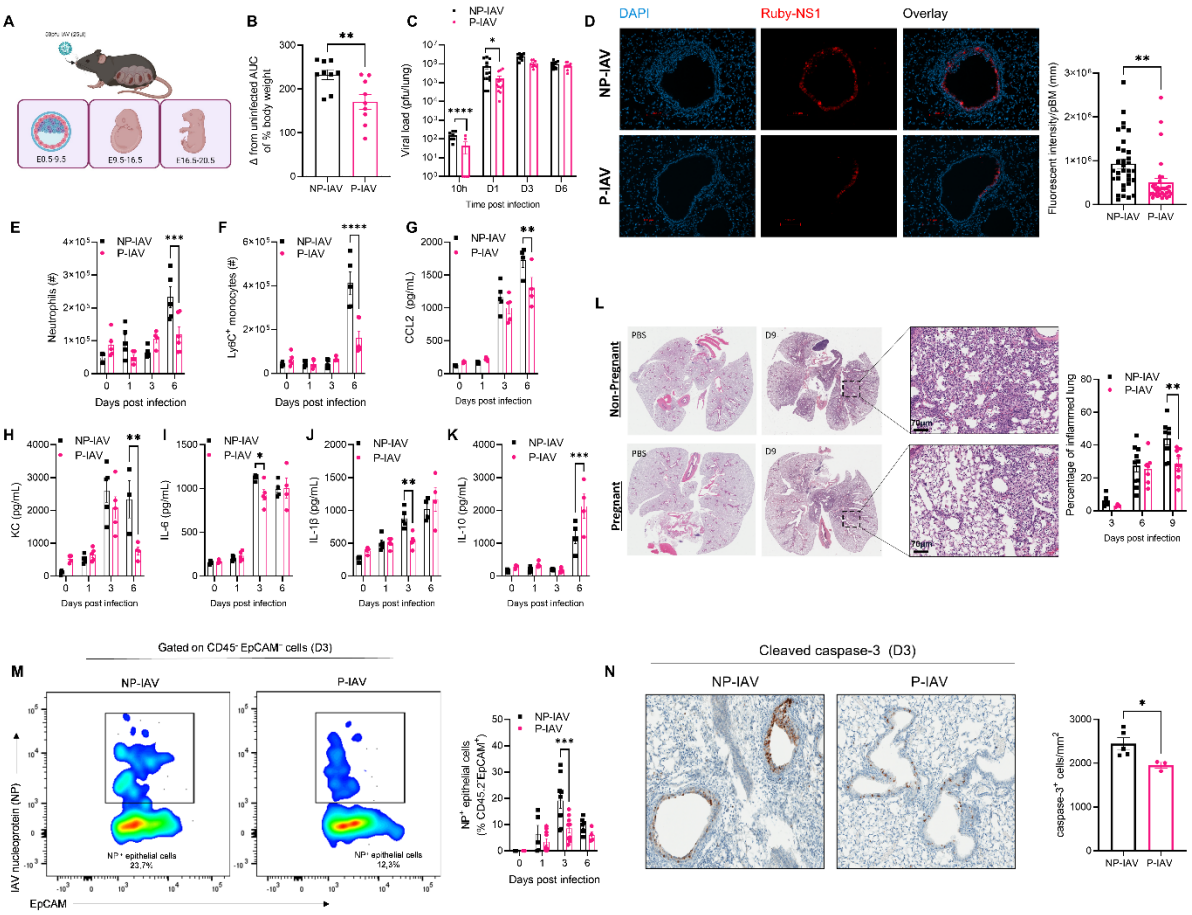


Figure 1. Mid-gestation pregnancy provides enhanced host resistance to IAV infection and limits IAV-induced pulmonary pathology. (A) Graphical representation of experimental model. Non-pregnant (NP) and pregnant (P) (E10; 2nd trimester-equivalent) C57BL/6 mice were intranasally (*i.n.*) infected with a sublethal dose of the A/Puerto Rico/8/34 H1N1 (PR8) strain of influenza (25-50pfu). (B) Body weight was monitored for 12 days post infection represented by area under the curve (AUC) as a measure of differences in the change in body weight compared to respective uninfected controls (n=8-9). (C) Kinetics of the viral load was measured by standard MDCK plaque assay on lung homogenates (n=8-12). (D) Fluorescence microscopy of infected airways in the lungs of non-pregnant and pregnant mice 3 days post infection *i.n.* with a sublethal dose of the reporter Ruby-NS1 PR8 strain (500pfu in 25µl PBS; n=4-5). Scale bar = 100µm. (E) Neutrophils and (F) $Ly6C^+$ monocyte absolute numbers were measured in lung homogenates of non-pregnant and pregnant IAV-infected mice by flow cytometry at various times post infection (n=4-5). (G-K) CCL2, KC, IL-6, IL-1β, and IL-10 concentrations of non-pregnant and pregnant IAV-infected mice measured by ELISA at various time points post infection (n=4-5). (L) Representative histology images and quantification of lungs from non-pregnant and pregnant naïve and IAV-infected mice at day 9 post infection stained with hematoxylin and eosin (n=8-9). Scale bar = 70µm. (M) Representative FACS plot and quantification of pulmonary infected epithelial cells gated on $CD45.2^-EpCAM^+NP^+$ (n=4-10). (N) Immunohistochemistry images from lung sections 3 days post IAV infection stained with anti-caspase-3 and respective quantification (n=3-5). Data are presented as mean ± s.e.m. Data were analyzed using Unpaired Student's T-test (B, D, L, N) or Two-way ANOVA followed by Sidak's multiple comparisons test (C, E-K, M).

Figure 2.

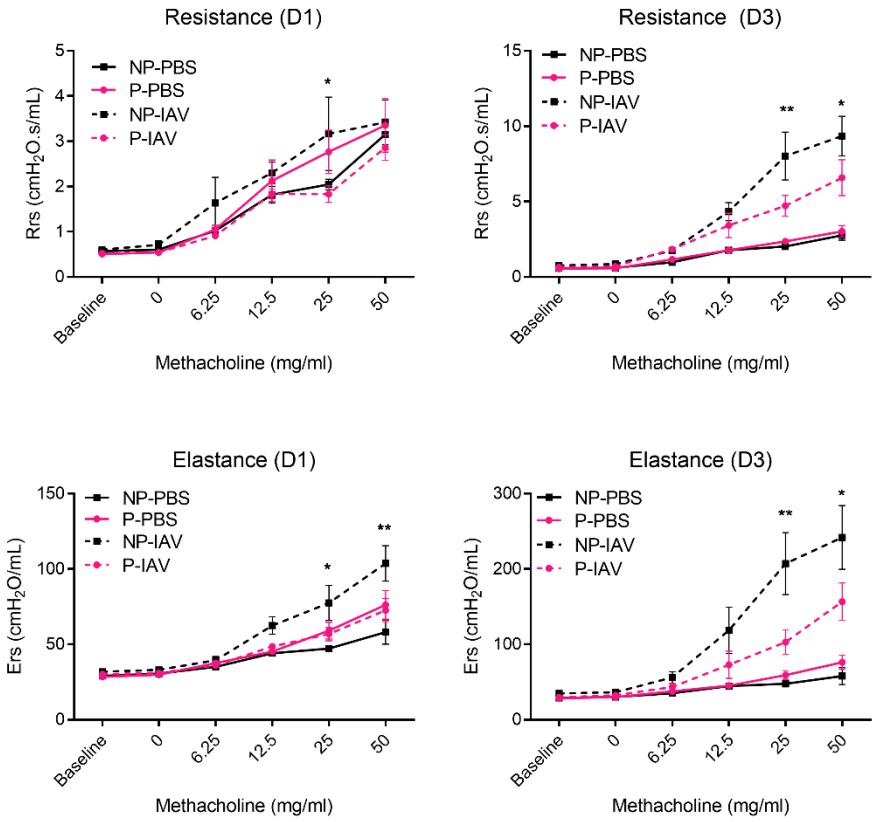


Figure 2. Pregnant mice are protected from IAV-induced AHR. Airway mechanics measured at days 1 and 3 post IAV-infection using flexiVent™ following challenge with increasing doses of methacholine (n=3-9). Data are presented as mean ± s.e.m. Data were analyzed using Two-way ANOVA followed by Sidak's multiple comparison.

Figure 3.

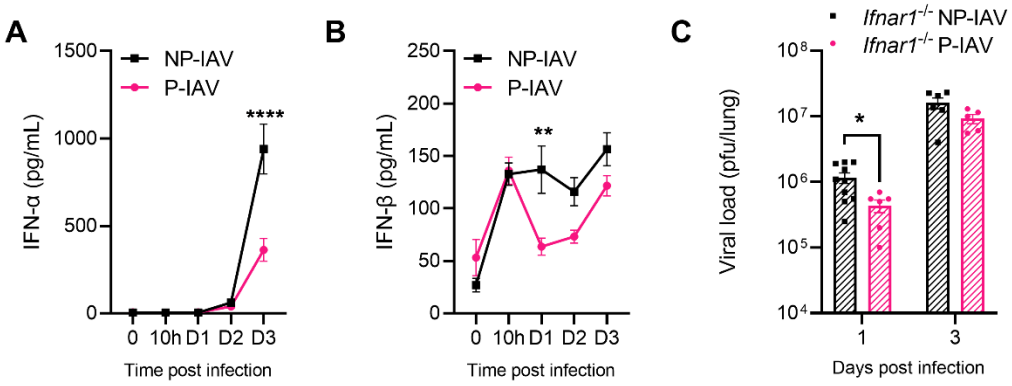


Figure 3. Maternal resistance is independent on pulmonary type I IFN-mediated antiviral immunity. (A) IFN-α and (B) IFN-β concentrations measured in lung homogenates of non-pregnant and pregnant IAV-infected mice (50pfu IAV; E10) by ELISA (n=4-6). (C) Kinetics of pulmonary viral load in *Ifnar1*$^{-/-}$ mice measured by standard MDCK plaque assay (n=5-10). Data are presented as mean ± s.e.m. Data were analyzed using Two-way ANOVA followed by Sidak's multiple comparisons test.

Figure 4.

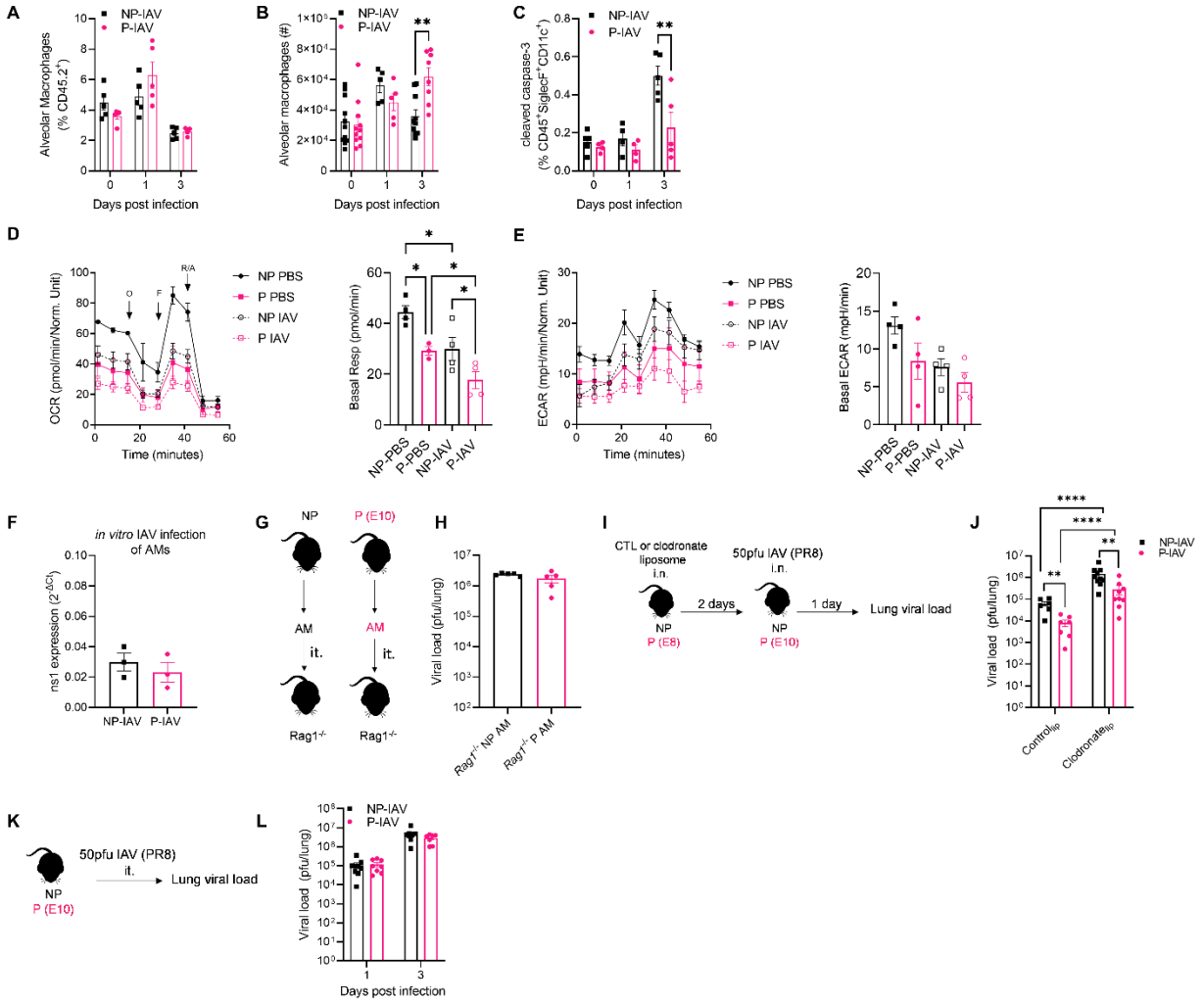

A
Alveolar Macrophages (% CD45.2+)
NP-IAV
P-IAV
Days post infection
B
Alveolar macrophages (#)
C
cleaved caspase-3 (% CD45+SiglecF+CD11c+)
D
OCR (pmol/min/Norm. Unit)
NP PBS
P PBS
NP IAV
P IAV
Time (minutes)
Basal Resp (pmol/min)
E
ECAR (mpH/min/Norm. Unit)
Basal ECAR (mpH/min)
F
in vitro IAV infection of AMs
ns1 expression (2-ΔCt)
G
NP
P (E10)
AM
it.
Rag1-/-
H
Viral load (pfu/lung)
Rag1-/- NP AM
Rag1-/- P AM
I
CTL or clodronate liposome i.n.
2 days
50pfu IAV (PR8) i.n.
1 day
Lung viral load
NP (E8)
NP (E10)
J
Controlliposome
Clodronateliposome
K
50pfu IAV (PR8)
it.
Lung viral load
L

Figure. 4. Maternal resistance to IAV infection is independent of AM function. AM (A) frequency and (B) total cell number ($CD45.2^+SiglecF^+CD11c^+$) measured in lung homogenates of non-pregnant and pregnant mice at various time points post IAV infection (50pfu IAV; E10) by flow cytometry (n=5-10). (C) Frequency of pulmonary apoptotic AMs ($CD45.2^+SiglecF^+CD11c^+Caspase\text{-}3^+$) at various time points post IAV infection (n=4-5). Quantification of basal (D) OCR and (E) ECAR of AMs subjected to a Seahorse™ assay measuring cellular metabolism before and after 24hr of infection with HIN1 (MOI 1) (n=8-10 pooled and plated in replicates). (F) Naïve AMs derived from the BAL fluid of non-pregnant and pregnant mice infected *in vitro* with IAV (MOI 1) for 24h and viral *Ns1* expression quantified by RT-PCR (AMs from n=8-10 mice pooled and plated in triplicates). (G) Murine adoptive transfer model. Naïve AMs derived from the BAL fluid of non-pregnant and pregnant mice intra-tracheally transferred (5×10^4 cells per 50 µl) into *Rag1*$^{-/-}$ recipient mice and infected two hours post transfer intranasally (50pfu IAV). (H) Pulmonary viral load of *Rag1*$^{-/-}$ recipient mice 3 days post infection by MDCK plaque assay (n=5). (I) Murine AM depletion model via administration of 70µl control liposome or clodronate liposome intranasally two days prior to infection (50pfu IAV; E10). (J) Pulmonary viral load 1 day post IAV infection measured by standard MDCK plaque assay following AM depletion (n=6-10). (K) Murine intra-tracheal IAV infection (50pfu in 25µl PBS). (L) Kinetics of pulmonary viral titers by plaque assay following intra-tracheal IAV infection (n=7-9). Data are presented as mean ± s.e.m. Data were analyzed using Two-way ANOVA followed by Sidak's multiple comparison (A-C, L), unpaired Student's T test (F, H) one-way ANOVA followed by Holm-Sidak's multiple comparisons test (D, E, J).

Figure 5.

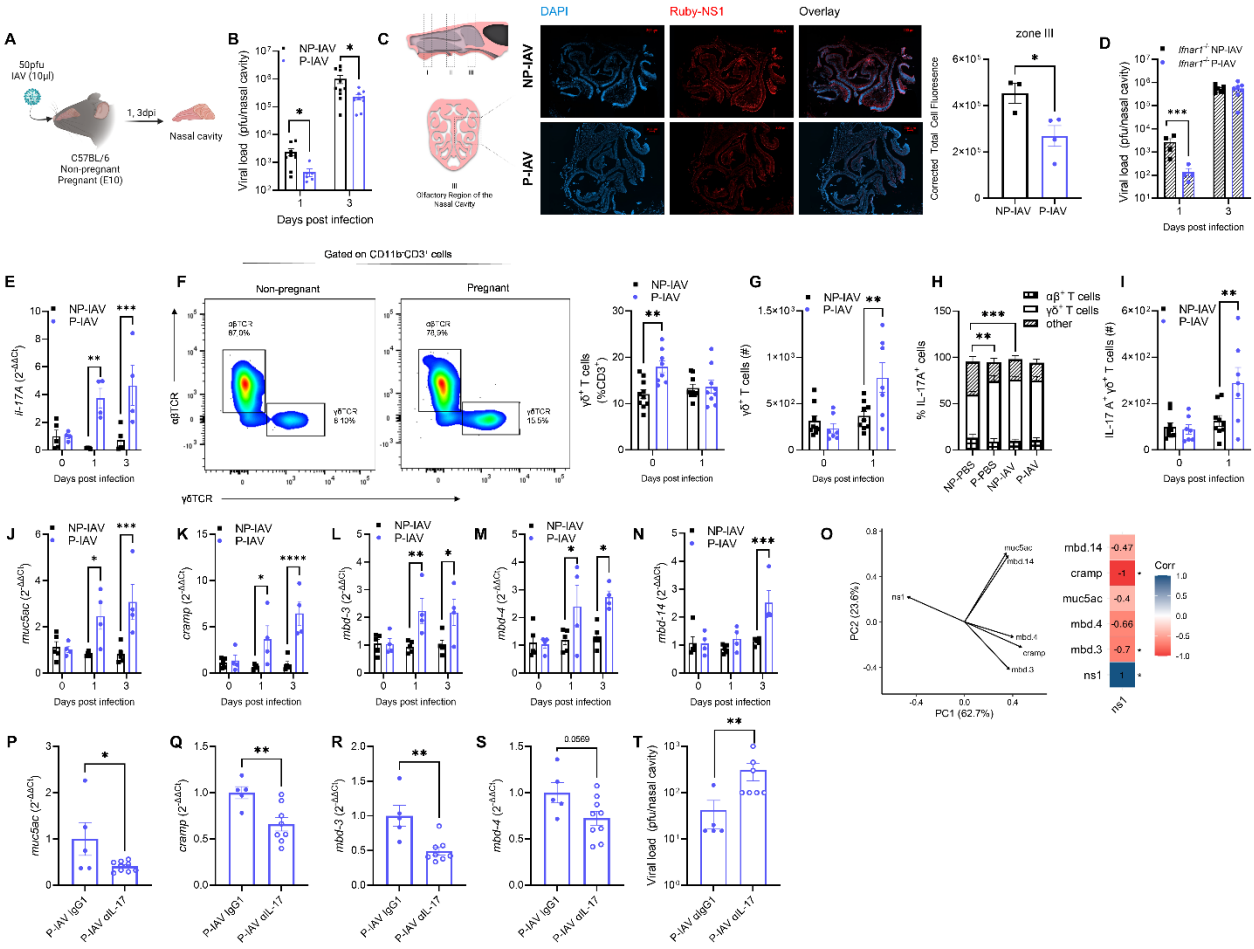
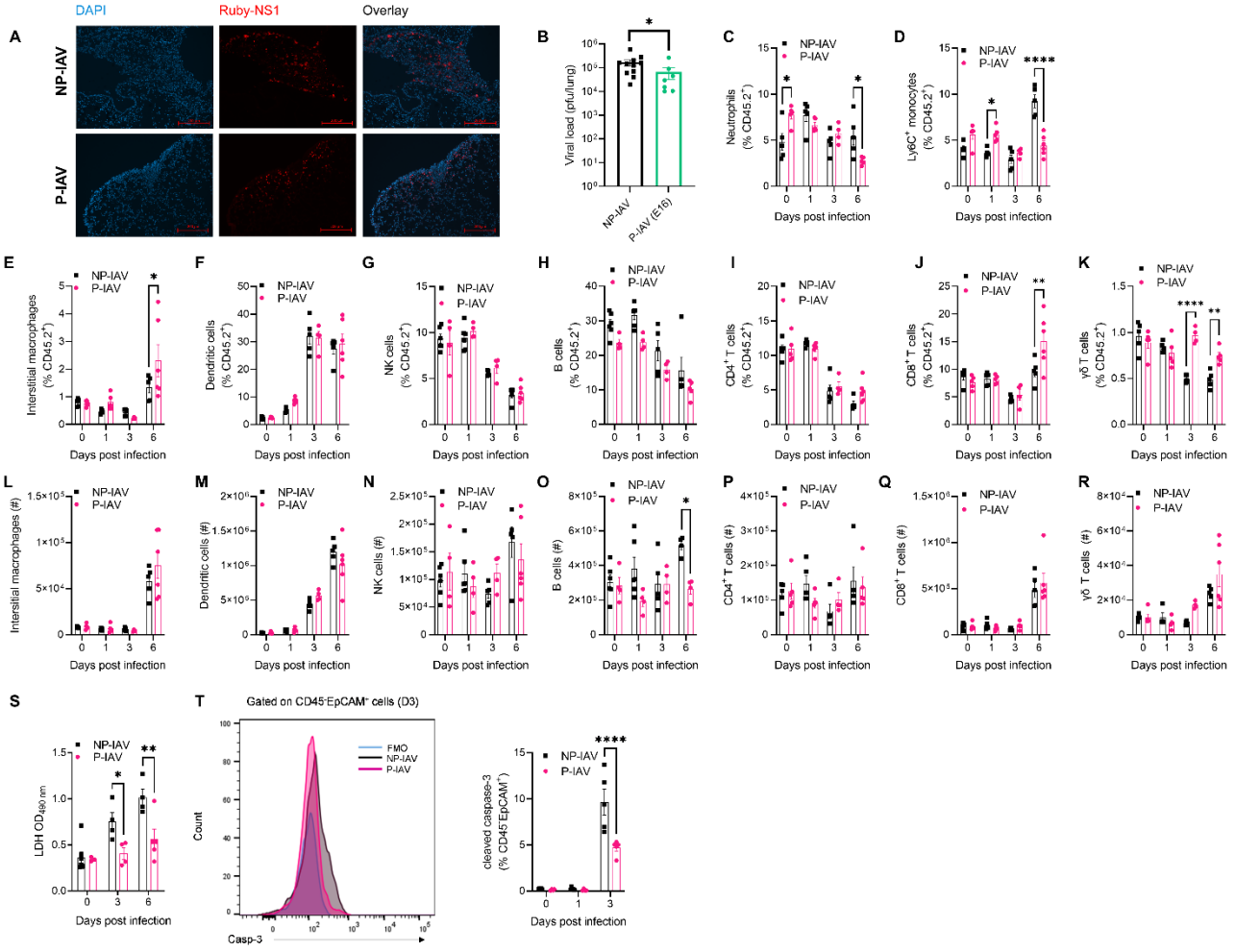


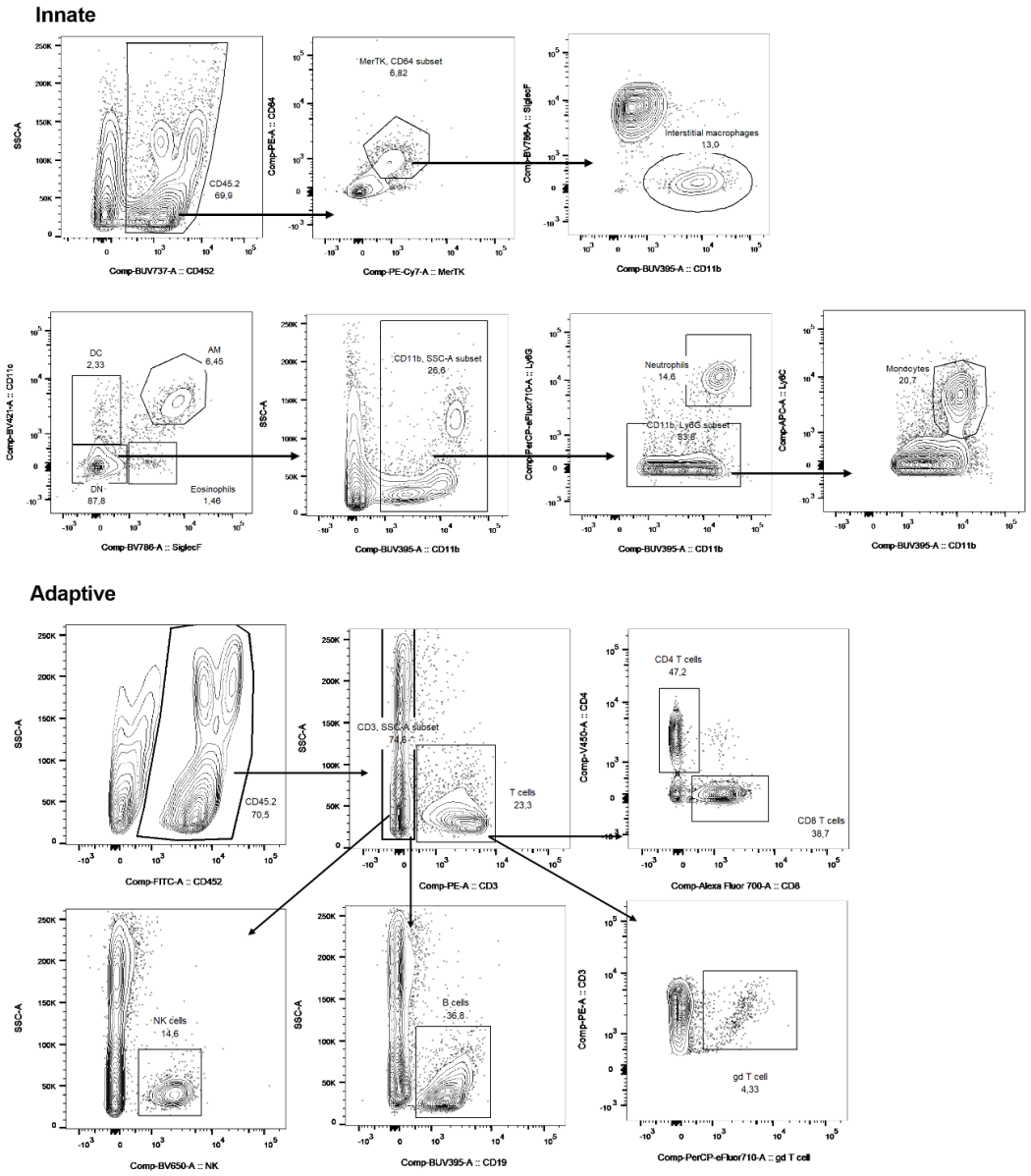
Figure 5. $\gamma\delta^+$-IL-17-induced AMPs production restricts IAV replication in the upper airways in pregnant-infected mice. (A) Graphical representation of upper airway infection. Infection of non-pregnant and pregnant (E10) C57BL/6 mice with a sublethal dose of PR8 H1N1 strain of influenza (50pfu in 10µl PBS) *i.n.* (B) Kinetics of the viral load measured by standard MDCK plaque assay on nasal cavities tissues of wildtype non-pregnant and pregnant mice (n=7-10). (C) Adapted graphical representation of various nasal tissue regions [80] and fluorescence microscopy of infected olfactory epithelial cells (zone III) 3 days post infection with Ruby-NS1 (500pfu in 10µl PBS) (n=3-4). Corrected total fluorescence calculated as integrated density – (area of selected cell × mean fluorescence of background readings). Scale bar = 200 µm. (D) Kinetics of viral load measured by standard MDCK plaque assay on nasal cavity tissues of non-pregnant and pregnant mice *Ifnar1*$^{-/-}$ (n=4-8). (E) Kinetics of nasal *il-17* expression measured by RT-PCR (n=4-5). (F) Representative FACS plots and frequency of $\gamma\delta^+$ T cells ($CD45^+CD11b^-CD3^+\gamma\delta TCR^+$) measured in cells harvested from nasal cavities of non-pregnant and pregnant mice 0- and 1-day post IAV infection (n=8-10). (G) Absolute number of $\gamma\delta^+$ T cells at days 0- and 1- post IAV infection measured by flow cytometry (n=8-10). (H) Proportion of IL-17-producing cells and (I) total number of $IL17^+\gamma\delta^+$ T cells in the nasal cavities of non-pregnant and pregnant mice at 0- and 1-day post infection following 4h stimulation with rIL-23 (10ng/ml) and rIL-1β (10ng/ml) (n=8-10). (J-N) *Muc5ac, Cramp,* and *Mbd-3, -4, -14,* expression in the nasal cavities of non-pregnant and pregnant IAV-infected mice measured by RT-PCR at various timepoints post infection (n = 4-5). (O) Loading plot and correlation coefficients of *Ns1* and AMP expression 3 days post IAV infection (n=4-5). (P-S) Quantification of nasal *Muc5ac, Cramp*, *Mbd-3,* and *Mbd-4* expression 1 day post IAV infection in pregnant mice by RT-PCR and (T) nasal viral titers quantified by MDCK plaque assay 1 day post IAV-infection following intraperitoneal *(i.p.)* administration of control αIgG1 or αIL-17A (200µg/mouse in 200µl PBS) (n=5-9). Data are presented as mean ± s.e.m. Data are presented as mean ± s.e.m. Data were analyzed using Two-way ANOVA followed by Sidak's multiple comparison (B, D-N), Pearson's correlation (O) or Unpaired Student's T test (C, P-T).

Supplemental Figure 1.



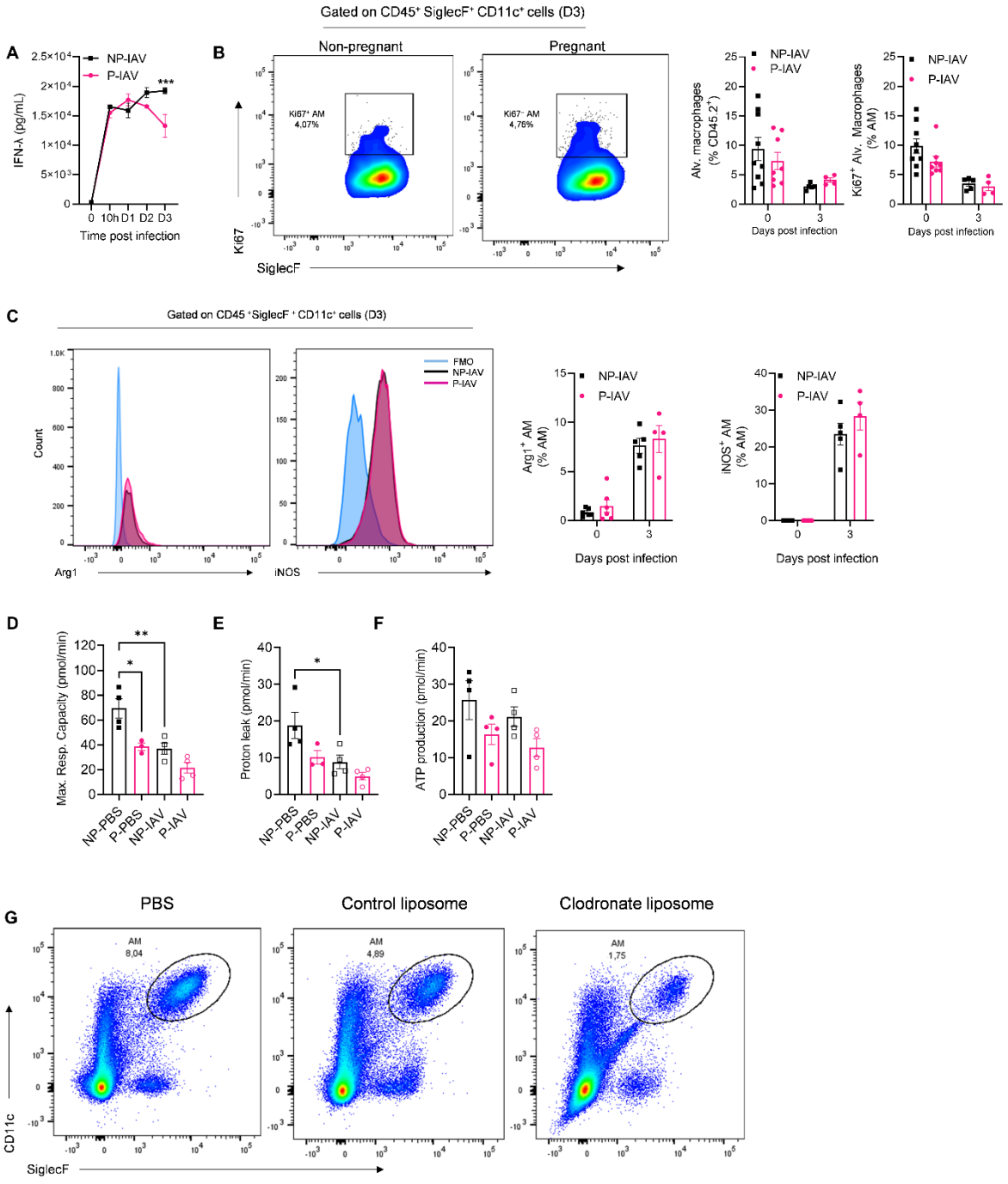
Supp. Fig. 1. Reduction of viral replication and inflammatory responses in pregnant mice. (A) Fluorescence microscopy of infected cells in the lung parenchyma of non-pregnant and pregnant mice 3 days post *i.n.* infection with Ruby-NS1 PR8 (500pfu) (n=4-5). Scale bar = 200μm. (B) Viral quantification by MDCK plaque assay of non-pregnant and pregnant (E16; 3rd trimester equivalent) C57BL/6 mice 1 day post IAV infection (50pfu). Frequency of pulmonary (C) neutrophils and (D) Ly6C^{+} monocytes at various time points post IAV (n=4-5). (E-K) Frequency and (L-R) absolute numbers of various innate and adaptive cells in the lungs of non-pregnant and pregnant mice at multiple timepoints post IAV-infection (n=4-6). Data analysed by FlowJo software. (S) Kinetics of lactate dehydrogenase released in BALF of non-pregnant and pregnant mice at various timepoints post IAV-infection (n=4-5). (T) Histogram plot and frequency of caspase-3^{+} epithelial cells (CD45^{-}EpCAM^{+}Casp3^{+}) in the lungs of non-pregnant and pregnant mice at various times post IAV infection (n=4-5). Data are presented as mean ± s.e.m. Data were analyzed using Unpaired Student's T test (B) or Two-way ANOVA followed by Sidak's multiple comparison (C-T).

Supplemental Figure 2.



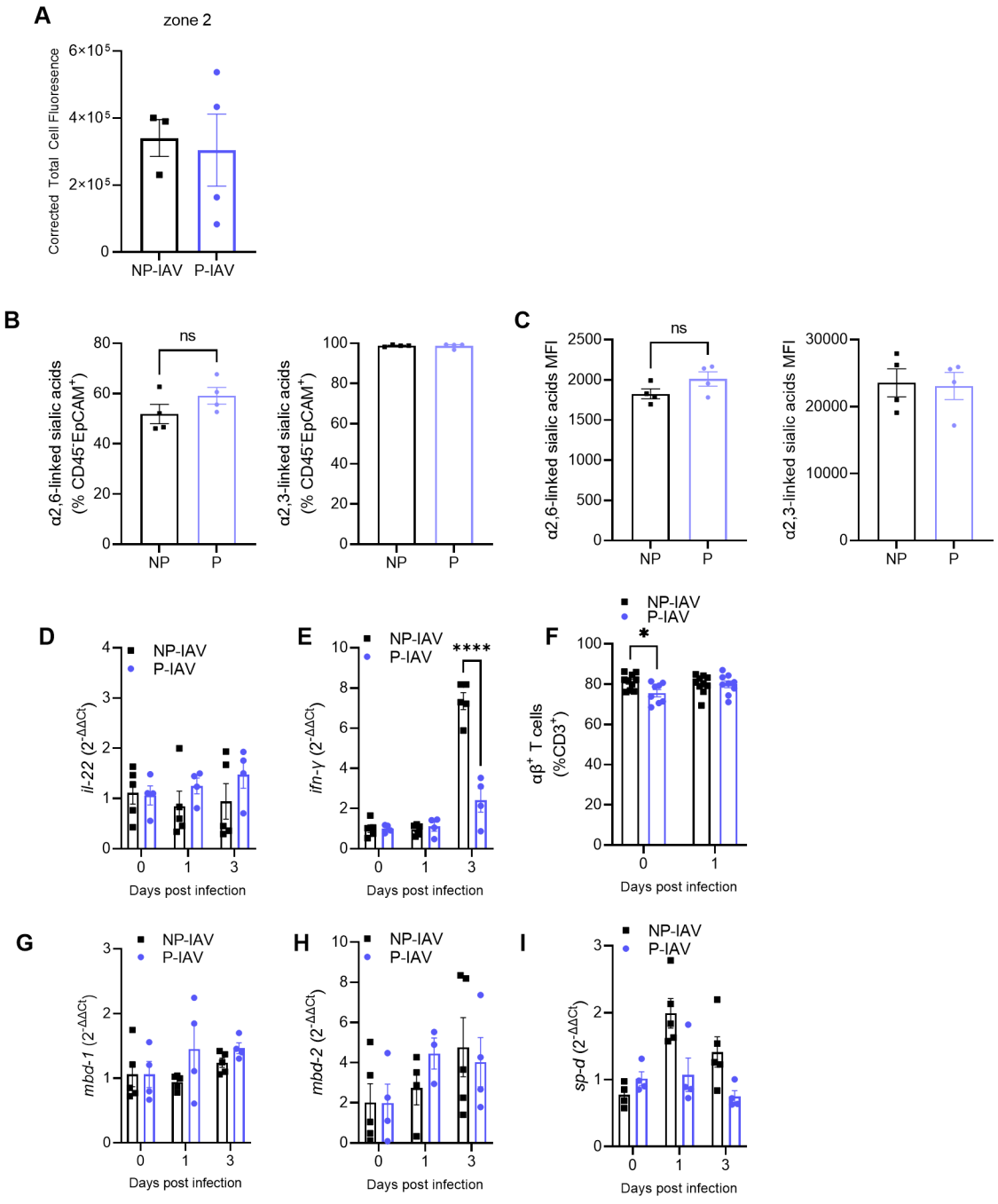
Supp. Fig. 2. Gating strategy for innate and adaptive immune cells in the lungs. Gating strategy for immune cells in the lungs. All populations were gated from single viable cells; interstitial macrophages ($CD45^+MerTK^+CD64^+SiglecF^-CD11b^+$), alveolar macrophages ($CD45^+CD11c^+SiglecF^+$), dendritic cells ($CD45^+CD11c^+SiglecF^-$), eosinophils ($CD45^+$, $CD11c^-$, $SiglecF^+$), neutrophils ($CD45^+SiglecF^-CD11c^-CD11b^+Ly6G^+$), inflammatory monocytes ($CD45^+$, $SiglecF^-$, $CD11c^-$, $CD11b^+$, $Ly6G^-$, $Ly6C^+$), T cells ($CD45^+$ $CD3^+$), B cells ($CD45^+$, $CD3^-$, $CD19^+$), NK cells ($CD45^+$, $CD3^-$, $NK1.1^+$), $CD4^+$ T cells ($CD45^+$, $CD3^+$, $CD4^+$, $CD8^-$), $CD8^+$T cells ($CD45^+$, $CD3^+$, $CD4^-$, $CD8^+$), $\gamma\delta^+$ T cells ($CD45^+$, $CD3^+$, $\gamma\delta TCR^+$).

Supplemental Figure 3.



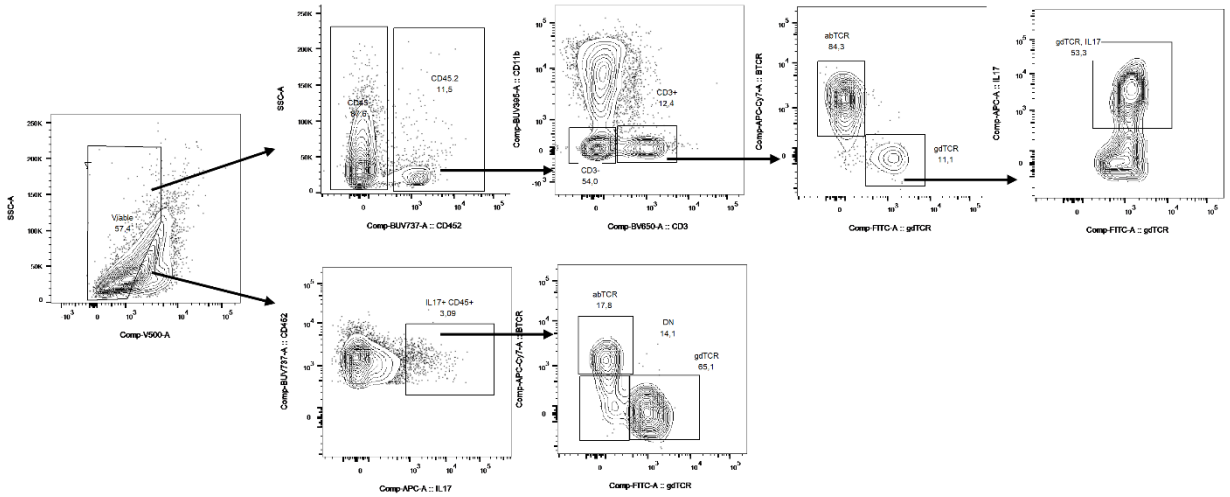
Supp. Fig. 3. **Maternal resistance is not conferred by IFN- λ, AM phenotype or metabolic profile.** Kinetics of IFN-λ concentrations measured in lung homogenates of non-pregnant and pregnant IAV-infected mice (50pfu IAV) by ELISA (n=4-6). (B) Representative FACS plots and frequency of AMs ($CD45^+SiglecF^+CD11c^+$) and $Ki67^+$ AMs ($CD45^+SiglecF^+CD11c^+Ki67^+$) at days 0- and 3-post IAV infection (n=4-9). (C) Frequency of $iNOS^+$ and $Arg1^+$ AMs at days 0- and 3-post IAV infection with representative histograms measured by flow cytometry ($CD45^+SiglecF^+CD11c^+iNOS^+/Arg1^+$) (n=4-5). (D-F) Maximal respiratory capacity, proton leak, and ATP production of AMs derived from the BALF of non-pregnant and pregnant mice infected *in vitro* with IAV (MOI 1) or medium for 24h (AMs from n=8-10 mice pooled and plated in replicates). (G) Representative FACS plots of AM population in the lungs ($CD45^+SiglecF^+CD11c^+$) following administration of PBS, control liposome, and clodronate liposome of naïve non-pregnant mice (70µl). Data are presented as mean ± s.e.m. Data were analyzed using Two-way ANOVA followed by Tukey's multiple comparison (A-C), or one-way ANOVA followed by Holm-Sidak's multiple comparisons test (D-F).

Supplemental Figure 4.



Supp. Fig. 4. Characterization of immune and antimicrobial constituents in nasal tissues. (A) Quantification of infected respiratory epithelial cells (zone II) 3 days post infection with Ruby-NS1 (500pfu in 10µl PBS) (n=3-4). (B) Frequency and (C) mean fluorescence intensity of α2,3- and α 2,6-sialic acids on the surface of nasal cavity epithelial cells in naïve non-pregnant and pregnant mice measured by flow cytometry. (n = 4). (D-E) Expression levels of *il-22* and *ifn-γ* in the nasal cavities of non-pregnant and pregnant IAV-infected mice determined by RT-PCR at various timepoints post infection (n =4- 5). (F) Frequency of $\alpha\beta^+$ T cells in nasal cavities of non-pregnant and pregnant mice at 0-, and 1- days post IAV infection (n=8-10). (G-I) Expression levels of *Mbd-1, -2, and Sp-d* in the nasal cavities of non-pregnant and pregnant IAV-infected mice determined by RT-PCR at various timepoints post infection (n =4- 5). Data were analyzed using Unpaired Student's T test (A-C) or Two-way ANOVA followed by Sidak's multiple comparison (D-I).

Supplemental Figure 5.



Supp. Fig 5. Gating strategy for IL-17^{+} γδ^{+} T cells in the nasal cavity. Sample obtained from naïve non-pregnant mouse. γδ^{+} T cell frequency and numbers were obtained from gating on single and viable cells, CD45^{+}CD11b^{-}CD3^{+}βTCR^{-}γδTCR^{+}IL-17A^{+}. The proportion of IL-17^{+} cells was obtained by gating on single and viable cells, CD45^{+} IL-17A^{+}. Data analyzed by FlowJo software.

2.11 References

1. Saunders-Hastings, P.R. and D. Krewski, *Reviewing the History of Pandemic Influenza: Understanding Patterns of Emergence and Transmission.* Pathogens, 2016. **5**(4).
2. Kucuk, M.P., et al., *Management of Acute Respiratory Distress Syndrome with H1N1 Influenza Virus in Pregnancy: Successful Mechanical Ventilation and Weaning with Airway Pressure Release Ventilation.* Turk J Anaesthesiol Reanim, 2018. **46**(1): p. 62-65.
3. Mor, G., P. Aldo, and A.B. Alvero, *The unique immunological and microbial aspects of pregnancy.* Nat Rev Immunol, 2017. **17**(8): p. 469-482.
4. Le Gars, M., et al., *Increased Proinflammatory Responses of Monocytes and Plasmacytoid Dendritic Cells to Influenza A Virus Infection During Pregnancy.* J Infect Dis, 2016. **214**(11): p. 1666-1671.
5. Engels, G., et al., *Pregnancy-Related Immune Adaptation Promotes the Emergence of Highly Virulent H1N1 Influenza Virus Strains in Allogenically Pregnant Mice.* Cell Host Microbe, 2017. **21**(3): p. 321-333.
6. Medzhitov, R., D.S. Schneider, and M.P. Soares, *Disease tolerance as a defense strategy.* Science, 2012. **335**(6071): p. 936-41.
7. Iwasaki, A. and P.S. Pillai, *Innate immunity to influenza virus infection.* Nat Rev Immunol, 2014. **14**(5): p. 315-28.
8. Littauer, E.Q., et al., *H1N1 influenza virus infection results in adverse pregnancy outcomes by disrupting tissue-specific hormonal regulation.* PLoS Pathog, 2017. **13**(11): p. e1006757.
9. Marcelin, G., et al., *Fatal outcome of pandemic H1N1 2009 influenza virus infection is associated with immunopathology and impaired lung repair, not enhanced viral burden, in pregnant mice.* J Virol, 2011. **85**(21): p. 11208-19.
10. Vermillion, M.S., et al., *Pregnancy preserves pulmonary function following influenza virus infection in C57BL/6 mice.* Am J Physiol Lung Cell Mol Physiol, 2018.
11. Jacobsen, H., et al., *Offspring born to influenza A virus infected pregnant mice have increased susceptibility to viral and bacterial infections in early life.* Nat Commun, 2021. **12**(1): p. 4957.
12. Heaton, N.S., et al., *Targeting Viral Proteostasis Limits Influenza Virus, HIV, and Dengue Virus Infection.* Immunity, 2016. **44**(1): p. 46-58.
13. Gui, R. and Q. Chen, *Molecular Events Involved in Influenza A Virus-Induced Cell Death.* Front Microbiol, 2021. **12**: p. 797789.
14. Simpson, J.L., et al., *Use of induced sputum for the diagnosis of influenza and infections in asthma: a comparison of diagnostic techniques.* J Clin Virol, 2003. **26**(3): p. 339-46.
15. Chang, Y.J., et al., *Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity.* Nat. Immunol, 2011. **12**(7): p. 631-638.
16. Schneider, C., et al., *Alveolar macrophages are essential for protection from respiratory failure and associated morbidity following influenza virus infection.* PLoS Pathog, 2014. **10**(4): p. e1004053.
17. Cardani, A., A. Boulton, T.S. Kim, and T.J. Braciale, *Alveolar Macrophages Prevent Lethal Influenza Pneumonia By Inhibiting Infection Of Type-1 Alveolar Epithelial Cells.* PLoS Pathog, 2017. **13**(1): p. e1006140.
18. Ghoneim, H.E., P.G. Thomas, and J.A. McCullers, *Depletion of alveolar macrophages during influenza infection facilitates bacterial superinfections.* J Immunol, 2013. **191**(3): p. 1250-9.

19. Coulombe, F., et al., *Targeted prostaglandin E2 inhibition enhances antiviral immunity through induction of type I interferon and apoptosis in macrophages.* Immunity, 2014. **40**(4): p. 554-68.
20. Pernet, E., et al., *Neonatal imprinting of alveolar macrophages via neutrophil-derived 12-HETE.* Nature, 2023. **614**(7948): p. 530-538.
21. Yan, J. and T. Horng, *Lipid Metabolism in Regulation of Macrophage Functions.* Trends Cell Biol, 2020. **30**(12): p. 979-989.
22. Viola, A., et al., *The Metabolic Signature of Macrophage Responses.* Front Immunol, 2019. **10**: p. 1462.
23. Lauzon-Joset, J.F., et al., *Pregnancy Induces a Steady-State Shift in Alveolar Macrophage M1/M2 Phenotype That Is Associated With a Heightened Severity of Influenza Virus Infection: Mechanistic Insight Using Mouse Models.* J Infect Dis, 2019. **219**(11): p. 1823-1831.
24. Freemerman, A.J., et al., *Metabolic reprogramming of macrophages: glucose transporter 1 (GLUT1)-mediated glucose metabolism drives a proinflammatory phenotype.* J Biol Chem, 2014. **289**(11): p. 7884-96.
25. Divangahi, M., I.L. King, and E. Pernet, *Alveolar macrophages and type I IFN in airway homeostasis and immunity.* Trends Immunol, 2015. **36**(5): p. 307-14.
26. Giri, T., et al., *Upregulated influenza A viral entry factors and enhanced interferon-alpha response in the nasal epithelium of pregnant rats.* Heliyon, 2022. **8**(5): p. e09407.
27. Palomino-Segura, M., I. Latino, Y. Farsakoglu, and S.F. Gonzalez, *Early production of IL-17A by gammadelta T cells in the trachea promotes viral clearance during influenza infection in mice.* Eur J Immunol, 2020. **50**(1): p. 97-109.
28. Sutton, C.E., et al., *Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity.* Immunity, 2009. **31**(2): p. 331-41.
29. Archer, N.K., et al., *Interleukin-17A (IL-17A) and IL-17F Are Critical for Antimicrobial Peptide Production and Clearance of Staphylococcus aureus Nasal Colonization.* Infect Immun, 2016. **84**(12): p. 3575-3583.
30. Borkner, L., et al., *Correction: IL-17 mediates protective immunity against nasal infection with Bordetella pertussis by mobilizing neutrophils, especially Siglec-F(+) neutrophils.* Mucosal Immunol, 2021. **14**(5): p. 1218-1219.
31. Solans, L., et al., *IL-17-dependent SIgA-mediated protection against nasal Bordetella pertussis infection by live attenuated BPZE1 vaccine.* Mucosal Immunol, 2018. **11**(6): p. 1753-1762.
32. Liang, S.C., et al., *Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides.* J Exp Med, 2006. **203**(10): p. 2271-9.
33. Chong, K.T., R.R. Thangavel, and X. Tang, *Enhanced expression of murine beta-defensins (MBD-1, -2,- 3, and -4) in upper and lower airway mucosa of influenza virus infected mice.* Virology, 2008. **380**(1): p. 136-43.
34. LeMessurier, K.S., Y. Lin, J.A. McCullers, and A.E. Samarasinghe, *Antimicrobial peptides alter early immune response to influenza A virus infection in C57BL/6 mice.* Antiviral Res, 2016. **133**: p. 208-17.
35. Klotman, M.E. and T.L. Chang, *Defensins in innate antiviral immunity.* Nat Rev Immunol, 2006. **6**(6): p. 447-56.

36. Delaveris, C.S., et al., *Membrane-tethered mucin-like polypeptides sterically inhibit binding and slow fusion kinetics of influenza A virus.* Proc Natl Acad Sci U S A, 2020. **117**(23): p. 12643-12650.
37. Currie, S.M., et al., *Cathelicidins Have Direct Antiviral Activity against Respiratory Syncytial Virus In Vitro and Protective Function In Vivo in Mice and Humans.* J Immunol, 2016. **196**(6): p. 2699-710.
38. Tripathi, S., et al., *The human cathelicidin LL-37 inhibits influenza A viruses through a mechanism distinct from that of surfactant protein D or defensins.* J Gen Virol, 2013. **94**(Pt 1): p. 40-49.
39. Robinson, D.P. and S.L. Klein, *Pregnancy and pregnancy-associated hormones alter immune responses and disease pathogenesis.* Horm Behav, 2012. **62**(3): p. 263-71.
40. Dawood, F.S., et al., *Incidence of influenza during pregnancy and association with pregnancy and perinatal outcomes in three middle-income countries: a multisite prospective longitudinal cohort study.* Lancet Infect Dis, 2021. **21**(1): p. 97-106.
41. Malinowski, A.K., A. Noureldin, and M. Othman, *COVID-19 susceptibility in pregnancy: Immune/inflammatory considerations, the role of placental ACE-2 and research considerations.* Reprod Biol, 2020. **20**(4): p. 568-572.
42. Knight, M., et al., *Characteristics and outcomes of pregnant women admitted to hospital with confirmed SARS-CoV-2 infection in UK: national population based cohort study.* BMJ, 2020. **369**: p. m2107.
43. Song, D., et al., *Passive and active immunity in infants born to mothers with SARS-CoV-2 infection during pregnancy: Prospective cohort study.* medRxiv, 2021.
44. Sutton, D., K. Fuchs, M. D'Alton, and D. Goffman, *Universal Screening for SARS-CoV-2 in Women Admitted for Delivery.* N Engl J Med, 2020. **382**(22): p. 2163-2164.
45. Adhikari, E.H., et al., *Pregnancy Outcomes Among Women With and Without Severe Acute Respiratory Syndrome Coronavirus 2 Infection.* JAMA Netw Open, 2020. **3**(11): p. e2029256.
46. Jaworska, J., et al., *NLRX1 prevents mitochondrial induced apoptosis and enhances macrophage antiviral immunity by interacting with influenza virus PB1-F2 protein.* Proc Natl Acad Sci U S A, 2014. **111**(20): p. E2110-9.
47. Hussell, T. and T.J. Bell, *Alveolar macrophages: plasticity in a tissue-specific context.* Nat Rev Immunol, 2014. **14**(2): p. 81-93.
48. Xue, C., et al., *Vgamma4(+)gammadeltaT Cells Aggravate Severe H1N1 Influenza Virus Infection-Induced Acute Pulmonary Immunopathological Injury via Secreting Interleukin-17A.* Front Immunol, 2017. **8**: p. 1054.
49. Misiak, A., M.M. Wilk, M. Raverdeau, and K.H. Mills, *IL-17-Producing Innate and Pathogen-Specific Tissue Resident Memory gammadelta T Cells Expand in the Lungs of Bordetella pertussis-Infected Mice.* J Immunol, 2017. **198**(1): p. 363-374.
50. Sobiesk, J.L. and S. Munakomi, *Anatomy, Head and Neck, Nasal Cavity*, in *StatPearls*. 2023: Treasure Island (FL).
51. King, A.E., D.C. Fleming, H.O. Critchley, and R.W. Kelly, *Differential expression of the natural antimicrobials, beta-defensins 3 and 4, in human endometrium.* J Reprod Immunol, 2003. **59**(1): p. 1-16.
52. King, A.E., et al., *Innate immune defences in the human uterus during pregnancy.* Placenta, 2007. **28**(11-12): p. 1099-106.
53. King, A.E., et al., *Expression of natural antimicrobials by human placenta and fetal membranes.* Placenta, 2007. **28**(2-3): p. 161-9.

54. van Riel, D., et al., *Influenza pathogenicity during pregnancy in women and animal models.* Semin Immunopathol, 2016. **38**(6): p. 719-726.
55. Hegewald, M.J. and R.O. Crapo, *Respiratory physiology in pregnancy.* Clin Chest Med, 2011. **32**(1): p. 1-13.
56. Orban, N., E. Maughan, and N. Bleach, *Pregnancy-induced rhinitis.* Rhinology, 2013. **51**(2): p. 111-9.
57. Baudoin, T., et al., *Redefining Pregnancy-Induced Rhinitis.* Am J Rhinol Allergy, 2021. **35**(3): p. 315-322.
58. Madanchi, H., M. Shoushtari, H.H. Kashani, and S. Sardari, *Antimicrobial peptides of the vaginal innate immunity and their role in the fight against sexually transmitted diseases.* New Microbes New Infect, 2020. **34**: p. 100627.
59. Wira, C.R., et al., *Innate immunity in the human female reproductive tract: endocrine regulation of endogenous antimicrobial protection against HIV and other sexually transmitted infections.* Am J Reprod Immunol, 2011. **65**(3): p. 196-211.
60. Frew, L., et al., *Human cathelicidin production by the cervix.* PLoS One, 2014. **9**(8): p. e103434.
61. Bagri, P., Yu, E., Karushic, C., *Mucosal immunology of the female reproductive tract and its regulation by female sex hormones*. Reproductive Immunology, ed. G. Mor. 2021: Academic Press.
62. Anipindi, V.C., et al., *IL-17 Production by gammadelta(+) T Cells Is Critical for Inducing T(h)17 Responses in the Female Genital Tract and Regulated by Estradiol and Microbiota.* Immunohorizons, 2019. **3**(7): p. 317-330.
63. Kim, J.O., H.R. Cha, E.D. Kim, and M.N. Kweon, *Pathological effect of IL-17A-producing TCRgammadelta(+) T cells in mouse genital mucosa against HSV-2 infection.* Immunol Lett, 2012. **147**(1-2): p. 34-40.
64. Kang, S., et al., *Tissue Resident Memory gammadeltaT Cells in Murine Uterus Expressed High Levels of IL-17 Promoting the Invasion of Trophocytes.* Front Immunol, 2020. **11**: p. 588227.
65. Pinget, G.V., et al., *The majority of murine gammadelta T cells at the maternal-fetal interface in pregnancy produce IL-17.* Immunol Cell Biol, 2016. **94**(7): p. 623-30.
66. Nishimura, H., et al., *Intraepithelial gammadelta T cells may bridge a gap between innate immunity and acquired immunity to herpes simplex virus type 2.* J Virol, 2004. **78**(9): p. 4927-30.
67. Rakasz, E., A. Mueller, S. Perlman, and R.G. Lynch, *Gammadelta T cell response induced by vaginal Herpes simplex 2 infection.* Immunol Lett, 1999. **70**(2): p. 89-93.
68. Monin, L., et al., *gammadelta T cells compose a developmentally regulated intrauterine population and protect against vaginal candidiasis.* Mucosal Immunol, 2020. **13**(6): p. 969-981.
69. Alcaide, M.L., et al., *Bacterial Vaginosis Is Associated with Loss of Gamma Delta T Cells in the Female Reproductive Tract in Women in the Miami Women Interagency HIV Study (WIHS): A Cross Sectional Study.* PLoS One, 2016. **11**(4): p. e0153045.
70. De, Y., et al., *LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells.* J Exp Med, 2000. **192**(7): p. 1069-74.
71. Yang, D., et al., *Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6.* Science, 1999. **286**(5439): p. 525-8.

72. Hsieh, I.N. and K.L. Hartshorn, *The Role of Antimicrobial Peptides in Influenza Virus Infection and Their Potential as Antiviral and Immunomodulatory Therapy.* Pharmaceuticals (Basel), 2016. **9**(3).
73. Chen, K., et al., *Requirement of CRAMP for mouse macrophages to eliminate phagocytosed E. coli through an autophagy pathway.* J Cell Sci, 2021. **134**(5).
74. Minns, D., et al., *The neutrophil antimicrobial peptide cathelicidin promotes Th17 differentiation.* Nat Commun, 2021. **12**(1): p. 1285.
75. Gaush, C.R. and T.F. Smith, *Replication and plaque assay of influenza virus in an established line of canine kidney cells.* Appl Microbiol, 1968. **16**(4): p. 588-94.
76. Creisher, P.S., et al., *Adverse outcomes in SARS-CoV-2 infected pregnant mice are gestational age-dependent and resolve with antiviral treatment.* bioRxiv, 2023.
77. Pizzolla, A., et al., *Resident memory CD8(+) T cells in the upper respiratory tract prevent pulmonary influenza virus infection.* Sci Immunol, 2017. **2**(12).
78. Team, R.C. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing 2023; Available from: https://www.R-project.org/.
79. McGovern, T.K., et al., *Evaluation of respiratory system mechanics in mice using the forced oscillation technique.* J. Vis. Exp, 2013(75): p. e50172.
80. *Nasal Cavity Sections*. Revised Guides for Organ Sampling and Trimming in Rats and Mice; Available from: https://www.niehs.nih.gov/research/resources/visual-guides/guides/l1-post/index.cfm.

Preface to Chapter 3

In the 19th century, Paul Ehrlich was the first to describe the concept of passive immunity and show that mothers had the ability to transmit antibodies (known at the time as *"antitoxins"*) to their offspring, laying the foundation of neonatal protection [264, 265]. Some of his early findings in mice showed that offspring born to immunized mothers against ricin or abrin toxins inherited immunity that was further sustained post-natally through suckling [264, 265]. This passive immunity was also acquired in the progeny born to non-immune mothers that were fostered by immunized mothers. We know from chapter 2 that influenza caused mild disease in pregnant C57BL/6 dams. With this came the origin of chapter 3 which was to understand how IAV-infected dams would confer protection to their offspring and which route of antibody transfer, (*i.e.*, placenta or milk) conferred the most protection. We found that maternal IgG1 antibodies transferred both *in utero* and through suckling were necessary to provide adequate protection to progeny against IAV infection. In addition, we observed different subsets of antibodies generated from pre-conception infection versus infection mid-gestation which may possess qualitative differences in their functions, highlighting unique antibody immunomodulation during pregnancy. Thus, safely boosting maternal antibody titers during reproductive ages and during pregnancy and assuring adequate nursing post-natally is essential in long-lasting protection against IAV infection in the offspring.

Transplacental and Breast Milk Transfer of IgG1 Are Both Required for Prolonged Protection of Offspring Against Influenza A Infection

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3.1 Abstract

The immune system during pregnancy teeters between maintaining fetal tolerance and providing protection against pathogens, such as influenza A virus (IAV). Due to this delicate balance, pregnant women and their offspring often have increased susceptibilities to infection. During the first year of life, infant immunity against infection is mainly mediated via passively transferred maternal antibodies. However, our understanding of the route of transfer of the maternal antibodies for conferring protection to IAV infection in offspring is incomplete. Here we demonstrated that offspring from IAV-infected mice were significantly protected against IAV infection. This remarkable increase in survival is mediated via the elevated maternal serum IgG1. By cross-fostering, we further showed that this enhanced host resistance was only achieved in mice born to and nursed by IAV-infected mothers. Collectively, our data suggest that the prolonged protection of offspring against IAV infection requires maternal IgG1 from both the placenta and breast milk.

3.2 Introduction

Pregnancy has been long thought of as a state of immunosuppression, however, emerging evidence is drawing attention to unique and dynamic processes that differently influence the severity of maternal responses to infectious disease and vaccine outcomes [1, 2]. Therefore, strategic intervention during specific gestational periods which support the generation of abundant and efficient pathogen-specific antibodies will protect both the mother and infant against severe illness.

Infants under one year of age are at the highest risk of increased morbidity and hospitalization following infection with pathogens like influenza A virus (IAV), therefore, vaccination is recommended but only beyond 6 months of age [3, 4]. During this vaccination gap when children are immunologically immature and harbour a respiratory system that is still developing, maternal immunoglobulins (Ig) are critical for conferring protection against pathogens [5]. The magnitude of protection in the offspring is directly correlated with previous maternal exposure to influenza and the concentration of maternal antibodies resulting from natural infection or from vaccination to specific subtypes (with some degree of cross-protection) [5-7]. Notably, in humans, maternal vaccination during the second and early third trimester yields higher antibody titres in cord blood and consequently prolongs the window of protection in offspring against influenza and pertussis [8, 9]. As a result, public health strategies, especially during the current COVID-19 pandemic, are reinforcing the basis that maternally transferred antibodies can protect newborns from potentially fatal respiratory illness [8, 10, 11].

The lower and upper respiratory tract are kept under surveillance by IgG and IgA, respectively [12]. IgG is the most abundant antibody in the serum of which IgG1 and IgG2 subtypes have been implicated in the protection against severe influenza infection in animal models through their ability to activate complement, neutralize viral particles, and mediating antibody-dependent cell-

mediated cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis [13-17]. Due to differences in Fc receptor binding and structure, there is a transfer efficiency hierarchy to the neonate among the IgG subclasses with IgG1 being most abundantly transferred [5, 12, 13, 18, 19].

Natural IAV infection is known to trigger longer-lasting antibody responses compared to vaccination [12]. Although transplacental maternal antibodies circulate in the infant blood circulation for a limited time, passive immunity continues after birth through nursing where IgA and IgG, are transferred via breast milk [5, 12, 20, 21]. IgA provides mucosal immunity in the upper airways against IAV infection through comparable mechanisms to IgG in the lungs [12, 22]. Though maternal antibodies are known to protect infants from IAV infection [23], whether protection is mediated through transplacental or colostrum IgG and/or IgA and the duration of protection remains unclear. Thus, understanding the underlying mechanism of maternal antibody-mediated protection in offspring can be used for developing vaccine strategies to boost immunogenicity in pregnant women to generate and enhance a specific antibody profile. Using a model of mid-gestation IAV infection and pre-conception infection, we have provided evidence that offspring born to IAV-infected mothers are significantly protected against IAV infection. Furthermore, by cross fostering, we demonstrated that maternal IgG1 antibodies passively transferred through both the placenta and the colostrum are required to confer the prolonged protection in offspring infected with IAV.

3.3 Materials and Methods

Mice and litter swaps

8-10-week-old female C57Bl/6J mice were purchased from Jackson Laboratories, housed, and bred at the animal facility of the Research Institute of the McGill University Health Centre. Infections with IAV were performed 10 days after mating (E10) following visual identification of a copulation plug or two weeks prior to mating for pre-conception infection. All protocols were approved by the Animal Care Committee of McGill University. In the litter swap experiments, pups were cross fostered at 3 days old. In the long-term experiments, pups were weaned at 3-4 weeks and housed by sex. Survival experiments performed on female offspring while all other experiments were performed on both male and females at 6-8 weeks of age.

Infection

Experiments were performed using the mouse adapted H1N1 influenza virus A/Puerto Rico/8/34 (PR8) virus provided by J.A. McCullers (St. Jude Children's Research Hospital). The virus was propagated in eggs and titrated in Madin-Darby Canine Kidney (MDCK) cells by plaque assays. Mice were intranasally challenged with a sublethal dose of 25 or 250 pfu and LD_{50} dose of 500 pfu for survival experiments. Two different stocks derived from the same parental strain were thawed and titrated to determine the LD_{50} and the sublethal doses to be used for experimentation. Viral titres were determined in lung homogenates using standard MDCK plaque assays on days 3 and 6 as previously described [24-27]

Type I IFN assays

Total bioactive IFN-α and IFN-β were measured in bronchoalveolar lavage fluid (BALF) and lung homogenates using the murine B16-Blue IFN-α/-β reporter cell line (InvivoGen, San Diego, CA)

which monitors the activation of JAK/STAT/ISGF3 and/or IRF3 pathways via recognition of IFNAR by type I IFN. The assay was performed according to the manufacturer's specifications on days 0, 3, and 6 post infection as previously described [24-27].

Anti-influenza antibody quantification

BALF was collected with a 26-gauge needle though a tracheal cannula using 3 x 1 mL cold PBS and spun at 15,000 rpm for 10 minutes. Lung tissue was homogenized in 500µl RPMI and spun at 15,000 rpm for 5 minutes. BALF and lung supernatants were stored at -80ºC until use. Whole blood was collected by cardiac puncture into microtainer separator tubes (BD # 365967, BD Biosciences, Franklin Lakes, NJ) and was centrifuged at 13,300 rpm for 2 minutes to separate serum. ELISA plates (96 well medium binding microplates, Corning, NY) were coated overnight at 4°C with $2x10^7$ pfu of purified H1N1. Plates were washed and blocked for 2 h with 1% BSA in PBS. Serum, BALF, and lung samples of naïve mice were added, and plates were incubated at room temperature for 2 h. Anti-mouse IgG1 (Southern Biotech #1070-05, Birmingham, AL), anti-mouse IgG2α (Southern Biotech #1081-05), and goat anti-mouse IgA (Southern Biotech #1040-05) secondary antibodies (1:1000) were added. After 2 h incubation at room temperature, reactions were developed with 3,3',5,5' tetramethylbenzidine, halted using a sulfuric acid stop solution, and read at 450 nm absorbance on a plate reader.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9 (GraphPad Software, San Diego, California, USA). All data are presented as mean ± SEM. Statistical differences determined by two-way ANOVA followed by Sidak's multiple comparison test, Unpaired Student T test or Multiple Student T-tests followed by Bonferroni correction for multiple comparison identified in figure legends.

3.4 Results

Maternal IAV-infection confers IgG1-mediated protection to IAV-infected offspring

Peak antibody transfer in humans following maternal vaccination occurs in the second and early third trimesters (Supplemental Figure 1A), although the exact timing is still unclear [28]. Poor placental transfer of maternal antibodies during natural infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and influenza virus have been observed in women infected in their final trimester, however, transfer efficiency increases if infection occurs at an earlier gestational period [2, 8, 29, 30]. In a mouse model, embryonic day 10 (E10) is the initial phase of type 2 immunity and fetal development in mice [1] and IgG1 antibodies which are the primary neutralizing antibodies during influenza infection are known to be produced in a Th2 environment [12]. Therefore, we infected pregnant mice with a sublethal dose of the mouse adapted H1N1 A/Puerto Rico/8/34 (PR8) on E10, mirroring this critical window of pregnancy that allows for optimal and selective antibody transfer (Supplemental Figure 1A). Following maternal infection, antibodies were assessed in naïve offspring at 4 and 8 weeks after birth as well as the immune response to secondary IAV infection at 8 weeks (Figure 1A). Offspring born to IAV-infected dams had a significant survival advantage following infection with a lethal dose of PR8 coupled with negligible weight loss compared to control offspring (Figure 1B). Offspring born to IAV-infected dams also showed significantly reduced pulmonary viral burden (Figure 1C), which was correlated with a lower induction of type I interferons (IFNs) in the BALF and in the lung tissues (Figure 1D).

To determine the link between protection and specific serum antibodies, IgG1, IgG2α, and IgA levels were measured in dams 4 weeks postpartum and in offspring at 4 weeks of age and prior to IAV infection at 8 weeks of age. Serum IgG1 levels were elevated in the IAV-infected dams

(Figure 1E) and was associated with an elevated serum concentration of IgG1 in naïve offspring at 4 weeks old which was maintained even at 8 weeks of age (Figure 1F), for both male and female offspring (Supplemental Figure 1B).

Pre-conception infection confers IgG1-specific protection to IAV-infected offspring

To determine whether IgG1 antibodies generated against IAV infection prior to pregnancy persist and confer protection to offspring, 8-week-old female mice were infected with IAV two-weeks prior to mating. Offspring from control and pre-conception infected dams (PreC-IAVm) were then infected with IAV at 8 weeks of age (Figure 2A). Offspring born to PreC-IAVm dams had a reduced pulmonary viral burden (Figure 2B) with reduced induction of type I IFNs in the BALF and lungs (Figure 2C). Unlike infection during pregnancy, pre-conception infection generated a broader antibody profile with elevated maternal IgG1, IgG2α, and IgA in the serum 3 weeks postpartum (Figure 2D) which mirrored elevated levels in the 3-week-old offspring (Figure 2E). However, only IgG1 antibodies were maintained prior to infection in the 8-week-old offspring (Figure 2E).

Antibodies from placenta or colostrum alone are insufficient to confer protection to IAV-infected offspring

To determine the route of antibody transfer mediating protective immunity, pups from control and IAV-infected dams were cross fostered. Naïve offspring nursed by IAV-infected dams (IAVm dams + CTLm offspring) and offspring born to IAV-infected dams nursed by control dams (CTLm dam + IAVm offspring) were then infected with IAV at 8 weeks of age (Figure 3A). The levels of pulmonary viral load were comparable in both CTLm offspring and IAVm offspring (Figure 3B). Similarly, there was no difference in the levels of type I IFNs in the BALF and lungs (Figure 3C). Although IAV-infected dams had significantly higher levels of IgG1 compared to control dams

(Figure 3D), this was not reflected in the offspring at 3 weeks old nor prior to IAV infection at 8 weeks old (Figure 3E). There was also no difference in IgA levels detectable in the BALF and lungs of offspring at 8 weeks of age (Supplemental Figure 1C). Collectively, these results indicate that the IgG1-mediated protection in IAV-infected offspring required both passive transfer of the antibody from placenta and colostrum of IAV-infected mother.

3.5 Discussion

In our study, passively transferred IgG1 through both the placenta and colostrum provided protection in 6-8-week-old offspring from IAV-infected dams. Studies have shown protection against IAV in the offspring following maternal vaccination from 2 weeks in ferrets [31] to 5 weeks of age in mice, with maternal antibody titers in the murine study declining after 2 weeks of age [7, 16]. In our model of mid-gestation IAV infection, we found significant levels of maternal IgG1 in the offspring at 3 weeks old which persisted even at 8 weeks of age. Although antibodies produced after viral infections are predominantly of the IgG2α subtype [9, 14-16, 32, 33], our data are in accordance with previous studies which highlight the critical role of IgG1 during IAV infection [12, 14, 17]. Although IgG:Fc interactions were not assessed in this study, IgG1 has the longest half-life of about 3 weeks in the newborn and the highest affinity for Fcγ receptors, and thus IgG1 is particularly efficient in neutralizing virus and mediating FcR-dependent effector functions [12].

It has been previously shown that vaccination against pertussis during pregnancy enhances transplacental transfer of IgG1 and subsequent protection to offspring compared to immunization pre-conception [34]. In our model, offspring from IAV-infected dams were better protected and presented with a lower pulmonary viral burden from subsequent IAV infection than offspring from PreC-IAVm infected mice, despite the production of a broader antibody profile including IgG1,

IgG2α, and IgA in the latter. Although IgG1 is predominantly associated with a Th2 response, it has higher neutralizing abilities against influenza virus compared to the other IgG subtypes [12, 17]. While it is not possible to directly compare pregnancy and pre-conception IAV infection due to the differences in immune status of the female, the maternal Th2 environment at E10 could potentially bias antibody production towards IgG1. Additionally, the observed differences in degree of protection may be due to qualitative differences in IgG1 that differentially modulate the offspring immune system [13]. In pertussis infection, it has been shown that Fc glycan modifications during pregnancy allow for the specific placental selection and transfer of NK-activating antibodies mediated by the neonatal Fc receptor and Fc gamma receptor IIIa which provide immunity to offspring [35]. In addition, recent studies of vaccination against SARS-CoV-2 highlighted the importance of qualitatively distinct antibody profiles with altered kinetics of pregnant and lactating women compared to the general population [2, 36]. Glycan modifications regulate IgG-Fc receptor interactions yielding differences in antibody effector functions and transfer efficiency [9, 12, 37] and are influenced by inflammation, female reproductive hormones, and epigenetic modifications during pregnancy [37-41]. Therefore, antibodies generated in a pregnant environment encompass different Fc glycovariants than those generated pre-conception with different binding affinities to specific Fc receptor subtypes and may account for the differences seen in the viral load in their offspring following secondary infection. Thus, Fc glycan profiles and their interactions with placental Fc receptors in the context of IAV infection requires further investigation. Furthermore, in the current study there is a difference in the time elapsed from infection of the mother to the assessment of antibodies in the offspring between infection in pre-conception and during pregnancy. This may also impact the magnitude of antibody subtype produced.

To determine the route of IgG1 transfer conferring protection, pups from control and IAV-infected dams were cross-fostered. Unlike reported protection through the colostrum against infection by H5N1 and H3N2 influenza strains [16, 31, 42], our data suggest that antibody transfer from either nursing or through the placenta alone is insufficient to provide prolonged protective immunity against the H1N1 influenza subtype. The differences in the route, length, and degree of protection among studies may be due to the strain of influenza and the animal model used. Additionally, the abundance and contribution of IgG and IgA in mediating protection in these studies could reflect differences in vaccine-induced immunity vs. naturally acquired immunity [36].

3.6 Conclusion

In conclusion, pregnant women and their offspring are vulnerable to both seasonal and pandemic strains of influenza, however, vaccine-elicited immunity and the subsequent transfer of antibodies to the fetus is influenced by the immune status of the mother [2, 9, 29]. Boosting maternal IgG1 antibody titers during critical gestational windows in tandem with breast feeding can provide robust and extended protection to offspring against subsequent IAV infection. Understanding the unique immunological milieu during pregnancy and deciphering the maternal-fetal dialogue can guide the development of vaccine regiments to optimize specific maternal antibody profiles and generate long-lasting protection in mother and offspring.

3.7 Acknowledgments

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3.8 Author Contributions

JC performed experiments, analyzed data, and wrote the manuscript with inputs from JM and MD. JC, JM, and MD conceptualized and designed the experiments and discussed the results. JM and MD conceived the project.

3.9 Funding

This work was supported by the Richard and Edith Strauss Foundation.

3.10 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.11 Figures and Figure Legends

Figure 1.

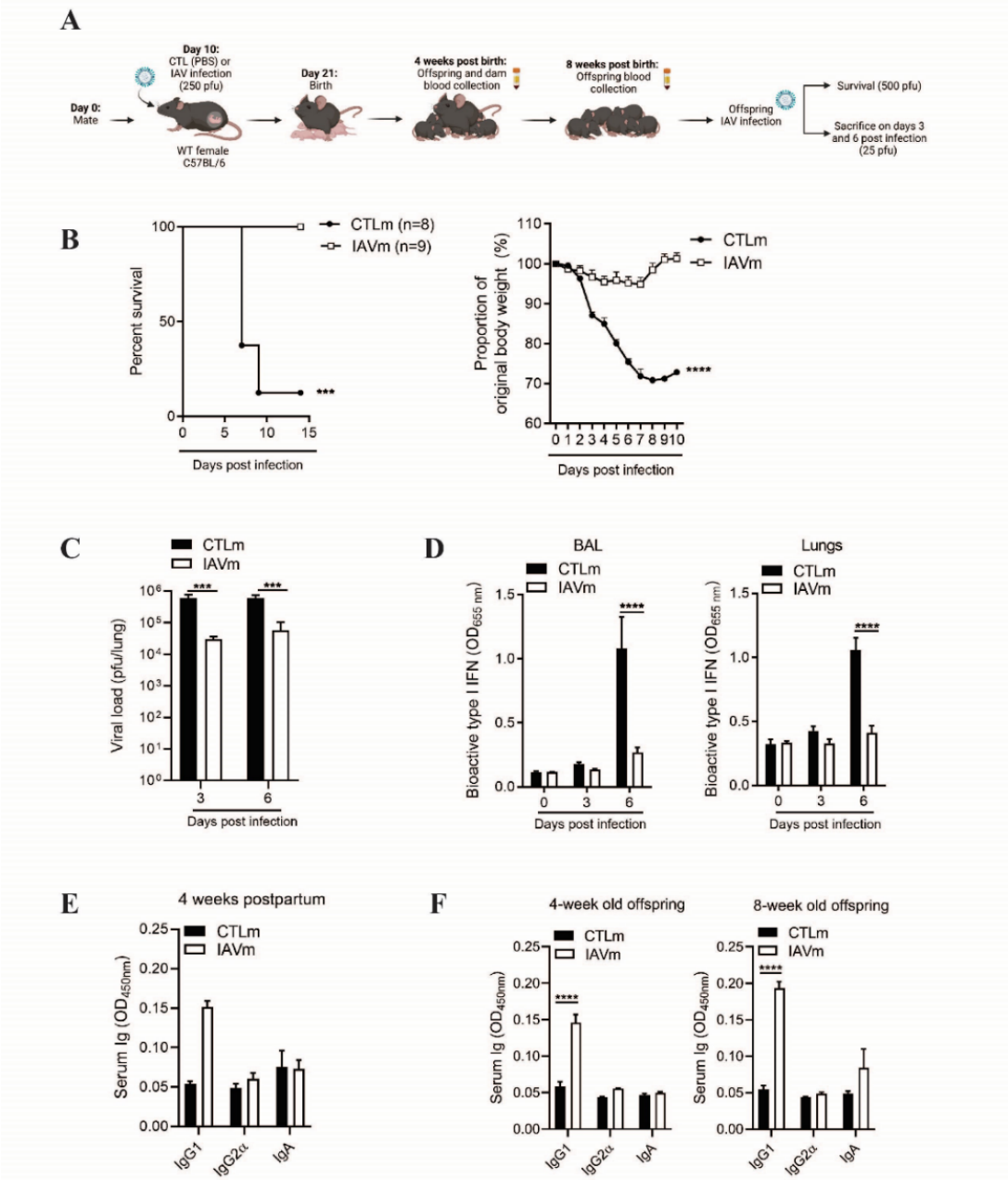


Figure 1. Maternal IAV-infection confers IgG1-mediated resistance to offspring. (A) Schematic representation of experimental design. 8–10-week-old C57BL/6 females were given PBS (CTLm; n=2 dams) or infected with 250 pfu A/Puerto Rico/8/34 (PR8) (IAVm; n=3 dams) intranasally on E10. (B) Survival curve and morbidity graph following lethal infection (500 pfu PR8) of 6-week-old females born to control dams (n=8 offspring) or IAV-infected dams (n=9 offspring). (C) Kinetics of viral load in whole lung homogenates by MDCK plaque assay and (D) type I IFNs in BALF and lung by B16 assay of 6-week-old offspring infected with 25 pfu PR8 (n=3-5 offspring). (E) Serum antibody levels of control and IAV-infected dams 4 weeks postpartum (n=2). (F) Serum antibody levels of naïve offspring at 4 weeks and 8 weeks of age prior to secondary infection (n=3-4). Data pooled for male and female offspring. Two-way ANOVA *** $p<0.001$, **** $p<0.0001$.

Figure 2.

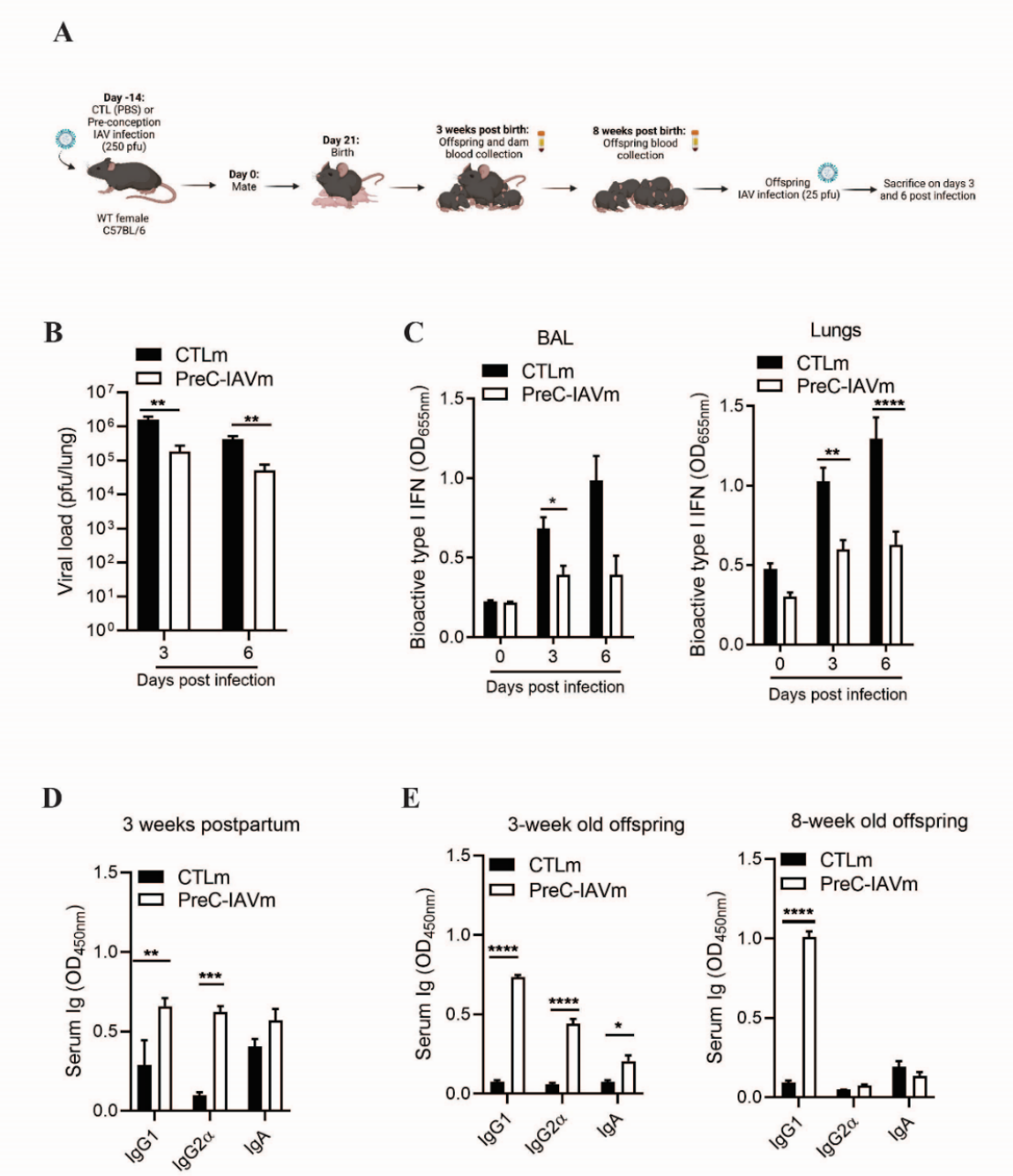

A
Day -14:
CTL (PBS) or
Pre-conception
IAV infection
(250 pfu)
WT female
C57BL/6
Day 0:
Mate
Day 21:
Birth
3 weeks post birth:
Offspring and dam
blood collection
8 weeks post birth:
Offspring blood
collection
Offspring
IAV infection (25 pfu)
Sacrifice on days 3
and 6 post infection
B
CTLm
PreC-IAVm
Viral load (pfu/lung)
Days post infection
C
BAL
Lungs
CTLm
PreC-IAVm
Bioactive type I IFN (OD655nm)
Days post infection
D
3 weeks postpartum
CTLm
PreC-IAVm
Serum Ig (OD450nm)
IgG1
IgG2α
IgA
E
3-week old offspring
8-week old offspring
CTLm
PreC-IAVm
Serum Ig (OD450nm)
IgG1
IgG2α
IgA

Figure 2. Pre-conception infection confers IgG1-specific protection to offspring. (A) Schematic representation of experimental design. 8–10-week-old C57BL/6 females were given PBS (CTLm; n=3 dams) or infected with 250 pfu PR8 (PreC-IAVm; n=4 dams) intranasally two weeks prior to mating. (B) Kinetics of viral load in whole lung homogenates by MDCK assay (n=3-4 offspring) and (C) type I IFNs in BALF and lungs by B16 assay of 8-week-old offspring born to control or PreC-IAV dams infected with 25 pfu PR8 (n=4-10 offspring). Multiple Student T-tests with Bonferroni correction for multiple comparisons were performed on BALF on day 0 and day 3 *$p<0.05$. No statistical analysis was performed on day 6 (CTLm n=2). (D) Serum antibody levels of control and PreC-IAVm dams 3 weeks postpartum (n=3-4). (E) Serum antibody levels of naïve offspring at 3 weeks and 8 weeks of age prior to secondary infection (n=3-7 offspring). Data pooled for male and female offspring. Two-way ANOVA *$p<0.05$, **$p<0.01$, *** $p<0.001$, ****<0.0001.

Figure 3.

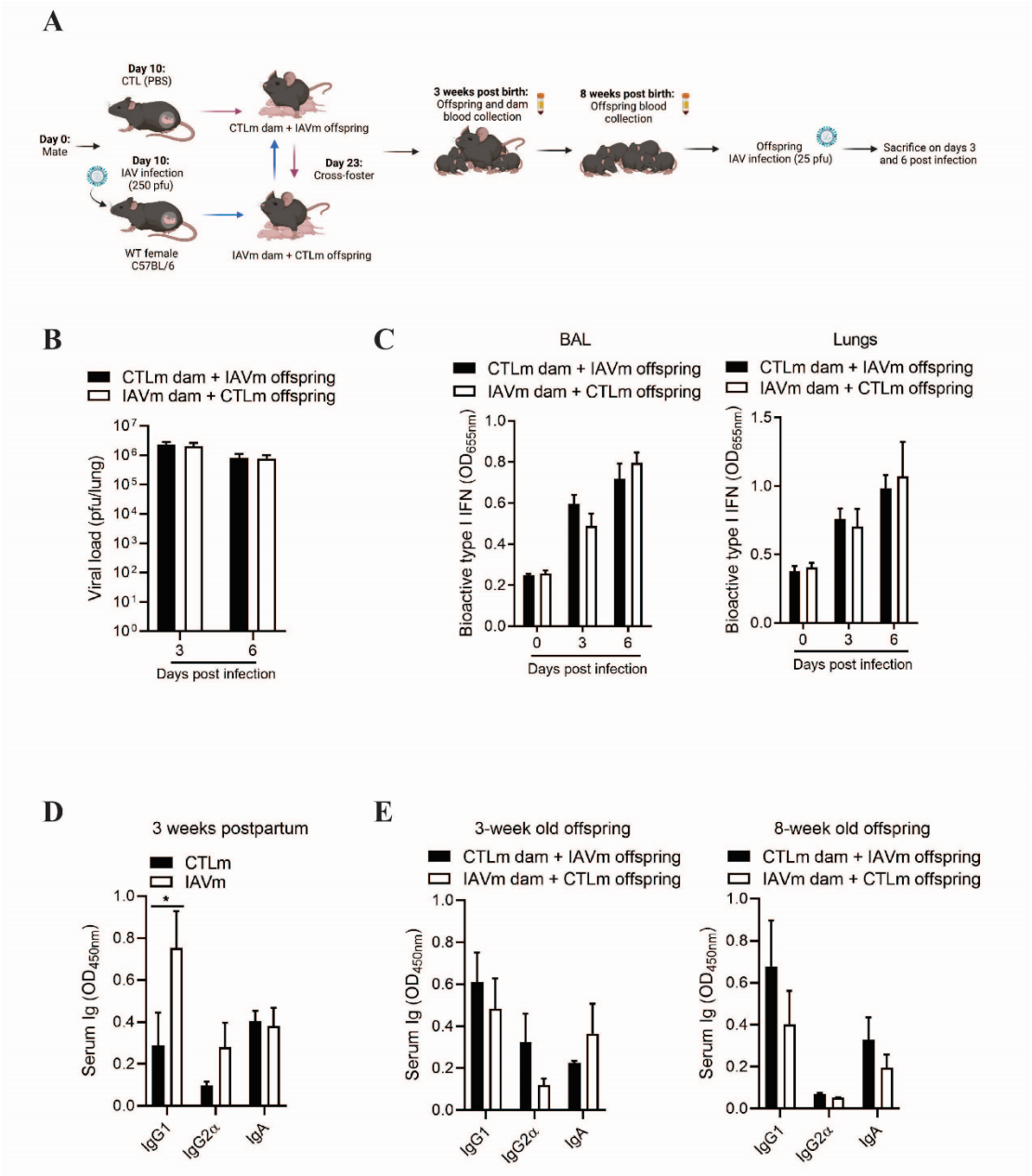
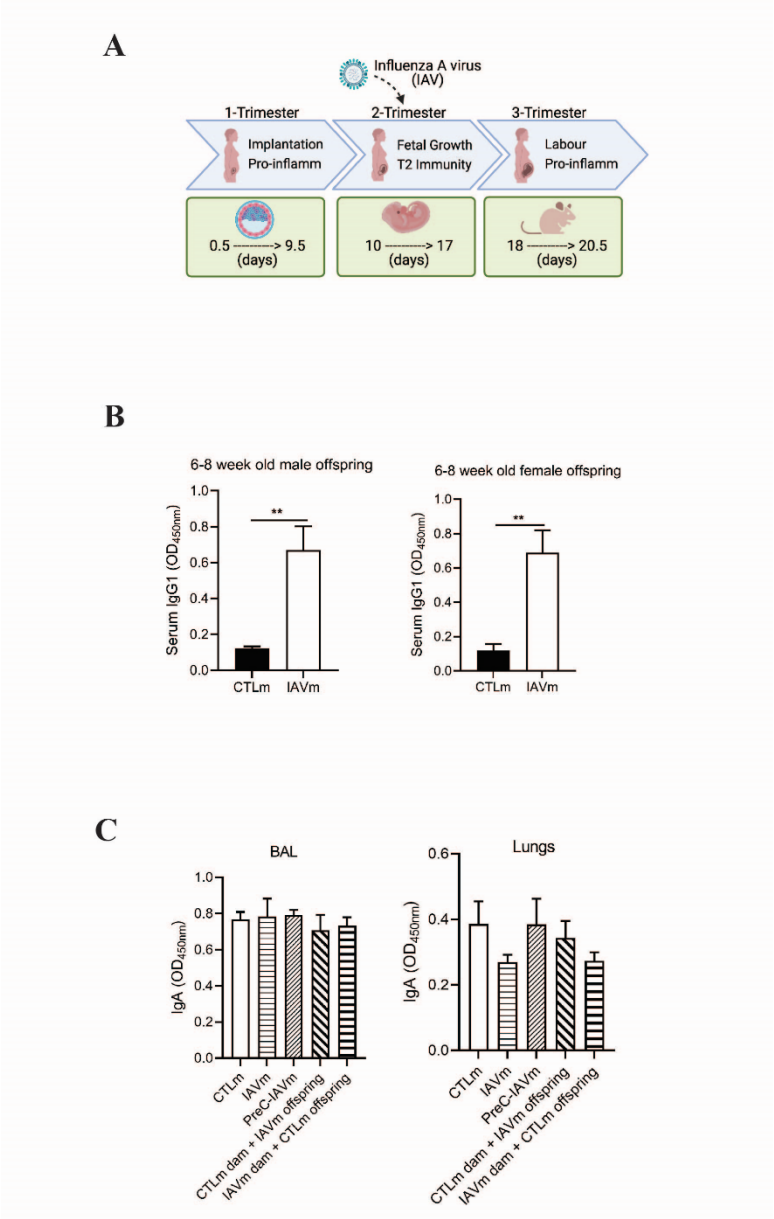

A
Day 10:
CTL (PBS)
Day 0:
Mate
Day 10:
IAV infection
(250 pfu)
WT female
C57BL/6
CTLm dam + IAVm offspring
IAVm dam + CTLm offspring
Day 23:
Cross-foster
3 weeks post birth:
Offspring and dam
blood collection
8 weeks post birth:
Offspring blood
collection
Offspring
IAV infection (25 pfu)
Sacrifice on days 3
and 6 post infection
B
CTLm dam + IAVm offspring
IAVm dam + CTLm offspring
Viral load (pfu/lung)
Days post infection
C
BAL
Lungs
CTLm dam + IAVm offspring
IAVm dam + CTLm offspring
Bioactive type I IFN (OD655nm)
Days post infection
D
3 weeks postpartum
CTLm
IAVm
Serum Ig (OD450nm)
IgG1
IgG2α
IgA
E
3-week old offspring
8-week old offspring
CTLm dam + IAVm offspring
IAVm dam + CTLm offspring
Serum Ig (OD450nm)
IgG1
IgG2α
IgA

Figure 3. Antibodies transferred solely through placenta or colostrum are insufficient in conferring prolonged protection to offspring against IAV infection. (A) Schematic representation of experimental design. 8–10-week-old C57BL/6 females were given PBS (CTLm; n=3 dams) or infected with 250 pfu PR8 (IAVm; n=3 dams) intranasally on E10. Three days after birth, pups were cross fostered. (B) Kinetics of viral load in whole lung homogenates by MDCK assay (n=4-8 offspring) and (C) type I IFNs in BALF and lungs by B16 assay of 8-week-old offspring born to IAV dams and fostered by control dams (CTLm dam + IAVm offspring) and of offspring born to control dams and fostered by IAV dams (IAVm dam + CTLm offspring) infected with 25 pfu PR8 (n=4-6 offspring). (D) Serum antibody levels of control and IAV-infected dams 3 weeks postpartum (n=3). (E) Serum antibody levels of naïve cross-fostered offspring at 3 weeks and 8 weeks of age prior to secondary infection (n=4-6 offspring). Data pooled for male and female offspring. Two-way ANOVA *$p<0.05$.

Supplemental Figure 1.



Supp. Fig. 1. (A) Schematic comparison of the immunology of pregnancy in humans versus mice. (B) IgG1 antibody levels of naïve 6-8-week-old male (n=7-13) and female (n=5-7) offspring born to control (n=5) and IAV-infected dams (n=6). (C) IgA antibody levels in BALF and lungs of offspring from CTLm (n=9), IAVm (n=3), PreC-IAVm (n=5), CTLm dam + IAVm offspring (n=6), IAVm dam + CTLm offspring (n=5). Unpaired Student T-test **p<0.005.

3.12 References

1. Mor, G., P. Aldo, and A.B. Alvero, *The unique immunological and microbial aspects of pregnancy.* Nat Rev Immunol, 2017. **17**(8): p. 469-482.
2. Atyeo, C., et al., *COVID-19 mRNA vaccines drive differential antibody Fc-functional profiles in pregnant, lactating, and non-pregnant women.* Sci Transl Med, 2021. **13**: p. eabi8631.
3. Centers for Disease, C. *Flu & Young Children*. September 14, 2021; Available from: https://www.cdc.gov/flu/highrisk/children.htm.
4. Thompson, M.G., et al., *Underdetection of laboratory-confirmed influenza-associated hospital admissions among infants: a multicentre, prospective study.* Lancet Child Adolesc Health, 2019. **3**(11): p. 781-794.
5. Niewiesk, S., *Maternal antibodies: clinical significance, mechanism of interference with immune responses, and possible vaccination strategies.* Front Immunol, 2014. **5**: p. 446.
6. Mbawuike, I.N., H.R. Six, T.R. Cate, and R.B. Couch, *Vaccination with inactivated influenza A virus during pregnancy protects neonatal mice against lethal challenge by influenza A viruses representing three subtypes.* J Virol, 1990. **64**(3): p. 1370-4.
7. van der Lubbe, J.E.M., et al., *Maternal antibodies protect offspring from severe influenza infection and do not lead to detectable interference with subsequent offspring immunization.* Virol J, 2017. **14**(1): p. 123.
8. Zhong, Z., et al., *The impact of timing of maternal influenza immunization on infant antibody levels at birth.* Clin Exp Immunol, 2019. **195**(2): p. 139-152.
9. Fouda, G.G., D.R. Martinez, G.K. Swamy, and S.R. Permar, *The Impact of IgG transplacental transfer on early life immunity.* Immunohorizons, 2018. **2**(1): p. 14-25.
10. Reuman, P.D., E.M. Ayoub, and P.A. Small, *Effect of passive maternal antibody on influenza illness in children: a prospective study of influenza A in mother-infant pairs.* Pediatr Infect Dis J, 1987. **6**(4): p. 398-403.
11. Zaman, K., et al., *Effectiveness of maternal influenza immunization in mothers and infants.* N Engl J Med, 2008. **359**(15): p. 1555-64.
12. Krammer, F., *The human antibody response to influenza A virus infection and vaccination.* Nat Rev Immunol, 2019. **19**(6): p. 383-397.
13. Vidarsson, G., G. Dekkers, and T. Rispens, *IgG subclasses and allotypes: from structure to effector functions.* Front Immunol, 2014. **5**: p. 520.
14. Hocart, M.J., J.S. Mackenzie, and G.A. Stewart, *The immunoglobulin G subclass responses of mice to influenza A virus: the effect of mouse strain, and the neutralizing abilities of individual protein A-purified subclass antibodies.* J Gen Virol, 1989. **70 (Pt 9)**: p. 2439-48.
15. Markine-Goriaynoff, D., et al., *IFN-gamma-independent IgG2a production in mice infected with viruses and parasites.* Int Immunol, 2000. **12**(2): p. 223-30.
16. Hwang, S.D., et al., *Protection of pregnant mice, fetuses and neonates from lethality of H5N1 influenza viruses by maternal vaccination.* Vaccine, 2010. **28**(17): p. 2957-64.
17. Huber, V.C., et al., *Distinct contributions of vaccine-induced immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza.* Clin Vaccine Immunol, 2006. **13**(9): p. 981-90.
18. Clements, T., et al., *Update on Transplacental Transfer of IgG Subclasses: Impact of Maternal and Fetal Factors.* Front Immunol, 2020. **11**: p. 1920.

19. Schroeder, H.W., Jr. and L. Cavacini, *Structure and function of immunoglobulins.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S41-52.
20. Atyeo, C. and G. Alter, *The multifaceted roles of breast milk antibodies.* Cell, 2021. **184**(6): p. 1486-1499.
21. Sumaya, C.V. and R.S. Gibbs, *Immunization of pregnant women with influenza A/New Jersey/76 virus vaccine: reactogenicity and immunogenicity in mother and infant.* J Infect Dis, 1979. **140**(2): p. 141-6.
22. Freyn, A.W., et al., *Influenza hemagglutinin-specific IgA Fc-effector functionality is restricted to stalk epitopes.* Proc Natl Acad Sci U S A, 2021. **118**(8).
23. Albrecht, M. and P.C. Arck, *Vertically Transferred Immunity in Neonates: Mothers, Mechanisms and Mediators.* Front Immunol, 2020. **11**: p. 555.
24. Coulombe, F., et al., *Targeted prostaglandin E2 inhibition enhances antiviral immunity through induction of type I interferon and apoptosis in macrophages.* Immunity, 2014. **40**(4): p. 554-68.
25. Pernet, E., et al., *Leukotriene B4-type I interferon axis regulates macrophage-mediated disease tolerance to influenza infection.* Nat Microbiol, 2019. **4**(8): p. 1389-1400.
26. Jaworska, J., et al., *NLRX1 prevents mitochondrial induced apoptosis and enhances macrophage antiviral immunity by interacting with influenza virus PB1-F2 protein.* Proc Natl Acad Sci U S A, 2014. **111**(20): p. E2110-9.
27. Downey, J., et al., *RIPK3 interacts with MAVS to regulate type I IFN-mediated immunity to Influenza A virus infection.* PLoS Pathog, 2017. **13**(4): p. e1006326.
28. Vermillion, M.S. and S.L. Klein, *Pregnancy and infection: using disease pathogenesis to inform vaccine strategy.* NPJ Vaccines, 2018. **3**: p. 6.
29. Atyeo, C., et al., *Compromised SARS-CoV-2-specific placental antibody transfer.* Cell, 2021. **184**(3): p. 628-642 e10.
30. Blanchard-Rohner, G., et al., *Influenza vaccination given at least 2 weeks before delivery to pregnant women facilitates transmission of seroprotective influenza-specific antibodies to the newborn.* Pediatr Infect Dis J, 2013. **32**(12): p. 1374-80.
31. Sweet, C., K.J. Jakeman, and H. Smith, *Role of milk-derived IgG in passive maternal protection of neonatal ferrets against influenza.* J Gen Virol, 1987. **68 (Pt 10)**: p. 2681-6.
32. Coutelier, J.P., et al., *Virally induced modulation of murine IgG antibody subclasses.* J Exp Med, 1988. **168**(6): p. 2373-8.
33. Coutelier, J.P., et al., *IgG2a restriction of murine antibodies elicited by viral infections.* J Exp Med, 1987. **165**(1): p. 64-9.
34. Healy, C.M., M.A. Rench, and C.J. Baker, *Importance of timing of maternal combined tetanus, diphtheria, and acellular pertussis (Tdap) immunization and protection of young infants.* Clin Infect Dis, 2013. **56**(4): p. 539-44.
35. Jennewein, M.F., et al., *Fc Glycan-Mediated Regulation of Placental Antibody Transfer.* Cell, 2019. **178**(1): p. 202-215 e14.
36. Gray, K.J., et al., *Coronavirus disease 2019 vaccine response in pregnant and lactating women: a cohort study.* Am J Obstet Gynecol, 2021. **225**(3): p. 303 e1-303 e17.
37. Bordt, E.A., et al., *Maternal SARS-CoV-2 infection elicits sexually dimorphic placental immune responses.* Sci Transl Med, 2021. **13**(617): p. eabi7428.
38. Prados, M.B., et al., *Progesterone induces a switch in oligosaccharyltransferase isoform expression: consequences on IgG N-glycosylation.* Immunol Lett, 2011. **137**(1-2): p. 28-37.
39. Chen, G., et al., *Human IgG Fc-glycosylation profiling reveals associations with age, sex, female sex hormones and thyroid cancer.* J Proteomics, 2012. **75**(10): p. 2824-34.

40. Selman, M.H., et al., *Changes in antigen-specific IgG1 Fc N-glycosylation upon influenza and tetanus vaccination.* Mol Cell Proteomics, 2012. **11**(4): p. M111 014563.
41. Alter, G., T.H.M. Ottenhoff, and S.A. Joosten, *Antibody glycosylation in inflammation, disease and vaccination.* Semin Immunol, 2018. **39**: p. 102-110.
42. Reuman, P.D., C.M. Paganini, E.M. Ayoub, and P.A. Small, Jr., *Maternal-infant transfer of influenza-specific immunity in the mouse.* J Immunol, 1983. **130**(2): p. 932-6.

Chapter 4: Discussion, Conclusions, and Future Directions

4.1 Maternal Resistance

Pregnant women are at a greater risk of hospitalization and intensive care unit admissions as seen during the 1918, 1957, and 2009 influenza pandemics, and in some cases, death due to ARDS or pneumonia [243, 247]. The majority of murine pregnancy studies have been modeled in BALB/c mice and in allogeneic pregnancies with established susceptibility to IAV [17, 249, 252-254]. Results from such studies model well the subset of pregnant women that is hospitalized or suffer severe disease during IAV pandemics. However, a protective effect of pregnancy against airway dysfunction caused by IAV infection has also been shown using C57BL/6 mice [255]. In addition, early antiviral treatment against IAV reduced the duration of hospitalization, severity of illness, and complications in pregnant women, suggesting that early control of viral replication is vital [266]. Increased susceptibility to viral illness is not a generalized phenomenon; it is important to note that a number of pregnant women infected with SARS-CoV-2 seemed to experience more mild illness compared to the general population [259-263, 267]. Therefore, it is likely that the IAV-affected women that have been reported to have severe disease carry a specific susceptibility and different mouse strains could be used to study both protected and susceptible populations. Therefore, to date, our dearth of understanding of immunity to maternal IAV infection may be due, in part, to our bias towards the study of susceptibility and the lack of attention to resistance to identify mechanisms of protection, the latter of which has been shown to have provided invaluable insights into host resistance in the field of HIV [258].

The goal of this work was to further dissect mechanisms of maternal immunity with a unique focus on host resistance in hopes of better understanding how pregnancy-related immunomodulation influences pulmonary immune responses, outcomes of disease, and offspring immunity. The overarching aims of this thesis were to: (1) better understand maternal immunity

in response to IAV infection and (2) optimize passive immunity to the offspring following IAV infection.

Pregnancy is formidably challenging to the mother's immune system, teetering between tolerance to a semi-allogeneic fetus, withstanding infection, and passing immunity to the fetus. Recent studies have shed light on the complexity of immune changes over the course of gestation and it is now understood that the maternal immune system starts as pro-inflammatory to allow for embryo implantation, then settles into this Th2/anti-inflammatory environment for fetal growth, and finally reverts to a pro-inflammatory stage that promotes labor and placental rejection [4]. However, this same pregnancy-associated immunomodulation of systemic inflammation and a shift to a type-1 immune response at an inappropriately early time-point leads to deleterious pregnancy outcomes, including pre-term birth, spontaneous abortion, intrauterine growth restriction, and small for gestational age babies [268-271]. Elevated pro-inflammatory cytokines such as IL-6 and TNF-α in mid-gestation showed an approximate 50% increase in the likelihood of pre-term labour [272]. In a prospective study, even pregnant women with mild influenza infection experienced a reduction in infant birthweights, specifically when infection occurred in the second trimester [239]. In our study conducted in chapter 2, we explored mid-gestation maternal resistance following IAV infection at the detriment of optimal birth outcomes presented as a reduction in the number of live pups **(Appendix 1B)** and pup weight upon birth **(Appendix 1C)**. The latter observation was mirrored and expanded upon in a recent study which demonstrated that low birth weights following mid-gestation IAV infection were a consequence of a reduction in progesterone concentration and signalling resulting in placental inflammation [273]. Altogether, our study likely models cases of mild maternal illness at the expense of favourable birth outcomes and reiterates the notion that maternal susceptibility is not a general quality of this population but rather depends on individual health. Moreover, this evolutionary trade-off illustrates the interplay

between maternal and fetal fitness and the delicate balance required to assume the survival of both with minimal adverse consequences.

Virulent strains of influenza have the capacity to evade upper airway defenses resulting in lower respiratory tract infections causing pneumonia and severe disease. Therefore, most studies have focused on the pulmonary immune response to IAV and the engagement of the alveolar compartment and have largely disregarded the influence of upper airway immunity on lower airway responses. In chapter 2, we revealed that during pregnancy, IL-17, predominantly produced by $\gamma\delta^+$ T cells in the upper airways, increased antimicrobial immunity during early IAV infection. Conceptually, this enhanced upper airway immunity elegantly highlights the relationship between host resistance and disease tolerance. Disease tolerance was contingent on early resistance mechanisms; that is, upper airway antimicrobial defenses in the pregnant mice drip-feeding IAV to the lower airways ultimately reduced excessive pulmonary inflammatory responses and airway dysfunction. In the words of Paracelsus: *"the dose makes the poison."*

AMPs are primeval innate defense molecules that are preserved in eukaryotes. Their production following exposure to invading pathogens is quicker and less energy-consuming than lymphocyte and antibody-mediated responses [274]. Their importance is highlighted in invertebrates which completely lack adaptive immune responses and rely solely on innate defenses and antimicrobial immunity to clear invading microbes [274]. However, AMPs are not traditionally known for their antiviral potential and have been generally overlooked in their contribution to upper airway resistance during viral infections, especially in the context of pregnancy. AMP expression has been documented within the most vulnerable sites of the human body that are exposed to the external environment including the respiratory tract and female reproductive tract. The expression of mucins, cathelicidins, and defensins in the vagina and cervicovaginal fluid function as essential barriers to the progression of infection into the uterine

cavity during pregnancy [57, 204]. Conceivably, pregnancy may upregulate antimicrobial immunity in the nasal epithelium, mimicking the enhanced mucosal defenses in the female reproductive tract to prevent propagation of infection at another barrier site that serves as an entry point for pathogens. These enhanced barrier defenses may be primitive characteristics, truly reflecting the critical role of AMPs in innate immunity, which evolved to ensure maternal survival.

The basis for these observed changes in the upper airways during pregnancy is unknown. However, there is growing appreciation for the microbial changes that occur during pregnancy [275] and we cannot exclude the role of female reproductive hormones in modulating immune responses in favour of host resistance. Barrier properties of the cervicovaginal mucus plug and AMP production are influenced by the vaginal microbiome and vary during the menstrual cycle and during pregnancy [202, 276]. Furthermore, progesterone has been shown to regulate $\gamma\delta^+$ T cell populations in the uterus [211, 277]. Interestingly, IL-17 production by $\gamma\delta^+$ T cells is regulated by gut microbiota-derived metabolites [278]. Potentially, changes to the nasal respiratory epithelial microbiota during pregnancy alters IL-17 production by $\gamma\delta^+$ T cells in favour of upper airway antimicrobial immunity [279, 280]. Although we provide one pathway of maternal resistance, as we've discussed in this thesis, pregnancy is a complex immunological period of heavily interdependent networks with substantial crosstalk. We suggest further studies to delineate additional regulators of upper airway maternal resistance against IAV infection, with a particular emphasis on the nasal microbiome and sex steroid hormones.

An important caveat in our study in chapter 2 is the characterization of pulmonary immune responses in the presence of unequal levels of pulmonary viral titers and therefore viral burden. One of the key findings in our study was that intra-tracheal infection resulted in equal pulmonary viral loads between non-pregnant and pregnant mice, demonstrating that resistance to being infected was indeed being exerted by the upper airways during pregnancy. Dose-response

experiments resulting in equivalent viral loads between pregnant and non-pregnant mice would allow for the comparison of lower airway responses to IAV. Alternatively, a comparison of intranasal infection to direct intratracheal infection would permit the assessment of the interaction of the upper and lower airways in response to infection by isolating the lower airway immune response. Such experiments would be required in order to determine both host resistance and disease tolerance mechanisms associated with pregnancy.

The literature surrounding pregnancy and IAV infection is often contradictory supporting either the lack of viral clearance or exacerbated inflammation. These discrepancies can be attributed to strain-specific differences of either the mouse model or influenza used, the gestational period of infection, and the mating strategy. Murine allogeneic pregnancies result in semi-allogeneic fetuses expressing foreign paternal antigens [281, 282], thus prompting unique maternal immunomodulation to prevent fetal rejection. These robust tolerogenic adaptations pose a greater challenge to the maternal immune system, specifically in the presence of infectious pathogens. However, some preliminary work in chapter 2 provided evidence that maternal resistance is, in part, conserved using allogeneic mating strategies. C57BL/6 females mated with BALB/c males had lower pulmonary viral titers **(Appendix 2A)** and better airway mechanics than non-pregnant mice 3 days following IAV infection **(Appendix 2B).** The expression of *cramp* in the nasal mucosa of allogeneic IAV-infected pregnant mice was also upregulated compared to their non-pregnant counterparts **(Appendix 2C).** Thus, maternal resistance to IAV infection in mid-gestation is not unique to syngeneic pregnancy but is also maintained in females following allogeneic mating. Importantly, pregnant IAV-infected females from both mating strategies had better airway mechanics, a functional parameter that is seldomly assessed to evaluate maternal disease severity in murine pregnancy studies. In consideration, it is worth exploring whether part of the susceptibility of pregnant BALB/c mice or other models of allogeneic pregnancies stems from

inadequate upper airway antimicrobial immunity. Evidently, the need for studies modelling maternal resistance is of paramount importance in achieving a better understanding of maternal immunity and protection from lower respiratory tract infections. In like manner, the induction of antimicrobial immunity in the nasal mucosa must be considered in the development of novel therapeutics or vaccine strategies for the general population to prevent exuberant pulmonary inflammatory responses and respiratory insufficiency.

Much of the notion of susceptibility during pregnancy stems from an assumption that pregnant mice, particularly BALB/c [283], are more Th2 biased. However, there has not been any characterization of murine immune responses throughout pregnancy thus far to clearly designate specific immune profiles to each trimester. Therefore, it still unclear whether pregnant mice adopt distinct immunological states that recapitulate each human trimester equivalent. Mid-gestation C57BL/6 mice in our model did not reveal any characteristics indicative of a Th2-skewed immune system. In fact, we observed maternal resistance depicted by a reduction in viral burden in both the second (E10) and third (E16) trimesters. However, the elaboration of immune responses to IAV infection and the mechanisms providing resistance in the third trimester require further study. Therefore, in order to gain a better understanding of the immune responses to infection during pregnancy, more efforts must go into immune profiling the upper and lower airways over different gestational periods to map the basal immunological landscape within these compartments in each trimester.

Broadly, chapter 2 showed that upper airway immunity is key to the observed maternal resistance to IAV infection. As we will discuss in the following section, increasing maternal immunity not only benefits the mother but also serves as a conduit for passing immunity to the fetus. Optimising passive immunity to the offspring is the most effective strategy against

preventing severe illness during early life as newborns are highly susceptible to infectious pathogens.

4.2 Passive Immunity to Offspring

After birth and during their first months of life, human newborns are not yet equipped with a fully matured immune system and therefore, highly susceptible to infectious pathogens, such as measles, pertussis, rubella, and influenza [226]. For this same reason, vaccination containing live pathogens is not recommended for children under one years old. Even tetanus-diphtheria-pertussis vaccine and the inactivated influenza vaccines are not recommended until 2 or 6 months of age [284, 285]. During that period of vulnerability, the baby acquires immunity through the trans-placental transfer of pathogen-specific antibodies (mostly IgG) *in utero* as of mid-gestation followed by postnatal breast-milk derived immunity (mostly IgA) [286]. Increasing maternal antibody concentrations enhances the levels of antibody transfer and, in consequence, the degree of passive immunity to the baby. The importance of maternal antibodies on neonatal protection is seen in premature or formula-fed babies that have a higher risk of infection [287, 288]. Hence, vaccination during pregnancy and during child-bearing years is essential to increase antibody titers against infectious disease. Even without vaccination, maternal exposure to non-lethal pathogens will result in the production of transferrable neutralizing antibodies [219].

In chapter 3, we showed that non-lethal IAV infection during mid-gestation generated high levels of circulating maternal IgG1 which was mirrored in the pup circulation upon weaning age (3 weeks) and persisted until adulthood. At 6-8 weeks old, offspring from IAV-infected dams were infected with a homologous strain of IAV and had a substantial survival advantage due to a 2-log reduction in viral burden. Interestingly, when females were infected pre-conception, a broader antibody profile was generated with the emergence of not only IgG1, but also IgG2α, and IgA in the mother and the neonate. A caveat in this study was the direct comparison between pre-

conception-infected dams and dams infected during mid-gestation as the time elapsed between infection and assessment of antibody levels and subsets was different. Since pre-conception females were bred two weeks following IAV infection, it could have allowed enough time for the dam to develop a more complete antibody repertoire. Alternatively, it has been shown that pregnant women generate distinct antibody profiles with altered kinetics following vaccination [289, 290]. In fact, antibodies generated during pregnancy have been recently referred to as *"super antibodies"* [291], due to specific alterations in IgG composition whereby certain sialic acids are deacetylated, expanding the repertoire of IgG-Fc receptor interactions [292]. In addition, altered IgG glycan profiles, which have been shown to be regulated in part by estrogen and progesterone [229, 293, 294], influence placental IgG transfer efficiency during normal healthy pregnancies and during maternal infection [234, 295]. Therefore, pregnancy modulates IgG Fc characteristics, which can be influenced by the presence of maternal infection, to optimize selective placental transfer efficiency and extend greater protective immunity to the newborn. However, glycan profiling and IgG-Fc receptor interactions were not assessed in this study. Therefore, the consequences of maternal IAV infection on the structural and functional properties of IgG potentially driving the selective transfer of IgG1 to the offspring remains to be determined.

Mothers continue passing immunity postnatally through breast milk. Our question was then whether *in utero* or breast milk antibodies were providing protection to the offspring. Following a series of cross-fostering experiments, our data revealed that progeny receiving solely *in utero* antibodies or milk antibodies were insufficiently protected

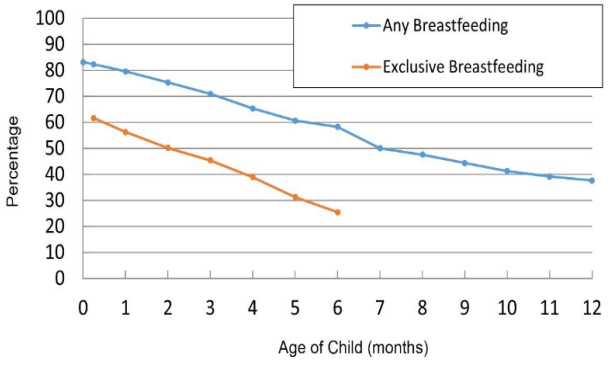


Figure 4.2. Percentage of breastfed children in the United States born in 2020 during the first year of life. Graph taken from [8]. Source: CDC

against IAV. Epidemiological data are showing an alarming decline in breast-fed neonates by 12 months **(Figure 4.2)** [296]. In addition, between 2019 and 2020, United States estimates showed only 61.2% of pregnant women received the influenza vaccine [297, 298]. Altogether, strategies to concurrently foster antibody production, either through mild maternal infection or vaccination regimens in conjunction with breast feeding, will protect infants from early-life infections.

There is a paucity of studies characterizing immunological and physiological changes in neonates that persist later in life due to maternal IAV infection [248, 249]. Interestingly, a recent study demonstrated that IAV-induced maternal immune activation during early gestation profoundly impacts offspring immunity and predisposes to early life infection. Jacobsen et al., showed that 6-week-old male, but not female, progeny born to IAV-infected dams experienced increased lethality when infected with a heterologous strain of influenza [249]. Moreover, male offspring had a slight reduction in lung function following homologous IAV infection [249]. Some findings that emerged from our study in chapter 3 were the functional consequences of mid-gestation IAV infection on lung physiology of 6-week-old progeny. Female offspring born to IAV-infected dams were protected from IAV-induced AHR whereas male offspring were not **(Appendix 3)**. IAV infection is known to exacerbate asthma symptoms in humans [299] and increase airway responses to inhaled methacholine in murine models [300]. Maternal asthma [301, 302] and infection [303] are risk factors for the development of asthma specifically in male offspring, however the mechanisms of sex-specific patterns of inheritance are unclear. Male fetuses reportedly have increased IgE levels which may uniquely predispose male offspring to atopy [304, 305]. Moreover, akin to antibodies, maternal immune cells are vertically transferred to the fetus through the placenta and influence offspring immunity and the outcome of infection in early life [219]. Maternal microchimerism, which is the presence of maternal cells within the fetal circulation, is documented to be more prevalent in female progeny and has been suggested to

protect against asthma, although the mechanisms remain unknown [306]. This may provide an additional explanation for the higher incidence of asthma in boys during childhood and may be an interesting avenue to explore based on the findings presented in Appendix 3.

Taken together, the impact of systemic inflammatory responses during pregnancy in response to IAV infection on the developing fetus requires further investigation. Although IAV is a non-congenital infection, maternal inflammation during infection, albeit mild, has long-lasting consequences on fetal immune development. These findings warrant further research into the impact of gestational IAV infection on the development of the fetal immune system and the consequences of such changes for the predisposition of airway disorders such as asthma or the susceptibility to infection during childhood and adulthood.

4.3 Concluding Remarks

The *"immunological paradox of pregnancy,"* as coined by Peter Medawar in 1953 [307], has puzzled the scientific community for decades and we've since come to appreciate just how complex it is, encompassing distinct immunological periods across trimesters which inevitably affect maternal disease and fetal outcomes. Several factors account for the uncertainty surrounding the spectrum of immune responses of pregnant women to IAV infection. Pregnant women are often excluded from research studies and the majority of studies are case series that are aggregates of a biased population of pregnant women with severe disease. Additionally, levels of circulating Th1 or Th2 cytokines are used as a proxy measure for the immune profile during pregnancy [308], which may not be representative of the unique immune landscape within different tissues. Thus, our understanding of maternal host defenses, specifically outside of the local maternal-fetal environment, is still limited. Here, we outline the intricate balance between maternal resistance and fetal tolerance in order to ensure maternal fitness in a mid-gestation model of IAV infection. Additionally, we show that mild maternal disease provides an immune advantage to the offspring through the generation and transfer of IgG1 antibodies, categorically through the placenta and breast milk, to provide protection against IAV infection later in life.

The work presented in this thesis provides novel evidence of maternal resistance to IAV and the class and route of antibody transfer optimizing protection to the offspring:

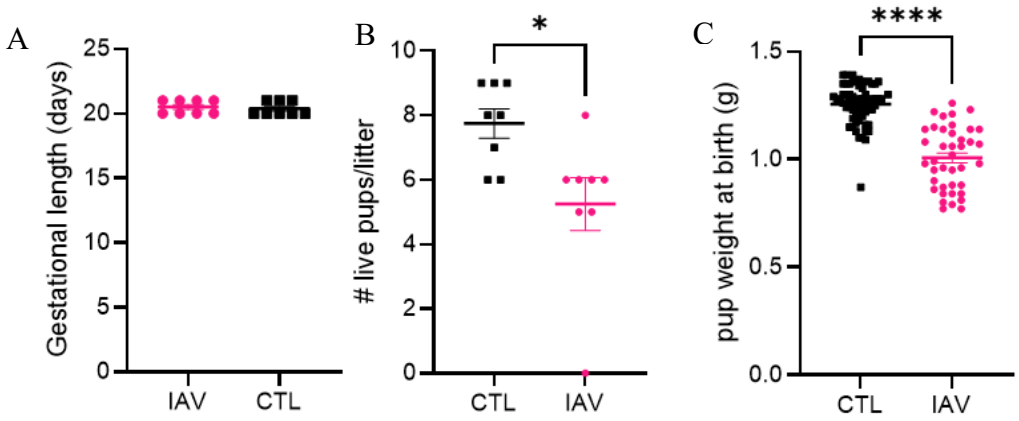
1. Maternal upper airway antimicrobial immunity to IAV infection
    - → Pregnancy enhances antiviral immunity via recruiting IL-17-producing $\gamma\delta^+$ T cells into the nasal mucosa.

2. Consequences of maternal IAV infection on offspring immunity

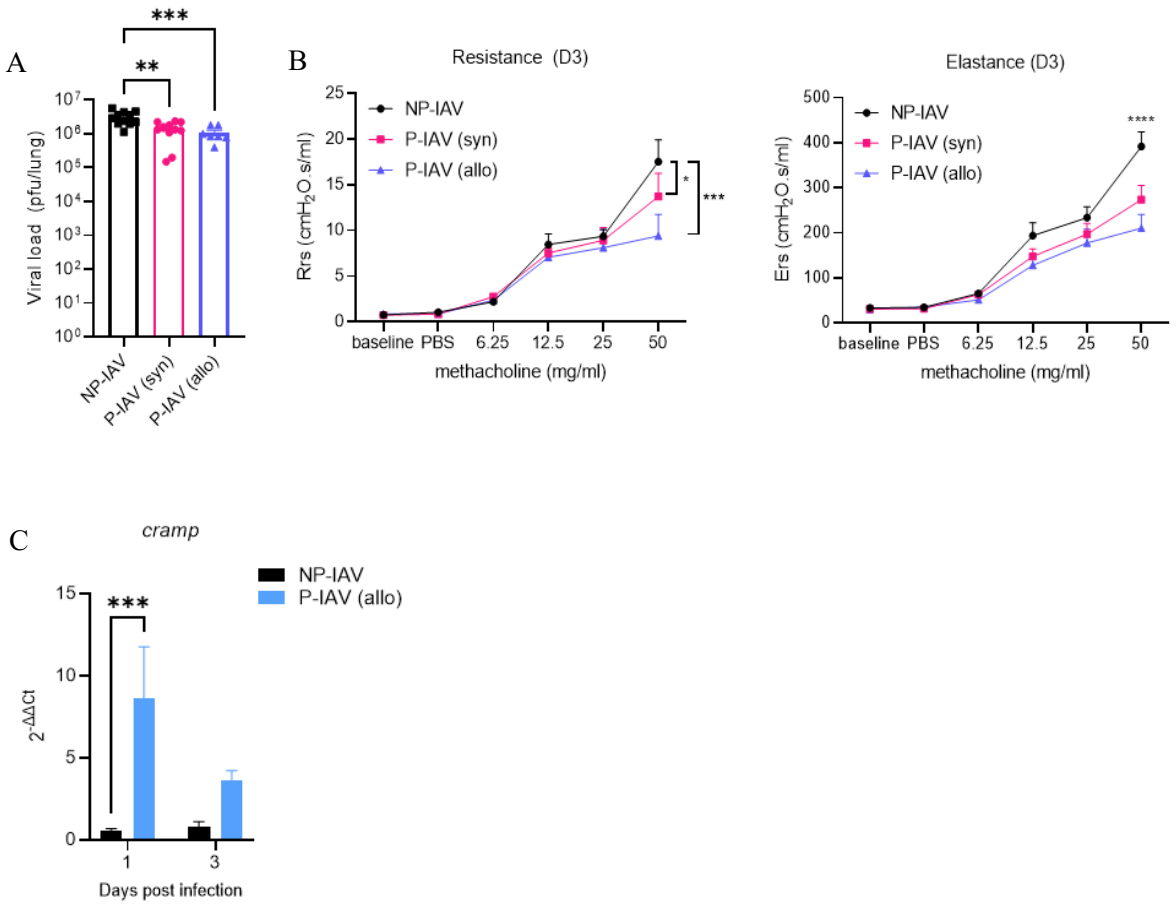
→ Transplacental and Breast Milk Transfer of IgG1 Are Both Required for Prolonged Protection of Offspring Against Influenza A Infection.

Taken together, we highlight the importance of upper airway immunity during early IAV infection and the significance in pursuing models of resistance. If pregnancy is categorized based on the immune responses needed to support a healthy pregnancy, it requires a robust, dynamic, and responsive immune system engaged at specific periods over the course of gestation and is not an immunosuppressed event. Surely, not all pregnant women are hospitalized or succumb to infection because if the maternal immune system was already predisposed to infection, or lacked the necessary tools to fight it, the survival of our species would be challenged. To quote scientific historian and philosopher, Thomas Kuhn: "*prevailed paradigm freezes science."* The pregnancy paradigm has shaped our way of thinking about maternal immunity in response to infection. Perhaps the perplexity of maternal immune responses to IAV infection is due to the singular focus on susceptibility. By neglecting the preponderance of pregnant women with mild disease, we are overlooking upper airway resistance as being a determining factor of disease severity against respiratory pathogens. It is our hope that this study will encourage novel research paths that challenge the current paradigm in order to reveal a more comprehensive understanding of the breadth of immune adaptations during pregnancy and their impact on outcomes of maternal disease.

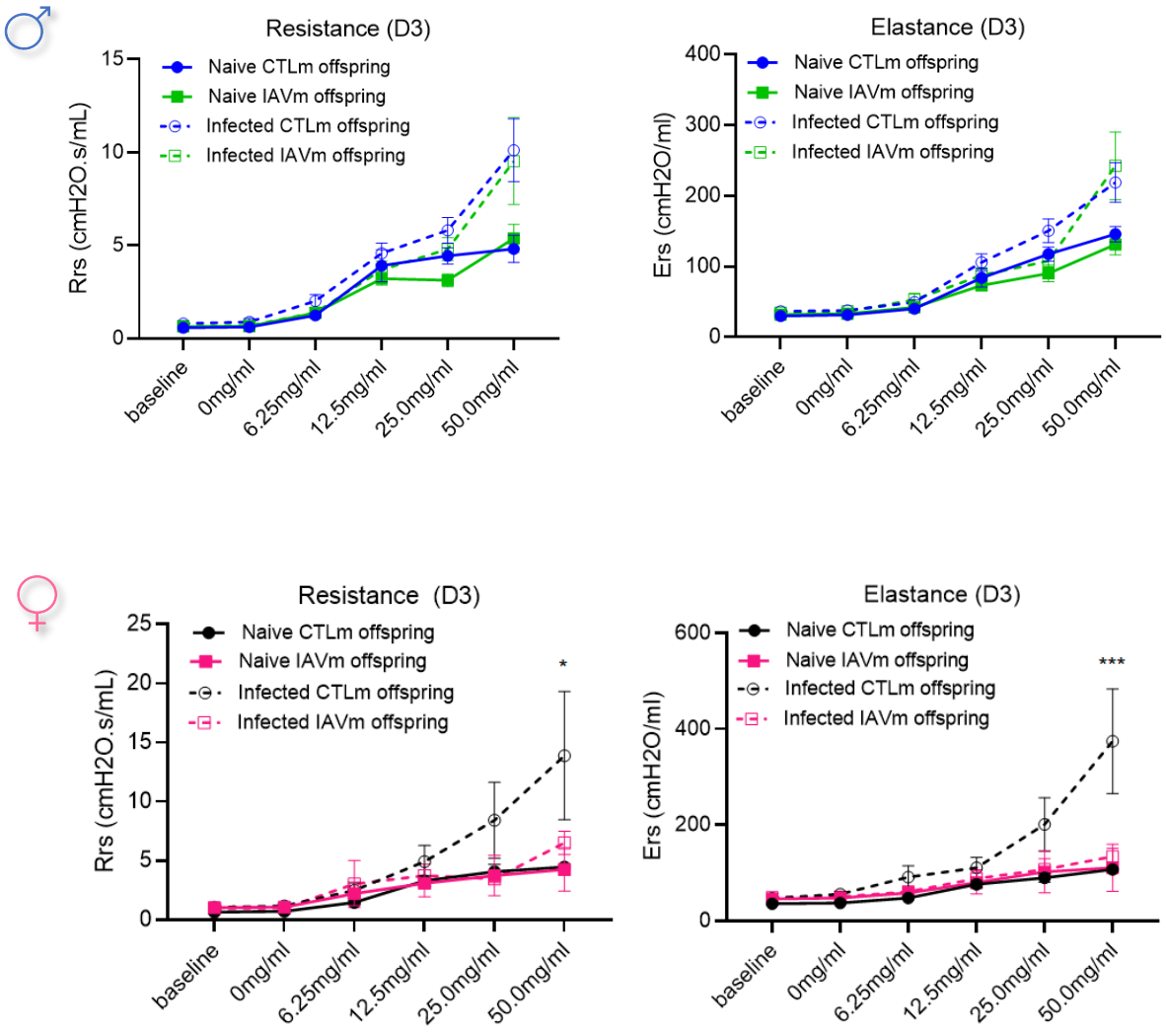
Appendices



Appendix 1. Birth outcomes of naïve and IAV-infected dams. (A) Gestation length (days), (B) litter size, and (C) pup weight at birth following maternal infection with a sublethal dose of IAV (50pfu) during mid-gestation (E10). n=7-9 dams. Data represented as mean ± SEM. Unpaired Student's T test.



Appendix 2. Upper airway antimicrobial immunity provides protection against IAV infection in allogeneically mated pregnant mice. (A) Viral load measured in lung homogenates and (B) airway mechanics measurements taken 3 days post IAV infection (50pfu) of non-pregnant, syngeneic pregnant (E10), and allogeneic pregnant mice (E10). n=5-11. (C) Kinetics of *cramp* expression in the nasal cavities of non-pregnant and allogeneic pregnant mice following IAV infection by RT-PCR. n=3-5. Data represented as mean ± SEM. Two-way or one-way ANOVA followed by Tukey's multiple comparisons test.



Appendix 3. Sex-specific differences in IAV-induced AHR in offspring born to naïve and IAV-infected dams. Resistance and elastance measured in 6-week-old offspring (male and female) born to CTL dams (CTLm) or IAV-infected dams (IAVm) 3 days following IAV infection by Flexivent. n=4-10 offspring. Data represented as mean ± SEM. Two-way ANOVA followed by Tukey's multiple comparisons test.

References

1. Krammer, F., et al., *Influenza.* Nat Rev Dis Primers, 2018. **4**(1): p. 3.
2. Alvites, R.D., et al., *The Nasal Cavity of the Rat and Mouse-Source of Mesenchymal Stem Cells for Treatment of Peripheral Nerve Injury.* Anat Rec (Hoboken), 2018. **301**(10): p. 1678-1689.
3. Wallace, L.E., et al., *Respiratory mucus as a virus-host range determinant.* Trends Microbiol, 2021. **29**(11): p. 983-992.
4. Mor, G., P. Aldo, and A.B. Alvero, *The unique immunological and microbial aspects of pregnancy.* Nat Rev Immunol, 2017. **17**(8): p. 469-482.
5. Vornhagen, J., K.M. Adams Waldorf, and L. Rajagopal, *Perinatal Group B Streptococcal Infections: Virulence Factors, Immunity, and Prevention Strategies.* Trends Microbiol, 2017. **25**(11): p. 919-931.
6. Langel, S.N., C.E. Otero, D.R. Martinez, and S.R. Permar, *Maternal gatekeepers: How maternal antibody Fc characteristics influence passive transfer and infant protection.* PLoS Pathog, 2020. **16**(3): p. e1008303.
7. Denney, L. and L.P. Ho, *The role of respiratory epithelium in host defence against influenza virus infection.* Biomed J, 2018. **41**(4): p. 218-233.
8. Centers for Disease, C. *Breast Feeding Rates*. Available from: https://www.cdc.gov/breastfeeding/data/nis_data/results.html.
9. Bagri, P., Yu, E., Karushic, C., *Mucosal immunology of the female reproductive tract and its regulation by female sex hormones*. Reproductive Immunology, ed. G. Mor. 2021: Academic Press.
10. Iwasaki, A. and P.S. Pillai, *Innate immunity to influenza virus infection.* Nat Rev Immunol, 2014. **14**(5): p. 315-28.
11. Crofts, K.F. and M.A. Alexander-Miller, *Challenges for the Newborn Immune Response to Respiratory Virus Infection and Vaccination.* Vaccines (Basel), 2020. **8**(4).
12. Lloyd, C.M. and S. Saglani, *Opening the Window of Immune Opportunity: Treating Childhood Asthma.* Trends Immunol, 2019. **40**(9): p. 786-798.
13. Morens, D.M. and A.S. Fauci, *The 1918 influenza pandemic: insights for the 21st century.* J Infect Dis, 2007. **195**(7): p. 1018-28.
14. Wack, A., E. Terczynska-Dyla, and R. Hartmann, *Guarding the frontiers: the biology of type III interferons.* Nat Immunol, 2015. **16**(8): p. 802-9.
15. Sones, J.L. and R.L. Davisson, *Preeclampsia, of mice and women.* Physiol Genomics, 2016. **48**(8): p. 565-72.
16. Le Gars, M., et al., *Increased Proinflammatory Responses of Monocytes and Plasmacytoid Dendritic Cells to Influenza A Virus Infection During Pregnancy.* J Infect Dis, 2016. **214**(11): p. 1666-1671.
17. Engels, G., et al., *Pregnancy-Related Immune Adaptation Promotes the Emergence of Highly Virulent H1N1 Influenza Virus Strains in Allogenically Pregnant Mice.* Cell Host Microbe, 2017. **21**(3): p. 321-333.
18. Barry, J.M., *The great influenza The epic story of the deadliest plague in history*. 2009, United States of America: Penguin Books. 527.
19. Saunders-Hastings, P.R. and D. Krewski, *Reviewing the History of Pandemic Influenza: Understanding Patterns of Emergence and Transmission.* Pathogens, 2016. **5**(4).
20. Taubenberger, J.K. and D.M. Morens, *The pathology of influenza virus infections.* Annu Rev Pathol, 2008. **3**: p. 499-522.

21. Crosby, A.W., *America's forgotten pandemic : the influenza of 1918*. 1989, Cambridge England ; New York: Cambridge University Press. xiv, 337 p.
22. Oxford, J.S. and D. Gill, *Unanswered questions about the 1918 influenza pandemic: origin, pathology, and the virus itself.* Lancet Infect Dis, 2018. **18**(11): p. e348-e354.
23. Worobey, M., J. Cox, and D. Gill, *The origins of the great pandemic.* Evol Med Public Health, 2019. **2019**(1): p. 18-25.
24. Guarner, J. and R. Falcon-Escobedo, *Comparison of the pathology caused by H1N1, H5N1, and H3N2 influenza viruses.* Arch Med Res, 2009. **40**(8): p. 655-61.
25. Hammond, J., Rolland, W., Shore, THG., *Purulent bronchitis: a study of cases occurring amongst the British troops at a base in France.* Lancet, 1919. **2**: p. 41-45.
26. Dou, D., et al., *Influenza A Virus Cell Entry, Replication, Virion Assembly and Movement.* Front Immunol, 2018. **9**: p. 1581.
27. Hussell, T. and T.J. Bell, *Alveolar macrophages: plasticity in a tissue-specific context.* Nat Rev Immunol, 2014. **14**(2): p. 81-93.
28. Neupane, A.S., et al., *Patrolling Alveolar Macrophages Conceal Bacteria from the Immune System to Maintain Homeostasis.* Cell, 2020. **183**(1): p. 110-125 e11.
29. Wessner, D.R., *The Origins of Viruses.* Nature Education 2010. **3**(9): p. 37.
30. Eigen, M. and P. Schuster, *The hypercycle. A principle of natural self-organization. Part A: Emergence of the hypercycle.* Naturwissenschaften, 1977. **64**(11): p. 541-65.
31. Tumpey, T.M., et al., *Characterization of the reconstructed 1918 Spanish influenza pandemic virus.* Science, 2005. **310**(5745): p. 77-80.
32. Pappas, C., et al., *Single gene reassortants identify a critical role for PB1, HA, and NA in the high virulence of the 1918 pandemic influenza virus.* Proc Natl Acad Sci U S A, 2008. **105**(8): p. 3064-9.
33. Conenello, G.M., et al., *A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence.* PLoS Pathog, 2007. **3**(10): p. 1414-21.
34. Hao, W., L. Wang, and S. Li, *Roles of the Non-Structural Proteins of Influenza A Virus.* Pathogens, 2020. **9**(10).
35. Tahamtan A, C.J., Hoseini Shokouh S J, Barati, M., *An Overview of Various Generations of Vaccines.* Journal of Archives in Military Medicine, 2017. **5**(3).
36. Principi, N., et al., *Drugs for Influenza Treatment: Is There Significant News?* Front Med (Lausanne), 2019. **6**: p. 109.
37. Shornick, L.P., et al., *Airway epithelial versus immune cell Stat1 function for innate defense against respiratory viral infection.* J Immunol, 2008. **180**(5): p. 3319-28.
38. Cole, A.M., P. Dewan, and T. Ganz, *Innate antimicrobial activity of nasal secretions.* Infect Immun, 1999. **67**(7): p. 3267-75.
39. Zhang, L.J. and R.L. Gallo, *Antimicrobial peptides.* Curr Biol, 2016. **26**(1): p. R14-9.
40. Kaliner, M.A., *Human nasal host defense and sinusitis.* J Allergy Clin Immunol, 1992. **90**(3 Pt 2): p. 424-30.
41. Vareille, M., E. Kieninger, M.R. Edwards, and N. Regamey, *The airway epithelium: soldier in the fight against respiratory viruses.* Clin Microbiol Rev, 2011. **24**(1): p. 210-29.
42. Sobiesk, J.L. and S. Munakomi, *Anatomy, Head and Neck, Nasal Cavity*, in *StatPearls*. 2023: Treasure Island (FL).
43. Frey, A., et al., *More Than Just a Barrier: The Immune Functions of the Airway Epithelium in Asthma Pathogenesis.* Front Immunol, 2020. **11**: p. 761.

44. de Vries, E., W. Du, H. Guo, and C.A.M. de Haan, *Influenza A Virus Hemagglutinin-Neuraminidase-Receptor Balance: Preserving Virus Motility.* Trends Microbiol, 2020. **28**(1): p. 57-67.
45. Linden, S.K., et al., *Mucins in the mucosal barrier to infection.* Mucosal Immunol, 2008. **1**(3): p. 183-97.
46. Roy, M.G., et al., *Muc5b is required for airway defence.* Nature, 2014. **505**(7483): p. 412-6.
47. Ehre, C., et al., *Overexpressing mouse model demonstrates the protective role of Muc5ac in the lungs.* Proc Natl Acad Sci U S A, 2012. **109**(41): p. 16528-33.
48. Barbier, D., et al., *Influenza A induces the major secreted airway mucin MUC5AC in a protease-EGFR-extracellular regulated kinase-Sp1-dependent pathway.* Am J Respir Cell Mol Biol, 2012. **47**(2): p. 149-57.
49. Delaveris, C.S., et al., *Membrane-tethered mucin-like polypeptides sterically inhibit binding and slow fusion kinetics of influenza A virus.* Proc Natl Acad Sci U S A, 2020. **117**(23): p. 12643-12650.
50. Zanin, M., P. Baviskar, R. Webster, and R. Webby, *The Interaction between Respiratory Pathogens and Mucus.* Cell Host Microbe, 2016. **19**(2): p. 159-68.
51. Baudoin, T., et al., *Redefining Pregnancy-Induced Rhinitis.* Am J Rhinol Allergy, 2021. **35**(3): p. 315-322.
52. Orban, N., E. Maughan, and N. Bleach, *Pregnancy-induced rhinitis.* Rhinology, 2013. **51**(2): p. 111-9.
53. Toppozada, H., et al., *The human respiratory nasal mucosa in pregnancy. An electron microscopic and histochemical study.* J Laryngol Otol, 1982. **96**(7): p. 613-26.
54. Ulkumen, B., et al., *Impact of Pregnancy and Glucocorticoid Treatment on NF-kappaB and MUC5AC in Mucosa of Rat Larynx.* J Voice, 2021. **35**(3): p. 342-348.
55. Tecle, T., S. Tripathi, and K.L. Hartshorn, *Review: Defensins and cathelicidins in lung immunity.* Innate Immun, 2010. **16**(3): p. 151-9.
56. Hsieh, I.N. and K.L. Hartshorn, *The Role of Antimicrobial Peptides in Influenza Virus Infection and Their Potential as Antiviral and Immunomodulatory Therapy.* Pharmaceuticals (Basel), 2016. **9**(3).
57. Ueda, Y., et al., *Cervical MUC5B and MUC5AC are Barriers to Ascending Pathogens During Pregnancy.* J Clin Endocrinol Metab, 2022. **107**(11): p. 3010-3021.
58. Muniz, L.R., C. Knosp, and G. Yeretssian, *Intestinal antimicrobial peptides during homeostasis, infection, and disease.* Front Immunol, 2012. **3**: p. 310.
59. Eisenhauer, P.B. and R.I. Lehrer, *Mouse neutrophils lack defensins.* Infect Immun, 1992. **60**(8): p. 3446-7.
60. Son, G.H., J.J. Lee, Y. Kim, and K.Y. Lee, *The Role of Antimicrobial Peptides in Preterm Birth.* Int J Mol Sci, 2021. **22**(16).
61. Chong, K.T., R.R. Thangavel, and X. Tang, *Enhanced expression of murine beta-defensins (MBD-1, -2,- 3, and -4) in upper and lower airway mucosa of influenza virus infected mice.* Virology, 2008. **380**(1): p. 136-43.
62. LeMessurier, K.S., Y. Lin, J.A. McCullers, and A.E. Samarasinghe, *Antimicrobial peptides alter early immune response to influenza A virus infection in C57BL/6 mice.* Antiviral Res, 2016. **133**: p. 208-17.
63. Agakidou, E., et al., *Antimicrobial Peptides in Early-Life Host Defense, Perinatal Infections, and Necrotizing Enterocolitis-An Update.* J Clin Med, 2022. **11**(17).
64. White, J.H., *Emerging Roles of Vitamin D-Induced Antimicrobial Peptides in Antiviral Innate Immunity.* Nutrients, 2022. **14**(2).

65. Castillo, J.A., et al., *Vitamin D-induced LL-37 modulates innate immune responses of human primary macrophages during DENV-2 infection.* Pathog Dis, 2022. **80**(1).
66. Currie, S.M., et al., *Cathelicidins Have Direct Antiviral Activity against Respiratory Syncytial Virus In Vitro and Protective Function In Vivo in Mice and Humans.* J Immunol, 2016. **196**(6): p. 2699-710.
67. Tripathi, S., et al., *The human cathelicidin LL-37 inhibits influenza A viruses through a mechanism distinct from that of surfactant protein D or defensins.* J Gen Virol, 2013. **94**(Pt 1): p. 40-49.
68. Jadhav, N.J., et al., *Immunomodulatory effect of 1, 25 dihydroxy vitamin D(3) on the expression of RNA sensing pattern recognition receptor genes and cytokine response in dengue virus infected U937-DC-SIGN cells and THP-1 macrophages.* Int Immunopharmacol, 2018. **62**: p. 237-243.
69. Barlow, P.G., et al., *Antiviral activity and increased host defense against influenza infection elicited by the human cathelicidin LL-37.* PLoS One, 2011. **6**(10): p. e25333.
70. Zhang, Y., et al., *The human Cathelicidin LL-37 induces MUC5AC mucin production by airway epithelial cells via TACE-TGF-alpha-EGFR pathway.* Exp Lung Res, 2014. **40**(7): p. 333-42.
71. Ishimoto, H., et al., *Different effects of telithromycin on MUC5AC production induced by human neutrophil peptide-1 or lipopolysaccharide in NCI-H292 cells compared with azithromycin and clarithromycin.* J Antimicrob Chemother, 2009. **63**(1): p. 109-14.
72. Rivas-Santiago, C.E., R. Hernandez-Pando, and B. Rivas-Santiago, *Immunotherapy for pulmonary TB: antimicrobial peptides and their inducers.* Immunotherapy, 2013. **5**(10): p. 1117-26.
73. De, Y., et al., *LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells.* J Exp Med, 2000. **192**(7): p. 1069-74.
74. Yang, D., et al., *Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6.* Science, 1999. **286**(5439): p. 525-8.
75. Palomino-Segura, M., I. Latino, Y. Farsakoglu, and S.F. Gonzalez, *Early production of IL-17A by gammadelta T cells in the trachea promotes viral clearance during influenza infection in mice.* Eur J Immunol, 2020. **50**(1): p. 97-109.
76. Borkner, L., et al., *Correction: IL-17 mediates protective immunity against nasal infection with Bordetella pertussis by mobilizing neutrophils, especially Siglec-F(+) neutrophils.* Mucosal Immunol, 2021. **14**(5): p. 1218-1219.
77. Selsted, M.E. and A.J. Ouellette, *Mammalian defensins in the antimicrobial immune response.* Nat Immunol, 2005. **6**(6): p. 551-7.
78. Iwasaki, A., E.F. Foxman, and R.D. Molony, *Early local immune defences in the respiratory tract.* Nat Rev Immunol, 2017. **17**(1): p. 7-20.
79. Liang, S.C., et al., *Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides.* J Exp Med, 2006. **203**(10): p. 2271-9.
80. Archer, N.K., et al., *Interleukin-17A (IL-17A) and IL-17F Are Critical for Antimicrobial Peptide Production and Clearance of Staphylococcus aureus Nasal Colonization.* Infect Immun, 2016. **84**(12): p. 3575-3583.
81. Mulcahy, M.E., et al., *Interleukin-22 regulates antimicrobial peptide expression and keratinocyte differentiation to control Staphylococcus aureus colonization of the nasal mucosa.* Mucosal Immunol, 2016. **9**(6): p. 1429-1441.

82. Miles, K., et al., *Dying and necrotic neutrophils are anti-inflammatory secondary to the release of alpha-defensins.* J Immunol, 2009. **183**(3): p. 2122-32.
83. Scott, M.G., et al., *The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses.* J Immunol, 2002. **169**(7): p. 3883-91.
84. Brook, M., et al., *Neutrophil-derived alpha defensins control inflammation by inhibiting macrophage mRNA translation.* Proc Natl Acad Sci U S A, 2016. **113**(16): p. 4350-5.
85. Takahashi, M., et al., *The Antimicrobial Peptide Human beta-Defensin-3 Accelerates Wound Healing by Promoting Angiogenesis, Cell Migration, and Proliferation Through the FGFR/JAK2/STAT3 Signaling Pathway.* Front Immunol, 2021. **12**: p. 712781.
86. Suzuki, K., et al., *Human anti-microbial cathelicidin peptide LL-37 suppresses the LPS-induced apoptosis of endothelial cells.* Int Immunol, 2011. **23**(3): p. 185-93.
87. Heilborn, J.D., et al., *The cathelicidin anti-microbial peptide LL-37 is involved in re-epithelialization of human skin wounds and is lacking in chronic ulcer epithelium.* J Invest Dermatol, 2003. **120**(3): p. 379-89.
88. Tate, M.D., et al., *Playing hide and seek: how glycosylation of the influenza virus hemagglutinin can modulate the immune response to infection.* Viruses, 2014. **6**(3): p. 1294-316.
89. Hiemstra, P.S., P.B. McCray, Jr., and R. Bals, *The innate immune function of airway epithelial cells in inflammatory lung disease.* Eur Respir J, 2015. **45**(4): p. 1150-62.
90. Eisele, N.A. and D.M. Anderson, *Host Defense and the Airway Epithelium: Frontline Responses That Protect against Bacterial Invasion and Pneumonia.* J Pathog, 2011. **2011**: p. 249802.
91. LeVine, A.M., et al., *Absence of SP-A modulates innate and adaptive defense responses to pulmonary influenza infection.* Am J Physiol Lung Cell Mol Physiol, 2002. **282**(3): p. L563-72.
92. LeVine, A.M., et al., *Surfactant protein D enhances clearance of influenza A virus from the lung in vivo.* J Immunol, 2001. **167**(10): p. 5868-73.
93. Reading, P.C., L.S. Morey, E.C. Crouch, and E.M. Anders, *Collectin-mediated antiviral host defense of the lung: evidence from influenza virus infection of mice.* J Virol, 1997. **71**(11): p. 8204-12.
94. Vigerust, D.J., et al., *N-linked glycosylation attenuates H3N2 influenza viruses.* J Virol, 2007. **81**(16): p. 8593-600.
95. Hartshorn, K.L., et al., *Reduced influenza viral neutralizing activity of natural human trimers of surfactant protein D.* Respir Res, 2007. **8**(1): p. 9.
96. LeVine, A.M. and J.A. Whitsett, *Pulmonary collectins and innate host defense of the lung.* Microbes Infect, 2001. **3**(2): p. 161-6.
97. To, K.K.W., et al., *Surfactant protein B gene polymorphism is associated with severe influenza.* Chest, 2014. **145**(6): p. 1237-1243.
98. Herrera-Ramos, E., et al., *Surfactant protein A genetic variants associate with severe respiratory insufficiency in pandemic influenza A virus infection.* Crit Care, 2014. **18**(3): p. R127.
99. Kim, J.K., et al., *Expression and localization of surfactant proteins in human nasal epithelium.* Am J Physiol Lung Cell Mol Physiol, 2007. **292**(4): p. L879-84.
100. Schicht, M., et al., *Detection of surfactant proteins A, B, C, and D in human nasal mucosa and their regulation in chronic rhinosinusitis with polyps.* Am J Rhinol Allergy, 2013. **27**(1): p. 24-9.

101. Wootten, C.T., R.F. Labadie, A. Chen, and K.F. Lane, *Differential expression of surfactant protein A in the nasal mucosa of patients with allergy symptoms.* Arch Otolaryngol Head Neck Surg, 2006. **132**(9): p. 1001-7.
102. Choi, Y.S. and N. Baumgarth, *Dual role for B-1a cells in immunity to influenza virus infection.* J Exp Med, 2008. **205**(13): p. 3053-64.
103. Baumgarth, N., et al., *The role of B-1 and B-2 cells in immune protection from influenza virus infection.* Curr Top Microbiol Immunol, 2000. **252**: p. 163-9.
104. Baumgarth, N., et al., *B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection.* J Exp Med, 2000. **192**(2): p. 271-80.
105. Baumgarth, N., et al., *Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system.* Proc Natl Acad Sci U S A, 1999. **96**(5): p. 2250-5.
106. Jayasekera, J.P., E.A. Moseman, and M.C. Carroll, *Natural antibody and complement mediate neutralization of influenza virus in the absence of prior immunity.* J Virol, 2007. **81**(7): p. 3487-94.
107. Caron, J., L.A. Ridgley, and M. Bodman-Smith, *How to Train Your Dragon: Harnessing Gamma Delta T Cells Antiviral Functions and Trained Immunity in a Pandemic Era.* Front Immunol, 2021. **12**: p. 666983.
108. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nat Immunol, 2010. **11**(5): p. 373-84.
109. Pernet, E., et al., *Leukotriene B4-type I interferon axis regulates macrophage-mediated disease tolerance to influenza infection.* Nat Microbiol, 2019. **4**(8): p. 1389-1400.
110. Le Goffic, R., et al., *Cutting Edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells.* J Immunol, 2007. **178**(6): p. 3368-72.
111. Rappe, J.C.F., et al., *A TLR7 antagonist restricts interferon-dependent and -independent immunopathology in a mouse model of severe influenza.* J Exp Med, 2021. **218**(11).
112. Koyama, S., et al., *Differential role of TLR- and RLR-signaling in the immune responses to influenza A virus infection and vaccination.* J Immunol, 2007. **179**(7): p. 4711-20.
113. Heer, A.K., et al., *TLR signaling fine-tunes anti-influenza B cell responses without regulating effector T cell responses.* J Immunol, 2007. **178**(4): p. 2182-91.
114. Peteranderl, C. and S. Herold, *The Impact of the Interferon/TNF-Related Apoptosis-Inducing Ligand Signaling Axis on Disease Progression in Respiratory Viral Infection and Beyond.* Front Immunol, 2017. **8**: p. 313.
115. Sato, M., et al., *Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction.* Immunity, 2000. **13**(4): p. 539-48.
116. Downey, J., et al., *RIPK3 interacts with MAVS to regulate type I IFN-mediated immunity to Influenza A virus infection.* PLoS Pathog, 2017. **13**(4): p. e1006326.
117. Jaworska, J., et al., *NLRX1 prevents mitochondrial induced apoptosis and enhances macrophage antiviral immunity by interacting with influenza virus PB1-F2 protein.* Proc Natl Acad Sci U S A, 2014. **111**(20): p. E2110-9.
118. Coulombe, F., et al., *Targeted prostaglandin E2 inhibition enhances antiviral immunity through induction of type I interferon and apoptosis in macrophages.* Immunity, 2014. **40**(4): p. 554-68.
119. Kugel, D., et al., *Intranasal administration of alpha interferon reduces seasonal influenza A virus morbidity in ferrets.* J Virol, 2009. **83**(8): p. 3843-51.

120. Guerrero-Plata, A., et al., *Activity and regulation of alpha interferon in respiratory syncytial virus and human metapneumovirus experimental infections.* J Virol, 2005. **79**(16): p. 10190-9.
121. Mordstein, M., et al., *Lambda interferon renders epithelial cells of the respiratory and gastrointestinal tracts resistant to viral infections.* J Virol, 2010. **84**(11): p. 5670-7.
122. Galani, I.E., et al., *Interferon-lambda Mediates Non-redundant Front-Line Antiviral Protection against Influenza Virus Infection without Compromising Host Fitness.* Immunity, 2017. **46**(5): p. 875-890 e6.
123. Klinkhammer, J., et al., *IFN-lambda prevents influenza virus spread from the upper airways to the lungs and limits virus transmission.* Elife, 2018. **7**.
124. Lazear, H.M., J.W. Schoggins, and M.S. Diamond, *Shared and Distinct Functions of Type I and Type III Interferons.* Immunity, 2019. **50**(4): p. 907-923.
125. Balachandran, S., et al., *Activation of the dsRNA-dependent protein kinase, PKR, induces apoptosis through FADD-mediated death signaling.* EMBO J, 1998. **17**(23): p. 6888-902.
126. Sadler, A.J. and B.R. Williams, *Interferon-inducible antiviral effectors.* Nat Rev Immunol, 2008. **8**(7): p. 559-68.
127. Gotz, V., et al., *Influenza A viruses escape from MxA restriction at the expense of efficient nuclear vRNP import.* Sci Rep, 2016. **6**: p. 23138.
128. Manz, B., et al., *Pandemic influenza A viruses escape from restriction by human MxA through adaptive mutations in the nucleoprotein.* PLoS Pathog, 2013. **9**(3): p. e1003279.
129. Cardani, A., A. Boulton, T.S. Kim, and T.J. Braciale, *Alveolar Macrophages Prevent Lethal Influenza Pneumonia By Inhibiting Infection Of Type-1 Alveolar Epithelial Cells.* PLoS Pathog, 2017. **13**(1): p. e1006140.
130. He, W., et al., *Alveolar macrophages are critical for broadly-reactive antibody-mediated protection against influenza A virus in mice.* Nat Commun, 2017. **8**(1): p. 846.
131. Ghoneim, H.E., P.G. Thomas, and J.A. McCullers, *Depletion of alveolar macrophages during influenza infection facilitates bacterial superinfections.* J Immunol, 2013. **191**(3): p. 1250-9.
132. Sanders, C.J., P.C. Doherty, and P.G. Thomas, *Respiratory epithelial cells in innate immunity to influenza virus infection.* Cell Tissue Res, 2011. **343**(1): p. 13-21.
133. Chen, X., et al., *Host Immune Response to Influenza A Virus Infection.* Front Immunol, 2018. **9**: p. 320.
134. Lin, K.L., et al., *CCR2+ monocyte-derived dendritic cells and exudate macrophages produce influenza-induced pulmonary immune pathology and mortality.* J Immunol, 2008. **180**(4): p. 2562-72.
135. Peiro, T., et al., *Neutrophils drive alveolar macrophage IL-1beta release during respiratory viral infection.* Thorax, 2018. **73**(6): p. 546-556.
136. Narasaraju, T., et al., *Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis.* Am J Pathol, 2011. **179**(1): p. 199-210.
137. Kim, H.Y., et al., *Innate lymphoid cells responding to IL-33 mediate airway hyperreactivity independently of adaptive immunity.* J Allergy Clin Immunol, 2012. **129**(1): p. 216-27 e1-6.
138. Chang, Y.J., et al., *Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity.* Nat Immunol, 2011. **12**(7): p. 631-8.
139. Monticelli, L.A., et al., *Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus.* Nat Immunol, 2011. **12**(11): p. 1045-54.

140. Qin, Y., et al., *Interleukin-13 stimulates MUC5AC expression via a STAT6-TMEM16A-ERK1/2 pathway in human airway epithelial cells.* Int Immunopharmacol, 2016. **40**: p. 106-114.
141. Kanoh, S., T. Tanabe, and B.K. Rubin, *IL-13-induced MUC5AC production and goblet cell differentiation is steroid resistant in human airway cells.* Clin Exp Allergy, 2011. **41**(12): p. 1747-56.
142. Jiao, J., et al., *Epidermal growth factor upregulates expression of MUC5AC via TMEM16A, in chronic rhinosinusitis with nasal polyps.* Allergy Asthma Clin Immunol, 2020. **16**: p. 40.
143. Sabbaghi, A., et al., *Role of gammadelta T cells in controlling viral infections with a focus on influenza virus: implications for designing novel therapeutic approaches.* Virol J, 2020. **17**(1): p. 174.
144. Pellicci, D.G., H.F. Koay, and S.P. Berzins, *Thymic development of unconventional T cells: how NKT cells, MAIT cells and gammadelta T cells emerge.* Nat Rev Immunol, 2020. **20**(12): p. 756-770.
145. Hayes, S.M., R.M. Laird, and P.E. Love, *Beyond alphabeta/gammadelta lineage commitment: TCR signal strength regulates gammadelta T cell maturation and effector fate.* Semin Immunol, 2010. **22**(4): p. 247-51.
146. Ribot, J.C., N. Lopes, and B. Silva-Santos, *gammadelta T cells in tissue physiology and surveillance.* Nat Rev Immunol, 2021. **21**(4): p. 221-232.
147. Laky, K., et al., *Enterocyte expression of interleukin 7 induces development of gammadelta T cells and Peyer's patches.* J Exp Med, 2000. **191**(9): p. 1569-80.
148. Kanamori, Y., et al., *Identification of novel lymphoid tissues in murine intestinal mucosa where clusters of c-kit+ IL-7R+ Thy1+ lympho-hemopoietic progenitors develop.* J Exp Med, 1996. **184**(4): p. 1449-59.
149. Hu, Y., et al., *Single-cell analysis reveals the origins and intrahepatic development of liver-resident IFN-gamma-producing gammadelta T cells.* Cell Mol Immunol, 2021. **18**(4): p. 954-968.
150. Hayakawa, S., et al., *Expression of the recombinase-activating gene (RAG-1) in murine early embryogenesis.* Immunol Cell Biol, 1996. **74**(1): p. 52-6.
151. Kimura, M., et al., *Synchronous expansion of intermediate TCR cells in the liver and uterus during pregnancy.* Cell Immunol, 1995. **162**(1): p. 16-25.
152. Mincheva-Nilsson, L., et al., *Gamma delta T cells of human early pregnancy decidua: evidence for local proliferation, phenotypic heterogeneity, and extrathymic differentiation.* J Immunol, 1997. **159**(7): p. 3266-77.
153. Nielsen, M.M., D.A. Witherden, and W.L. Havran, *gammadelta T cells in homeostasis and host defence of epithelial barrier tissues.* Nat Rev Immunol, 2017. **17**(12): p. 733-745.
154. Wang, X., et al., *Host-derived lipids orchestrate pulmonary gammadelta T cell response to provide early protection against influenza virus infection.* Nat Commun, 2021. **12**(1): p. 1914.
155. Wang, X., et al., *IL-17A Promotes Pulmonary B-1a Cell Differentiation via Induction of Blimp-1 Expression during Influenza Virus Infection.* PLoS Pathog, 2016. **12**(1): p. e1005367.
156. Guo, X.J., et al., *Lung gammadelta T Cells Mediate Protective Responses during Neonatal Influenza Infection that Are Associated with Type 2 Immunity.* Immunity, 2018. **49**(3): p. 531-544 e6.

157. Misiak, A., M.M. Wilk, M. Raverdeau, and K.H. Mills, *IL-17-Producing Innate and Pathogen-Specific Tissue Resident Memory gammadelta T Cells Expand in the Lungs of Bordetella pertussis-Infected Mice.* J Immunol, 2017. **198**(1): p. 363-374.
158. Hassane, M., et al., *Interleukin-7 protects against bacterial respiratory infection by promoting IL-17A-producing innate T-cell response.* Mucosal Immunol, 2020. **13**(1): p. 128-139.
159. Li, C., et al., *IL-17 response mediates acute lung injury induced by the 2009 pandemic influenza A (H1N1) virus.* Cell Res, 2012. **22**(3): p. 528-38.
160. Xue, C., et al., *Vgamma4(+)gammadeltaT Cells Aggravate Severe H1N1 Influenza Virus Infection-Induced Acute Pulmonary Immunopathological Injury via Secreting Interleukin-17A.* Front Immunol, 2017. **8**: p. 1054.
161. Crowe, C.R., et al., *Critical role of IL-17RA in immunopathology of influenza infection.* J Immunol, 2009. **183**(8): p. 5301-10.
162. Wiche Salinas, T.R., B. Zheng, J.P. Routy, and P. Ancuta, *Targeting the interleukin-17 pathway to prevent acute respiratory distress syndrome associated with SARS-CoV-2 infection.* Respirology, 2020. **25**(8): p. 797-799.
163. McGeachy, M.J., D.J. Cua, and S.L. Gaffen, *The IL-17 Family of Cytokines in Health and Disease.* Immunity, 2019. **50**(4): p. 892-906.
164. Robinson, D.P. and S.L. Klein, *Pregnancy and pregnancy-associated hormones alter immune responses and disease pathogenesis.* Horm Behav, 2012. **62**(3): p. 263-71.
165. Pozzilli, C., M. Pugliatti, and M.S.G. Paradig, *An overview of pregnancy-related issues in patients with multiple sclerosis.* Eur J Neurol, 2015. **22 Suppl 2**: p. 34-9.
166. Adams, K.M., Z. Yan, A.M. Stevens, and J.L. Nelson, *The changing maternal "self" hypothesis: a mechanism for maternal tolerance of the fetus.* Placenta, 2007. **28**(5-6): p. 378-82.
167. Fallon, P.G., et al., *IL-4 induces characteristic Th2 responses even in the combined absence of IL-5, IL-9, and IL-13.* Immunity, 2002. **17**(1): p. 7-17.
168. Ashkar, A.A., J.P. Di Santo, and B.A. Croy, *Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy.* J Exp Med, 2000. **192**(2): p. 259-70.
169. Mor, G. and I. Cardenas, *The immune system in pregnancy: a unique complexity.* Am J Reprod Immunol, 2010. **63**(6): p. 425-33.
170. Medina-Estrada, I., N. Alva-Murillo, J.E. Lopez-Meza, and A. Ochoa-Zarzosa, *Immunomodulatory Effects of 17beta-Estradiol on Epithelial Cells during Bacterial Infections.* J Immunol Res, 2018. **2018**: p. 6098961.
171. Robinson, D.P., M.E. Lorenzo, W. Jian, and S.L. Klein, *Elevated 17beta-estradiol protects females from influenza A virus pathogenesis by suppressing inflammatory responses.* PLoS Pathog, 2011. **7**(7): p. e1002149.
172. Giraud, S.N., et al., *Estradiol inhibits ongoing autoimmune neuroinflammation and NFkappaB-dependent CCL2 expression in reactive astrocytes.* Proc Natl Acad Sci U S A, 2010. **107**(18): p. 8416-21.
173. Wira, C.R., et al., *Sex hormone regulation of innate immunity in the female reproductive tract: the role of epithelial cells in balancing reproductive potential with protection against sexually transmitted pathogens.* Am J Reprod Immunol, 2010. **63**(6): p. 544-65.
174. Cvoro, A., et al., *Selective estrogen receptor-beta agonists repress transcription of proinflammatory genes.* J Immunol, 2008. **180**(1): p. 630-6.
175. Pazos, M., R.S. Sperling, T.M. Moran, and T.A. Kraus, *The influence of pregnancy on systemic immunity.* Immunol Res, 2012. **54**(1-3): p. 254-61.

176. Pazos, M.A., T.A. Kraus, C. Munoz-Fontela, and T.M. Moran, *Estrogen mediates innate and adaptive immune alterations to influenza infection in pregnant mice.* PLoS One, 2012. **7**(7): p. e40502.
177. Harding, A.T., et al., *GPER1 is required to protect fetal health from maternal inflammation.* Science, 2021. **371**(6526): p. 271-276.
178. Su, L., et al., *Progesterone inhibits Toll-like receptor 4-mediated innate immune response in macrophages by suppressing NF-kappaB activation and enhancing SOCS1 expression.* Immunol Lett, 2009. **125**(2): p. 151-5.
179. Vassiliadou, N., L. Tucker, and D.J. Anderson, *Progesterone-induced inhibition of chemokine receptor expression on peripheral blood mononuclear cells correlates with reduced HIV-1 infectability in vitro.* J Immunol, 1999. **162**(12): p. 7510-8.
180. Ferguson, K.K., et al., *Longitudinal profiling of inflammatory cytokines and C-reactive protein during uncomplicated and preterm pregnancy.* Am J Reprod Immunol, 2014. **72**(3): p. 326-36.
181. Jarmund, A.H., et al., *Cytokine Patterns in Maternal Serum From First Trimester to Term and Beyond.* Front Immunol, 2021. **12**: p. 752660.
182. Ander, S.E., M.S. Diamond, and C.B. Coyne, *Immune responses at the maternal-fetal interface.* Sci Immunol, 2019. **4**(31).
183. Manchorova, D., et al., *Human decidual gamma/delta T cells possess unique effector and TCR repertoire profiles during pregnancy.* Cell Immunol, 2022. **382**: p. 104634.
184. Mor, G., I. Cardenas, V. Abrahams, and S. Guller, *Inflammation and pregnancy: the role of the immune system at the implantation site.* Ann N Y Acad Sci, 2011. **1221**(1): p. 80-7.
185. Parker, E.L., R.B. Silverstein, S. Verma, and I.U. Mysorekar, *Viral-Immune Cell Interactions at the Maternal-Fetal Interface in Human Pregnancy.* Front Immunol, 2020. **11**: p. 522047.
186. Hofmann, A.P., S.A. Gerber, and B.A. Croy, *Uterine natural killer cells pace early development of mouse decidua basalis.* Mol Hum Reprod, 2014. **20**(1): p. 66-76.
187. Guimond, M.J., B. Wang, and B.A. Croy, *Engraftment of bone marrow from severe combined immunodeficient (SCID) mice reverses the reproductive deficits in natural killer cell-deficient tg epsilon 26 mice.* J Exp Med, 1998. **187**(2): p. 217-23.
188. Care, A.S., et al., *Macrophages regulate corpus luteum development during embryo implantation in mice.* J Clin Invest, 2013. **123**(8): p. 3472-87.
189. Svensson, J., et al., *Macrophages at the fetal-maternal interface express markers of alternative activation and are induced by M-CSF and IL-10.* J Immunol, 2011. **187**(7): p. 3671-82.
190. Svensson-Arvelund, J., et al., *The human fetal placenta promotes tolerance against the semiallogeneic fetus by inducing regulatory T cells and homeostatic M2 macrophages.* J Immunol, 2015. **194**(4): p. 1534-44.
191. Anipindi, V.C., et al., *IL-17 Production by gammadelta(+) T Cells Is Critical for Inducing T(h)17 Responses in the Female Genital Tract and Regulated by Estradiol and Microbiota.* Immunohorizons, 2019. **3**(7): p. 317-330.
192. Anipindi, V.C., et al., *Estradiol Enhances CD4+ T-Cell Anti-Viral Immunity by Priming Vaginal DCs to Induce Th17 Responses via an IL-1-Dependent Pathway.* PLoS Pathog, 2016. **12**(5): p. e1005589.
193. Monin, L., et al., *gammadelta T cells compose a developmentally regulated intrauterine population and protect against vaginal candidiasis.* Mucosal Immunol, 2020. **13**(6): p. 969-981.

194. Figueiredo, A.S. and A. Schumacher, *The T helper type 17/regulatory T cell paradigm in pregnancy.* Immunology, 2016. **148**(1): p. 13-21.
195. Gomez-Lopez, N., et al., *Regulatory T Cells Play a Role in a Subset of Idiopathic Preterm Labor/Birth and Adverse Neonatal Outcomes.* Cell Rep, 2020. **32**(1): p. 107874.
196. Wu, H.X., et al., *Decidual stromal cells recruit Th17 cells into decidua to promote proliferation and invasion of human trophoblast cells by secreting IL-17.* Cell Mol Immunol, 2014. **11**(3): p. 253-62.
197. Braga, A., et al., *Th17/Regulatory T cells ratio evolution: A prospective study in a group of healthy pregnant women.* J Reprod Immunol, 2022. **149**: p. 103468.
198. Hussain, T., et al., *Understanding the Immune System in Fetal Protection and Maternal Infections during Pregnancy.* J Immunol Res, 2022. **2022**: p. 7567708.
199. Zaga-Clavellina, V., et al., *Central role of the placenta during viral infection: Immuno-competences and miRNA defensive responses.* Biochim Biophys Acta Mol Basis Dis, 2021. **1867**(10): p. 166182.
200. Megli, C.J. and C.B. Coyne, *Infections at the maternal-fetal interface: an overview of pathogenesis and defence.* Nat Rev Microbiol, 2022. **20**(2): p. 67-82.
201. Delorme-Axford, E., et al., *Human placental trophoblasts confer viral resistance to recipient cells.* Proc Natl Acad Sci U S A, 2013. **110**(29): p. 12048-53.
202. Yarbrough, V.L., S. Winkle, and M.M. Herbst-Kralovetz, *Antimicrobial peptides in the female reproductive tract: a critical component of the mucosal immune barrier with physiological and clinical implications.* Hum Reprod Update, 2015. **21**(3): p. 353-77.
203. Zhai, Y.J., Y. Feng, X. Ma, and F. Ma, *Defensins: defenders of human reproductive health.* Hum Reprod Update, 2023. **29**(1): p. 126-154.
204. Madanchi, H., M. Shoushtari, H.H. Kashani, and S. Sardari, *Antimicrobial peptides of the vaginal innate immunity and their role in the fight against sexually transmitted diseases.* New Microbes New Infect, 2020. **34**: p. 100627.
205. Wira, C.R., et al., *Innate immunity in the human female reproductive tract: endocrine regulation of endogenous antimicrobial protection against HIV and other sexually transmitted infections.* Am J Reprod Immunol, 2011. **65**(3): p. 196-211.
206. Frew, L., et al., *Human cathelicidin production by the cervix.* PLoS One, 2014. **9**(8): p. e103434.
207. Lim, R., G. Barker, and M. Lappas, *Human cathelicidin antimicrobial protein 18 (hCAP18/LL-37) is increased in foetal membranes and myometrium after spontaneous labour and delivery.* J Reprod Immunol, 2015. **107**: p. 31-42.
208. Marchini, G., et al., *The newborn infant is protected by an innate antimicrobial barrier: peptide antibiotics are present in the skin and vernix caseosa.* Br J Dermatol, 2002. **147**(6): p. 1127-34.
209. Iwasaki, A., *Antiviral immune responses in the genital tract: clues for vaccines.* Nat Rev Immunol, 2010. **10**(10): p. 699-711.
210. Kang, S., et al., *Tissue Resident Memory gammadeltaT Cells in Murine Uterus Expressed High Levels of IL-17 Promoting the Invasion of Trophocytes.* Front Immunol, 2020. **11**: p. 588227.
211. Mincheva-Nilsson, L., *Pregnancy and gamma/delta T cells: taking on the hard questions.* Reprod Biol Endocrinol, 2003. **1**: p. 120.
212. Kim, J.O., H.R. Cha, E.D. Kim, and M.N. Kweon, *Pathological effect of IL-17A-producing TCRgammadelta(+) T cells in mouse genital mucosa against HSV-2 infection.* Immunol Lett, 2012. **147**(1-2): p. 34-40.

213. Pinget, G.V., et al., *The majority of murine gammadelta T cells at the maternal-fetal interface in pregnancy produce IL-17.* Immunol Cell Biol, 2016. **94**(7): p. 623-30.
214. Nishimura, H., et al., *Intraepithelial gammadelta T cells may bridge a gap between innate immunity and acquired immunity to herpes simplex virus type 2.* J Virol, 2004. **78**(9): p. 4927-30.
215. Rakasz, E., A. Mueller, S. Perlman, and R.G. Lynch, *Gammadelta T cell response induced by vaginal Herpes simplex 2 infection.* Immunol Lett, 1999. **70**(2): p. 89-93.
216. Alcaide, M.L., et al., *Bacterial Vaginosis Is Associated with Loss of Gamma Delta T Cells in the Female Reproductive Tract in Women in the Miami Women Interagency HIV Study (WIHS): A Cross Sectional Study.* PLoS One, 2016. **11**(4): p. e0153045.
217. Robbins, J.R. and A.I. Bakardjiev, *Pathogens and the placental fortress.* Curr Opin Microbiol, 2012. **15**(1): p. 36-43.
218. Krammer, F., *The human antibody response to influenza A virus infection and vaccination.* Nat Rev Immunol, 2019. **19**(6): p. 383-397.
219. Albrecht, M. and P.C. Arck, *Vertically Transferred Immunity in Neonates: Mothers, Mechanisms and Mediators.* Front Immunol, 2020. **11**: p. 555.
220. Vidarsson, G., G. Dekkers, and T. Rispens, *IgG subclasses and allotypes: from structure to effector functions.* Front Immunol, 2014. **5**: p. 520.
221. Hocart, M.J., J.S. Mackenzie, and G.A. Stewart, *The immunoglobulin G subclass responses of mice to influenza A virus: the effect of mouse strain, and the neutralizing abilities of individual protein A-purified subclass antibodies.* J Gen Virol, 1989. **70 (Pt 9)**: p. 2439-48.
222. Markine-Goriaynoff, D., et al., *IFN-gamma-independent IgG2a production in mice infected with viruses and parasites.* Int Immunol, 2000. **12**(2): p. 223-30.
223. Hwang, S.D., et al., *Protection of pregnant mice, fetuses and neonates from lethality of H5N1 influenza viruses by maternal vaccination.* Vaccine, 2010. **28**(17): p. 2957-64.
224. Huber, V.C., et al., *Distinct contributions of vaccine-induced immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza.* Clin Vaccine Immunol, 2006. **13**(9): p. 981-90.
225. Borghi, S., et al., *FcRn, but not FcgammaRs, drives maternal-fetal transplacental transport of human IgG antibodies.* Proc Natl Acad Sci U S A, 2020. **117**(23): p. 12943-12951.
226. Niewiesk, S., *Maternal antibodies: clinical significance, mechanism of interference with immune responses, and possible vaccination strategies.* Front Immunol, 2014. **5**: p. 446.
227. Clements, T., et al., *Update on Transplacental Transfer of IgG Subclasses: Impact of Maternal and Fetal Factors.* Front Immunol, 2020. **11**: p. 1920.
228. Schroeder, H.W., Jr. and L. Cavacini, *Structure and function of immunoglobulins.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S41-52.
229. Prados, M.B., et al., *Progesterone induces a switch in oligosaccharyltransferase isoform expression: consequences on IgG N-glycosylation.* Immunol Lett, 2011. **137**(1-2): p. 28-37.
230. Chen, G., et al., *Human IgG Fc-glycosylation profiling reveals associations with age, sex, female sex hormones and thyroid cancer.* J Proteomics, 2012. **75**(10): p. 2824-34.
231. Selman, M.H., et al., *Changes in antigen-specific IgG1 Fc N-glycosylation upon influenza and tetanus vaccination.* Mol Cell Proteomics, 2012. **11**(4): p. M111 014563.
232. Bordt, E.A., et al., *Maternal SARS-CoV-2 infection elicits sexually dimorphic placental immune responses.* Sci Transl Med, 2021. **13**(617): p. eabi7428.

233. Alter, G., T.H.M. Ottenhoff, and S.A. Joosten, *Antibody glycosylation in inflammation, disease and vaccination.* Semin Immunol, 2018. **39**: p. 102-110.
234. Jennewein, M.F., et al., *Fc Glycan-Mediated Regulation of Placental Antibody Transfer.* Cell, 2019. **178**(1): p. 202-215 e14.
235. Gould, V.M.W., et al., *Nasal IgA Provides Protection against Human Influenza Challenge in Volunteers with Low Serum Influenza Antibody Titre.* Front Microbiol, 2017. **8**: p. 900.
236. Suzuki, T., et al., *Relationship of the quaternary structure of human secretory IgA to neutralization of influenza virus.* Proc Natl Acad Sci U S A, 2015. **112**(25): p. 7809-14.
237. Harris, J.W., *INFLUENZA OCCURRING IN PREGNANT WOMEN*

A STATISTICAL STUDY OF THIRTEEN HUNDRED AND FIFTY CASES. JAMA 1919. **72**(14): p. 978-980.
238. Song, J.Y., et al., *Paradoxical long-term impact of maternal influenza infection on neonates and infants.* BMC Infect Dis, 2020. **20**(1): p. 502.
239. Dawood, F.S., et al., *Incidence of influenza during pregnancy and association with pregnancy and perinatal outcomes in three middle-income countries: a multisite prospective longitudinal cohort study.* Lancet Infect Dis, 2021. **21**(1): p. 97-106.
240. Reid, A., *The effects of the 1918-1919 influenza pandemic on infant and child health in Derbyshire.* Med Hist, 2005. **49**(1): p. 29-54.
241. Siston, A.M., et al., *Pandemic 2009 influenza A(H1N1) virus illness among pregnant women in the United States.* JAMA, 2010. **303**(15): p. 1517-25.
242. Mosby, L.G., S.A. Rasmussen, and D.J. Jamieson, *2009 pandemic influenza A (H1N1) in pregnancy: a systematic review of the literature.* Am J Obstet Gynecol, 2011. **205**(1): p. 10-8.
243. Kourtis, A.P., J.S. Read, and D.J. Jamieson, *Pregnancy and infection.* N Engl J Med, 2014. **370**(23): p. 2211-8.
244. Louie, J.K., D.A. Wadford, A. Norman, and D.J. Jamieson, *2009 pandemic influenza A (H1N1) and vaccine failure in pregnancy.* Obstet Gynecol, 2011. **117**(2 Pt 2): p. 470-2.
245. Louie, J.K., et al., *Severe 2009 H1N1 influenza in pregnant and postpartum women in California.* N Engl J Med, 2010. **362**(1): p. 27-35.
246. Rolland-Harris, E., et al., *Hospitalization of pregnant women with pandemic A(H1N1) 2009 influenza in Canada.* Epidemiol Infect, 2012. **140**(7): p. 1316-27.
247. Kucuk, M.P., et al., *Management of Acute Respiratory Distress Syndrome with H1N1 Influenza Virus in Pregnancy: Successful Mechanical Ventilation and Weaning with Airway Pressure Release Ventilation.* Turk J Anaesthesiol Reanim, 2018. **46**(1): p. 62-65.
248. Van Campen, H., et al., *Maternal Influenza A Virus Infection Restricts Fetal and Placental Growth and Adversely Affects the Fetal Thymic Transcriptome.* Viruses, 2020. **12**(9).
249. Jacobsen, H., et al., *Offspring born to influenza A virus infected pregnant mice have increased susceptibility to viral and bacterial infections in early life.* Nat Commun, 2021. **12**(1): p. 4957.
250. Creisher, P.S., et al., *Adverse outcomes in SARS-CoV-2 infected pregnant mice are gestational age-dependent and resolve with antiviral treatment.* bioRxiv, 2023.
251. Littauer, E.Q., et al., *H1N1 influenza virus infection results in adverse pregnancy outcomes by disrupting tissue-specific hormonal regulation.* PLoS Pathog, 2017. **13**(11): p. e1006757.

252. Chan, K.H., et al., *Wild type and mutant 2009 pandemic influenza A (H1N1) viruses cause more severe disease and higher mortality in pregnant BALB/c mice.* PLoS One, 2010. **5**(10): p. e13757.
253. Marcelin, G., et al., *Fatal outcome of pandemic H1N1 2009 influenza virus infection is associated with immunopathology and impaired lung repair, not enhanced viral burden, in pregnant mice.* J Virol, 2011. **85**(21): p. 11208-19.
254. Lauzon-Joset, J.F., et al., *Pregnancy Induces a Steady-State Shift in Alveolar Macrophage M1/M2 Phenotype That Is Associated With a Heightened Severity of Influenza Virus Infection: Mechanistic Insight Using Mouse Models.* J Infect Dis, 2019. **219**(11): p. 1823-1831.
255. Vermillion, M.S., et al., *Pregnancy preserves pulmonary function following influenza virus infection in C57BL/6 mice.* Am J Physiol Lung Cell Mol Physiol, 2018. **315**(4): p. L517-L525.
256. Davis, S.M., et al., *Estradiol and progesterone influence on influenza infection and immune response in a mouse model.* Am J Reprod Immunol, 2017. **78**(4).
257. Hall, O.J., et al., *Progesterone-Based Therapy Protects Against Influenza by Promoting Lung Repair and Recovery in Females.* PLoS Pathog, 2016. **12**(9): p. e1005840.
258. Deng, H., et al., *Identification of a major co-receptor for primary isolates of HIV-1.* Nature, 1996. **381**(6584): p. 661-6.
259. Malinowski, A.K., A. Noureldin, and M. Othman, *COVID-19 susceptibility in pregnancy: Immune/inflammatory considerations, the role of placental ACE-2 and research considerations.* Reprod Biol, 2020. **20**(4): p. 568-572.
260. Knight, M., et al., *Characteristics and outcomes of pregnant women admitted to hospital with confirmed SARS-CoV-2 infection in UK: national population based cohort study.* BMJ, 2020. **369**: p. m2107.
261. Song, D., et al., *Passive and active immunity in infants born to mothers with SARS-CoV-2 infection during pregnancy: Prospective cohort study.* medRxiv, 2021.
262. Sutton, D., K. Fuchs, M. D'Alton, and D. Goffman, *Universal Screening for SARS-CoV-2 in Women Admitted for Delivery.* N Engl J Med, 2020. **382**(22): p. 2163-2164.
263. Adhikari, E.H., et al., *Pregnancy Outcomes Among Women With and Without Severe Acute Respiratory Syndrome Coronavirus 2 Infection.* JAMA Netw Open, 2020. **3**(11): p. e2029256.
264. Browning, C.H., *Emil Behring and Paul Ehrlich: their contributions to science.* Nature, 1955. **175**(4458): p. 616-9; concl.
265. Silverstein, A.M., *Paul Ehrlich: the founding of pediatric immunology.* Cell Immunol, 1996. **174**(1): p. 1-6.
266. Oboho, I.K., et al., *Benefit of Early Initiation of Influenza Antiviral Treatment to Pregnant Women Hospitalized With Laboratory-Confirmed Influenza.* J Infect Dis, 2016. **214**(4): p. 507-15.
267. Crovetto, F., et al., *Seroprevalence and presentation of SARS-CoV-2 in pregnancy.* Lancet, 2020. **396**(10250): p. 530-531.
268. Kemp, M.W., *Preterm birth, intrauterine infection, and fetal inflammation.* Front Immunol, 2014. **5**: p. 574.
269. Ragsdale, H.B., et al., *Regulation of inflammation during gestation and birth outcomes: Inflammatory cytokine balance predicts birth weight and length.* Am J Hum Biol, 2019. **31**(3): p. e23245.

270. Hsu, P. and R.K. Nanan, *Innate and adaptive immune interactions at the fetal-maternal interface in healthy human pregnancy and pre-eclampsia.* Front Immunol, 2014. **5**: p. 125.
271. Fried, M., et al., *Systemic Inflammatory Response to Malaria During Pregnancy Is Associated With Pregnancy Loss and Preterm Delivery.* Clin Infect Dis, 2017. **65**(10): p. 1729-1735.
272. Keenan-Devlin, L.S., et al., *Using principal component analysis to examine associations of early pregnancy inflammatory biomarker profiles and adverse birth outcomes.* Am J Reprod Immunol, 2021. **86**(6): p. e13497.
273. Creisher, P.S., et al., *Suppression of progesterone by influenza A virus causes adverse maternal and fetal outcomes in mice.* bioRxiv, 2023.
274. Groisman, E.A., *How bacteria resist killing by host-defense peptides.* Trends Microbiol, 1994. **2**(11): p. 444-9.
275. Nuriel-Ohayon, M., H. Neuman, and O. Koren, *Microbial Changes during Pregnancy, Birth, and Infancy.* Front Microbiol, 2016. **7**: p. 1031.
276. Zierden, H.C., et al., *Cervicovaginal mucus barrier properties during pregnancy are impacted by the vaginal microbiome.* Front Cell Infect Microbiol, 2023. **13**: p. 1015625.
277. Szekeres-Bartho, J., et al., *The role of gamma/delta T cells in progesterone-mediated immunomodulation during pregnancy: a review.* Am J Reprod Immunol, 1999. **42**(1): p. 44-8.
278. Dupraz, L., et al., *Gut microbiota-derived short-chain fatty acids regulate IL-17 production by mouse and human intestinal gammadelta T cells.* Cell Rep, 2021. **36**(1): p. 109332.
279. Koskinen, K., et al., *The nasal microbiome mirrors and potentially shapes olfactory function.* Sci Rep, 2018. **8**(1): p. 1296.
280. Mahdavinia, M., et al., *A comprehensive review of the nasal microbiome in chronic rhinosinusitis (CRS).* Clin Exp Allergy, 2016. **46**(1): p. 21-41.
281. Aluvihare, V.R., M. Kallikourdis, and A.G. Betz, *Regulatory T cells mediate maternal tolerance to the fetus.* Nat Immunol, 2004. **5**(3): p. 266-71.
282. Arck, P.C. and K. Hecher, *Fetomaternal immune cross-talk and its consequences for maternal and offspring's health.* Nat Med, 2013. **19**(5): p. 548-56.
283. Mosmann, T.R. and R.L. Coffman, *TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties.* Annu Rev Immunol, 1989. **7**: p. 145-73.
284. Centers for Disease, C. *Diphtheria, Tetanus, and Pertussis Vaccines*. Available from: https://www.cdc.gov/vaccinesafety/vaccines/dtap-tdap-vaccine.html.
285. Centers for Disease, C. *Influenza (Flu)Vaccines*. Available from: https://www.cdc.gov/vaccinesafety/vaccines/flu-vaccine.html.
286. Palmeira, P., et al., *IgG placental transfer in healthy and pathological pregnancies.* Clin Dev Immunol, 2012. **2012**: p. 985646.
287. Stuebe, A., *The risks of not breastfeeding for mothers and infants.* Rev Obstet Gynecol, 2009. **2**(4): p. 222-31.
288. Field, C.J., *The immunological components of human milk and their effect on immune development in infants.* J Nutr, 2005. **135**(1): p. 1-4.
289. Atyeo, C., et al., *COVID-19 mRNA vaccines drive differential antibody Fc-functional profiles in pregnant, lactating, and non-pregnant women.* Sci Transl Med, 2021. **13**: p. eabi8631.
290. Gray, K.J., et al., *Coronavirus disease 2019 vaccine response in pregnant and lactating women: a cohort study.* Am J Obstet Gynecol, 2021. **225**(3): p. 303 e1-303 e17.

291. Center, C.C.s.H.M. *"Pregnant women produce super antibodies to protect newborns, now scientists know how."*. 2022; Available from: www.sciencedaily.com/releases/2022/06/220608112456.htm.
292. Erickson, J.J., et al., *Pregnancy enables antibody protection against intracellular infection.* Nature, 2022. **606**(7915): p. 769-775.
293. Mijakovac, A., et al., *Effects of Estradiol on Immunoglobulin G Glycosylation: Mapping of the Downstream Signaling Mechanism.* Front Immunol, 2021. **12**: p. 680227.
294. Ercan, A., et al., *Estrogens regulate glycosylation of IgG in women and men.* JCI Insight, 2017. **2**(4): p. e89703.
295. Martinez, D.R., et al., *Fc Characteristics Mediate Selective Placental Transfer of IgG in HIV-Infected Women.* Cell, 2019. **178**(1): p. 190-201 e11.
296. Centers for Disease, C. *Breastfeeding Report Card*. Available from: https://www.cdc.gov/breastfeeding/data/reportcard.htm.
297. Razzaghi, H., et al., *Influenza and Tdap Vaccination Coverage Among Pregnant Women - United States, April 2020.* MMWR Morb Mortal Wkly Rep, 2020. **69**(39): p. 1391-1397.
298. Centers for Disease, C. *COVID-19 vaccination among pregnant people aged 18-49 years overall, by race and ethnicity, and date reported to CDC - Vaccine Safety Datalink,* United States*. Available from: https://covid.cdc.gov/covid-data-tracker/#vaccinations-pregnant-women.
299. Simpson, J.L., et al., *Use of induced sputum for the diagnosis of influenza and infections in asthma: a comparison of diagnostic techniques.* J Clin Virol, 2003. **26**(3): p. 339-46.
300. Chang, Y.J., et al., *Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity.* Nat. Immunol, 2011. **12**(7): p. 631-638.
301. Lebold, K.M., D.B. Jacoby, and M.G. Drake, *Inflammatory mechanisms linking maternal and childhood asthma.* J Leukoc Biol, 2020. **108**(1): p. 113-121.
302. de Gouveia Belinelo, P., et al., *Maternal asthma is associated with reduced lung function in male infants in a combined analysis of the BLT and BILD cohorts.* Thorax, 2021. **76**(10): p. 996-1001.
303. Tarrant, I. and B.B. Finlay, *Like mother, like child: The maternal microbiome impacts offspring asthma.* Cell Rep Med, 2022. **3**(8): p. 100722.
304. Liu, C.A., et al., *Prediction of elevated cord blood IgE levels by maternal IgE levels, and the neonate's gender and gestational age.* Chang Gung Med J, 2003. **26**(8): p. 561-9.
305. Mohammadzadeh, I., M. Haghshenas, S. Asefi, and R. Alizadeh-Navaei, *IgE level in newborn umbilical cord and its relationship with some maternal factors.* Clin Mol Allergy, 2019. **17**: p. 11.
306. Thompson, E.E., et al., *Maternal microchimerism protects against the development of asthma.* J Allergy Clin Immunol, 2013. **132**(1): p. 39-44.
307. Medawar, P.B., *The Nobel Lectures in Immunology. The Nobel Prize for Physiology or Medicine, 1960. Immunological tolerance.* Scand J Immunol, 1991. **33**(4): p. 337-44.
308. Saito, S., A. Nakashima, T. Shima, and M. Ito, *Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy.* Am J Reprod Immunol, 2010. **63**(6): p. 601-10.