Review

The Power of First Impressions: Can Influenza Imprinting during Infancy Inform Vaccine Design?

Melissa Rioux [1], Mara McNeil [1], Magen E. Francis [1], Nicholas Dawe [1], Mary Foley [1], Joanne Langley [2, 3], and Alyson A. Kelvin[1, 2, 3, 4] *

[1] Department of Microbiology and Immunology, Faculty of Medicine, Dalhousie University, Halifax, NS B3H 4R2, Canada. melissa.rioux@dal.ca, mara.mcneil@dal.ca, m.francis@dal.ca, nc350339@dal.ca, mr471027@dal.ca, akelvin@dal.ca.

[2] Department of Pediatrics, Division of Infectious Disease, Faculty of Medicine, Dalhousie University, Halifax, NS B3K 6R8, Canada. Joanne.langley@dal.ca, akelvin@dal.ca.

[3] Canadian Centre for Vaccinology, IWK Health Centre, Halifax, NS B3K 6R8, Canada. Joanne.langley@dal.ca, akelvin@dal.ca.

[4] Vaccine and Infectious Disease Organization - International Vaccine Centre (VIDO-InterVac), Saskatoon, Saskatchewan, Canada. Alk308@usask.ca

* Correspondence:Correspondence: akelvin@dal.ca; Tel.: 001-647-963-2035 (A. A. K.)

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Abstract: Influenza virus infection causes severe respiratory illness in people worldwide, disproportionately affecting infants. The immature respiratory tract coupled with the developing immune system is thought to synergistically play a role in the increased disease severity in younger age groups. Although vaccines remain the best solution for protecting this vulnerable population, no vaccines are available for those under 6 months, and for infants aged 6 months to 2 years, the vaccine elicits a dampened immune response. Dampened immune responses may be due to unique features of the infant immune system and a lack of pre-existing immunity. Unlike older children and adults, the infant immune system is Th2 skewed and has less antigen presenting cells and soluble immune factors. Paradoxically, we know that a person's first infection with the influenza virus during infancy or childhood leads to the establishment of life-long immunity toward that particular virus strain. This is called *influenza imprinting*. To provide better protection against influenza virus infection and disease in infants, more research must be conducted to understand the *imprinting event*. We contend that by understanding influenza imprinting in the context of the infant immune system and the infant's immature respiratory tract, we will be able to design more effective influenza vaccines for both infants and adults. Working through the lens of imprinting, using infant influenza animal models such as mice and ferrets which have proven useful for infant immunity studies, we will gain a better understanding of imprinting and its implications regarding vaccine design. This review examines literature regarding infant immune development, current vaccine strategies, respiratory development, and the importance of researching the imprinting event in infant animal models to develop more effective and protective vaccines for all including young children.

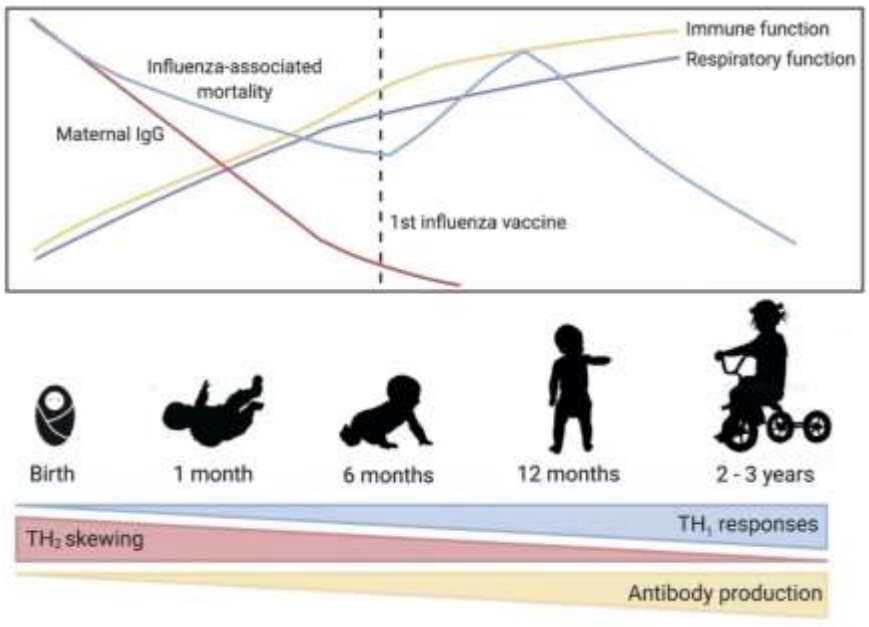
Keywords: influenza virus, immune response, infant immunity, imprinting, orthomyxoviridae, vaccination

1. Introduction

Influenza virus is a negative-sense RNA virus and a major burden on global health [1]. Influenza viruses mutate rapidly due to antigenic shift and antigenic drift, and new strains emerge each year leading to continual disease in humans [2]. The influenza virus types A and B are currently circulating in humans, and are estimated to infect between 5-10% of adults and 20-30% of children each year [3].

Clinical symptoms of influenza virus infection can vary from mild coughing and sneezing to complicated pneumonia and possibly multi-organ failure and death [4]. Influenza virus complications are most frequent in children and the elderly [4], with some of the highest hospitalization and death rates due to influenza occurring in these ages groups [5,6]. There are differences between infant and adult immune systems that are thought to be responsible for the increased rates of infection, hospitalization, and death seen in the younger age groups. These include deficiencies in soluble immune factors [7,8], low levels of antigen presenting cells [9], a skewed Th2 response [10], and lower levels of immunoglobulins [11,12]. These immune system differences may also contribute to the different vaccination outcomes experienced in infants and young children compared to adults. Furthermore, infants do not have pre-existing immunity to influenza viruses which may play a role in disease susceptibility and vaccine responses. Since influenza viruses mutate rapidly leading to new seasonal virus strains that can evade pre-existing immunity, the annual influenza vaccine requires continual updating with the current circulating strains. There are several vaccine platforms currently available and approved for use including inactivated (IIV), live attenuated (LAIV), recombinant, and adjuvanted vaccines [13]. Only IIV are authorized for children aged 6 months – 2 years, and no vaccine is authorized for use earlier than 6 months of age [13]. Figure 1 highlights some of the important trends in early immunology and influenza virus-associated mortality. One's first exposure to influenza virus, whether that be by infection or vaccination, is a significant event which we refer to as the *imprinting event* [14]. In particular, the first influenza virus infections shapes the immune system with respect to influenza virus antigens, and thus defines the immune response in subsequent influenza virus infections and vaccinations [14]. More work is needed to understand the full extent with which vaccination of infants imprints the immune system. Understanding the imprinting event in the context of the infant immune system will be important in designing more effective vaccines to protect this vulnerable population.

Figure 1. Trends in early-life immune development and influenza virus-associated mortality. As



immune and respiratory function improve over the first few years of life, influenza-associated mortality decreases. Maternal immunoglobulin G (IgG) peaks at birth and decreases rapidly over the first few months of life, leaving a window of vulnerability to infection before autonomous antibody production is maximized. At birth, the immune system is biased toward T helper cell type 2 responses, but maturation over the first 2-3 years of life is characterized by increased T helper cell type 1 responses and increased antibody production so it is no longer skewed but able to elicit a balanced response to antigens.

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Researchers have used several infant animal models to study the infant immune response to influenza virus infection, including mice and ferrets. A literature review of infant mice studies has shown decreased T cell immune responses in infant animals [15,16], but studies investigating humoral and antibody responses are currently lacking. Reviewing literature from infant ferret studies has demonstrated that outcomes of influenza virus infection may be significantly dependent on ferret age post-partum [17–20]. These models, discussed in detail below, may be useful for future studies involving imprinting and development of immune memory so that infant-specific vaccines can be developed.

Below we review the literature surrounding infant immune imprinting in the context of influenza vaccination. We will first discuss influenza virology, clinical outcomes, and vaccine platforms. We will then discuss how this pertains to infant infection and vaccination. Finally, we will end with a discussion of possible animal models for further investigation of infant influenza virus immunity and vaccination.

2. Influenza virus

2.1. Virology

Despite pre-exiting immunity acquired through previous infection and vaccination, influenza viruses continue to cause significant morbidity and mortality worldwide. Seasonal influenza epidemics result in an estimated 200,00 to 645,000 deaths every year [21]. As well, epidemics have a large economic impact and may incur large costs to the healthcare system through healthcare provider visits, hospital admissions, and workdays lost.

Influenza viruses are negative-sense RNA viruses belonging to the *Orthomyxoviridae* family [22]. As shown in Figure 2, there are four types of influenza viruses, but only influenza A virus (IAV) and influenza B virus (IBV) cause seasonal epidemics in humans [23], and only IAV has historically caused pandemics. Influenza A subtypes are classified by the glycoproteins hemagglutinin (HA) and neuraminidase (NA) found on the outer viral membrane [24]. To date, 18 different HA subtypes and 11 different NA subtypes have been identified [24], with the H1N1 and H3N2 subtypes currently circulating in humans [25]. The IAV is further divided into groups based on the HA protein similarity in the membrane proximal domain of the protein: group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17 and H18) and group 2 (H3, H4, H7, H10, H14, H15) [26]. Due to the various combinations of subtype and strain exposures, unique immune histories are created per individual.

Influenza viruses are a major global health burden as new strains emerge seasonally due to antigenic drift causing disease in humans and animals [27]. Influenza viruses have two main mechanisms of mutation: antigenic drift and antigenic shift. Antigenic drift occurs as a result of small genetic changes in the virus as it replicates. Over time, these small changes can accumulate and produce virus strains that are antigenically different than their predecessors [2]. Another way new influenza virus strains can emerge is through antigenic shift in which new combinations of virus proteins emerge due to swapping of genomic segments while two influenza viruses infect the same cell at the same time [2]. Antigenic shift is the main mode of pandemic virus development but it is due to antigenic drift that new influenza virus vaccines need to be made each year.

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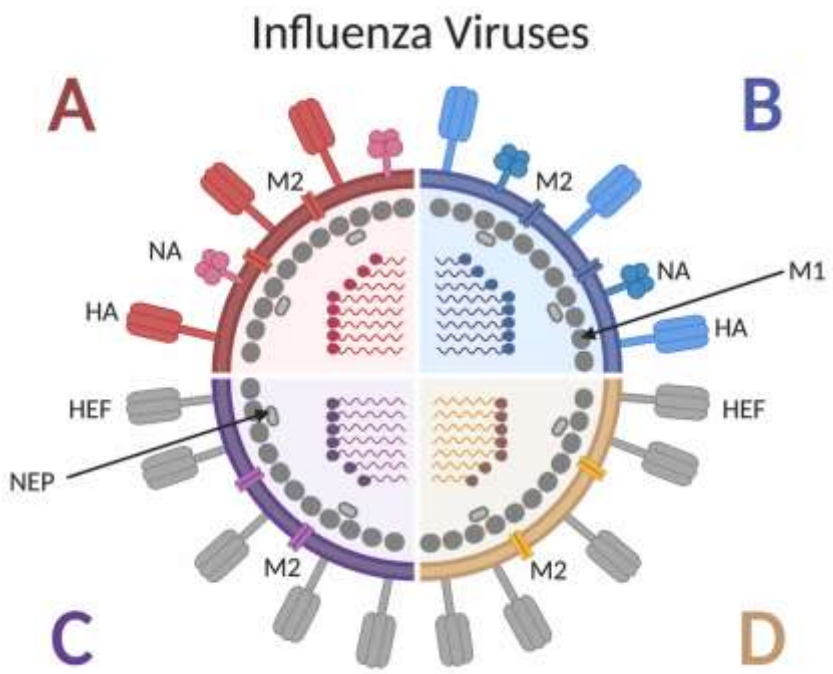

Figure 2. Schematic of influenza A, B, C and D virus structure. Influenza A and B viruses express surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), as well as the M2 ion channel. Both A and B viruses have 8 genomic segments coding for at least 10 proteins. Influenza C and D viruses express the surface glycoprotein hemagglutinin-esterase fusion (HEF), as well as the M2 ion channel. Both C and D viruses have 7 genomic segments coding for 9 proteins. All four types of influenza viruses express the M1 protein along the inner surface on the envelope, adjacent to the nuclear export protein (NEP).

IAV is a spherical, enveloped virus covered in membrane proteins HA, NA, and matrix 2 (M2). The viral envelope is supported by the matrix 1 (M1) protein, and the segmented RNA genome is found inside [28]. The eight segments of single-stranded, negative-sense RNA encode for at least 11 viral proteins: HA, NA, M1, M2, nucleoprotein (NP), non-structural protein 1 (NS1), nuclear export protein (NEP), polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase basic protein 1-F2 (PB1-F2) [28]. Each segment is contained in a rod-shaped, viral ribonucleoprotein (vRNP) complex that contains viral RNA wrapped around copies of NP, as well as the heterotrimeric viral polymerase made of PA, PB1, and PB2 [29].

The infection process begins with binding of IAV hemagglutinin to host sialic acid residues, facilitating viral endocytosis into respiratory epithelial cells [22]8/17/2020 6:04:00 PM. The low host endosomal pH causes conformational changes in the viral HA which exposes a viral fusion peptide, leading to fusion of the viral envelope with the host endosomal membrane to form a pore [30]. At the same time, ions are pumped through the M2 ion channel into the virion, leading to acidification of the virion and release of vRNPs through an endosomal pore into the host cell [22,30]. The vRNPs follow nuclear localization signals to the host cell nucleus, where the viral RNA polymerase synthesizes mRNA and cRNA from the negative-sense viral RNA [31]. The mRNA is 5′ capped and 3′ polyadenylated and can be exported and translated in a similar process as host mRNAs utilizing the host cell machinery [28]. The viral proteins M1 and NEP regulate nuclear export of mRNA [28]. Structural viral proteins are synthesized on ribosomes and transported to the host plasma membrane via the secretory pathway [32], where accumulation of HA, NA, and M2 induces a curvature in the membrane [33]. Non-structural proteins are synthesized on free ribosomes in the cytosol. The structural and non-structural proteins meet at the membrane for assembly.

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The virion is considered to be fully infectious once a full genome is incorporated and packaged within the envelope. Current findings suggest that IAV uses selective genome packaging to ensure a fully infectious virion [34]. After budding, sialidase activity possessed by the NA protein facilitates release of the virion into the extracellular space [33]. The released virion moves between cells of the respiratory epithelium, where HA can attach to the sialic acids of the next target cell to facilitate infection. As the virion moves outside of the cell, NA continues to play a role by cleaving sialic acid residues from mucins that could trap the virus and prevent infection [33].

2.2. Clinical Outcomes and Pathology

The pathology caused by seasonal influenza virus infection is primarily localized to the respiratory tract where the virus infects respiratory epithelial cells. Damage to the respiratory tract is mainly caused by the host inflammatory response to the virus occurring after infection [35]. Clinical presentation varies widely depending on the specific host being infected and the virus strain. Some influenza viral strains are able to infect both the upper and lower respiratory tract while other viruses remain only in the upper [36]. The tropism of the virus often dictates disease since when the virus is able to infect lower in the respiratory lung function can be compromised. Symptoms of infection can range from mild upper respiratory symptoms to lower respiratory tract involvement manifested by bronchitis, bronchiolitis, and/or complicated pneumonia [36]. Diffuse alveolar damage is possible, which can lead to respiratory dysfunction, endothelial leakage, precipitating in multi-organ failure and even death [4]. Specifically, physiological failure of the lungs occurs due to airway obstruction, loss of alveolar structure, degradation of the lung extracellular matrix, and epithelial cell death [35]. Approximately 30-40% of hospitalized patients with laboratory-diagnosed influenza also develop acute pneumonia, which is overrepresented by patients below 5 years and above 65 years of age [35].

Clinical data consistently points to children 0 to 2 years old as a high-risk group for severe influenza infection and associated complications. Influenza virus complications are associated with high rates of hospitalization and death occurring in infants and young children [5,6]. Interestingly, both viral load and the duration of shedding are shown to be higher in children compared to adults, suggesting children act as super-spreaders of the virus [35]. In support of increased influenza disease in childhood, children less than 1 year of age made up the largest proportion of H1N1 infections in Italy during the 2012-2013 season where they commonly presented with high fever, apnea, respiratory symptoms, otitis, laryngitis, and pharyngitis [37]. Another publication also reported that the influenza hospitalization rate was also greater for infants compared to older children [38]. In Argentina during the 2009 H1N1 pandemic season, 75% of children admitted to hospitals due to influenza virus infection were less than 2 years old [39]. In addition, the highest death rate during the 2009 H1N1 pandemic in Argentina was among infants as the infant case fatality rate was 7.6 deaths per 100 000 compared to 1.1 per 100 000 among all children [39]. Moreover, children less than 6 months of age had the highest mortality rate due to influenza virus in the US between 2010 and 2016 [40]. Influenza vaccination has been linked to decreased severe disease and death in children. Despite knowing this, lower vaccination rates are also commonly observed in infants [4]. Data from the US showed that infants who were vaccinated for influenza were less likely to die of complications compared to those that were not vaccinated, as 74% of the infants who died from influenza virus in 2010 to 2014 had not been vaccinated [41]. It is clear that infants are a high-risk group to influenza virus infection and more research needs to be done to understand the unique infant immune system and if vaccines can be developed specifically for the young that can leave long-lasting broad immunity.

3. Current vaccination strategies

Annual vaccination is the best way to prevent infection by emerging seasonal influenza strains [42] and has been supported by the Centers for Disease Control and Prevention since 2010 [43]. Although vaccination is the best means of protection against influenza virus morbidity and mortality, no vaccine platform offers long-lasting protection against influenza viruses because influenza viruses rapidly mutate [44]. Due to the continual antigenic changes in the circulating influenza viruses, the

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vaccine formulations must be updated yearly. The World Health Organization selects the IAV and IBV strains to be included in seasonal influenza vaccines twice a year (once per Northern and Southern hemisphere). The strain selections are based on data of circulating influenza strains in the opposite hemisphere [45]. Importantly, vaccine responses and the outcome of vaccination in terms of effectiveness are highly dependent on the host's age. The elderly and infants both have decreased responses to vaccination. Here we summarize approved vaccine platforms and the age specific responses, especially in the young.

The purpose of an influenza vaccine is to deliver viral antigens, typically the HA protein from the predominant strain of the season. Historically, the influenza virus was chemically inactivated following harvest from embryonated chicken eggs to produce whole-virion inactivated vaccines [46]. However, split-virion or subunit vaccines are now more commonly used since they cause less reactogenicity than whole virus vaccines after administration [45]. There are several influenza vaccine platforms currently in use that offer benefits to specific hosts. These platforms include inactivated influenza (IIV), live-attenuated (LAIV), recombinant, and adjuvanted influenza vaccines. The recommended influenza vaccine platform differs by age group, as shown in Table 1.

Table 1. Recommended influenza vaccine platforms by age group. [43,54,56].

Age Group	Recommended vaccine	Live vaccine (Yes/No)	Reason for recommendation
Infants aged 0 - 6 months	None; maternal vaccination during pregnancy recommended	No	No licensed influenza vaccines for infants less than 6 months of age
Infants aged 6 months - 2 years	TIV or QIV	No	LAIV is not authorized for use in children less than two years of age
Children aged 2-17 years	TIV, QIV or LAIV	Yes	All vaccines authorized for age group
Adults aged 18-59 years	TIV, QIV or LAIV	Yes	All vaccines authorized for age group
Adults 60 - 64 years	TIV and QIV	No	Nasal spray vaccines not recommended
Adults aged 65 and over	Adjuvanted TIV or high dose TIV	No	Unadjuvanted vaccines are poorly immunogenic in elderly populations

IIVs can be chemically inactivated whole virus or digested virus called split virion vaccines [46]. Split virion vaccines are now preferred over whole virus. After the viruses have been expanded, they are inactivated and then digested with detergents, which is where the term split virion originates [46]. After splitting the virus, components undergo minimal protein isolation, meaning the vaccine can contain elements of the other viral proteins in the vaccine dose. IIVs are typically administered intramuscularly and are available in trivalent and quadrivalent formulations [13]. In Canada, five trivalent inactivated influenza vaccines (TIV) and three quadrivalent inactivated influenza vaccines (QIV) were licensed for use in the 2019-2020 season [47]. Trivalent inactivated influenza vaccines are composed of the two IAV strains currently circulating in humans (H1N1 and H3N2), and only one IBV strain (either Yamagata or Victoria lineage), while quadrivalent formulations contain two IAV strains and two IBV strains [42]. Quadrivalent inactivated vaccines have become increasingly popular to reduce mismatch between IBV strains in circulation, which ultimately leads to better protection from influenza virus infection [48].

Unlike split-virion vaccines, subunit vaccines are either purified post-inactivation or are manufactured to contain only the antigenically reactive components desired. The most common

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component is the viral surface protein HA [49]. The manufacturing of the subunit vaccine results in a lack of the internal proteins of the influenza virus such as the nucleoprotein, polymerases and matrix proteins which may reduce off target antibody responses [50]. T cell responses are often directed towards internal viral proteins and may aid in influenza infection clearance by directed cell killing [50]. Split-virion vaccines which contain internal proteins may unintendingly elicit a cellular immune response while subunit vaccines which comprise of a single peptide do not [50]. Regardless of type, younger age groups have decreased immune responses after immunization.

Live attenduate Influenza Vaccine (LAIV) is composed of live influenza viruses that have been attenuated. The LAIV has been recommended in individuals 2 to 59 years old who do not have immune compromising conditions [47]. The vaccine may come in trivalent or quadrivalent forms and is administered intranasally as a spray [43]. It is hypothesized that the direct immunization in the respiratory mucosa is the mechanism driving immune responses in the younger age groups. To update the LAIV formulation each year, reassortants are made with the HA and NA genes of circulating strains, which are reassorted with the backbone of internal genes from cold adapted master strains. The master strain for influenza A and influenza B reassortants are the 1960 A/Ann Arbor/6/60 and the B/Ann Arbor/1/66, respectively [51,52]. By using these internal genes, the resulting reassortant vaccine viruses are cold-adapted, temperature sensitive, and attenuated. The cold-adaptation and temperature sensitivity ensures the virus will only replicate in the cooler temperatures of the nasal cavities in the upper respiratory and not at higher temperatures in the lungs [43]. Since LAIV is given intranasally, it has the potential to induce mild upper respiratory symptoms such as sneezing and nasal congestion and may not be appropriate for persons with risk factors for influenza-related complications such as children with asthma [43].

An adjuvanted vaccine contains a substance, the *adjuvant*, which helps increase vaccine immunogenicity. Three of the most common adjuvants used in influenza vaccine formulation studies include Alum or Aluminum salts and the two oil-in-water emulsions MF59 and AS03 [53]. Although there are several adjuvants in development, only one, MF59, is approved for use in Canada in the Fluad and Fluad pediatric vaccines [54]. The addition of an adjuvant has shown to be an effective way to enhance vaccine efficacy in children and the elderly [55]. MF59 in particular has shown to elicit greater antibody and cellular responses in children after vaccination [53]. In addition, adjuvants added to vaccine can significantly reduce the amount of adjuvant needed for each vaccine dose. This is important when vaccine adjuvants are laborious or costly to make or when vaccines need to be produced quick, such as during a pandemic.

This section serves to highlight the vaccine platforms that are currently available and the age groups that they are recommended for with specific focus on younger age groups. Because influenza viruses rapidly mutate and pre-existing immunity is not protective, vaccines are agreed to be the best way to protect against infection from new circulating influenza virus strains. However, a review of the current vaccine platforms demonstrates the reduced vaccine availability for infants and young children, a group that is at high risk. It might be necessary to develop vaccine platforms that cater directly to infant immune systems, rather than attempting to use the same vaccine platform as adults. In order to develop vaccines better suited to protect infants, we need to understand how their immune systems differ from adults. We also ask the question about what we can learn from infant influenza imprinting and if imprinting mechanisms can be leveraged for the development of the next generation of more effective influenza vaccines.

4. The predictive power of influenza virus immune imprinting

In order to elicit the best immune response at vaccination, we need to consider how the influenza virus first imprints the immune system and subsequently how the imprinted immune system influences vaccine responses throughout a lifetime. Evidence shows that memory B and T cells produced during early exposures to the influenza virus still circulate in adult life [57]. Here we examine the shaping of the immune system by the first influenza virus infection and the subsequent consequences on later infections and vaccinations to give insight into using the mechanisms of immune imprinting for vaccine design. Insight into imprinting will be beneficial for both the design

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of more effective infant influenza vaccines and also for vaccines with increased longevity and broadness for adults. The imprinted immune system can be beneficial or deleterious during a later influenza virus infection depending on the relatedness of the viruses [27].

When building an influenza virus immune history, not all infections are created equally. The significance of one's first influenza virus exposure in early life on subsequent immune responses to infection and vaccination has been described for decades. The term *Original Antigenic Sin* (*OAS*) was coined in the 1950s by Thomas Francis to describe the dominating effect of the first IAV infection on immune responses later in life [58]. The *sin* refers to the preference of the immune system to recall pre-existing antibodies instead of eliciting new ones against a novel antigen during infection [12]. The *sin* in OAS highlights the possible deleterious effect of the first infection on subsequent immune responses, but as understanding of pre-exiting immunity increases, OAS is now frequently referred to as *antigenic seniority* to capture possible protective effects that may occur as well [59].

The first exposure to influenza virus and the resulting immune response are referred to as the *imprinting event* [14]. In the context of influenza virus, imprinting leads to life-long immunological memory that will be recalled at later infections and vaccinations, which can skew the immune system in ways that are beneficial or deleterious to the host [14]. Evidence shows that people possess higher antibody titers to influenza virus strains encountered in childhood, regardless of their age [60]. As a consequence, during adulthood certain influenza virus infections will elicit more robust antibody responses. This is likely due to the childhood exposure to an antigenically similar virus [59]. For example, individuals imprinted with group 1 HAs and then later infected with group 2 HAs still show an increase in antibody response to group 1 HAs [59].

Imprinting has been demonstrated repeatedly in studies as age cohorts are differentially impacted by emerging influenza viruses. For example, elders were least affected by the 2009 H1N1 pandemic due to imprinting with similar viruses in childhood from the 1918 pandemic [61,62]. From data such as this we are able to predict which age population will be most affected by emerging viruses based on imprinting year [60]. Animal studies have shown that despite antibodies elicited during heterologous infection being primarily directed towards the original imprinting strain and not the challenge strain, these antibodies are still able to induce protection through non-neutralizing cross-reactions [63]. B-cell clonotypes from early exposures can be elicited and improved with subsequent exposures, leading to improved antibody response [14]. Stalk antibodies can be broadly reactive and are a common platform for universal vaccine designs. Arevalo et al. showed that natural infection in humans and experimental infection in ferrets can lead to stalk antibodies that one encountered during the imprinting event, despite exposures to antigenically distinct subtypes [64].

It has also been shown that negative vaccine effects due to immune imprinting are possible. The 2020 study by Skowroksi et al. examined age-specific vaccine outcomes of the 2018/209 H3N2 influenza epidemic [65]. This study revealed age-specific decrease in vaccine effectiveness in a non-elderly cohort. Authors hypothesized that this group of adults aged 25-54 specifically were likely imprinted with an H3N2 virus in clade 3C.3a [65]. Upon vaccination against H3N2 viruses in 3C.2a, an antigenically distinct clade, authors proposed based on their 'I-REV' (Imprint-Regulated Effect of Vaccine) hypothesis that exposure to this antigenically distant virus interfered with the imprinted immune response leading to poor vaccine outcomes [65]. Studies such as these are important to help us understand the immune mechanisms that occur during childhood imprinting that which affect vaccination outcomes into adulthood [14]. Taken together, childhood imprinting has been shown to have a substantial impact on later exposures to antigens via vaccination. Understanding both the negative and positive impacts of imprinting is essential in improving future vaccine outcomes.

Given the life-long implications of the first influenza virus exposure, it follows that the first exposure could be optimized through improved vaccination strategies. The importance of eliciting a robust immune response at vaccination in infancy is therefore not limited to the infant population. Strategic vaccination tailored to the early immune system would offer better protection from influenza virus morbidity and mortality in infants, but importantly, this protection may be long-lasting and improve infection outcomes in adults as well.

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5. Infant immune development

To understand the imprinting event and how it can be applied to optimize infant influenza vaccination, we must first consider the unique features of the early immune system. These features, along with the still-developing respiratory tract, provide the physiological context in which the imprinting event occurs. The greatest challenge facing the neonatal immune system is the transition from the relatively sterile intrauterine environment to a world of tremendous antigenic variation. During this transition, infants have two seemingly contradictory challenges: they must learn to coexist with commensal microbes, but also learn to eliminate invasive pathogens [66]. Neonates are known to be particularly susceptible to infection for a number of possible reasons. One reason may be the lack of immunological memory that is necessary to mount efficient immune responses to specific antigens [10]. Another reason may be that infants possess fewer immune cells overall at peripheral lymphoid tissues compared to adults, which has been shown in mice [10,67]. Finally, some evidence suggests that neonatal immune cells are qualitatively different from adults; immune cell subtypes are not only present in different proportions, but there are phenotypic differences as well [7].

Neonatal immune systems are often described as immature. Indeed, *in vitro* and *in vivo* studies have shown some deficiency and immune deviation among infant B cells, T cells, and antigen presenting cells, see Figure 3 [10]. However, three studies in 1996 showed that infant mice were capable of mature T-cell responses under the right circumstances [68–70]. It is thus important to distinguish the unique features of the infant immune system from immaturity or immunodeficiency.

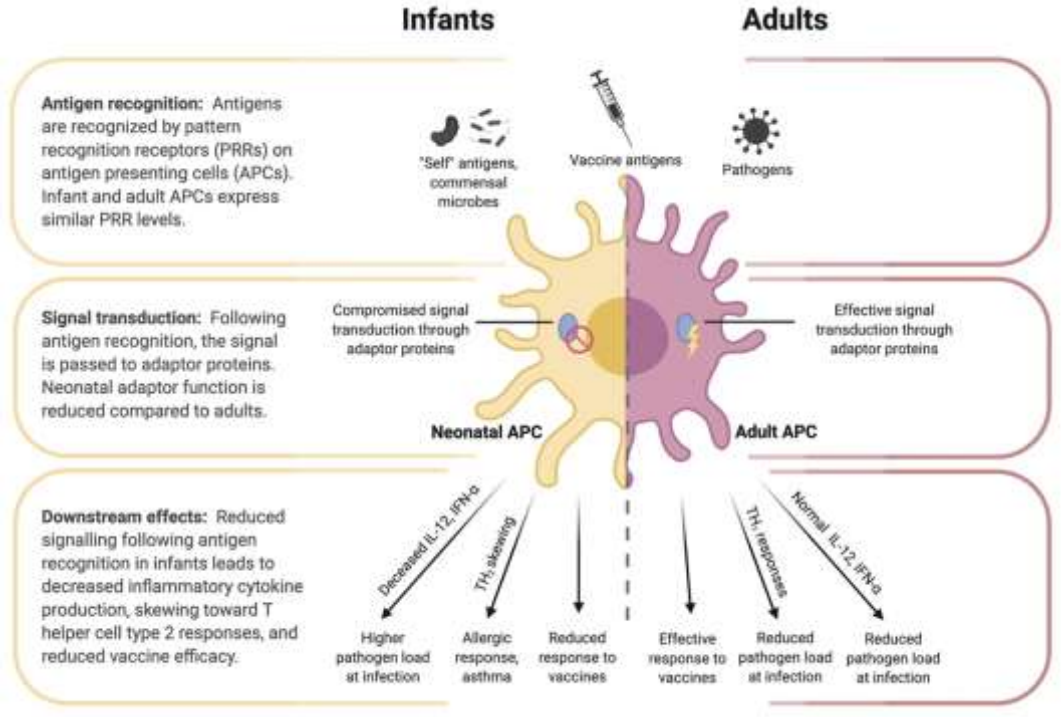

Figure 3. Differences in antigen presenting cell (APC) function between infants and adults. Despite similar pattern recognition receptor (PRR) expression between infant and adult APCs, the downstream signaling following antigen recognition by PRRs differs. Reduced adaptor protein function (e.g. IRF-4) in neonatal APCs contributes to tolerance of self-antigens and commensal microbes, while also reducing responsiveness to pathogens and vaccine antigens, decreased inflammatory cytokine production, and skewing toward a T helper cell type 2 response.

5.1. Passive immunity through maternal antibodies

Early immunity against specific antigens is partially conferred by maternal immunoglobulins (Ig). During the third trimester of pregnancy, significant amounts of maternal IgG are transported

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across the placenta [71]. This protection is short-lived, as maternal IgG levels in the infant decrease over the first few months of life [72]. Breast-fed infants also obtain maternal IgA produced by mammary gland lymphocytes post-partum [71]. Because maternal antibodies are still present in the first 6 months of life, influenza infection is also typically more frequent in infants 6 to 12 months old compared to newborns to 6 months, because of decreased immunity [73]. Studies have shown that influenza vaccination during pregnancy can be protective to infants in the first few months of life. In addition, infants born to influenza-vaccinated mothers have significantly increased hemagglutinin inhibition antibody titres at birth and 2-3 months of age than those born to unvaccinated mothers[74].

5.2. Innate immunity of the infant

There are some major similarities and differences between adult and infant innate immune system components. In response to infection, neonates can produce interleukin (IL)-6 at the same or greater levels than adults [75,76]. During respiratory infection in premature and newborn infants, the antimicrobial peptides human beta-defensin 1, human beta-defensin 2 and the cathelicidin LL-37/hCAP-18 are already present and significantly increased in the lungs [77]. Levels of these peptides were correlated with each other and with levels of IL-8 and TNF-α [77]. Unfortunately, newborns are deficient in other soluble immune factors such as components of the complement system, particularly in pre-term and low birth weight infants [7,8]. As newborns age, whole complement activity and components of the classical (C1q, C4, C3) and alternative pathway (factor B, properdin) increase significantly [78]. In addition, neonatal neutrophils are present in lower numbers and lacking in functionality. Neonatal antigen-presenting cells, such as macrophages and dendritic cells, show decreased expression of co-stimulatory molecules, major histocompatibility complex (MHC)-II, and toll-like receptors (TLR) [9]. Innate lymphoid cells (ILCs) may have a compensatory role during this time; ILCs appear and are programmed long before birth, during the embryonic stage [79–81]. ILCs show higher levels of activity in infants compared to adults [9].

5.3. Adaptive immune development in early life

As early as 14 weeks gestation, mature B and T cells with a remarkable range of antigenic diversity are already circulating, although there is relatively little antigen present [82]. Infants are capable of both cell-mediated and humoral immunity at birth. This includes production of all Ig isotypes, development of Th1 and Th2 subsets, and cytotoxic T cell responses [83,84]. Hours after birth, colonization of the infant gastrointestinal tract begins [85] and within a week, microbe-specific IgA produced in the intestine can be detected [86].

Infants were once believed to be immunodeficient because of limited production of IL-2; however, the distinction between Th1 and Th2 T helper cell subsets showed that infant immune responses are simply biased toward the Th2 lineage [10]. The Th2-skewed infant immune response is associated with decreased cell-mediated immunity and may be due to ineffective activation of the innate immune system in early life. Infants have decreased Toll-like receptor (TLR) responses which leads to skewed downstream cytokine production. For example, after TLR stimulation, infant immune cells have decreased production of Th1 cytokines such as interferon (IFN)-α and IFN-γ, and increased Th2 cytokines such as IL-10 [87,88].

Infant B cell responses are considered to be deficient compared to older children and adults – they do not respond fully to T cell-independent antigens until about two years of age [11]. Mutated IgM and IgD B cells undergo somatic hypermutation at the rate of adults by age two [12] while IgG and IgA subtypes only acquire mutations at 60-75% of adult frequencies by age three [12]. The costimulatory receptors CD40, CD80 and CD86 are expressed in lower levels on neonatal naïve B cells compared to adult B cells [89]. In combination with immature B cell, dendritic cell and T cell interactions, B cell activation is limited [89]. This can affect the levels of activated B cells that proliferate, undergo somatic hypermutation, undergo affinity maturation and switch from IgM to IgG, IgA and IgE producing cells [89]. In addition, infant germinal center B cells favor the induction of memory B-cell responses over antibody-secreting plasma cells [89]. The reduction in plasma cells

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results in lower peak IgG titres. Specifically, plasma B cells exhibit limited IgG response to protein antigens under 12 months of age and polysaccharide antigens under 18-24 months of age [89].

5.4. The developing respiratory tract

The immunological landscape during infancy provides some context for the imprinting event but does not provide a complete physiological picture at the time of the first influenza infection or vaccination. It is necessary to also consider the features of the early respiratory tract, and how that may differ from older children and adults.

The respiratory tract is a major organ system that serves for gas exchange and respiration for vertebrate organisms. To provide efficient gas exchange, the lungs require high surface area and a mechanical force. The surface area allows for thee maximum gas to be exchanged while the mechanical force moves air in and out of the system [90]. Importantly, the continuous air flow allows ample opportunity for internal exposure to pathogens and other environmental threats. With this in mind, the respiratory system must also be able to respond to continual antigen exposure. For humans and other vertebrates including mice and ferrets, the respiratory tract is in an immature state at birth that requires additional development.

During embryogenesis, the conducting airways are the first to develop with the initiation of a lung bud and repetitive branching also called branching morphogenesis on each side [91]. Epithelial differentiation then occurs which allows the major formation and eventual enlargement of the gas exchange surface. The lungs are not fully developed at full gestation. Despite this, the lungs are still capable of gas exchange even prior to birth but may be the cause of respiratory vulnerability in the young [91]. Fetal lung development is characterized by three stages: the pseudoglandular stage, the conalicular stage, and the saccular stage, and alveolarization (the formation of the majority of the lung surface area for gas exchange). Importantly the alveolarization along with the microvascular of endothelial vessel maturation does not begin until week 36 of embryonic development and continues after birth [91]. Alveolarization specifically is the formation of the new inter-airspace walls, or the alveolar septa. The Alveolar septa divides the alveolar duct airspaces and respiratory bronchioles allowing more surface area to be developed. The resulting air spaces are the alveoli. In order to finish development for mature gas exchange and maximum surface area, the alveolar septa eventually thins out and microvasculature develops to provide a thin barrier and easy access from the blood to air within alveolar space. Recent studies have shown that alveolarization actually continues into young adulthood, and so alveolarization and microvascular maturation occur concurrently [91]. Considering that the lung is the site of antigen exposure and first immune interaction for respiratory infections such as influenza viruses, the immature state of the lung may also play a role in influenza imprinting during infancy and the acquisition of life-long immunity to specific influenza viruses.

6. Infant immune responses to vaccination

While we have discussed the development of the immune system through infancy, it is important to understand how this pertains to the infant response to influenza virus vaccination. If we are able to identify how an infant immune response to vaccination differs from an adult, infant-specific vaccines could be designed to target these possible limitations and elicit a more protective immune response.

During gestation, maternal influenza vaccination is shown to decrease the risk of influenza infection in infants up to 6 months post-partum, and protection does not seem to differ significantly with the timing of maternal vaccination [92]. Influenza vaccines are the most common vaccine administered during pregnancy [93]. At birth, infants are capable of protective immune responses to vaccination [84]. While vaccines preferentially elicit an IgG response, the predominant infant immune response is IgM [84]. The ratio of IgG2 to IgG1 is also much lower when compared to adults [84]. In terms of an antibody response, these responses before 12 months of age are usually shorted in duration [84]. It has also been shown that there is an age dependent increase in the seroconversion and antibody concentration in infants after vaccination [84]. There is an age dependent limitation of

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infant antibody response and the adult levels of the protective IgG and IgA are only reached at 12 months or later [84].

Due to the fact that interactions between antigen-presenting cells and T cells seem to be suboptimal during infancy, efficient immune response to vaccination requires the use of specific adjuvants or delivery systems [84]. Specifically, the infant immune system can respond appropriately to protein antigens (*i.e.*, immune responses that require T cell help), but have limited responses to carbohydrate (T-cell independent) antigens [11]. Thus IIV and subunit vaccines have limited efficacy in infants – this has been overcome by the use of conjugate vaccines to engage infant T helper cells [82]. Influenza vaccination in infants and young children induces a dampened immune response in terms of cell reactivity and response magnitude, where immunity often wanes within months of vaccination [57,60]. Conversely, the LAIV platform has been shown to be most effective in the younger age groups [94]. This may possibly be due to the live viruses present in the vaccine formulation as well as the presentation directly to the respiratory tract [51]. More work needs to be done to determine if vaccination in the young with LAIV can also lead to imprinting.

Vaccination in infants does not elicit the same level of protective immune response as vaccination in adults. It will be important to consider possible mechanistic differences that lead to the different vaccination response, and to do so, proper animal models for infant immune studies must be established.

7. Modeling influenza virus infection and vaccination

Experimental animal studies have been the cornerstone for biological discoveries. We have limited ability to conduct ethical and controlled experiments in humans, and even less ability in infant humans. The respiratory tract as well as the immune system of infants is significantly different from that of the adult, which are the probable causes of differing responses during infection and vaccination. [95]. To explore the differences between adult and infant immune responses during influenza virus infection, researchers have often turned to the use of infant animal models to gain insight into the severe respiratory disease of infants. With the use of infant animals, the nuances of adaptive immune regulation such as the imbalance of Th2 responses can be discovered through blood and tissue collection and subsequent leukocyte population and immune mediator analysis. Below we review the work of investigators using infant animal models such as the mouse and ferret for influenza virus infection studies and highlight the experimental specifics. The purpose of this section is to bring to light the use of infant animal models to be used for future imprinting studies and development of immune memory for possible vaccine development.

7.1. Mouse Models

While the use of infant animals for influenza virus infection and vaccination research has not been standardized, mouse models are the main experimental animal for other immune studies. Mice are preferred due to their small size, relatively low main maintenance cost, and ability to reproduce quickly [96]. The gestation of a pregnant mouse is typically 21 days, and after birth neonatal and young mice are milk fed from their mothers until weaning at 3-4 weeks old [97]. Similarly to humans, mice must undergo additional development of the immune system and respiratory system after birth since they are born with some limitations [98]. For example, at birth, T cells in humans and rodents have reduced ability to help immunoglobulin production due to reduction in IL-2, IL-4, and INF-gamma secretion [98]. In respect to B cells in humans and rodents, both heavy and light chain rearrangement has been shown to be fully intact in the fetal spleen but with reduced expression of lymphopoiesis enzymes [98]. Notably, circulating IgM concentrations are reduced to 10% of that of the adult reaching adult levels of IgM at 2 years and IgG at 4-6 years in humans [98]. Although there are many similarities between human and mouse immune systems, there are also differences that should be taken into consideration when designing infant mouse experiments. For example, unlike humans, it has been noted that mice have very reduced lymphocyte levels at birth [99,100] and the T cells seem to have a memory phenotype. Another element that must be considered is the corresponding ages of infant mice and infant humans. The 3-day-old mice neonates are considered

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representative of late term human neonates of 22-26 weeks gestation [101,102]. Therefore, many infant influenza studies using the mouse model have studied mice in the neonatal phase 2-7 days post-birth which is actually modeling humans in the second trimester of development [102–106]. It has also been reported that the mouse immune makeup at two weeks postpartum most resembles that of the infant human [107], and thus many other infant mice model studies of influenza virus infection have been done at two weeks postpartum [107].

With this in consideration, the use of neonatal and infant mice has been instrumental in learning about the infant immune responses post influenza virus infection. Firstly, results have suggested that neonatal/infant mice are able to clear low levels of viral infection by immune responses regulated differently than compared to adults. Specifically, γδ T cells have been shown to protect neonatal mice against mortality following influenza virus infection [103]. In one study, wild-type 7-day old mice infected with A/HKx31 (H3N2) influenza virus recovered from intranasal infection, while γδ T cell deficient neonatal mice had a greater percentage succumb to infection [103]. Further investigation suggested that the γδ T cell protective role is due to IL-17A secretion, which contributes to the infiltration of group 2 innate lymphoid cells and regulatory T cells that can promote lung repair during neonatal influenza virus infection [103]. Another study suggested that infant mice, 2 weeks postpartum, had decreased ability to generate tissue-resident memory (TRM) T cells following influenza virus infection despite a robust CD4+ and CD8+ respiratory localized response for virus clearance [107]. This is interesting because adult mice are able to elicit lung-localized TRM T cells following respiratory virus infection or vaccination, which allows for a rapid response to secondary challenge [108,109]. Another study observed differences in the migration of T cells into the murine respiratory tract following influenza virus infection in infants compared to adults [104]. In this study, mice were infected at 2 days old, differing from the 2-week-old mice in the previous study. Despite the difference in mouse ages, the young mice had decreased ability to establish TRM T cells due to decreased access of T cells to the lung alveoli and instead remained in the interstitium. The lack of migration of T cells into the airways in infants may possibly be due to differential regulation of CXCR3 ligands CXCL9 and CCL2, which were detected in the adult lung but not in the neonatal lung [104]. Neonatal mice infected with influenza virus have also been shown to have increased expression of CD31 on T cells, which was shown to inhibit T cell activation in the lung compared to adults [105]. These studies may have shed some light on the mechanisms of influenza virus imprinting. Follow up on why infant mice have an absence of localized memory responses should be viewed in relation to influenza imprinting and the establishment of a broader peripheral immune memory.

In addition to the above work, studies have shown that neonates have an increased ability to develop inducible Bronchus Associated Lymphoid Tissue (iBALT) within the lungs [15]. iBALT is an ectopic tertiary lymphoid tissue that forms in the lung following respiratory insult and serves as an area for local antigen capture and T and B cell stimulation [20]. It is composed of follicular dendritic cells, resident dendritic cells, high endothelial venules and lymphocytes, and has previously been shown to be established in infants during respiratory infection. A study using neonatal mice showed that IL-17A producing T cells were essential for iBALT establishment in the neonatal lung after antigen exposure [15]. Interestingly, the number of T cells and B cells in the alveolar space were decreased in neonatal mice and the lymphocytes were organized in the iBALT structure in the alveoli [15].

Looking at these studies together, there is much evidence to support the decreased activity, recruitment, and memory establishment of T lymphocytes in infants following influenza virus infection in the mouse model. These studies show how the infant regulates the immune response to prevent an overactive filtration of T cells into the delicate immature lung. We first recognize from this review of the literature that there are few studies investigating the humoral response and antibody evolution following influenza virus infection in the infant mouse model. Furthermore, we see a trend of decreased T cell immune responses as expected from what is understood of the Th2 polarization of the infant immune system. It is also evident that there is decreased recruitment of T cells into the alveolar space, which may be a clue to the establishment of influenza imprinting. The connection of these mechanisms inhibiting T cell recruitment and retention in central immunity to the development of broad influenza virus immunity and viral imprinting should be investigated.

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7.2. Ferret Model

The ferret model has also been used to investigate age-related host response and disease severity to influenza virus infection both for the old [110] and young [17,18,111,112]. Ferrets were first used to investigate infant responses to influenza virus infection in the late 1970s by a group from the University of Birmingham in the United Kingdom [19,113–117]. In adult ferrets, influenza virus infection with seasonal influenza virus strains typically leads to a non-fatal disease characterized by weight loss, temperature increase, and respiratory symptoms including coughing and sneezing [118,119] at varying degrees dependent on the strain. In infant ferrets, studies have shown a trend that suggests that influenza disease severity is dependent on ferret age throughout immaturity prior to adulthood. As we examine the following studies using the ferret model, we will see how this model has contributed to a better understanding of the infant response to influenza virus infection.

Firstly, a study by Collie and colleagues found that seasonal influenza virus infection with a recombinant H3N2 virus strain led to mortality in newborn ferrets, significant viral replication within the respiratory tract, and evidence of severe respiratory pathology including collapsed alveolar spaces and necrotizing bronchiolitis [117]. In a follow up study that compared influenza illness in newborn ferrets to 15-day old suckling ferrets, it was found that while newborn ferrets succumb to illness, 15-day old ferrets seemed resilient to fatal disease and developed pathology similar to adults [120]. Furthermore, the increased disease severity in infants was suggested to be due to an increased proportion of ciliated epithelium-lined airway when compared to the adult and 15-day old ferret lungs [19]. This suggested a rapid development of the ferret respiratory tract, which may influence influenza severity. In our studies, using infant (4-week old) and newly-weaned (8-week old) ferrets inoculated with the 2009 H1N1 pandemic virus, it was found that infant ferrets had a 100% mortality rate while the 8-week old newly weaned ferrets did not display any significant clinical symptoms [17,18]. Pathological and virological assessments of the respiratory tracts indicated similar levels of replicating virus but the infant ferret lungs had evidence of pathology with significant T cell infiltration into the submucosal glands [17]. Interestingly, the newly weaned animals had minimal signs of leukocyte infiltration into the respiratory tract. Clear alveolar spaces were noted with structures surrounding the bronchi similar to iBALT with organized T cell and B cell zones. This organization has also been observed in infant respiratory infection and insult [18,20]. Similar results were found in another study inoculating the newly weaned age group with pandemic H1N1 influenza A virus strains [112]. Taken together, these studies suggest that due to the respiratory tract development in ferrets post-partum, the outcome of influenza virus infection may be significantly dependent on the age of the ferret by week postpartum. It is known that the ferret respiratory tract is not fully developed until at least 8 weeks of age which coincides with weaning. Considering the direct susceptibility of the ferret to human strains of influenza viruses, the newborn, infant, and newly weaned ferret may serve as an appropriate model for determining strain and age-specific outcomes of new influenza virus strains and the understanding of imprinting mechanisms.

The purpose of this section was to highlight the importance of using appropriate animal models for influenza virus infection studies. After reviewing studies conducted in both infant mice and ferrets, it is clear that these models are able to provide valuable information regarding the infant immune system but more development needs to be done. We need an infant animal model that shares the unique immune features of a human infant in order to understand the influenza virus imprinting event so we can design vaccines that better cater to the infant immune response.

Concluding statements

Infant immune systems and respiratory tracts differ significantly from those of adults. Infants and young children have increased rates of influenza virus infection, hospitalization, and mortality. Due to some of these immune differences, current vaccination strategies are not appropriate for infant use, and others result in dampened immune responses in infants, providing inadequate protection to future influenza virus infection. In this review, we outlined the importance of the imprinting event and how this significant event can shape the immune response in future infections. Currently there is no standardized animal model for infant influenza and immune research. Both mice and ferrets

have been used in these previous studies. There are some gaps as the models primarily examine cell-mediated immunity rather than humoral immunity and many of the studies are focused on infection rather than vaccination in infants. In order to move forward, studies investigating infant immune response to vaccination should be conducted in infant animal models of appropriate age as well as in humans. Understanding how vaccination can serve as the imprinting event will inform the development of more efficacious vaccines and better infant vaccination policies. This is necessary work if we wish to keep infants as safe as possible during seasonal influenza epidemics and may also provide keys to developing a broadly reactive and long-live universal influenza vaccine that will continue to provide protection into adulthood.

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