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## Chapter 59

# Respiratory Virus Vaccines

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### INTRODUCTION

As we breathe, we sample an estimated 10L of air per minute (Kohlmeier and Woodland, 2009). The mucosa of our respiratory system is, therefore, in direct and continual contact with the environment and, as such, is highly exposed to microorganisms, some of which may be pathogenic. Respiratory infections are among the leading causes of acute illness and mortality worldwide, being responsible for nearly 4 million deaths annually, the majority of which occur in infants and children in developing countries

(Girard et al., 2005). The main viruses responsible for acute respiratory infection include respiratory syncytial virus (RSV), influenza virus, human parainfluenza virus (HPIV), human metapneumovirus (HMPV), human rhinovirus (HRV), coronaviruses, and adenoviruses. However, despite the public health importance of these infections, licensed vaccines are currently available only for influenza viruses.

Protective immunity against respiratory virus infection is a complex interplay between systemic and mucosal responses. However, immune responses generated during a

natural infection may not provide complete protection from reinfection and may actually contribute to the pathogenesis of disease (reviewed in [Sections Pathogenesis and Immune Responses to Respiratory Virus Infection](#)). Vaccine-induced immune responses must, therefore, aim to be more protective and less pathogenic than those induced naturally. In addition, our understanding of the relative contribution of mucosal and systemic immunity to protection remains incomplete. For example, it is well known that inactivated vaccines against influenza given intramuscularly (i.m.) are protective owing to the induction of systemic humoral immunity in the absence of a robust mucosal immune response, and current guidelines for vaccine licensure require influenza vaccines to induce systemic immune responses. However, it is also evident that some intranasal vaccines are protective owing to the induction of mucosal immunity, despite less impressive systemic immune responses (reviewed in [Section Respiratory Virus Vaccines](#)). Unfortunately, standardized methods of measuring mucosal immune responses are lacking, and reliable correlates of protection for vaccines that protect through mucosal immunity have not been identified. In this chapter, we review the main viral pathogens of the respiratory tract, the immune responses they induce, current vaccines, and vaccines that are in development to control them.

## VIROLOGY

The viruses that infect the respiratory tract belong to various families and vary in their genome composition, the presence or absence of an envelope, and their replicative cycles.

### Paramyxoviruses

The majority of respiratory viruses that are responsible for acute respiratory infection belong to the *Paramyxoviridae* family and include RSV, HPIV, and HMPV. These viruses infect cells lining the respiratory tract by first attaching to the cell through the interaction of viral envelope glycoproteins, with one or more cellular receptors in the host cell plasma membrane. For example, the HPIV envelope protein HN binds sialic acid residues extending from host cells ([Schomacker et al., 2012](#)), and the G protein of RSV and HMPV binds to glycosaminoglycans (GAGs) that comprise long chains of disaccharides that form part of the cellular glycocalyx ([Feldman et al., 1999](#)). The RSV and HMPV F protein are also known to bind GAGs, and findings indicate that the F protein of these viruses is involved in attachment by interacting with the cellular proteins nucleolin and integrin  $\alpha\beta 1$ , respectively ([Tayyari et al., 2011](#); [Cseke et al., 2009](#)). Upon binding to the host cell, the F protein undergoes a conformational change that exposes a hydrophobic fusion peptide, which is responsible for the fusion of the paramyxovirus envelope and the host cell plasma

membrane. After viral fusion, the genome is released into the cytoplasm, viral genes are transcribed, and viral genomes are replicated ([Collins and Crowe, 2007](#); [Collins and Melero, 2011](#)).

The paramyxovirus genome comprises single-stranded, negative-sense, nonsegmented RNA. The viral RNA (vRNA) must first be transcribed into positive-sense messenger RNA (mRNA) before viral proteins can be translated by the host cell machinery. This is achieved by a viral RNA-dependent RNA polymerase (the large, L, protein) that is packaged into the virion and enters the host cell upon infection. The L protein is also responsible for genome replication, in which positive-sense complementary RNA (cRNA) serves as an intermediate template for the production of vRNA. An essential cofactor for the L protein is the phospho (P) protein, which tethers the polymerase so it can reach the bases in the vRNA and also binds the N protein, which encapsidates the vRNA and cRNA. There is also evidence that transcription is enhanced by the M2-1 protein and that the switch from transcription to replication is mediated by the M2-2 protein ([Collins and Crowe, 2007](#); [Collins and Melero, 2011](#)).

Once transcribed, viral structural proteins assemble and newly synthesized viral genomes are packaged into virions that bud from the plasma membrane. The matrix, or M protein, lines the inner surface of the viral envelope and may play a role in budding ([Henderson et al., 2002](#); [Teng and Collins, 1998](#)). In addition, the HN protein of HPIV is also involved in budding and in clearing sialic acid residues from the plasma membrane ([Karron and Collins, 2007](#)).

To complete the replication cycle, paramyxoviruses have evolved multiple mechanisms to prevent the activation of cellular defenses in response to infection, such as the nonstructural (NS) proteins 1 and 2 of RSV ([Collins and Crowe, 2007](#)) and the C or V proteins of HPIV ([Karron and Collins, 2007](#)).

One additional protein found in paramyxoviruses is the short transmembrane glycoprotein (SH) that is anchored to the envelope and shares structural features with viroporins, a group of hydrophobic molecules that insert into the membrane of infected cells and increase their permeability to small molecules and ions ([Gonzalez and Carrasco, 2003](#)).

### Influenza Viruses

The *Orthomyxoviridae* family includes influenza viruses, which bind to terminal sialic acid–galactose linkages by the hemagglutinin (HA) envelope glycoprotein. Orthomyxovirus attachment to the host cell initiates receptor-mediated endocytosis and endosome acidification. Protons are permitted to enter the influenza virion via the M2 ion channel, and acidification results in a conformational change in the HA protein, revealing the fusion peptide that initiates membrane fusion between the viral envelope and the endosome membrane

(reviewed by Palese and Shaw (2007)). The HA is synthesized as a precursor (HA0) that is cleaved into its active form (HA1 and HA2) by cellular proteases, and the amino acid sequence at the cleavage site determines the type of protease that is able to activate the HA. If trypsin-like proteases are required for cleavage, the virus is limited in its tissue tropism to the respiratory tract of humans and the gastrointestinal tract of birds; however, the presence of multiple basic residues at the cleavage site extends the range of proteases that can cleave the HA, resulting in a disseminated, often lethal, infection in poultry (Wright et al., 2007b).

Once the virus envelope has fused with the endosome, the influenza genome enters the cytoplasm. The orthomyxovirus genome comprises seven or eight segments of single-stranded, negative-sense RNA, and each segment encodes one or more proteins. Each segment is encapsidated in nucleoprotein (NP) and forms a panhandle comprising the 5' and 3' ends, to which a polymerase complex is attached. Together, these are known as the viral ribonucleoprotein complex. In the nucleus, orthomyxovirus vRNA is either transcribed into mRNA or replicated by means of a positive-sense cRNA intermediate. Viral mRNA molecules exit the nucleus and are translated in the cytoplasm by the host cell machinery. Structural proteins assemble at the plasma membrane, where newly synthesized viral genomes are packaged and virions bud (Palese and Shaw, 2007). How the individual segments traffic to the plasma membrane and are packaged remain active areas of research. The matrix (M1) protein lines the virion beneath the envelope and may be important for morphology and viral assembly at the plasma membrane. In addition, the neuraminidase (NA) protein permits budding by cleaving sialic acid residues from the host cell plasma membrane (Palese and Shaw, 2007).

To complete the replication cycle, influenza viruses inhibit interferon (IFN) production and signaling. This is achieved by the NS protein 1 (Palese and Shaw, 2007) (Section Adenoviruses).

## Coronaviruses and Rhinoviruses

The genomes of coronaviruses and rhinoviruses comprise positive-sense, single-stranded RNA that can be translated by the host cell machinery in the cytoplasm (Kennedy et al., 2012). Coronaviruses, which belong to the *Coronaviridae* family, are enveloped and attach to host epithelial cells by the spike (S) envelope proteins (Blau and Holmes, 2001). Fusion occurs at the plasma membrane, or after endocytosis, and the genome is translated into a polyprotein, which is then posttranslationally processed into structural proteins that form viral particles and nonstructural proteins that aid in viral genome replication (Lai et al., 2007).

Rhinoviruses, which belong to the *Picornaviridae* family, are not enveloped and instead have a capsid of icosahedral symmetry comprising four proteins, VP1–4 (reviewed

by Greenberg (2011), Kennedy et al. (2012)). The majority of rhinoviruses bind to intercellular adhesion molecule-1 (ICAM-1) (Greve et al., 1989), and binding leads to a conformational change in the capsid that creates a pore, through which the genome enters the cytoplasm to be translated and replicated (Bella and Rossmann, 2000).

## Adenoviruses

Adenoviruses are nonenveloped and possess a capsid of icosahedral symmetry. At each of the 12 corners, a fiber protrudes from the capsid that makes contact with the host cell receptor to initiate receptor-mediated endocytosis. Acidification of the endosome results in conformational changes in the capsid that lead to viral uncoating and the release of the double-stranded DNA genome into the cell. The genome is transported into the nucleus, where it is transcribed into RNA, which is alternatively spliced into monocistronic mRNAs that are translated by the host cell machinery into early gene products. Early gene products remodel the intracellular environment to favor viral replication and are responsible for viral replication. The late phase of the viral life cycle is concerned with the production of structural proteins in sufficient quantities to ensure adequate packaging of the newly synthesized genomes and maximizing the production of progeny virions, which are released by cell lysis (Berk, 2007).

## CLINICAL FEATURES AND EPIDEMIOLOGY

Respiratory viruses can infect various parts of the respiratory tract and cause a range of illness. Mild upper respiratory tract (URT) infection (URTI) can be complicated by sinusitis or otitis media, and a lower respiratory tract (LRT) infection (LRTI) can lead to bronchiolitis or pneumonia and possible postinfectious respiratory complications such as sensitization to asthma. The major public health impact is from LRTIs, and RSV is responsible for the majority of cases in infants. HMPV, HPIV, and influenza can also lead to LRTI, with HPIV3 and HMPV affecting infants almost as young as those afflicted with RSV, whereas HPIV1, HPIV2, and influenza are often diagnosed in children 6 months of age or older. RSV and influenza are also recognized as an important cause of LRTIs in the elderly and in those with cardiopulmonary disease or immunosuppression (Schmidt, 2011). Moreover, influenza pandemics occur at irregular and unpredictable intervals with widespread morbidity and mortality and economic consequences. In addition, although there have been no cases of severe acute respiratory syndrome (SARS) since 2004, several novel coronaviruses have been identified, including the virus responsible for Middle East respiratory syndrome (MERS) (Zaki et al., 2012). Given the clinical significance of these infections, and the fact that licensed vaccines are available only for influenza viruses, there is an unmet need for vaccines.

## RSV

Humans are the only natural host for RSV, with infections occurring in annual epidemics during winter and spring months in temperate climates and the rainy season in the tropics (Girard et al., 2005). The virus is highly contagious, with most children being infected in the first year of life. The peak of severe disease usually occurs before 6 months of age, with the peak incidence of hospitalization in 2- to 3-month-old infants (Collins and Melero, 2011). Reinfection is also common. In one study, among children who had been infected in their first year of life, 47% were reinfected in their second year, and 45% in their third year of life (Glezen et al., 1986). Moreover, reinfection is independent of antigenic changes in the virus, implying that the protective immunity mounted during an infection does not protect against subsequent reinfection (Collins and Melero, 2011). This is of note when attempting to induce protective immune responses by vaccination.

Globally, there were an estimated 34 million cases of LRTI caused by RSV in children under 5 years of age in 2005, with 10% requiring hospitalization (Martinez et al., 1997). In the United States, one study estimated that 2.1 million children under 5 years of age require medical attention each year owing to RSV (Botosso et al., 2009), and another study estimated that RSV was responsible for 18,000–75,000 hospitalizations per year (Girard et al., 2005). In the United Kingdom, the total annual incidence of hospitalization attributed to RSV was 28.3 per 1000 children under 1 year of age and 1.3 per 1000 children between 1 and 4 years of age (Waris, 1991). More than half of the hospitalizations for RSV occur in previously healthy, full-term infants, and children who experienced severe LRTI caused by RSV were at increased risk of wheezing and asthma later in life (Girard et al., 2005).

Pediatric mortality from RSV in the United States was estimated to be between 5.3 per 100,000 per year in infants under 1 year of age and 0.9 per 100,000 per year in children 1 to 4 years of age in one study (Cooney et al., 1975), and in another U.S. study, RSV was estimated to be responsible for 90–1900 deaths per year (Girard et al., 2005). However, an estimated 99% of RSV-associated deaths occur in developing countries, possibly because of limited health-care resources (Martinez et al., 1997).

In healthy adults, reinfection rates are approximately 5–10% per year, though hospitalizations are rare. Morbidity and mortality are more pronounced in the elderly and it has been estimated that RSV causes an average of 17,358 deaths annually in the United States, with 78% occurring in individuals over 65 years of age (Cooney et al., 1975). In addition, the cost of caring for patients with severe LRTI from RSV and its sequelae are substantial (Girard et al., 2005).

RSV infection induces antibodies against the two main antigens, the F and G envelope glycoproteins. The

G protein is the most variable protein in RSV and is the basis for the separation of strains into two antigenic groups (A and B). Moreover, sites of positive selection that partially coincide with epitopes recognized by anti-G-protein monoclonal antibodies (mAbs) suggest immune-driven RSV evolution (Botosso et al., 2009). However, most anti-G mAbs do not neutralize infectivity (Martinez et al., 1997) and the selection pressure is, therefore, weak. This favors a slow coevolution of several RSV lineages, and multiple genotypes within each group can cocirculate within the same season, with shifts in the predominance of groups A and B occurring in 1- to 2-year cycles (Waris, 1991). In contrast, the sequence of the F gene is highly conserved among RSV isolates, despite the identification of a number of neutralizing mAbs against the protein that should impart a selection pressure for mutation (Collins and Melero, 2011). This implies that the function of the F protein confers structural restrictions that may limit antigenic diversity.

## HPIV

Human parainfluenza viruses are also a common cause of acute respiratory infection, with 80% of children seropositive by 5 years of age (Cooney et al., 1975). As with RSV, reinfection is common (Schomacker et al., 2012). There are four serotypes of HPIV (HPIV 1–4), with each serotype associated with a broad spectrum of upper and lower respiratory symptoms. HPIV1 and 2 are, however, more frequently associated with croup (laryngotracheobronchitis), and HPIV3 is more likely to cause bronchiolitis, pneumonia, and LRTI resembling disease caused by RSV (Schomacker et al., 2012). HPIV4 is a less frequent cause of clinically significant disease, though a study found HPIV4 in 10% of HPIV-positive samples in a day-care setting (Fairchok et al., 2011).

HPIV LRTI is a major cause of hospitalization in children under 5 years of age, second only to RSV, though infection is usually self-limiting and rarely fatal, unless an individual is immunosuppressed. Severe infection may have long-term effects on lung function, but this remains unclear (Schomacker et al., 2012).

## HMPV

HMPV belongs to the same subfamily as RSV, and two major groups (A and B) and four minor subgroups (A1, A2, B1, and B2) have been identified, based on sequence variability in the G and F glycoproteins (Kroll and Weinberg, 2011). As with RSV, and HPIV, by 5 years of age, most children will have been infected with HMPV, and reinfections are common (Kroll and Weinberg, 2011). The virus also has a seasonal distribution, with the main occurrence in winter and spring (Kahn, 2006).

HMPV typically leads to flu-like symptoms in otherwise healthy adults, but is responsible for 5–15% of hospitalizations for LRTI in children and can lead to severe disease in the elderly or immunocompromised hosts (Papenburg and Boivin, 2010; Boivin et al., 2007).

## Influenza Viruses

Influenza viruses are divided into types A, B, and C based on antigenic differences in the NP and M genes. Influenza A viruses are the most clinically significant and are divided into subtypes based on antigenic differences in the HA and NA genes. To date, 16 HA and 9 NA subtypes have been identified from waterfowl (Palese and Shaw, 2007) and 17th and 18th subtypes of HA have been identified from bats in Guatemala and Peru (Tong et al., 2012, 2013).

Influenza viruses cause a spectrum of clinical illness associated with infection of the upper and lower respiratory tract, with more severe disease associated with LRTI. The viruses are spread by respiratory droplets or direct contact. Annual influenza epidemics have a seasonal distribution, with the main occurrence in winter months (seasonal influenza) in temperate climates (Girard et al., 2005). However, unlike RSV, HPIV, and HMPV, influenza A viruses have a broad host range that includes birds, pigs, dogs, horses, marine mammals, and humans, with the main reservoir for infection being aquatic birds (Palese and Shaw, 2007; Wright et al., 2007b). This broad host range, together with the segmented nature of the influenza virus genome, makes the epidemiology of influenza complex and gives rise to zoonotic infections and pandemics.

Pandemic influenza can arise if a novel virus emerges that readily transmits from person to person and if the majority of the population is susceptible to infection. If an avian or animal virus crosses the species barrier to circulate in humans, the population will probably be immunologically naïve and, therefore, susceptible to infection. However, the virus must be able to transmit efficiently from person to person for a pandemic to occur.

As the influenza virus genome is segmented, if a host is infected with two or more influenza viruses, the potential exists for the gene segments to reassort, such that a progeny virus containing genes from each parent virus can be produced (Wright et al., 2007b). If a virus that circulates within the human population reassorts with one that is novel for humans, the resultant virus may possess genes that allow it to replicate efficiently in humans, but with glycoproteins to which the population is immunologically naïve, and a pandemic could occur. The introduction of a virus with a novel HA subtype into the human population is known as “antigenic shift” (Wright et al., 2007b).

Three global influenza pandemics were recorded in the twentieth century from viruses of the subtypes H1N1, H2N2, and H3N2, respectively. In 2009, another pandemic H1N1

(pH1N1) influenza virus emerged in Mexico (Girard et al., 2010). This H1N1 virus was antigenically unrelated to previously circulating seasonal H1N1 viruses, and molecular studies revealed that it was a reassortant with genes derived from viruses that had been circulating in pigs: the North American H3N2 triple-reassortant, the classical swine H1N1, and the Eurasian “avian-like” swine H1N1 viruses. In most countries, the median age of infection during the 2009 pandemic was estimated to be 12–17 years, and in most individuals, infection led to a mild, self-limiting URTI. However, 2–5% of confirmed cases in the United States and Canada required hospitalization, and the case-fatality rate was 0.15–0.25%. Moreover, nearly one-third of the fatalities among hospitalized patients occurred in previously healthy individuals (Girard et al., 2010).

After each pandemic, the newly emerged subtype became established and caused annual seasonal influenza epidemics. In the United States, it has been estimated that 25–50 million cases of influenza occur annually, with approximately 225,000 requiring hospitalization (Lambert and Fauci, 2010). Current vaccines are aimed at the circulating H1N1 and H3N2 subtypes of influenza A and the predominant circulating strain of influenza B and are therefore trivalent vaccines. However, two antigenically distinct lineages of influenza B viruses (Victoria and Yamagata) cocirculate, and the World Health Organization recommended that influenza vaccines should contain both of these lineages. Clinical trials of quadrivalent vaccines containing the H1N1 and H3N2 influenza A viruses and the Victoria and Yamagata influenza B viruses have been conducted, and their use in the United States received an interim recommendation of the Advisory Committee on Immunization Practices for the 2013–2014 influenza season (Dolin, 2013).

Owing to the low fidelity of the RNA-dependent RNA polymerase of influenza, and immune selection pressure on the HA protein, viral replication can yield a quasi-species that may differ antigenically from the parent virus. Therefore, each season, the predominant circulating strain may be antigenically distinct from the previous year. This phenomenon is known as “antigenic drift” and leads to a need to update the influenza vaccine annually (Wright et al., 2007b).

Sporadic infections by H5N1, H9N2, H7N7, and H7N9 subtypes of influenza have been reported in humans who were in close direct contact with infected animals. Additionally, H3N2 variant viruses have infected humans, the majority of whom were in close contact with pigs (Epperson et al., 2013). So far, there has been limited transmission of these viruses between people, though there is concern that they may acquire mutations or gene segments that allow efficient spread from person to person.

## Coronaviruses

Coronaviruses are frequent causes of the common cold, causing URTIs throughout the world, in all age groups, leading

to millions of days of work and school absence, physician visits, and frequent inappropriate antibiotic use (Greenberg, 2011). Coronaviruses are transmitted by respiratory droplets and are reported to cause 7–30% of common colds, with a peak prevalence in late fall, winter, and early spring.

The first human coronaviruses (HCoV) to be recognized as significant respiratory pathogens, HCoV-229E and OC43, were identified in the 1960s (Greenberg, 2011). Whereas infection with the majority of coronaviruses is associated with self-limiting URT symptoms in otherwise healthy individuals, a coronavirus was identified as the agent responsible for SARS in 2003 (Drosten et al., 2003; Ksiazek et al., 2003). The SARS coronavirus (SARS-CoV) emerged in the Guangdong province of China in November 2002 and spread to 32 countries, leading to 8096 cases and 774 deaths worldwide by the time the outbreak was brought under control in June 2003 (WHO, 2004). Subsequently, heightened international surveillance for coronaviruses led to the identification of the strains HCoV-NL63, NH, and HKU1 in 2004–2005 (Greenberg, 2011) and the MERS coronavirus (MERS-CoV) in 2012 (Zaki et al., 2012). MERS-CoV was identified in 699 individuals between September 2012 and June 2014 with 209 fatalities (WHO, 2014).

## Rhinoviruses

Previously, HRVs were classified into two species, HRV-A (containing 75 serotypes) and HRV-B (containing 25 serotypes). In 2009, a novel species, HRV-C, was identified, which contains at least 50 serotypes (Jacobs et al., 2013). HRVs are spread by direct contact, hand-to-hand contact or aerosols. Traditionally, they have been associated with a URTI, causing between 25 and 50% of common colds (Makela et al., 1998). However, they are increasingly recognized as a cause of LRTI, particularly in patients with asthma and in infants, the elderly, and immunocompromised individuals (Jacobs et al., 2013). Bronchiolitis is a common clinical manifestation in hospitalized children infected with HRV, and HRV is also a common pathogen in viral community-acquired pneumonia. HRV has also been associated with exacerbations of asthma and chronic obstructive pulmonary disease (Jacobs et al., 2013).

## Adenoviruses

As of this writing, 52 serotypes and seven species of adenovirus have been identified. Tissue tropism and clinical manifestations vary between the serotypes, and the viruses are responsible for both febrile respiratory disease and gastrointestinal illness (reviewed by Lynch et al. (2011)). Adenoviruses are estimated to be responsible for 5–10% of pediatric and 1–7% of adult respiratory tract infections (Ison, 2006; Lee et al., 2010). They are spread primarily via respiratory droplets, direct contact, or fomites,

and more than 80% of cases are in children under 4 years of age (Lynch et al., 2011). However, epidemics have also been described in children and adults, especially in military recruits in closed or crowded settings. Most individuals develop a self-limiting URT infection that may be asymptomatic, but conjunctivitis, tonsillitis, otitis media, or croup can occur. Infection can also spread to cause bronchiolitis or pneumonia or disseminate to cause viral meningitis or encephalitis that can be fatal (Lynch et al., 2011).

## PATHOGENESIS

There are many factors that determine the pathogenesis of disease and clinical outcome, including factors pertaining to the virus, host genetics, host immune responses, and the environment.

### Viral Factors

The site of viral replication may influence the pathogenesis of disease and outcome of infection. For example, seasonal influenza viruses usually infect the epithelium of the URT, which is consistent with the most common clinical manifestations of seasonal influenza, whereas highly pathogenic avian influenza viruses of the H5N1 subtype show a stronger tropism for the LRT than for the URT (Kuiken et al., 2012). H5N1 viruses attach abundantly to “Clara” or club cells lining the bronchioles, type II pneumocytes lining the alveoli, and alveolar macrophages in the alveoli, consistent with the clinical manifestation of diffuse alveolar damage (Kuiken et al., 2012). RSV also targets both type I alveolar and nonbasilar epithelial cells and possibly alveolar macrophages, which may contribute to LRTI (van Drunen Littelvan den Hurk and Watkiss, 2012).

Tissue tropism is determined, in part, by the receptor preference of the virus. For example, cells lining the URT of humans predominantly possess sialic acid residues with a terminal  $\alpha 2, 6$  linkage to galactose, whereas cells lining the human LRT have both  $\alpha 2, 3$  and  $\alpha 2, 6$  linkages (Shinya et al., 2006). It is believed that the ability of an influenza virus HA to preferentially bind  $\alpha 2, 6$ - or  $\alpha 2, 3$ -linked sialosaccharides therefore partly determines the tissue tropism and hence the clinical outcome.

When host cells are infected, type I IFN and proinflammatory cytokines are expressed, cellular translation is suppressed, and an antiviral state is induced (discussed in Section Immune Responses to Respiratory Virus Infection). However, most viruses that infect the respiratory tract modulate the host response to infection by blocking IFN activation and/or signaling and inhibiting apoptosis. This prevents the host from effectively clearing virally infected cells and inducing an antiviral state in neighboring cells, thereby promoting viral replication in infected tissues that may contribute to the observed pathology. RSV is the most

effective paramyxovirus at subverting host cell responses, inhibiting apoptosis and type I IFN production and signaling by means of the NS1 and 2 proteins, inhibiting nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation through the binding and sequestration of cellular protein kinase R (PKR) by the viral N protein, and inhibiting the production of stress granules that can restrict viral replication (Collins and Melero, 2011). Influenza A viruses encode the NS1 protein that downregulates IFN production (Palese and Shaw, 2007), and HPIV encodes either a C protein or a V protein that suppresses IFN induction and signaling (Karron and Collins, 2007), whereas SARS-CoV encodes eight proteins that antagonize the IFN response by a variety of mechanisms (Totura and Baric, 2012).

In addition to blocking IFN activation and signaling, viruses employ other means of ensuring their replication in the face of host immunity. The RSV G protein is highly glycosylated, a feature that may inhibit the binding of antibodies, and the protein is highly variable, enabling a substantial population of immune-escape mutants to be produced during infection. Additionally, a truncated, soluble form of the G protein is produced during infection, which acts as a decoy antigen that can bind RSV-specific antibodies, thus reducing their availability to neutralize virus. RSV also infects antigen-presenting cells (APCs), such as dendritic cells (DCs), and can affect their maturation and antigen-presentation functions and can lead to dysregulation of adaptive immune responses (van Drunen Littel-van den Hurk and Watkiss, 2012).

A number of virulence determinants have also been identified in influenza viruses, including the HA and the polymerase complex. For example, the presence of a multibasic site in the HA gene renders viruses highly pathogenic in poultry, as they are able to replicate systemically (Bosch et al., 1981; Kawaoka and Webster, 1988). In addition, substitution of a glutamic acid (E) residue for a lysine (K) residue at amino acid position 627 in the PB2 protein (E627K) of the polymerase is associated with altered host range (Subbarao et al., 1993) and virulence in humans and in mice that are experimentally infected with avian H5N1 and H7N7 viruses (Hatta et al., 2001; Munster et al., 2007; Subbarao et al., 1993). In the absence of the E627K mutation, a PB2 D701N mutation is also correlated with increased virulence (Li et al., 2005), and in the absence of both the PB2 E627K and the D701N mutations, PB2 590S and 590R were found to contribute to 2009 pH1N1 influenza virus replication and virulence (Mehle and Doudna, 2009). In addition, an N66S mutation in the PB1 F2 protein has been shown to contribute to the virulence of H5N1 and 1918 pandemic H1N1 viruses (Conenello et al., 2007), and several virulence determinants have been identified in the NS1 protein, including D92E, P42S, L103F, and I106M, reviewed by Kuiken et al. (2012).

## Host Factors

There are several host factors that are known to contribute to the severity of respiratory virus disease and the outcome of infection. For example, it is known that the more severe RSV LRTI in infants is associated with prematurity, chronic lung disease, congenital heart disease, and T cell immunodeficiency. Other risk factors include low birth weight, multiple births, male gender, and low titers of maternally derived anti-RSV antibodies (Groothuis et al., 2011; van Drunen Littel-van den Hurk and Watkiss, 2012). Additionally, low levels of vitamin D in cord-blood of healthy neonates is correlated with an increased risk of RSV LRTI in the first year of life (Belderbos et al., 2011). Infants are also at a greater risk of LRT disease from HPIV infection than older children; this has been attributed to smaller airways that are more susceptible to obstruction, immature immune responses, and the presence of anti-HPIV maternal antibodies that can suppress primary antibody responses (Crowe and Williams, 2003; Karron and Collins, 2007). In adults, immunodeficiency, immunosuppression, or old age may lead to more severe illness (Collins and Melero, 2011).

In addition, a number of genetic polymorphisms have also been described in host genes that may affect the outcome of respiratory virus infection and disease severity. Single-nucleotide polymorphisms have been identified in genes that encode surfactant proteins, such as surfactant proteins A, B, C, and D; pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs); chemokines and cytokines, such as RANTES, IL-4, -5, -6, -8, -9, -10, -13, and -18, TNF- $\alpha$ , TGF- $\beta$ , and IFN- $\gamma$ ; chemokine and cytokine receptors, such as CCR5, IL-4RA, and IL-8RA; adhesion molecules, such as ICAM-1, VCAM-1, and E-selectin; and HLA molecules such as HLA-A and -B, among others (Miyairi and DeVincenzo, 2008; Poland et al., 2008). However, few consistent results have been obtained between studies, probably a result of differences in study design, sample size, etc., in addition to true variability. Clearly the contribution of genetic polymorphisms to disease outcome is complex and remains an active area of research.

## Immunopathology

As well as being responsible for viral clearance and protection against reinfection, the host innate and adaptive immune responses to respiratory viruses can lead to pathology and enhanced disease. This is especially important to bear in mind in vaccine development, as vaccine-induced immune responses must protect against infection without leading to immunopathology.

Excessive inflammation is an important component in the pathogenesis of respiratory virus infections. Upregulation of IL-8 leads to the recruitment of neutrophils to the site of infection, and although these cells may participate



in virus elimination, in high numbers they can also cause pathology. Upregulation of IL-8 is known to correlate with RSV disease severity (Goetghebuer et al., 2004) and, during an influenza infection, overproduction of cytokines such as TNF- $\alpha$ , IL-6, IL-8, and type I and II IFNs and chemokines can also result in the recruitment of immune cells to the site of infection and result in damage to lung tissue (Cheung et al., 2002; de Jong et al., 2006). Elevated IL-8/CXCL8, MIP1 $\alpha$  and  $\beta$ /CCL3 and 4, RANTES/CCL5, and CXCL9 have also been described in children with HPIV disease, with an association of more severe HPIV disease with high concentrations of IL-8 and IP-10 (reviewed by Schomacker et al. (2012)). Additionally, the RSV soluble G protein can lead to leukocyte recruitment by mimicking the chemokine fractalkine (Tripp et al., 2001) and this can further exacerbate inflammation.

Pathogenesis can also be enhanced by an insufficiency of anti-inflammatory immune responses in the lung, such as the cytokines IL-10 and TGF- $\beta$  (Carlson et al., 2010; LeBouder et al., 2009; Sun et al., 2009), or insufficient numbers of immunosuppressive resident alveolar macrophages (Rygiel et al., 2009; Snelgrove et al., 2008).

Dysregulation of adaptive immune responses can also lead to increased pathology, and a Th2-biased cellular immune response has been implicated in the immunopathogenesis of RSV disease (van Drunen Littel-van den Hurk et al., 2007).

## IMMUNE RESPONSES TO RESPIRATORY VIRUS INFECTION

Various defense mechanisms have evolved in the respiratory tract to prevent and control infection. Currently, there is considerable effort to develop or improve vaccines against respiratory viruses. However, achieving this goal has been complicated by an incomplete knowledge of how the immune system recognizes, contains, and eliminates respiratory viruses.

This section discusses the immune responses against respiratory virus infections, from the initiation of innate and adaptive responses following primary virus infection to the recall of immune responses during a secondary infection. In addition, advances in our understanding of respiratory mucosal immunity are discussed.

### Innate Immune Responses

A common feature of respiratory virus infections is that the initial infection is established in epithelial cells lining the respiratory tract. Epithelial cells, as well as alveolar macrophages and DCs, are exposed to the contents of the airway lumen and detect the presence of an invading virus through PRRs (Holt et al., 2008). The recognition of pathogen-associated molecular patterns (PAMPs) by these receptors initiates a cascade of signals that results in the production of

cytokines and chemokines. The release of these inflammatory mediators into the surrounding environment establishes a local antiviral state. In addition, chemokines provide the necessary signals for the recruitment of leukocytes to the site of infection. Finally, the combination of inflammatory cytokines and PRRs initiates the process of DC maturation and trafficking that is required for the induction of adaptive immune responses (Holt et al., 2008).

The best described of the PRRs are those of the TLR family. With respect to respiratory viruses, TLR3, 7, and 9 recognize various products of viral replication (double-stranded RNA, single-stranded RNA, and unmethylated CpG DNA, respectively) (Alexopoulou et al., 2001; Diebold et al., 2004; Hagglund et al., 2004; Lund et al., 2003), and TLR4 recognizes the F protein of RSV (Kurt-Jones et al., 2000). TLRs that recognize nucleic acids are located in late endosomes. This location optimizes the ability of TLRs to interact with viral nucleic acids while limiting their access to host-derived nucleic acids (Heil et al., 2003; Matsumoto et al., 2003). Although TLRs expressed on the cell surface or within the cell utilize different signaling pathways, each of these receptors can activate the transcription of IFN-inducing genes (Wang et al., 2007).

Viral RNA is also recognized by cytoplasmic sensors such as RNA helicases. The retinoic acid-inducible gene I protein interacts with 5'-triphosphate RNA and is important for early cytokine production in response to numerous RNA viruses (Hornung et al., 2006; Pichlmair et al., 2006; Yoneyama et al., 2004; Kato et al., 2005; Pothlichet et al., 2013; Graham et al., 2013). The melanoma differentiation-associated gene 5 protein is a related helicase that recognizes polyinosinic polycytidylic acid and is crucial for innate recognition for picornaviruses (Kato et al., 2006) and human metapneumovirus infection (Banos-Lara Mdel et al., 2013). Similar to signaling through TLRs, the pathways utilized by RNA helicases ultimately trigger IFN-regulatory factor and NF- $\kappa$ B activation (Le Goffic et al., 2007). The key difference between these molecules and TLRs is that the RNA helicases are localized throughout the cytosol, rather than being restricted to intracellular compartments. Thus, pathogens such as paramyxoviruses that do not enter endosomes can trigger innate immune responses via RNA helicases.

The innate recognition of viral components through PRRs described above leads to a program of gene expression that promotes a localized antiviral state and elicits the recruitment of inflammatory cells to the site of infection. Type I interferons, including IFN- $\alpha$  and IFN- $\beta$ , are most commonly associated with early antiviral responses in the lung, and numerous studies in mice and humans have shown that plasmacytoid DCs (pDC's) are the primary source of these cytokines after infection with viruses (Asselin-Paturel et al., 2001; McGill et al., 2009). However, there is a level of redundancy with respect to IFN production, with alveolar macrophages or pDCs predominating depending on the type

of viral infection (Pribul et al., 2008). The precise contribution of pDCs to lung antiviral immunity is also controversial; several *in vitro* studies show that respiratory viruses, including influenza viruses, can infect pDCs and pDCs can activate virus-specific CD4<sup>+</sup> T cells (Wikstrom and Stumbles, 2007; GeurtsvanKessel and Lambrecht, 2008), but evidence for a major role of pDCs in controlling influenza virus *in vivo* is absent.

Type I IFNs produced after respiratory virus infection act in concert with PRR signaling to form a feedback loop, by signaling through the IFN- $\alpha/\beta$  receptor to promote sustained production of proinflammatory cytokines such as TNF- $\alpha$ , IL-1, and IL-6 by lung-resident innate immune cells (Chan et al., 2005; Nakajima et al., 2013; Almansa et al., 2012). These proinflammatory cytokines and PRR-mediated signals also prompt alveolar macrophages, DCs, and epithelial cells to initiate a coordinated program of chemokine production after viral infection. For example, DCs secrete successive waves of chemokines after influenza virus infection, beginning with those capable of recruiting inflammatory cells such as neutrophils and natural killer (NK) cells to the lung, followed by chemokines associated with the recruitment of monocytes and T cells (Piqueras et al., 2006; Marois et al., 2012; Teijaro et al., 2010).

The cytokines IL-1 $\beta$ , IL-18, and IL-33 activate monocytes, macrophages, and neutrophils and drive the development of CD4<sup>+</sup> T cell adaptive responses in both mice and humans. CD4<sup>+</sup> T cell differentiation in the presence of IL-1 $\beta$ , IL-18, or IL-33 results in Th17, Th1, or Th2 effector cells, respectively (Chung et al., 2009; Dinarello, 1999; Lasiglie et al., 2011; Ohno et al., 2009). These cytokines are processed as a result of caspase-1 activation, and activation of caspase-1 is regulated by the inflammasome, a large multimeric structure (Martinon et al., 2002). A subgroup of the nucleotide-binding domain, leucine-rich repeat-containing proteins (NLRPs) are key mediators of the inflammasome.

Activation of the inflammasome can be divided into two categories: activation driven by host- and environment-derived molecules and activation driven by pathogen-associated activators, including PAMPs derived from bacteria, virus, fungus, and protozoa. Paramyxoviruses such as RSV and orthomyxoviruses such as influenza viruses can activate the NLRP3 inflammasome (Kanneganti et al., 2006; Segovia et al., 2012; Komune et al., 2011), which has been shown to play a critical antiviral role in influenza virus-infected mice (Thomas et al., 2009; Ichinohe et al., 2009; Allen et al., 2009).

## Adaptive Immune Responses during a Primary Infection

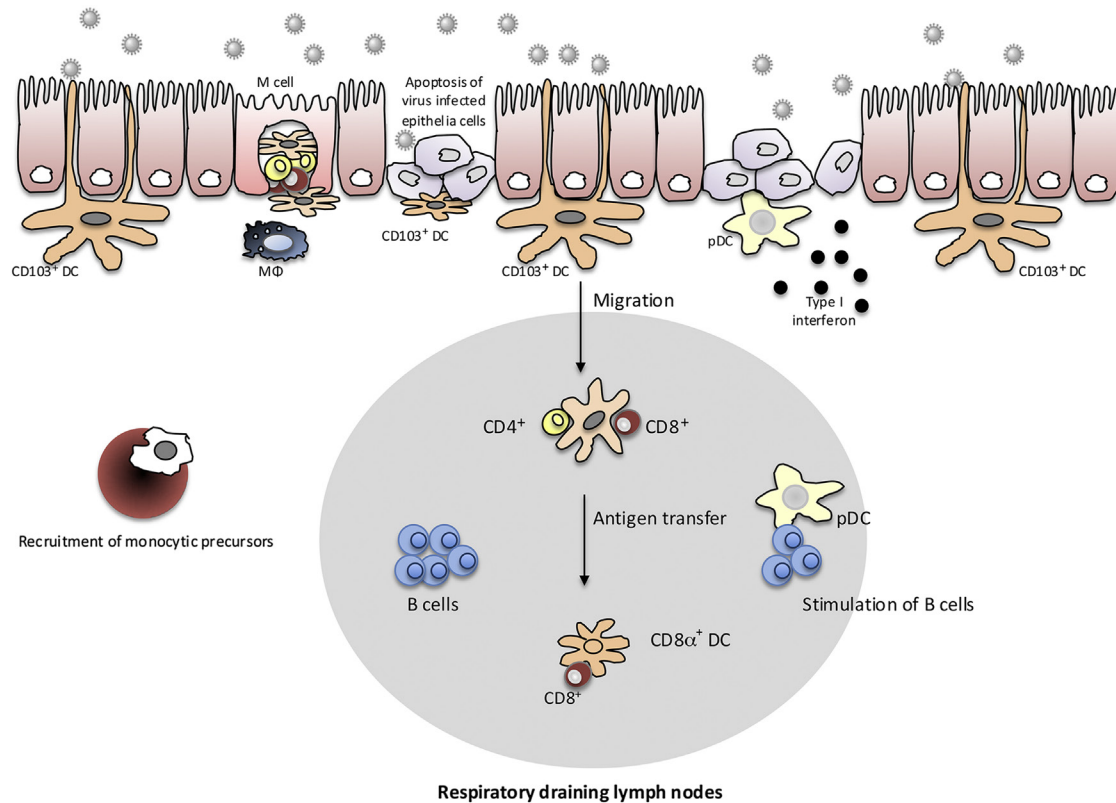
### T Cell Responses

After infection of the LRT, antigen-bearing mature DCs enter the lymph nodes draining the lung, where they form

stable interactions with naïve T cells specific for that antigen through T cell receptors (TCRs). Signals delivered by antigen recognition, in addition to accessory signals delivered through costimulatory molecules, result in T cell priming and the clonal expansion of antigen-specific effector T cells (Moon et al., 2007; Obar et al., 2008). The instructions delivered by DCs during the initial expansion phase can have a dramatic impact on the survival and function of the responding T cells. For instance, expression of FasL on DCs after influenza infection has been shown to regulate the magnitude of the CD8<sup>+</sup> T cell response (Legge and Braciale, 2005). Resident CD8 $\alpha^+$  conventional DCs in the mediastinal lymph node also mediate the induction of protective immunity to influenza virus, and these cells have been found to cross-present viral antigens to CD8<sup>+</sup> T cells without being directly infected by the virus (Belz et al., 2004).

After activation and clonal expansion of antigen-specific effector T cells in the draining lymph nodes, the cells lose their preference for the lymphoid tissue and migrate via the bloodstream to the site of infection, where the antiviral mechanisms described below are exerted. Analysis of chemokine expression in the lung during the adaptive phase of the immune response has shown elevated expression of numerous molecules associated with effector T cell trafficking (Monick et al., 2007). For instance, the trafficking of effector T cells during RSV infection is partially dependent on CXCR1, and this chemokine receptor may play a role in other paramyxovirus infections (Harcourt et al., 2006). The appearance of antigen-specific effector T cells at the site of virus infection is first observed around 6–7 days after infection with influenza and parainfluenza viruses in mice (Pommerenke et al., 2012; Kohlmeier and Woodland, 2009; Lawrence and Braciale, 2004; Roman et al., 2002). The continual migration of effector T cells from lymphoid tissues during acute infection results in a massive increase in the number of antigen-specific cells in the lung airways and parenchyma from 7 to 10 days after influenza virus infection (Flynn et al., 1998), and the arrival of effector T cells has an immediate and dramatic impact on the viral load (Kohlmeier et al., 2010).

The adaptive immune responses induced after a respiratory virus infection are shown in Figures 1–3. Upon arrival at the effector site (Figure 1), antigen-specific T cells first interact with APCs such as DCs (Shen et al., 2010). Moreover, a subset of DCs, classed as CD11c<sup>hi</sup>, present a crucial T cell survival factor (IL-15) to antiviral CD8<sup>+</sup> T cells *in trans* (McGill et al., 2010). Immune responses are determined by the cytokine milieu in the respiratory tract, as well as the type and level of costimulatory molecules expressed by APCs. For instance, lung DCs are biased to promote Th2 responses, most likely via the production of IL-6 in the absence of the Th1-prone IL12p70 cytokine or through the production of leukotriene LTC4 (Dodge et al., 2003; Barrett et al., 2011).



**FIGURE 1** Upon virus infection, viral antigen from dying respiratory epithelial cells is transported from the lung to the lymph node by migratory antigen-presenting cells such as mouse CD103<sup>+</sup> dendritic cells (DCs) or human monocyte-derived DCs that can stimulate both CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses. Viral antigen can also be transferred to lymph node-resident CD8 $\alpha$ <sup>+</sup> DCs and presented to naïve CD8<sup>+</sup> T cells. In addition, plasmacytoid dendritic cells (pDCs) are an early source of antiviral type I interferon via recognition of viral RNA.

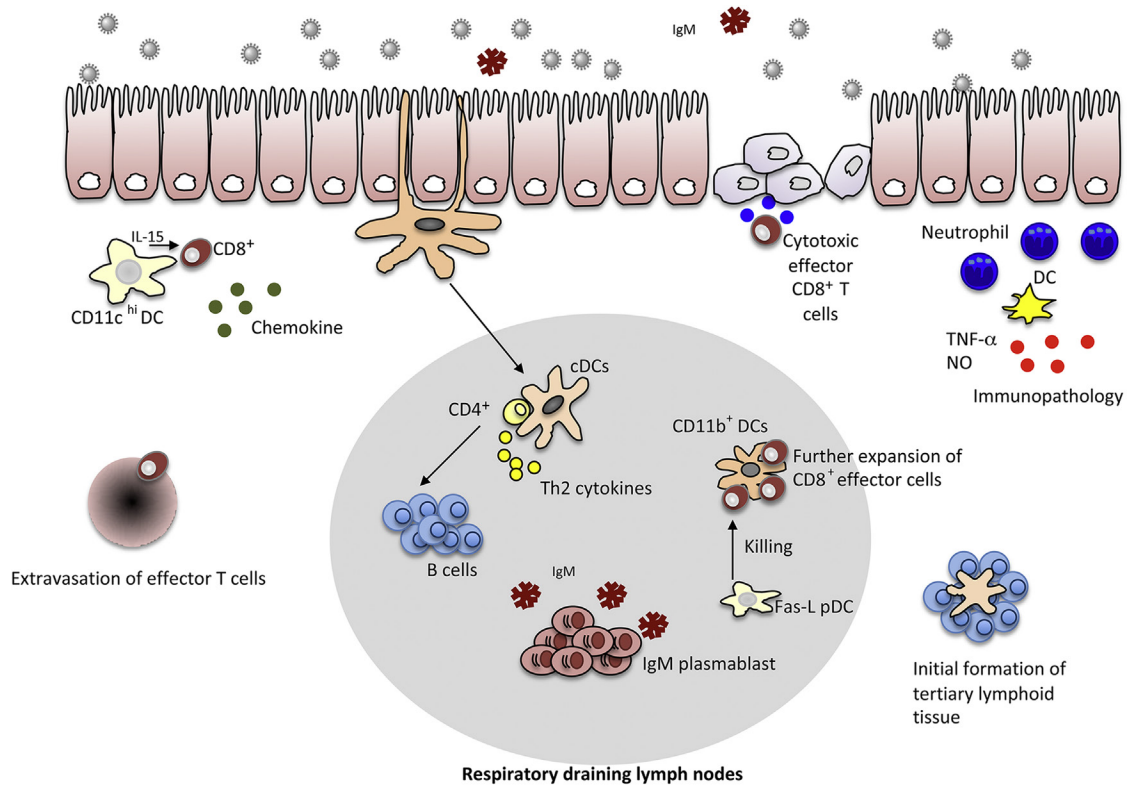
Effector T cells employ one of the following three antiviral mechanisms (Figure 2). First, T cells can promote the lysis of infected cells by exocytosis of granules containing perforin and granzyme (Trapani and Smyth, 2002; Hou and Doherty, 1995). Second, T cells can induce apoptosis of infected cells by expressing CD95 (Fas) ligand (FasL) (Topham and Doherty, 1997; Hou and Doherty, 1995) or TNF-related apoptosis-inducing ligand (TRAIL) (Brincks et al., 2011). Third, T cells can produce proinflammatory and regulatory mediators, such as IFN- $\gamma$ , after an encounter with virally infected cells (Hamada et al., 2013).

Several studies suggest a crucial role for the cytolytic functions of CD8 effector T cells in influenza virus infection. The direct lysis of infected cells requires TCR-mediated recognition of processed viral antigens on the infected target cell (Brincks et al., 2008; Topham and Doherty, 1997). In contrast, the release of proinflammatory mediators such as IFN- $\gamma$  by CD8<sup>+</sup> T cells has only a modest impact on virus clearance and recovery. There is also evidence from models of influenza infection that infected alveolar epithelial cells may be eliminated by the host response through the action of macrophages capable of triggering apoptosis through a TRAIL-dependent mechanism (Herold et al., 2008).

Effector CD4<sup>+</sup> T cells have also been found to exhibit cytotoxic activity *in vitro*, but the contribution of this mechanism to virus clearance *in vivo* is modest (Agrewala et al., 2007; Graham et al., 1994) and is restricted to the cytolysis of cells that bear viral antigens presented by major histocompatibility complex (MHC) class II molecules. Such cells include CD45<sup>+</sup> mononuclear phagocytic cells, and a few CD45<sup>-</sup> lung parenchymal cells, such as type II alveolar epithelial cells, that express MHC class II molecules in either a constitutive or an inducible manner (Debbabi et al., 2005). The primary role of antiviral CD4<sup>+</sup> T cells is to support the activation and differentiation of B cells, which leads to antibody production (Topham et al., 1996; Topham and Doherty, 1998) as discussed below.

Another aspect of effector T cells is their potential to exert cytotoxic activity and simultaneously produce cytokines such as IFN- $\gamma$ . For example, during an influenza infection, the interaction of primed CD8<sup>+</sup> T cells with lung DCs elicits both cytokine production and cytotoxic phenotypes (Hufford et al., 2011).

To summarize the cellular immune responses during a primary influenza infection, specific CD4<sup>+</sup> and CD8<sup>+</sup> effector T cells in the lung predominantly produce IFN- $\gamma$  and



**FIGURE 2** During the effector phase of the response, influenza-specific CD8<sup>+</sup> T cells elicit a cytotoxic response. TNF- $\alpha$  and nitric oxide (NO) produced from a subset of DCs and neutrophils contribute to both viral clearance and immunopathology. Lung CD11c<sup>hi</sup> DCs present the crucial T cell survival factor IL-15 to antiviral CD8<sup>+</sup> T cells *in trans* and promote the production of chemokines. In draining lymph nodes, CD11b<sup>+</sup> DCs contribute to the expansion of specific CD8<sup>+</sup> T cells, and conventional DCs (cDCs) induce the Th2 response. Plasmablasts initially produce IgM, and effector CD4<sup>+</sup> T cells provide help for virus-specific B cell/plasmablast differentiation and proliferation. pDCs also play a detrimental role by eliminating virus-specific CD8 T cells in a process involving FasL.

TNF- $\alpha$ , and CD4<sup>+</sup> effector T cells also produce IL-2 and IL-10 (Carding et al., 1993; Pipeling et al., 2008; Mayer et al., 2005). CD8<sup>+</sup> effector T cells localize to the respiratory epithelium and induce apoptosis of infected epithelial cells through Fas–FasL interactions or the exocytosis of cytolytic granules containing perforin and granzyme (Hou and Doherty, 1995; Tripp et al., 1995).

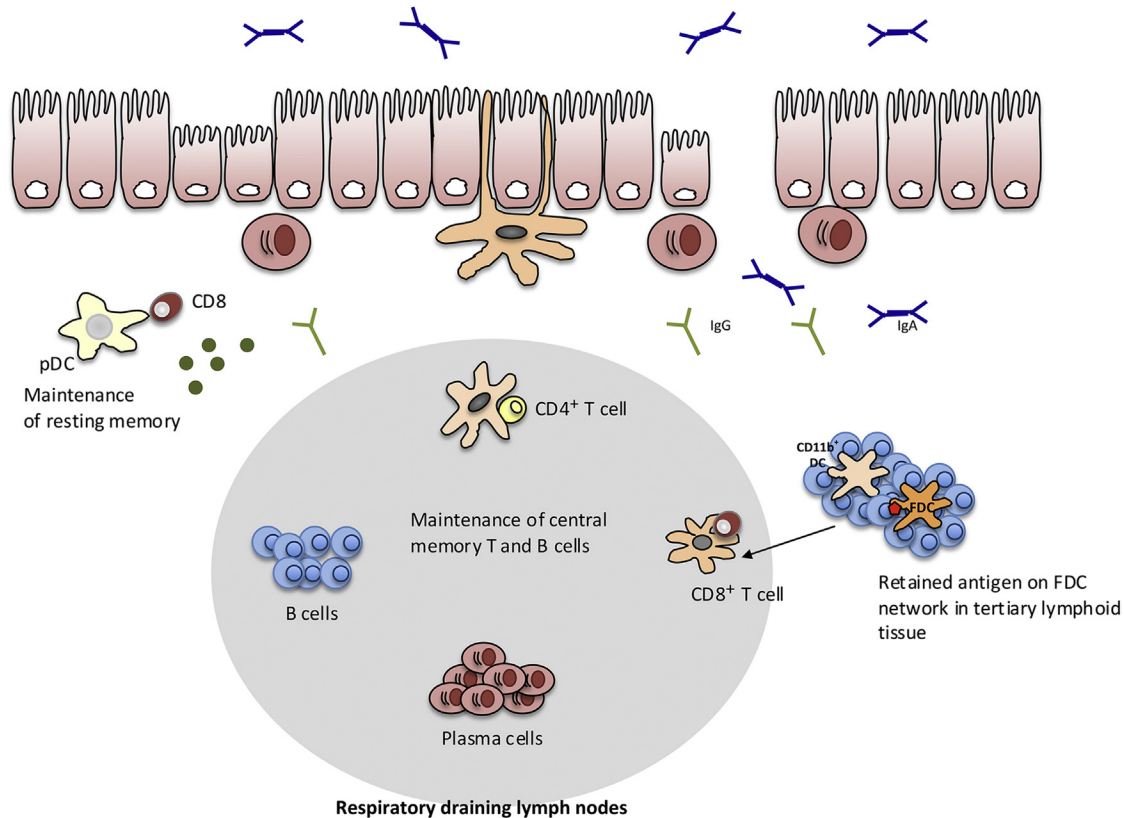
### B Cell Responses

B cells are found interspersed in the lung interstitium and in the cervical and mediastinal/bronchial lymph nodes that drain the upper and lower respiratory tract, respectively. During a respiratory virus infection, a tertiary lymphoid structure also forms along the branching point of the bronchial tree, called the bronchus-associated lymphoid tissue (BALT) (Brandtzaeg, 2010). The BALT contains organized B cell areas, germinal centers, and antibody-forming cells (Randall, 2010).

B cell responses can be classified into three categories: innate-like B cell responses, T-dependent B cell responses, and T-independent B cell responses. Innate-like B cell responses consist of antibodies produced almost exclusively

from B-1 cells, a small subset of B cells characterized by a unique developmental origin, phenotype, tissue distribution, and regulation, compared with conventional B cells (Baumgarth et al., 1999; Baumgarth, 2011). T-dependent B cell responses are B cell responses that are facilitated by CD4 T cells, whereas T-independent B cell responses are not. It has been shown that CD4 T cell deficiency results in a drastically reduced humoral response to influenza virus infection in mice (Mozdzanowska et al., 2005). However, mice lacking CD4 and CD8 T cells are still protected from lethal infection (Lee et al., 2005; Mozdzanowska et al., 2005), highlighting the importance of T-independent B cell responses.

B cell responses are critical for viral clearance in primary respiratory virus infections, such as influenza (Gerhard, 2001). Although control of early infection (3–6 days postinfection) is not impaired in B-cell-deficient mice, the mice fail to clear the virus and ultimately succumb to infection (Graham and Braciale, 1997; Lee et al., 2005). Studies in influenza virus-infected mice also show that serum antibody titers are first detected around 6–7 days postinfection, at least 3 days later than responses are detected in the respiratory tract. They steadily increase for about a month, after which relatively high



**FIGURE 3** In the memory phase, migratory lung DCs capture viral antigen retained on follicular DCs (FDCs) in tertiary lymphoid organs and present it to specific T cells in the respiratory draining lymph nodes. These stimulated T cells upregulate the expression of CD69, causing them to be retained in the lymph nodes that drain the site of primary infection. Resident DCs are able to reactivate memory responses in the lymph nodes. Lung DCs can promote the production of IgA in a process that depends on TGF- $\beta$ . The generation of B cell memory, particularly the generation of long-lived plasma cells, is dependent on cognate T–B cell interaction and CD40 signaling that occurs in the germinal center.

antibody titers are maintained for life. Virus-specific antibody-forming cells reside transiently in the spleen, from around 6 or 7 days postinfection, and persist for the long term in the bone marrow (Jones and Ada, 1987; Hyland et al., 1994).

### Recall Responses to Respiratory Viruses

After recovery from an infection (Figure 3), a state of immunological “memory” ensues, in which the individual is better able to control a subsequent infection with the same pathogen (Ahmed and Gray, 1996). Immunological memory is maintained by both T and B cell subsets, and there are profound differences in the generation, trafficking, and maintenance of T and B cell memory.

Antigen-specific memory T cells persist at increased frequencies, have a reduced requirement for costimulatory signals in comparison to naïve T cells, and respond quickly to antigenic restimulation (Woodland et al., 2002). In the case of influenza and parainfluenza (PIV) virus infections, it has been clearly established that both CD4<sup>+</sup> and CD8<sup>+</sup> memory T cell subsets respond to and control secondary infection (Woodland and Dutton, 2003).

B cell memory is characterized by two distinct populations: long-lived plasma cells that continually secrete antibody and memory B cells that persist in a quiescent state (Bachmann et al., 1994; Slifka and Ahmed, 1998). The generation of long-lived plasma cells is dependent on cognate T–B cell interaction and CD40 signaling that occurs in the germinal center (Noelle et al., 1992; Lee et al., 2003). Antigen-specific IgG and IgA antibodies are maintained long after infection and may be protective against heterologous strains of virus. For example, up to 96% of people born between 1909 and 1919 in Finland had preexisting antibodies to the 2009 pH1N1 influenza virus, probably because of its relationship to the 1918 H1N1 pandemic influenza virus that circulated in the first part of the twentieth century (Ikonen et al., 2010; Yu et al., 2008). The presence of cross-reactive antibodies contributed to the unexpectedly low numbers of the elderly with severe illness during the 2009 pandemic, compared with seasonal influenza virus strains (Monsalvo et al., 2011; O’Donnell et al., 2012).

Although neutralizing antibodies directed against the HA globular head are highly efficient at preventing and clearing influenza virus infection, they can also

provide a selective pressure for viral immune evasion. Cross-protection against various influenza A subtypes, termed heterosubtypic immunity, requires the immune system to recognize epitopes that are conserved between subtypes. Such epitopes can be found in the membrane-proximal stalk region of HA (Han and Marasco, 2011) or in internal proteins such as nucleoprotein or the M protein. Anti-stalk antibodies do not inhibit virion binding to mammalian host cells, but inhibit fusion between the viral envelope and the endosomal membrane. They have broadly neutralizing activity and passively protect mice from lethal challenge in vivo (Okuno et al., 1994; Throsby et al., 2008; Sui et al., 2009). Interestingly, cross-reactive anti-HA stalk monoclonal antibodies have been generated from the acute response to 2009 H1N1 pandemic virus and also from healthy subjects vaccinated with inactivated virus (Corti et al., 2011; Sui et al., 2009; Wrammert et al., 2008). How to induce high-titers of anti-HA stalk antibodies in humans remains an active area of universal influenza vaccine research.

Cellular immune responses to cross-reactive epitopes (often expressed on internal viral proteins) also provide a substantial degree of protection against serologically distinct viruses (Yewdell et al., 1985; Rimmelzwaan and Osterhaus, 1995), and although these heterosubtypic cellular responses are not able to prevent reinfection, they can ameliorate disease by reducing the maximal viral load, mediating faster viral clearance, and providing a substantial degree of protection against challenge with a lethal dose of virus in animal models (Hillaire et al., 2011). There is also some epidemiological evidence that heterosubtypic cellular immunity plays a role in the response to infection with novel influenza viruses in humans; however, the protective effect appears to be weak and may wane over time (Epstein, 2006; Epstein and Price, 2010). It has, therefore, been suggested that protective cellular immunity could be sustained by reinfection or annual immunization.

The effector mechanisms of heterosubtypic immunity remain ambiguous (Nguyen et al., 2001). In murine models of influenza A virus infection, heterosubtypic immunity is observed in the absence of antibodies that recognize influenza envelope glycoproteins and is thought to be mediated primarily by CD8<sup>+</sup> T cells, with a relatively small contribution by CD4<sup>+</sup> T cells (Doherty et al., 1997; McKinstry et al., 2012). Heterotypic influenza-specific CD8<sup>+</sup> T cells have also been shown to lyse influenza virus-infected cells (Nguyen et al., 1998). However, heterosubtypic immunity has been observed in CD8<sup>+</sup> T-cell-deficient mice, but not in mice lacking B cells (Nguyen et al., 2001), indicating that there is redundancy in the system. The same investigators found that the heterosubtypic immunity does not require IFN- $\gamma$  (Nguyen et al., 2000), but does require a properly diversified antibody repertoire (Nguyen et al., 2007).

## Mucosal Immune Responses to Respiratory Virus Infection

The mucosal immune system has been described in detail elsewhere in this book; however, there are some features unique to the respiratory tract that are worth noting. Like the immune system in general, the mucosal immune system of the respiratory tract uses innate and specific mechanisms to prevent and limit infection. Innate defenses include physical and chemical factors such as the secretion of mucus, which traps microorganisms and antigens and facilitates their transport out of the body by mucociliary motion. Mucosal secretions also contain chemically active substances, including acids, lactoferrin, and lysozyme, which inhibit the growth of microbes. In addition, the luminal side of the respiratory tract is physically protected by layers of epithelial cells that adhere to each other at tight junctions, using occludin and various members of the claudin family, and at adherent junctions using E-cadherin (Tsukita et al., 2008).

In humans, the respiratory tract can be divided anatomically into the URT, which comprises the nose, mouth, and pharynx, and the LRT, which includes the trachea, bronchi, and lungs, with the lymphoid tissue of Waldeyer's ring representing the line of demarcation. The unpaired nasopharyngeal tonsils (also called the adenoids) and the palatine and lingual tonsils constitute most of Waldeyer's ring, with the tubal tonsils and lateral pharyngeal bands as less prominent components (Dolen et al., 1990). This lymphoid tissue is functionally comparable to the nasal or nose-associated lymphoid tissue (NALT) in rodents, which is composed of two paired lymphoepithelial structures beside the nasopharyngeal duct, dorsal to the cartilaginous soft palate (Kuper et al., 1992; Fukuyama et al., 2002). However, rodents do not have tonsils.

Waldeyer's ring is more strategically situated than the NALT to generate mucosal immunity, because its elements are exposed to both airborne and alimentary antigens. In addition, human tonsils have deep antigen-retaining crypts, and tonsils express germinal centers shortly after birth, whereas the rodent NALT has a plain surface and requires an external stimulus to induce the expression of germinal centers (Brandtzaeg, 2010). Tissue equivalent to Waldeyer's ring has also been found in nonhuman primates (Loo and Chin, 1974; Harkema et al., 1987) and horses (Mair et al., 1987, 1988), but functional studies have not been performed in these species.

The mucosa-associated lymphoid tissues (MALT), including the NALT and BALT, consist of follicle-associated epithelium and T-cell- and B-cell-enriched areas. The initiation of antigen-specific immune responses occurs at special gateways, which comprise microfold (M) cells located in the epithelium overlying the MALT follicles. The cilia of the apical side of the M cells are shorter than those of conventional epithelial cells,

and on the basal side, there is a large pocket-like structure that can hold immunocompetent cells required for the generation of immune responses such as T cells, B cells, and APCs. As lysosome development in M cells is poor, in most cases antigens pass through the cells unmodified and are taken up by DCs in the pocket (Sato and Kiyono, 2012).

Upon encountering antigen, DCs migrate to the T cell region of the MALT and present peptide antigen via MHC molecules to naïve T cells. Antigen-specific T cells become primed, clonally expand, and leave the follicle to enter the circulation. They then home to effector sites to elicit mechanisms involved in viral clearance as described above. In the B cell region, a germinal center forms and antibody class switching occurs (Cerutti, 2008). A class switch to IgA predominantly occurs in the MALT owing to the action of the IgA-associated cytokine family of TGF- $\beta$ , IL-2, IL-4, IL-5, IL-6, and IL-10 (McGhee et al., 1989; Mestecky and McGhee, 1987; Cerutti, 2008). Postswitched IgA<sup>+</sup> B cells leave the MALT through efferent lymph vessels under the control of the lipid mediator, sphingosine-1-phosphate, and the cells then enter the circulatory system (Gohda et al., 2008; Kunkel and Butcher, 2003; Kunkel et al., 2003; Lazarus et al., 2003) and home to effector sites found in unorganized lymphatic tissue spread over the lamina propria that underlies the mucosal epithelium. In the lamina propria, B cells differentiate into plasma cells and secrete IgA, IgD, IgM, and IgG antibodies, although IgA is the major mucosal antibody isotype (Shikina et al., 2004; Shimoda et al., 2001).

Surgical removal of the murine NALT or cervical lymph nodes does not, however, abrogate cellular or antibody immune responses to experimental influenza infection, suggesting that there may be additional inductive sites other than the NALT and demonstrating that dissecting the relative contributions of anti-influenza immunity is difficult.

In the URT, IgA is the major mediator of immunity to influenza. In mice that had recovered from an influenza infection, immunity to reinfection was abrogated by the intranasal instillation of anti-IgA antibodies, but not anti-IgG or IgM (Renegar and Small, 1991a), and intravenous passive transfer of IgA resulted in IgA in nasal secretions that protected mice from intranasal challenge with influenza (Renegar and Small, 1991b). After passive transfer, nasal IgA titers that conferred protection were at a concentration equivalent to that seen in convalescent mice, whereas IgG transudation into the URT could be detected only after 2.5 times the normal convalescent serum titer had been passively transferred (Renegar et al., 2004). Moreover, recombinant IgA is sufficient to prevent influenza transmission in a guinea pig model (Seibert et al., 2013).

In humans, IgA is present in monomeric and dimeric forms. During transcytosis through mucosal epithelial cells, an extra polypeptide secretory component is added to

dimeric IgA and the resulting molecule is known as secretory IgA (S-IgA). Dimeric and S-IgA are 7–10 times more efficient than monomeric IgA at neutralizing influenza viruses (Renegar et al., 1998). Dimeric and S-IgA are represented by two subclasses, IgA1 and IgA2, with covalently or noncovalently joined dimers, respectively. Both subclasses are detected in nasal secretions after an influenza infection; however, HA preferentially stimulates an IgA1 response (Brown et al., 1985). S-IgA is not considered to be inflammatory because the Fc region is not available to activate immune cells or bind complement. S-IgA is also resistant to proteolysis and can neutralize viruses inside epithelial cells and transport viruses that have passed the epithelial barrier to the lamina propria back to the lumen (Sato and Kiyono, 2012). S-IgA may therefore be useful in preventing viruses from breaching the mucosal barrier, while avoiding immunopathology by not activating inflammatory responses directly and by limiting the number of antigen–antibody complexes in the lamina propria that can trigger inflammation.

It is currently thought that plasma IgG serves as a backup for S-IgA in the URT, whereas in the LRT, IgG is the dominant antibody involved in protection (Renegar et al., 2004). The Fc receptor for IgG mediates transport of IgG across epithelial barriers by transcytosis, permitting the transudation of IgG from the serum into the lung where it is able to neutralize viruses (Spiekermann et al., 2002). This explains why passively transferred IgG is effective at preventing severe disease from respiratory infections in experimental animals and why serum IgG antibodies are the main correlate of protection for parentally administered inactivated influenza vaccines in humans (Section Respiratory Virus Vaccines).

## Respiratory Virus Reinfection

Viruses that can cause repeated infection are typically characterized either by a failure to induce robust immunity or by significant antigenic diversity in the face of protective immune responses. Influenza viruses can reinfect hosts because the antigenic sites evolve and drift to avoid neutralization by prior immunity. Infection induces a strong homosubtypic neutralizing antibody response in healthy individuals that contributes to recovery and protection from repeat influenza virus infection with homologous virus or an antigenically similar virus (Wrämmert et al., 2008). Moreover, natural infection can lead to long-lasting immunity to the infecting virus. For example, when the influenza A H1N1 subtype reemerged in 1977, the most susceptible members of the population were those born after the time when similar H1N1 viruses had previously circulated, the 1950s (Shortridge et al., 1979). However, because influenza viruses undergo antigenic drift and shift, the effective period of protection may last only until an antigenic variant emerges.

In contrast to the robust strain-specific protection after influenza virus infection, primary infection with RSV, HPIV, and HMPV provides only partial protection from reinfection. RSV commonly reinfects the host even though genetic diversity in the virus is not extreme. In healthy adults challenged every few months with the same strain of RSV, about 25% were infected each time and about half of those became symptomatic (Hall et al., 1991). These studies are now being reproduced with experimental human challenge infection (DeVincenzo et al., 2010), and access to modern immunological techniques may provide some insight into the mechanism of immune evasion. Most reinfections are limited to the upper respiratory tract, unless subjects are immunocompromised. Reinfections may be the consequence of a highly prevalent and contagious virus, effective evasion of local and innate immunity, or a steep gradient for transudation of antibody from the serum to the nasal epithelium (Graham, 2011). Alternatively, RSV infection may alter the characteristics of the adaptive immune effectors and memory. Although RSV infection provides a sufficient antigenic stimulus to induce both antibody (Shinoff et al., 2008) and T cell responses (Heidema et al., 2008), the durability of the antibody is poor (Handforth et al., 2000; Dakhama et al., 1997; Collarini et al., 2009).

## RESPIRATORY VIRUS VACCINES

The most efficient means of preventing respiratory virus infections is vaccination. However, among respiratory viruses, licensed vaccines are available only for influenza. It seems logical to consider live attenuated vaccines delivered intranasally for protection against respiratory viruses, as they would induce a mucosal immune response. However, a systemic immune response can be protective if it is sufficiently robust, such as that induced by inactivated influenza vaccines administered by the i.m. route. Moreover, achieving an appropriate balance between sufficient attenuation and immunogenicity, especially in young infants who must be vaccinated in the face of maternal antibody, is a challenge.

This section describes licensed vaccines as well as vaccines that are currently in development.

### Influenza

Vaccination remains the primary strategy for the prevention and control of influenza (Lambert and Fauci, 2010). As described in [Section Immune Responses to Respiratory Virus Infection](#), after an influenza infection, both cell-mediated immunity and systemic and mucosal neutralizing antibodies are produced. Whereas cell-mediated immunity contributes significantly to the clearance of a primary influenza virus infection, and can ameliorate disease caused by reinfection, neutralizing antibodies play an important role in preventing reinfection. The goal of vaccination is to

prime the immune response to limit viral replication upon subsequent infection, and inactivated, recombinant hemagglutinin and live attenuated influenza vaccines (LAIVs) are licensed for use, with novel vaccines in varying stages of development. This section focuses on licensed vaccines and the immune responses they elicit, as determined from clinical trial data.

Owing to antigenic drift in circulating viruses (discussed in [Section Virology](#)), an influenza vaccine from one season may not be effective in subsequent seasons. Each year, the strains that are to be included in the vaccine for the next influenza season are chosen and vaccine seed viruses are generated (Lambert and Fauci, 2010). For inactivated vaccines, the influenza A seed viruses are reassortant viruses, with the HA and NA gene segments derived from the circulating virus and the internal protein genes derived from a vaccine donor strain that is adapted for high yield in eggs (A/Puerto Rico/8/34; PR8) (Kilbourne, 1969). Licensed LAIVs also contain the HA and NA from the circulating virus, combined with the internal protein genes from temperature-sensitive, cold-adapted, attenuated master donor viruses that limit replication of the vaccine viruses to the cooler upper respiratory tract (Maassab, 1967).

If the yield of the vaccine virus in eggs is poor, they may be “egg-adapted” through serial passage. Vaccine viruses are then amplified in hundreds of millions of eggs and purified. The inactivated vaccine viruses are treated with formalin or  $\beta$ -propiolactone and “split” with detergents before being formulated for clinical use, with or without thimerosal as a preservative (Fiore et al., 2013).

Until 2013, seasonal influenza vaccines were trivalent, containing two subtypes of influenza A viruses (H1N1 and H3N2) and one influenza B virus. However, from the 2013 winter season in the Northern Hemisphere, quadrivalent vaccines, containing two subtypes of influenza A viruses and two strains of influenza B viruses, have become available.

It takes several months from the generation of a seed virus to the manufacture and distribution of a vaccine. Typically, for seasonal influenza in the Northern Hemisphere, manufacturers amplify vaccine viruses and inactivate or purify them between February and late summer and formulate and distribute them for administration in the fall before the anticipated peak of the influenza season (Lambert and Fauci, 2010). In 2009, the H1N1 pandemic virus emerged in April, when the manufacture of seasonal trivalent vaccines was already under way. A monovalent H1N1 vaccine was produced as quickly as possible in addition to the seasonal vaccines, but the monovalent vaccine was not available for widespread use until after the pandemic had peaked in the Northern Hemisphere and was not available at all during the 2009 winter season in the Southern Hemisphere (Broadbent and Subbarao, 2011; Skowronski et al., 2011).

In addition, current global vaccine production capacity does not cover the high-risk population around the world



(Girard et al., 2005). One means of increasing capacity is to move toward using cell culture instead of eggs for vaccine production, and several companies are investigating this. Additionally, the amount of HA in each vaccine dose could be reduced and used with an adjuvant such as MF59 or AS03. However, adjuvanted seasonal influenza vaccines are not yet licensed in the United States.

Trivalent inactivated vaccines (TIVs) against seasonal influenza are administered i.m. and have an efficacy ranging from 60 to 100% in preventing influenza morbidity and mortality in healthy adolescents and adults (Osterholm et al., 2012). A trivalent LAIV consisting of an A or B Ann Arbor cold-adapted backbone together with the HA and NA of the target viruses (FluMist<sup>®</sup>, MedImmune), administered by a nasal spray, is licensed in several countries, including North America and Europe. In the United States, the seasonal LAIV is licensed for healthy individuals between 2 and 49 years of age, but it is currently not approved for use in children under 2 years of age, and it is not approved for use in the elderly or in immunocompromised individuals (Ambrose et al., 2011).

There is a substantial body of evidence from large-scale randomized clinical trials demonstrating the effectiveness of LAIVs, and a number of clinical studies have also shown that the efficacy of LAIVs is equivalent or superior to that induced by i.m. vaccination with TIVs in children (reviewed by Luke et al. (2013)). In adults, however, the majority of double-blind, randomized, placebo-controlled trials have shown TIVs to have a greater efficacy than LAIVs (Edwards et al., 1994; Monto et al., 2009; Ohmit et al., 2006, 2008). In the military, vaccine effectiveness was also found to be higher for TIVs than for LAIVs, except in new recruits (Eick-Cost et al., 2012; Wang et al., 2009).

### *Immune Responses Induced by TIVs and LAIVs in Healthy Adults and Children*

TIVs primarily induce serum antibodies against the influenza HA glycoprotein, which are typically measured by hemagglutinin inhibition (HI) assays, which serve as a surrogate for virus-neutralization assays. In healthy individuals who are immunologically primed by previous infection or vaccination, influenza-specific antibody-secreting cells in the peripheral blood peak 1 week after vaccination and serum antibody levels peak 2 to 4 weeks postvaccination. However, in unprimed individuals, for example, children, it may take 4 weeks or longer for serum antibody levels to peak after vaccination (Broks-tad et al., 1995; Cox et al., 1994; el-Madhun et al., 1998). The rise in serum antibody titer after TIV administration has been documented for multiple isotypes, including IgM, IgA, and IgG, with a more pronounced rise in IgG titers (Moldoveanu et al., 1995).

In contrast, vaccination with an LAIV leads to seroconversion more frequently in immunologically naïve individuals than in those who are immunologically primed. For example, in children, after a single dose of trivalent LAIV, seroconversion rates of 16–58%, 92–100%, and 88–100% have been reported against influenza A H1N1, H3N2, and B, respectively, which increased to 77% and 61% for H1N1 after a second dose at day 28 or 60, respectively (Belshe et al., 1998; Lee et al., 2004). However, in healthy adults, serum antibody titers are lower after LAIV than TIV vaccination (Moldoveanu et al., 1995), and in one study only 59% of LAIV recipients had an increase in serum IgG titer compared to 94% of TIV recipients (Clements et al., 1986). Protection mediated by inactivated vaccines therefore correlates with serum neutralizing antibody titers, whereas other immune mechanisms contribute to protection mediated by LAIV.

A higher percentage of LAIV recipients have mucosal antibodies and antibody-secreting cells than those receiving TIV. One study recorded that 83% of LAIV recipients had increased influenza-specific IgA in the mucosa, compared to only 38% of TIV recipients (Clements et al., 1986). Moreover, levels of IgA in nasal wash specimens correlated with protection against challenge with wild-type influenza viruses (Clements et al., 1986). Mucosal IgA in adults vaccinated with LAIV declined 6 months after vaccination (Clements and Murphy, 1986).

In addition to IgA, IgG has also been found in nasal secretions after LAIV (Moldoveanu et al., 1995). As described (Section Immune Responses to Respiratory Virus Infection), IgA is the major mediator of immunity to influenza infection in the URT, with IgG serving as a backup. This also appears to be the case after vaccination with LAIV.

The majority of antibodies induced by influenza vaccines that are associated with protection are directed against the globular head of the HA. Recently, neutralizing antibodies have also been identified that bind to a conserved epitope in the HA stem. The HA stem antibodies have been found to be broadly neutralizing, and there is much effort to generate vaccines that elicit these antibodies to produce a more broadly cross-protective vaccine (Corti et al., 2010; Ekiert et al., 2009; Kashyap et al., 2008; Okuno et al., 1993; Sui et al., 2009). In addition, antibodies directed against the NA protein are also generated after vaccination with both TIV and LAIV (Murphy et al., 1972). Anti-NA antibodies restrict virus release from infected cells and reduce the severity of disease by limiting spread (Murphy et al., 1972). However, currently licensed inactivated vaccines are standardized to HA, but not NA protein content. The amount of NA protein varies from vaccine to vaccine, and the contribution of vaccine-induced anti-NA antibodies to protection against influenza is not well understood (Hassantoufighi et al., 2010).

In addition to humoral immunity, influenza-specific CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) are associated with accelerated clearance of virus and recovery from infection. However, the extent to which the cellular immune response is protective against infection is unknown because the recall response is likely to occur after the peak of viral replication (Subbarao et al., 2006), and cell-mediated immunity induced after vaccination has been less well studied than the humoral response, and the results are variable. Studies have shown that immunization of healthy adults with whole-virus inactivated vaccine resulted in enhanced CTL responses in peripheral blood, whereas immunization with a subunit vaccine resulted in poor CTL responses, the duration of which varied from several months to years (Ennis et al., 1977; McMichael et al., 1981; Powers and Belshe, 1993). In addition, an increase in IFN- $\gamma$ -producing T cells was seen in children ages 6 months to 9 years of age who were vaccinated with inactivated vaccine; however, similar responses were not induced in adults (He et al., 2008). H5N1-specific CD4<sup>+</sup> T cells were also detected after a single dose of AS03-adjuvanted inactivated vaccine and was amplified by a second dose of vaccine (Moris et al., 2011). In addition, numerous studies have found a significant increase in IFN- $\gamma$ -producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells after vaccination with LAIV in both adults and children (Basha et al., 2011; Forrest et al., 2008; He et al., 2006a; Hoft et al., 2011; Lanthier et al., 2011); however, the role of cellular immune responses in LAIV-mediated protection needs further investigation.

### *Immune Responses Induced by TIVs and LAIVs in the Elderly*

More than 90% of annual influenza-related deaths in the United States occur in individuals 65 years of age or older (Thompson et al., 2003). Vaccinating elderly individuals is therefore a public health priority. However, a randomized placebo-controlled trial estimated the efficacy of the TIV to be 50% for the prevention of influenza in older adults (Govaert et al., 1994). Elderly individuals often have a significantly reduced antibody response to influenza vaccination (Goodwin et al., 2006). In a quantitative review of 4492 elderly subjects, 42%, 51%, and 35% seroconverted to H1N1, H3N2, and influenza B vaccination, respectively, compared to 60%, 62%, and 58% in younger subjects (Goodwin et al., 2006).

The impaired ability of the elderly to mount an adequate immune response to influenza vaccines has been attributed to immunosenescence. Immunosenescence is the decline in the body's ability to fight infection, mount novel immune responses, and recall responses (Targonski et al., 2007), and both innate and adaptive responses are implicated. Impaired function of costimulatory molecules, altered secretion of inflammatory cytokines, and

diminished function of natural killer cells, macrophages, and neutrophils have been observed in the elderly, as well as a decreased proliferative capacity of B cells and impaired T cell memory recall (Sullivan et al., 2010). In addition, thymic involution and a decline in T cell output are features of advancing age. This, together with a lifetime of exposure to a variety of pathogens, leads to a reduction in the naïve T cell pool and a relative increase in the proportion of memory T cells in the elderly compared with young adults. The most pronounced functional changes are in the CD8<sup>+</sup> T cell subset, in which progressive exhaustion occurs (Pawelec et al., 2005), whereas the CD4<sup>+</sup> T cell subset is less affected by replicative senescence (Czesnikiewicz-Guzik et al., 2008).

The TIV is standardized on the basis of the amount of HA protein, with one dose of 15  $\mu$ g per strain being recommended in healthy, previously primed individuals. Increasing the dose increases serum antibody response to the vaccine, and in an attempt to enhance immune responses to influenza vaccines in the elderly, a high-dose TIV (60  $\mu$ g HA protein per strain) was licensed in 2009 for use in persons ages 65 years and older. Postlicensure studies have shown enhanced immune responses in this age group, compared to the standard dose (Sullivan et al., 2010), and vaccine effectiveness studies are under way. In addition, an AS03-adjuvanted TIV (discussed below) containing 15  $\mu$ g HA protein per strain showed a 12% higher efficacy than a nonadjuvanted TIV in a phase 3 randomized clinical trial in the elderly, but the difference was not statistically significant (McElhaney et al., 2013). LAIVs are not licensed for use in the elderly at present; however, they have been evaluated in clinical studies in persons 50 years of age and older and are safe and well tolerated. In clinical trials, LAIVs were administered in addition to TIVs, and coadministration was reported to enhance local HA-specific IgA antibody responses. However, the efficacy of the combination was not greater than that of TIV alone (Gorse et al., 2004; Powers et al., 1989, 1991; Treanor et al., 1996).

### *Immune Responses Induced by TIVs in Immunocompromised Individuals*

In individuals with chronic or immunocompromising conditions, serological responses to TIV vaccination are typically lower than in healthy adults. Antibody responses against influenza were adequate in HIV-seropositive individuals who had no or minimal immunodeficiency or had responded well to antiretroviral therapy (Chadwick et al., 1994; Huang et al., 1987; Madhi et al., 2011; Staprans et al., 1995). However, in individuals with advanced HIV disease and low CD4 T cell counts, TIVs may not induce protective titers even after two doses (Kroon et al., 2000; Miotti et al., 1989). LAIVs are not licensed for use in immunocompromised individuals.

### *Immune Responses in Animal Models*

Although there is a large amount of data available on the immune responses to seasonal influenza vaccines in humans, improved seasonal and pandemic influenza vaccines are evaluated first in animal models.

The most commonly used models are mice and ferrets. Mice immunized with inactivated influenza vaccines develop serum HI and neutralizing antibodies, the titers of which correlate with protection from subsequent challenge (Luke and Subbarao, 2011). Although cellular immune responses are mounted during a secondary influenza infection (Woodland et al., 2002), passively transferred antibodies protected immunosuppressed mice, suggesting that cell-mediated immunity is not essential for protection if sufficient antibody is present (Virelizier, 1975). The goal of parenteral immunization with inactivated influenza vaccines is to induce sufficient serum antibody titers to limit influenza disease. This protection is mediated by serum IgG that transudes into the lower respiratory tract, neutralizing virus.

Passive transfer of immune serum to naïve mice reduced the replication of influenza virus in the lungs and protected recipient mice from lethal influenza pneumonitis, but did not prevent tracheitis or replication of the influenza virus in the URT (Ramphal et al., 1979). During a natural influenza infection of the URT, mucosal immune responses, including secretory IgA antibodies, play an important role in controlling disease (Section Immune Responses to Respiratory Virus Infection). Several studies have documented that higher levels of serum antibodies are required to provide protection against respiratory viruses in the URT compared to the LRT (Prince et al., 1985; Ramphal et al., 1979; Takiguchi et al., 1992). Additionally, influenza in ferrets is primarily a URT infection and vaccination with killed or inactivated influenza viruses does not protect against influenza infection unless administered with an adjuvant (Potter et al., 1972a,b). Adjuvants are probably required for parenterally administered inactivated vaccines to elicit the higher levels of serum IgG antibody that are needed to restrict viral replication in the URT, in the absence of robust mucosal immune responses.

In mice, LAIVs induce a range of systemic and pulmonary immune effectors and protect animals against challenge virus replication (Chen et al., 2011; Lau et al., 2011). The magnitude of the pulmonary IgA and memory CD8<sup>+</sup> T lymphocyte responses depends on the replication efficiency of the vaccine virus in the respiratory tract, but systemic immunity, such as serum antibody titers and memory CD8<sup>+</sup> T lymphocytes in the spleen, does not (Lau et al., 2011). After one dose of an H5N1 LAIV that replicated in the lungs of mice and induced local immunity, influenza-specific lymphocytes in the lung contributed to the clearance of challenge virus from the lungs, whereas the contribution of serum antibody and splenic CD8<sup>+</sup> T cells was negligible.

After two doses, complete protection was achieved and was associated with maturation of the antibody response (Lau et al., 2012).

Taken together, the data suggest that LAIV protects animals by inducing multiple arms of the immune response, including mucosal immunity and pulmonary and systemic antibody and cellular immune responses, in a manner similar to natural infection. However, inactivated vaccines aim to induce serum antibody alone. Although this does not mimic a natural infection, if antibody titers are sufficiently high, the inactivated vaccine will protect against disease caused by influenza viruses.

### *Correlates of Protection*

For inactivated influenza vaccines, serum anti-HA antibody titers correlate well with resistance to influenza infection in people as well as in animal models. Lower antibody titers are associated with an increased risk of illness, though a specific antibody titer that can guarantee protection from infection has not been identified. An HI titer of 1:40 represents the level at which it is anticipated that approximately 50% of persons will be protected (Hobson et al., 1972), and this benchmark forms the basis of the licensing criteria for inactivated vaccines (CBER, 2009). Although several papers refer to “seroprotection,” there is insufficient evidence to support the use of this term for vaccines.

The benchmark of an HI titer of 1:40 was defined in healthy adults who were experimentally challenged with an influenza virus (Hobson et al., 1972). However, antibody titers that correlate with protection in healthy adults may not translate to clinical improvements in influenza outcomes in the elderly (Gorse et al., 2004). In addition, LAIVs are effective despite inducing variable serum HI antibody titers. Therefore, alternative correlates of protection are needed. As IgA and cellular immune responses are generated in the lungs of mice vaccinated with LAIV, in addition to systemic antibody and cellular responses, the extent to which the different arms of the immune response contribute to LAIV-induced protection are beginning to be evaluated (Chen et al., 2011; Lau et al., 2011, 2012). LAIV-induced nasal wash IgG and IgA correlated with protection from virus replication, and in human challenge studies, either serum antibody or nasal wash IgA was a predictor of protection (Belshe et al., 2000b). Cellular immune responses have also been investigated as a correlate of LAIV-induced protection, and one study found a correlation between IFN- $\gamma$ -producing T cells (measured by ELISPOT) and protection from culture-confirmed influenza illness in young children (Forrest et al., 2008). In addition, it has been shown that LAIVs alter the expression of IFN-related genes, whereas TIVs do not, indicating that the innate immune response plays an important role in protection mediated by LAIVs (Nakaya et al., 2011).

### *Adjuvanted Influenza Vaccines*

Adjuvants are added to vaccine formulations to enhance immune responses to the antigen in the vaccine. Aluminum salts (alum) are the most commonly and historically used adjuvants worldwide. They act by capturing antigens at the injection site, so the antigen is slowly processed and presented by the immune system (the so-called depot effect), and they cause mild cell damage and inflammation that promotes a Th2 immune enhancement (Tetsutani and Ishii, 2012). Moreover, alum particles enter host cells and bind DNA, introducing it into the cytoplasm of antigen-bearing DCs, where it engages receptors that promote both MHC class II presentation and DC–T cell interactions (McKee et al., 2013).

Oil-in-water adjuvants, such as MF59 (Novartis) and AS03 (GlaxoSmithKline), are more effective at inducing high-titer antigen-specific serum antibody responses than alum and have been used with inactivated split-virion influenza vaccines in Europe. These adjuvants induce broad, cross-clade humoral responses and permit dose sparing, in which comparable immune responses are induced with a reduced amount of HA in the vaccine (Galli et al., 2009; Leroux-Roels et al., 2007).

Our understanding of the mechanism of action of MF59 and AS03 remains incomplete. Neither appears to act via a depot effect; instead they induce a local and transient proinflammatory cytokine and chemokine response at the injection site and draining lymph nodes that recruit immune cells from the circulation. In mice, AS03 induced the cytokine IL-6 and chemokine CXCL1, which peaked locally by 6h postvaccination. The neutrophil-mobilizing cytokine CSF3 and lymphocyte-mobilizing cytokines CCL2, 3, and 5 were induced by 24h postvaccination. In addition, the eosinophil-recruiting chemokine CCL11 and the cytokine IL-1 $\beta$  were induced at low levels, and IFN- $\gamma$ , CSF2 (GM-CSF), and TNF- $\alpha$  were induced at levels that were only marginally above background. IFN- $\alpha$  and - $\beta$  were not induced. The local cytokine response was paralleled by an enhanced recruitment of monocytes and granulocytes in the draining lymph node (Morel et al., 2011). MF59 also induces local upregulation of cytokines, chemokines, and other innate immunity genes, promoting the recruitment of immune cells such as monocytes, dendritic cells, and granulocytes. However, the mechanism of action of MF59 was found to be independent of the NLRP3 inflammasome, but required MyD88 for a TLR-independent signaling pathway (Seubert et al., 2011).

### *Inactivated Whole-Virion Vaccines*

Whereas the inactivated vaccine viruses are typically disrupted with detergents to make split-virion (subunit) vaccines, inactivated whole influenza virion (WIV) vaccines

have also been developed, in which the virions are left intact. These are less widely used because of increased reactivity and adverse events (Fiore et al., 2013). However, mice immunized with WIV vaccines consistently showed higher HI titers and virus-neutralizing antibody titers than subunit vaccines, as well as an increased production of proinflammatory cytokines by dendritic cells and IFN- $\alpha$  by plasmacytoid cells, resulting in a desired Th1 response (Geeraedts et al., 2008; Koyama et al., 2010). The approach of using inactivated whole influenza vaccines is being revisited with pandemic influenza vaccines. Whole-virion inactivated H1N1 and H5N1 vaccines administered with alum are immunogenic in humans (Kulkarni et al., 2012; Lagler et al., 2012; Lin et al., 2006; Nakayama et al., 2012).

### *DNA Influenza Vaccines*

DNA vaccines encoding one or several proteins of influenza viruses induce an immune response targeting the encoded proteins (Fynan et al., 1993; Ulmer et al., 1993; Wolff et al., 1990). DNA vaccines can be produced rapidly and at low cost; however, designing DNA vaccines is complex. Over the years, it has been shown that numerous factors play roles in the efficiency of expression, such as the promoter, the G/C content, supercoiling, polyadenylation, and codon optimization (Laddy and Weiner, 2006). In addition, safety remains a concern, as there might be a risk of integration into the host genome (Klinman et al., 1997).

Numerous studies have evaluated DNA vaccines expressing NP, M1, or HA proteins in animal models (Fu et al., 1999; Saha et al., 2006; Tao et al., 2009; Ulmer et al., 1998, 1996a,b). In mice, the administration of DNA vaccines encoding the NP protein of influenza induces a strong CTL response, which correlates with protection against challenge with homologous or heterologous viruses (Ulmer et al., 1993). In addition, one study showed that delivering the vaccine by *in vivo* electroporation instead of the classical epidermal route also induces protective humoral and cellular immune responses in mice, ferrets, and nonhuman primates (Laddy et al., 2008).

Recently, a phase 1 clinical trial with an adjuvanted plasmid DNA vaccine encoding influenza H5, HA, NP, and M2 elicited T cell responses against HA in the majority of the subjects and against NP and M2 in some (Smith et al., 2010).

### *Universal Influenza Vaccines*

Licensed influenza vaccines primarily elicit an immune response to the globular “head” region of the HA glycoprotein. However, immune selection pressure leads to antigenic drift (discussed in Section Clinical Features and Epidemiology), so new influenza vaccines need to be selected for each season as well as when a pandemic emerges.

It remains difficult to predict which strains will circulate in the upcoming influenza season, and rates of morbidity and mortality are greater in influenza epidemics when the virus and vaccine are “mismatched” (Pica and Palese, 2013). The expectation is that vaccines capable of protecting against a broad(er) spectrum of influenza viruses would result in less frequent updating of seasonal influenza vaccines and would provide a degree of preexisting immunity if a novel strain emerges.

Vaccines that aim to provide broad cross-protection against multiple subtypes of influenza are known as universal vaccines, and several platforms are in development, including those that target the HA, M2, NP, M1, and NA proteins (Subbarao and Matsuoka, 2013).

Unlike the head region of HA, the “stalk” domain has a high degree of sequence conservation between influenza viruses, and broadly neutralizing stalk-reactive antibodies have been identified. These antibodies may inhibit pH-induced conformational changes in the HA required for cellular entry, prevent the cleavage of HA0 into HA1 and HA2 (discussed in Section Virology), or act via antibody-dependent cell-mediated cytotoxicity (ADCC) or through the activation of complement (Krammer and Palese, 2013).

However, the HA stalk is less immunogenic than the head, and several techniques have been employed to elicit stalk-reactive antibodies, including removing the head, novel prime–boost strategies, and sequential vaccination with chimeric HA molecules that contain the same stalk region but “exotic” head domains that aim to boost antibodies to the conserved epitopes within the stalk (Krammer and Palese, 2013). These strategies have been tested in mice with varying degrees of success in generating protective immune responses against heterologous influenza viruses (Subbarao and Matsuoka, 2013). Taken together, HA stalk-based strategies show promise as universal influenza vaccine candidates.

The influenza M2 protein extends beyond the viral envelope, and antibodies against the M2 extracellular domain (M2e) may act via ADCC, NK cell activity, or complement-mediated lysis. In addition, the sequence of the M2e is conserved among human influenza A viruses, making it an attractive target for universal vaccines. However, the peptide is poorly immunogenic, and techniques to improve immunogenicity are under investigation in mice, including fusing the peptide to an immunogenic carrier protein or delivering the antigen in a virus-like particle (VLP) (reviewed by Subbarao and Matsuoka (2013)).

The influenza NP and M1 proteins are also conserved among influenza A viruses and have potential for use in a universal vaccine, but as they are not exposed on the surface of virions or infected cells, they mainly induce cellular immune responses. Phase 1 and 2 clinical trials with modified vaccinia virus Ankara (MVA) expressing NP and M1 proteins report a 60% reduction in laboratory-confirmed influenza

infection after experimental challenge (Lillie et al., 2012); however, larger studies are required to confirm this.

Immune responses directed against the influenza NA protein do not protect against infection, but rather limit the spread of infection and reduce the severity of disease. Recently, baculovirus-expressed VLPs containing N1 NA proteins were found to induce heterosubtypic NA antibodies in mice that protected them against homologous and heterologous challenge (Quan et al., 2012). A universal vaccine may therefore benefit from anti-NA immune responses.

It may be useful to combine several components into a universal vaccine, for example, NP and M1 to induce cellular responses and HA and M2e to induce humoral responses. However, there are several challenges to the development of a universal vaccine. As outlined above, most of the viral proteins that potentially induce broad heterosubtypic immunity are poorly immunogenic and require a large dose of antigen, multiple doses, addition of adjuvants, fusion to immunogenic carriers, or the use of vectors or VLPs. Additionally, regulatory challenges include how to determine and define the potency of the vaccine and the need to identify immune correlates of protection and develop validated assays to measure them. In addition, clinical trials will be challenging because it is likely that efficacy will be measured in terms of amelioration of disease rather than preventing infection (Subbarao and Matsuoka, 2013).

Given these hurdles, achieving a truly universal vaccine that protects against all types or subtypes of influenza will probably proceed in a stepwise manner, with the first step being a vaccine that is more broadly cross-protective than currently licensed vaccines.

## RSV

The primary target populations for RSV vaccination are young infants and the elderly, because hospitalization rates are the highest in these age groups and 78% of RSV-related deaths occur in individuals over 65 years of age (Thompson et al., 2003). There is a large body of evidence that protection against infection is conferred mainly by neutralizing antibodies (Collins and Melero, 2011); however, multiple doses of vaccine might be necessary in young infants, because of the immature immune system and the presence of maternal antibodies. In addition, protective immunity mounted during infection does not protect against subsequent reinfection. Reinfections are common and are independent of antigenic changes in the virus (Collins and Melero, 2011). These factors present challenges to RSV vaccination and, as yet, vaccines have not been licensed. This section discusses clinical trial data from various approaches to RSV vaccination.

The first approach to vaccination was the development of a formalin-inactivated RSV vaccine (F1-RSV) that was evaluated in the 1960s in infants and young children. A concentrated F1-RSV vaccine, given i.m. with alum, was well

tolerated and moderately immunogenic, but was poorly protective against infection. In fact, vaccinees who were exposed naturally to infection during the subsequent RSV season had immune-mediated enhancement of disease, with 80% of individuals requiring hospitalization and two fatalities (Fulginiti et al., 1969; Kapikian et al., 1969; Kim et al., 1969). Subsequent studies revealed that vaccine-induced immune responses were different from those elicited after natural infection, with poor induction of neutralizing antibodies (Murphy and Walsh, 1988) and an exaggerated CD4<sup>+</sup> T-cell response (Kim et al., 1976). Vaccine-mediated enhancement of disease also occurred in murine models, with poor induction of neutralizing antibodies probably due to denaturation of antigen in the vaccine or a lack of antibody affinity maturation after poor TLR stimulation (Delgado et al., 2009). In addition, an exaggerated Th2 response and a loss of regulatory T cells contributed to disease (Connors et al., 1992, 1994; de Swart et al., 2007; Loebbermann et al., 2013; Waris et al., 1996). Therefore, it is clear that in addition to a neutralizing antibody response, a balanced CD4 and CD8 T cell response is also desirable.

After this experience, inactivated RSV vaccines were considered unsuitable for pediatric use, and alternative approaches were sought. In the 1960s, a series of live attenuated vaccines (LAVs) was developed by serial passage of RSV at suboptimal temperatures (cold passage, cp) or by growth in the presence of mutagens to produce temperature-sensitive (ts) mutants. The LAV did not cause disease enhancement in animal models or in clinical trials (Wright et al., 2007a). The most promising vaccine generated by this method, cpts248/404, was well tolerated and immunogenic in seronegative infants and children greater than 6 months of age, but caused mild congestion in younger infants and was deemed to be insufficiently attenuated (Wright et al., 2000).

Several LAVs have also been developed using reverse genetics techniques and were evaluated in clinical trials. The most promising, rA2cp248/404/1030 $\delta$ SH (MEDI-559), was strongly ts and was well tolerated in infants (Karron et al., 2005). The first dose provided substantial reduction in replication of a second dose of vaccine given 2 months later. However, the majority of individuals did not have increases in serum antibody titers, indicating that other immune mechanisms might play a role in the protection from replication of the second dose of vaccine. As with live attenuated influenza vaccines, establishing correlates of protection for live RSV vaccines is an active area of research, made more challenging by the weak immune responses in infants and limitations on sampling. As of this writing, clinical trials are under way to further monitor tolerability and immunogenicity and the ability of candidate vaccines to induce protection against natural RSV exposure in the community (Collins and Melero, 2011).

Genetic changes have been employed to generate candidate LAVs, including deletion of the M2-2 coding

sequence, which increases transcription and antigen synthesis at the expense of viral replication (Bermingham and Collins, 1999), and deletion of the NS1 and NS2 genes, which increase IFN production and signaling and might limit viral replication and pathology, but increase immunogenicity (Teng et al., 2000).

The use of attenuated parainfluenza virus as a vector to express RSV F and/or G protein has also been considered as a pediatric vaccine against both HPIV and RSV, as PIV has the advantage of improved *in vitro* growth and stability compared with RSV. Bovine PIV3 is attenuated in humans and has been used as a vector into which the F and HN genes from human PIV3 and the F and/or G protein of RSV are incorporated to make a bivalent vaccine against both HPIV3 and RSV (Schmidt et al., 2002; Tang et al., 2008). One example of this approach is in clinical trials in children older than 6 months of age and who are seronegative to RSV and HPIV3 (Collins and Melero, 2011). The vaccine in this study comprises a bovine PIV3 vector into which the human PIV3 F and HN genes and the RSV F gene have been added (Tang et al., 2004). As an alternative to bovine PIV3, murine PIV1 (Sendai virus) is also being evaluated as a vaccine backbone into which RSV antigens are inserted (Jones et al., 2009). There is substantial antigenic cross-reactivity between Sendai virus and HPIV1, and the virus may be attenuated in humans.

Other approaches to live vaccines for RSV include the use of alphaviruses (Elliott et al., 2007) or replication-defective adenoviruses (Shao et al., 2009) as vectors for RSV antigens.

In addition to an RSV vaccine in infants, there is also a need for RSV vaccines in older children, adults, and the elderly. However, these individuals are likely to have been previously infected with RSV, and preexisting immunity may restrict the replication of LAVs. In addition, vectored vaccine approaches should not be based on common human pathogens, as preexisting immunity to the vector may limit vaccine virus replication and interfere with the development of immunity.

The use of protein-based vaccines in these populations is currently being evaluated. Disease enhancement has not been documented in adults who were previously exposed to RSV, and candidate RSV F protein-based vaccines are well tolerated in healthy adults, children over 12 months of age, pregnant women, and the elderly (Girard et al., 2005). In a phase 3 clinical trial involving 289 children 1–12 years of age with cystic fibrosis, a purified F protein-3 (PFP-3) vaccine induced a fourfold rise in serum antibody titer, but was not associated with significant protection against LRTI episodes compared to placebo (Piedra et al., 1998). In another approach, a subunit vaccine of copurified F, G, and M proteins from RSV-A was tested in adults and found to induce neutralizing antibodies in 76–93% of vaccinees, but titers waned after 1 year, suggesting that annual immunization might be necessary with this vaccine (Girard et al., 2005).

Interestingly, in a phase 1 clinical study of 35 pregnant women vaccinated with PFP-2, anti-F antibodies were persistently elevated in newborns of the vaccinated mothers, without enhancement of disease. Maternal immunization could, therefore, be a strategy to protect infants under 6 months of age, for whom the development of an RSV vaccine has been such a challenge (Durbin and Karron, 2003).

A recombinant postfusion form of the F protein with a deletion of the major hydrophobic regions was also produced. This subunit vaccine forms stable trimers that are recognized by neutralizing mAbs. High levels of neutralizing antibodies were induced in the sera of vaccinated rodents, and the animals were protected from challenge. This may represent an improved subunit vaccine (McLellan et al., 2011; Swanson et al., 2011).

Another protein-based vaccine, BBG2Na, has been evaluated in clinical trials. BBG2Na consists of a fragment of the RSV G protein that contains a central conserved domain fused to the albumin-binding domain of streptococcal G protein, expressed in bacteria. However, this vaccine was not very immunogenic in clinical trials and was associated with hypersensitivity reactions (purpura) in some individuals (Power et al., 2001).

VLPs have also been evaluated for use as an RSV vaccine, and one such formulation (RSV-F particle vaccine) is currently in clinical trials (Collins and Melero, 2011).

It is the hope that improved methods of producing RSV antigens or using VLPs will result in an effective vaccine that could be given to adults periodically, possibly with the annual influenza vaccine. However, in children, it remains to be seen whether a vaccine can be developed that is sufficiently attenuated, yet immunogenic enough to provide protection. However, even if complete protection is unrealistic, immunity that results in substantial reduction of virus replication may be sufficient to prevent severe disease.

## HPIV

As 80% of children are seropositive for HPIV by 5 years of age, and HPIV LRTI is a major cause of hospitalization in this age group (Schomacker et al., 2012), young infants and children are the target population for HPIV vaccination. Protective immunity against infection is mediated primarily by mucosal and serum neutralizing antibodies; however, reinfection is common owing to a difficulty in maintaining protective titers of S-IgA and IgG in the respiratory lumen, thus representing a challenge to vaccine development (Glezen et al., 1984). This section reviews data from clinical trials of inactivated, live, and vectored HPIV vaccines.

The first approach taken to HPIV vaccination was the use of formalin-inactivated PIV3 (FI-PIV3) in an intramuscular vaccine. Although vaccine-induced disease enhancement seen with formalin-inactivated RSV was not observed

with FI-PIV3, there was no evidence of protection either (Kim et al., 1969). Subsequent vaccine efforts, therefore, focused on the generation of live attenuated vaccines, and two approaches were investigated: the use of a ts HPIV3 strain and the use of a bovine PIV strain.

Successive passage at lower temperatures has been shown to attenuate HPIV. The most promising vaccine candidate using this approach, cp45, is designated by the number of times the virus was passaged at low temperature in African green monkey kidney cells (Belshe and Hisson, 1982; Ray et al., 1995). The vaccine was safe and immunogenic in adults, seropositive and seronegative children, and infants 1 month of age or older (Belshe et al., 2004b). In a phase 2 clinical trial in 6- to 18-month-old children, the vaccine was well tolerated and 84% of previously seronegative vaccinees had a fourfold or greater rise in serum geometric mean antibody titer (Belshe et al., 2004b). Moreover, coadministration of an RSV and cp45 PIV vaccine was successful, with little evidence of interference (Belshe et al., 2004a). However, the cp45 PIV vaccine was overattenuated in seropositive children and adults.

Bovine PIV3 makes an attractive LAV as it is known to be antigenically related to human PIV, is attenuated in humans, and protects monkeys against challenge with HPIV3. Clinical trials were conducted in adults, seropositive children, seronegative infants, and children 2–6 months of age with residual maternal antibodies. In seropositive individuals, the vaccine was overattenuated, but in seronegative children and infants, the vaccine virus was highly infectious. Despite replication of the vaccine virus, serum antibody responses to HPIV3 were low, consistent with an incomplete antigenic relatedness between the human and the bovine PIV HN genes (Greenberg et al., 2005; Karron et al., 1995). A phase 2 clinical trial of 192 children showed that seroconversion to bovine PIV3 occurred in 57–67% of vaccinated children after three doses, but that seroconversion to HPIV3 occurred in only 21–25% of vaccinees (Greenberg et al., 2005). Additionally, during the study, 47% of the placebo group experienced PIV3 infection, highlighting the ubiquitous nature of the pathogen.

After these studies, it was concluded that bovine PIV3 might better serve as a backbone for the insertion of HPIV3 genes. As described above, at this writing, one such vaccine, with HPIV3 HN and F genes and the RSV F gene inserted into a bovine PIV3 backbone, is being evaluated in clinical trials in seronegative children. This vaccine candidate was highly attenuated in seropositive children and adults.

Additional vaccine candidates include those generated by reverse genetics. One such vaccine contains mutations in the P/C gene, which reduces the virus's ability to inhibit type I IFN induction and signaling, and another contains ts and attenuating mutations from other paramyxoviruses (Sato and Wright, 2008).

## HMPV

By the age of 5 years, most children will have been infected with HMPV. Reinfections are common, and HMPV is responsible for 5–15% of childhood hospitalizations for LRTI. In addition, severe disease can also be seen in the elderly or immunocompromised individuals (Feuillet et al., 2012). Despite this clinical need, HMPV vaccines have not yet entered clinical trials, and the data reviewed in this section are from studies in animal models.

As with RSV, vaccination of macaques and mice with inactivated HMPV led to disease enhancement after challenge and a strong Th2-type immune response associated with a lack of neutralizing antibodies (Herfst et al., 2008a; Hamelin et al., 2007). Therefore, alternative approaches have been evaluated, including soluble protein-based subunit vaccines, live attenuated vaccines, or DNA vaccines encoding viral proteins.

Soluble F-protein subunit vaccines have been shown to generate high titers of neutralizing antibody in serum in golden Syrian hamsters and cotton rats, associated with protection against subsequent infection (Cseke et al., 2007; Herfst et al., 2007). However, stable and long-term immunity was not induced in monkeys (Herfst et al., 2008b).

Live attenuated vaccines have been generated either by cold passage or by reverse genetics. Temperature-sensitive viruses conferred complete protection against heterologous HMPV challenge in golden Syrian hamsters (Herfst et al., 2008a), and a recombinant HMPV lacking a region of the SH ( $\Delta^{101-103}$ SH), the G, or the M2-2 protein induced high titers of neutralizing antibody and protected hamsters and African green monkeys against PIV and HMPV challenge (Biacchesi et al., 2004; Buchholz et al., 2005, 2006). A chimeric vaccine has also been developed in which the F2 subunit of the HMPV fusion protein is inserted into a backbone of bovine PIV that also contains the HPIV3 F and HN genes (Tang et al., 2003). This B/HPIV3/HMPV F2 vaccine induced neutralizing antibodies and protected hamsters and African green monkeys against PIV and HMPV challenge (Tang et al., 2003, 2005).

## Coronavirus

Coronaviruses such as HCoV-229E and OC43 are frequent causes of respiratory illness throughout the world, and SARS-CoV and MERS-CoV represent significant public health threats. However, at present there are no licensed vaccines for human coronavirus infections, though a number of vaccines are licensed against animal coronaviruses, and several vaccine platforms have been developed for SARS-CoV that show great promise in preclinical studies.

Whole SARS-CoV particles inactivated with  $\beta$ -propiolactone, formalin, UV light, or a combination of two techniques have been evaluated in mice, ferrets, rabbits, and

nonhuman primates. Such vaccines have been administered by a variety of routes and tested in the presence or absence of various adjuvants. In general, vaccination elicited a serum IgG response in animal models, with higher doses of vaccine eliciting higher IgG antibody titers (Subbarao, 2010). Moreover, some studies demonstrated that inactivated SARS-CoV vaccines were efficacious in protecting mice (See et al., 2006; Spruth et al., 2006; Stadler et al., 2005) and nonhuman primates (Qin et al., 2006; Zhou et al., 2005), although many studies did not investigate protective efficacy. In addition, an inactivated SARS vaccine that was evaluated in clinical trials was safe and well tolerated and induced neutralizing antibodies (Lin et al., 2007).

Many subunit vaccines comprise purified, recombinant SARS-CoV S protein, as this protein is the target for protective neutralizing antibodies elicited by SARS-CoV infection. A truncated soluble form of the S protein that lacks the transmembrane domain also neutralized infectivity of SARS-CoV (Bisht et al., 2005; He et al., 2006b; Zhou et al., 2006) and substantially reduced the titer of challenge virus replication in the respiratory tract of mice (Bisht et al., 2005). A trimeric spike protein vaccine (TriSpike) was also immunogenic in mice and hamsters and provided protection against challenge in hamsters, with reduced occurrence and severity of pneumonitis and no evidence of pulmonary consolidation or SARS-CoV-associated hepatic cellular necrosis (Kam et al., 2007). However, sera from animals immunized with TriSpike were associated with a 100- to 1000-fold increase in virus entry into Fc $\gamma$ R2-positive (ACE2-negative) human B cells, which has led to concerns about antibody-dependent enhancement of disease (Kam et al., 2007).

Vectored vaccines have been developed against SARS-CoV. In this approach, SARS-CoV proteins are expressed by Venezuelan equine encephalitis virus replicon particles (VRPs), chimeric bovine/human PIV3 vectors, recombinant replication-defective human adenovirus-5 (Ad-5), poxvirus MVA, attenuated rabies viruses, or attenuated vesicular stomatitis virus (Subbarao, 2010). When animals were immunized with vectored vaccines expressing the S protein of SARS-CoV, neutralizing antibodies were induced, and vaccinated animals were protected from challenge. However, if other SARS-CoV proteins were expressed, animals were not protected from challenge. For example VRPs expressing the SARS-CoV S protein provided protection from challenge in mice; however, VRP expressing the SARS-CoV N protein (VRP-N) did not (Deming et al., 2006). Moreover, VRP-N vaccination resulted in a marked bronchiolitis, alveolitis, and interstitial accumulation of eosinophils and mononuclear leukocytes (Deming et al., 2006). In addition, chimeric bovine/human (B/H) PIV3 vectors expressing the SARS-CoV S protein or the S, M, and E proteins together also generated neutralizing antibodies in hamsters and protected animals from challenge, whereas B/H parainfluenza viruses expressing M, N, or E proteins were not protective (Buchholz et al., 2004).



The route of administration is also important in determining protective efficacy of a vaccine. An Ad-5 vector expressing the SARS-CoV S and N genes given i.m. had a limited effect in reducing pulmonary replication of challenge virus, despite eliciting higher serum IgG titers and a greater cellular immunity than when the vaccine was given intranasally (i.n.). However, the vaccine administered i.n. induced higher mucosal IgA responses than when given i.m. and provided protection from pulmonary replication, suggesting that mucosal immunity is important in mediating protection (See et al., 2006).

Mice, rabbits, and monkeys immunized with a modified vaccinia virus MVA-S vaccine were protected from replication of challenge virus (Bisht et al., 2004; Chen et al., 2005). However, in ferrets, MVA-S antibodies declined rapidly after vaccination, and the vaccine did not protect the animals from replication or spread of the challenge virus (Weingartl et al., 2004).

A recombinant live attenuated SARS-CoV vaccine virus in which expression of the E protein was abrogated by point mutations and a deletion in the nucleotide sequence was restricted *in vitro* and attenuated in hamsters (DeDiego et al., 2007). In addition, inactivation of the 3'-5' exonuclease attenuated SARS-CoV, and the resulting virus was protective in an aged immunocompromised mouse model of SARS infection (Graham et al., 2012).

DNA vaccines expressing SARS-CoV S protein (either without the cytoplasmic domain or without the cytoplasmic and transmembrane domains) also induced humoral and cellular immunity in mice and protected animals from replication of challenge virus (Yang et al., 2004). In addition, priming with a DNA vaccine and boosting with an inactivated vaccine resulted in an increase in CD4<sup>+</sup> T cells and a stronger antibody response (Zakhartchouk et al., 2005). The DNA vaccine was well tolerated and produced cellular immune responses and neutralizing antibody in a phase I clinical trial (Martin et al., 2008).

Despite these successes in preclinical vaccine development, as the SARS outbreak was declared over in 2003, the impetus was not sustained long enough for many of these products to be evaluated in clinical trials.

A cautionary note about SARS-CoV vaccines is the association of a variety of vaccine platforms with pulmonary immunopathology in mice after challenge with the virus, despite immunogenicity and protective efficacy (Tseng et al., 2012). The relevance of the rodent model to vaccines in humans remains uncertain.

## Adenovirus

Adenovirus epidemics have been described in adults, especially in military recruits in closed or crowded settings (Lynch et al., 2011). Live attenuated, orally administered adenovirus vaccines against serotypes 4 and 7, which cause

respiratory disease, were used by the U.S. military starting in 1971 (Top, 1975). During this time the incidence of disease fell substantially. However, when the manufacturer ceased vaccine production in 1995, epidemics reemerged in military facilities (Lynch et al., 2011). In 2011, a new live attenuated adenovirus vaccine was issued to military recruits during basic training, and there has since been a reduction in the rate of febrile respiratory illness (Armed Forces Health Surveillance, 2013). The vaccine is not available to children or the civilian adult population at present, though adenoviruses are estimated to account for 5–10% of pediatric and 1–7% of adult respiratory tract infections (Lynch et al., 2011).

In summary, the majority of studies evaluating respiratory virus vaccines measure serum antibody responses, because a large body of evidence indicates that, although both cellular and humoral responses contribute to the clearance of a primary infection, neutralizing antibodies protect against secondary infection. Humoral responses can be readily detected after vaccination with inactivated or subunit vaccines; however, fewer individuals seroconvert after vaccination with live vaccines. Alternative immune mechanisms such as mucosal antibody responses are probably responsible for protection by live attenuated vaccines, and immune correlates of protection are under investigation.

## NEW DEVELOPMENTS IN MUCOSAL VACCINATION

Because respiratory pathogens infect the host at mucosal surfaces, the induction of mucosal immunity by vaccination is desirable. Vaccines that are administered by intramuscular or subcutaneous injection induce protective immunity in the systemic immune compartments, but are generally poor at inducing mucosal immune responses. Mucosally delivered vaccines, in contrast, induce both mucosal and systemic immunity and are also more readily accepted because they do not require needles or syringes (Levine and Dougan, 1998; Yuki and Kiyono, 2009). Ongoing research into the molecular and cellular mechanisms of surface immunological barrier systems has provided practical strategies for the development of a new mucosal vaccine for the control of respiratory viruses.

### M-Cell-Targeted Vaccines

M cells, located in the follicle-associated epithelium of the MALT, play a pivotal role in the uptake of luminal antigens in the respiratory tract and the induction of antigen-specific immune responses in both the systemic and the mucosal compartments (Kiyono and Fukuyama, 2004). It is, therefore, logical and attractive to develop M-cell-targeted mucosal vaccines. Several molecules have been found to bind preferentially to M cells, such as *Ulex*

*europaeus* agglutinin-1 (UEA-1), which has specificity for  $\alpha(1,2)$ -fructose (Sharma et al., 1998), and rho-protein 1 derived from reovirus, which binds to a carbohydrate structure containing  $\alpha 2, 3$ -linked sialic acids on the plasma membranes of M cells (Helander et al., 2003). Moreover, intranasal vaccines conjugated to either UEA-1 (Manocha et al., 2005) or rho-protein induced not only strong antigen-specific plasma IgG and mucosal IgA responses, but also CTL immunity (Wu et al., 2001). In addition, a novel M-cell-specific monoclonal antibody has recently been identified that selectively recognizes M cells, but not goblet cells or epithelial cells. This has been used as a carrier for an M-cell-targeted mucosal vaccine (Wu et al., 2001; Nochi et al., 2007).

Concerns have been raised about the potential induction of unwanted mucosal inflammation associated with M cell targeting (Kuolee and Chen, 2008), and additional research is needed to better understand how M cells sample antigens and transcytose them to the basolateral membrane. However, most microparticles administered orally become trapped in the mucus, and only a small fraction of them enter mucosal inductive sites. New approaches that are being developed to transitionally or conditionally enhance the number and function of M cells (Neutra and Kozlowski, 2006) are likely to reduce these concerns.

## Synthetic Delivery Systems

There is substantial interest in the exploitation of nanoparticle technology for drug and vaccine delivery. Nanoparticles are solid particles ranging in size from 10 to 1000 nm in diameter that are often made from biodegradable materials. An antigen payload can be dissolved, entrapped, adsorbed, attached, or encapsulated into the matrix of the particle and released as the particle degrades over a period of time, which may vary from days to weeks, depending on the formulation (Adair, 2009).

Several types of nanoparticles have been investigated for vaccine delivery and have proven to be safe, nontoxic, and suitable for loading with antigens. These include the polyesters (poly(lactic acid), poly(glycolic acid), and their copolymers), polyorthoesters, polyanhydrides, and polycarbonates (Reddy et al., 2006). These materials can protect antigens from degradation, and the particles can be prepared in a chemically reproducible manner within a narrow size limit. In addition, some biopolymers exhibit a natural adjuvant behavior, for example, poly(lactide co-glycolide) (PLGA) appears to activate the maturation of DCs, possibly by providing a necessary stimulatory signal, though the exact mechanism is not fully elucidated (Bennewitz and Babensee, 2005; Yoshida and Babensee, 2004).

Surface modifications of nanoparticles that change their overall charge and hydrophobicity are aimed at improving mucoadherence properties (des Rieux et al., 2006). This can

be achieved by coating with stabilizers, other polymers, or surfactants. Polyethylene glycol has been used for its stabilizing properties and because it can enhance the affinity of the nanoparticles for mucosal surfaces. Molecules such as lectins and chitosan can also increase interaction with and transport across the mucosal surface. Interestingly, chitosan is reported to be able to open tight junctions between epithelial cells, facilitating the transport of encapsulated macromolecules across the epithelial layer (Illum and Davis, 2001; Illum et al., 2001). Nanoparticles coated with mannose (mannan) have also been produced with the aim of targeting mannose receptors on APCs, thus improving cell adhesion and uptake (Cui and Mumper, 2003).

A PLGA-coated nanoparticle vaccine encapsulating influenza virus proteins has been developed. The influenza HA protein retained its antigenicity after encapsulation, and mice vaccinated with PLGA particles of 2.2–10.8  $\mu\text{m}$  in diameter mounted both systemic and mucosal responses, which were protective against intranasal challenge with an H3N2 influenza virus (Amidi et al., 2007; Moldoveanu et al., 1993). Chitosan-coated nanoparticles containing purified influenza virus have also been tested in human volunteers, in which a fourfold or greater increase in anti-HA antibodies was observed in >40% of the volunteers (Illum and Davis, 2001). Moreover, a single dose of the intranasal vaccine resulted in high titers of nasal IgA antibody and strong systemic antigen-specific responses, which were greater than those induced after intramuscular inoculation with soluble influenza antigen (Amidi et al., 2007). Nanoparticle-conjugated RSV proteins have also been shown to generate strong systemic Th1 immunity in mice, associated with protection against wild-type RSV challenge (Kalkanidis et al., 2006; Xiang et al., 2006). In addition, PLGA-coated nanoparticles incorporating HPIV HN and F glycoproteins have been shown to induce virus-neutralizing antibodies, which were protective against challenge infection in hamsters (Ray et al., 1993).

## Mucosal Adjuvants

Studies in animal models and humans have shown that the choice of adjuvant can dramatically affect the immediate immune response and long-term protective effect of a vaccine (Ogra et al., 2001; Galli et al., 2009). In addition, the quality of the immune response, especially the development of high-affinity B cells, long-lived memory B cells, and plasma cells can be influenced by the choice of adjuvant (Galli et al., 2009). Although the mechanisms have not yet been fully elucidated, mucosal adjuvants can be broadly classified into two categories: those that act as immunostimulatory molecules and those that facilitate vaccine delivery (O'Hagan, 2001). The former include adjuvants that are toxin-based or cytokine-based

and molecules associated with innate immunity, for example, PAMPs. The latter contain immune-stimulating complexes, liposomes, live attenuated vectors, and chitosan (discussed above). From a mechanistic point of view, mucosal adjuvants modulate innate immune responses in the same way as parenterally administered vaccines (Lambrecht et al., 2009), and TLR agonists constitute a major category of mucosal adjuvants. These adjuvants are based on PAMPs and are often formulated as oil-in-water emulsions.

### *Virosomes and Immune Stimulating Complexes*

Virosomes are virus-like particles that have been investigated for their potential as vaccines (Huckriede et al., 2005; Bungener et al., 2005). They closely resemble native virus; however, they are nonreplicating and consist of reconstituted viral envelopes, generated by treatment with a detergent. They have a diameter of 100–200 nm and, as such, fall into the size range of small particles. Virosomal influenza vaccines are available commercially (Herzog et al., 2009) and have been shown to induce HA-specific antibodies after i.m. administration. Phase I clinical trials revealed the tolerability and immunogenicity of a Matrix<sup>TM</sup>-adjuvanted virosomal H5N1 DNA vaccine (Cox et al., 2011). Influenza virosomes that incorporate the RSV-fusion protein have also been constructed and have been shown to generate virus-specific IgA in the URT and LRT after i.n. administration with adjuvant in mice (Cusi et al., 2002).

Immune stimulating complexes (ISCOMs) are nonreplicating particles of ~40 nm diameter, comprising viral glycoproteins complexed with saponin derived from the bark of the tree *Quillaja saponaria* (Gregory et al., 2013). ISCOM antigens from a number of microorganisms, including viruses, bacteria, and parasites, have been shown to induce humoral and cell-mediated immunity; Th1 immune responses appear to predominate (Morein et al., 1998).

Extensive studies have been carried out with influenza virus ISCOMs in several species, including mice and monkeys, and an equine influenza ISCOM vaccine is available commercially (Mumford et al., 1994). An influenza ISCOM vaccine (Sundquist et al., 1988) stimulated high levels of virus-specific IgM and IgG serum antibodies and proliferative T cell responses in macaques, and the animals were completely protected from intratracheal challenge with the virus (Rimmelzwaan et al., 1997).

Intranasal immunization of mice with RSV ISCOMs induced very high levels of long-lasting RSV-specific IgA in both the URT and the LRT (Hu et al., 1998). Bovine RSV ISCOMs inoculated subcutaneously into calves were completely protective against challenge with virulent bovine RSV, whereas calves immunized with a conventional bovine RSV vaccine developed moderate to severe

respiratory disease after challenge (Hagglund et al., 2004). The ISCOM-vaccinated animals developed high titers of nasal and serum virus-specific IgG as well as serum IgA, which correlated with protection.

### *Toxin-Based Adjuvants*

Detoxified derivatives of cholera toxin (CT) and heat-labile enterotoxin (LT) produced by enterotoxigenic *Escherichia coli* are effective mucosal adjuvants that promote mucosal and systemic immunity to coadministered protein antigens via oral or nasal routes (Freitag and Clements, 1999; Mestecky et al., 1999). The results of murine studies demonstrated that both CT and LT induced the expression of B7-1 and/or B7-2 on APCs that deliver costimulatory signals to CD4<sup>+</sup> T cells (Freitag and Clements, 1999; Mestecky et al., 1999), but CT acts in the presence of IL-4 to induce predominantly Th2 responses, whereas LT supports Th1 responses with IFN- $\gamma$  production. Recent vaccine development efforts have focused on the nasal administration of antigen with CT and LT derivatives for the induction of mucosal IgA (Byun et al., 2001; Fujihashi et al., 2002).

Both CT and LT are AB<sub>5</sub>-type molecules, consisting of one A subunit and five B subunits. Chimeric molecules, made by the spontaneous association of the A subunit of CT with the B subunit of the LT, or vice versa, are effective mucosal adjuvants for protein vaccines. The type of T helper responses induced is dictated by the origin of the B subunit (Takahashi et al., 2009). One such example is the nasal administration of an influenza HA subunit vaccine together with a chimeric mutant (m) CT-A/LT-B adjuvant. In murine studies, this adjuvanted vaccine protected animals from influenza virus challenge (Kweon et al., 2002).

However, there are some concerns about the safety profile of toxin-derived adjuvants. Nasal administration of mCT-A/LT-B targeted neuronal tissues, though it did not affect trafficking of coadministered vaccine antigens into the neuronal tissues (Kweon et al., 2002).

Taken together, these findings suggest that although recombinant chimeric toxin-based molecules show promise as a new generation of mucosal adjuvants, the safety of the CT adjuvant may need to be improved. The inactivated LT-adjuvanted intranasal influenza vaccine (NasalFlu) was withdrawn because of concomitant facial nerve paralysis (Bell's palsy) that was noted as a potential adverse event caused by the CT adjuvant (Herzog et al., 2009; Kunzi et al., 2009; Garner-Spitzer et al., 2009).

### *Cytokine-Based Mucosal Adjuvants*

Several cytokines associated with innate immunity and inflammation support the generation of antigen-specific S-IgA and serum IgG/IgA and may be of use as mucosal adjuvants.

## IL-1

Mice nasally immunized with soluble antigens, including ovalbumin or tetanus toxoid (TT) plus IL-1 $\alpha$  and IL-1 $\beta$  developed antigen-specific antibody responses that were similar to those induced by coadministered CT adjuvant. The cytokines IL-1 $\alpha$  and IL-1 $\beta$  promoted antigen-specific IgG1 antibody responses initially, followed by IgG2b, with minimal IgG2a antibody responses, a pattern associated with a predominantly Th2-type response (Staats and Ennis, 1999; Thompson and Staats, 2011). Furthermore, levels of antigen-specific S-IgA antibody similar to those induced by CT were found in mucosal secretions of mice that received nasal IL-1. These results indicate that IL-1 could be a useful mucosal adjuvant.

## IL-6/IL-12

APCs are known to contribute to the creation of an appropriate cytokine environment for the growth and development of Th1 or Th2 responses. Two well-known cytokines secreted by APCs, IL-6 and IL-12, can influence the outcome of Th1 and Th2 cell subset-mediated immune responses (Vajdy et al., 1995; Rincon et al., 1997). When TT was used as an antigen, nasal coadministration of IL-6 or IL-12 induced antigen-specific serum IgG antibody responses and promoted protective immunity against lethal challenge with tetanus toxin. Interestingly, whereas nasal treatment with IL-12 promoted mixed Th1/Th2-type responses, IL-6 supported predominantly Th2-type responses. In addition, IL-12 but not IL-6 can induce antigen-specific mucosal IgA antibody (Boyaka et al., 1999). These findings suggest that IL-12 could be a potent mucosal cytokine for the upregulation of antigen-specific mucosal immune responses.

## Type I Interferons

Type I IFN affects the differentiation and function of immune cells, including T cells and DCs, and efficiently enhances a primary antibody response (Marrack et al., 1999; Luft et al., 1998). It was reported that type I IFN was effective as both a systemic and a mucosal adjuvant, promoting Th1-type immune responses (Proietti et al., 2002). Nasal administration of influenza vaccine with type I IFN was effective at inducing serum antigen-specific IgG2a and mucosal IgA antibody responses and at providing full protection against influenza virus challenge (Proietti et al., 2002).

## Immunization Routes

It is well known that i.n. vaccination stimulates immune responses in the NALT and is effective at inducing mucosal immunity in the respiratory tract (Brandtzaeg, 2011; Jabbal-Gill, 2010). Intranasal administration of the live attenuated influenza vaccine Flumist (MedImmune) has proven effective in protection against seasonal influenza virus infection and protects children against drifted influenza virus strains

(Belshe et al., 2000a; Mendelman et al., 2004). Furthermore, owing to the development of novel technologies, both aerosol spray and droplet delivery of vaccines are attractive possibilities (Jabbal-Gill, 2010).

In addition to the i.n. route of vaccine inoculation, the sublingual route has been explored (Shim et al., 2011; Czerkinsky et al., 2011). The sublingual epithelium harbors a dense lattice of DCs, and vaccine delivered via this route stimulates broad and disseminated mucosal and systemic immune responses, similar to intranasal inoculation (Czerkinsky et al., 2011). Sublingual vaccination with soluble or particulate antigens promotes strong mucosal IgA and systemic IgG antibody responses as well as CD8<sup>+</sup> T cell responses. Overall, sublingual immunization was comparable to nasal immunization in the magnitude, breadth, and anatomic dissemination of the induced immune responses. Importantly, sublingual administration did not redirect antigens and/or adjuvants to the brain (Czerkinsky et al., 2011).

Sublingual vaccination against influenza has been shown to protect mice against lung infection (Song et al., 2008). In addition, sublingual administration of inactivated influenza A/PR8 (H1N1) vaccine virus together with a mucosal adjuvant such as CT (Cuburu et al., 2007) or a nontoxic mCT-A/LT-B adjuvant induced both systemic and mucosal virus-specific antibody responses as well as CTL responses with protective immunity after respiratory challenge with the A/PR8 virus (Kweon et al., 2002; Song et al., 2008). These studies demonstrated that sublingual administration of an inactivated influenza virus with a toxin adjuvant such as CT did not migrate to or replicate in the central nervous system. Moreover, using mucosal adjuvant such as CT mobilizes DCs within the sublingual epithelium. These observations suggest that sublingual immunization may be another attractive and safe mucosal route for the administration of respiratory virus vaccines.

## CONCLUDING REMARKS

Given the global burden of respiratory tract infection, there remains an unmet need for effective methods of intervention. The most efficient means of preventing respiratory virus infections is vaccination. However, among respiratory viruses, licensed vaccines are available only for influenza.

Systemic immune responses can be protective in the absence of mucosal immunity if they are sufficiently robust, such as that induced by i.m. inactivated influenza vaccines; however, many vaccines in development for respiratory viruses are live attenuated viruses that are mucosally administered. Although live vaccines show promise, our understanding of the mechanism of protection at mucosal surfaces is incomplete, and there is currently a lack of adequate immune correlates of protection for these vaccines. In addition, immune responses mounted to a natural infection are often not sufficient to prevent reinfection and may

also be involved in the pathogenesis of disease. Achieving an appropriate balance between sufficient attenuation and immunogenicity, especially in young infants who must be vaccinated in the face of an immature immune system and maternal antibody, is a challenge.

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