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PATHOPHYSIOLOGY AND EPIDEMIOLOGY OF VIRUS-INDUCED ASTHMA

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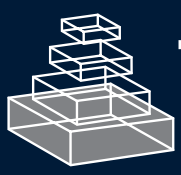
Hirokazu Kimura and Akihide Ryo



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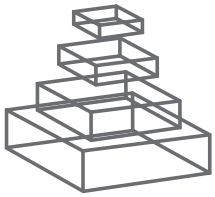

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PATHOPHYSIOLOGY AND EPIDEMIOLOGY OF VIRUS-INDUCED ASTHMA

Topic Editors:
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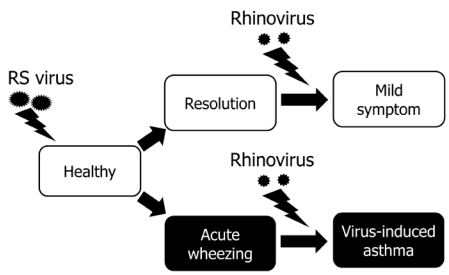

Respiratory viral infections and development of asthma.
Host-pathogen interactions that determine the severity of respiratory illnesses, and risk for subsequent asthma was increased by respiratory virus infections including RS virus and human rhinovirus (HRV). Most acute wheezing may spontaneously resolve within a few days, a history of wheezing and host immunological conditions (e.g., atopic features) heightens the risk for asthma. Once asthma is established, various viruses (ie; HRV) induce asthma symptoms in humans.

Virus-caused asthma, we now call a phenotype of asthma. Regardless of the significance and popularity of this disease, the etiology of the virus-induced asthma have not well understood. In addition, a few effective vaccines have been applied to prevent respiratory virus infection. To solve the issues, it is essential to clarify and delineate both aspects of the virus and host defense systems including acute/chronic inflammation and airway tissue remodeling. To deeply review and discuss pathophysiology and epidemiology of virus-induced asthma, this topics includes new findings of the host immunity, pathology, epidemiology, and virology of asthma/chronic obstructive pulmonary disease (COPD). We believe that these works are well summarized and informative to glimpse the field of virus-associated asthma and COPD, and may help understanding the basic and clinical aspects of the diseases.

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Pathophysiology and epidemiology of virus-induced asthma

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Keywords: virus-induced asthma, epidemiology, pathology, respiratory virus, human immunity

Many respiratory viruses are mainly responsible for common cold, bronchitis, bronchiolitis, and pneumonia. Furthermore, asthma and chronic obstructive pulmonary disease (COPD) are major cause of mortality. The prevalence of asthma in developed countries is approximately 10% in adults and even higher in children (Barnes, 2008). Thus, the medical costs for these diseases are a major burden in many countries. Respiratory virus infections also cause the most of acute exacerbation of asthma (virus-induced asthma) or COPD. Among them, human rhinoviruses (HRV) are detected in the two thirds of the cases with asthma exacerbations in children (Johnston et al., 1995). However, epidemiology and pathophysiology of asthma and COPD is not known. Furthermore, a few effective vaccines have been applied. Therefore, it may be important to better understand pathophysiology of virus-induced asthma or virus-induced COPD exacerbation. Both aspects of the virus agents and host defense systems including acute/chronic inflammation and airway tissue remodeling should be clarified. This e-book aims to review and discuss pathophysiology and epidemiology of virus-induced asthma and COPD focusing on new findings of the host immunity and virology.

This Research Topic contains 7 review articles and 3 original articles regarding pathophysiology of virus-induced asthma. As the first article, Kudo et al. (2013) reviewed pathology of asthma. This article globally covers from molecular histopathology involved in cytokine networks of asthma. The readers may easily understand molecular immunopathology of virus-induced asthma. In the second issue, Okayama (2013) presents cellular and humoral immunity of asthma. Accumulating evidence implicates that the genetic and environmental factors may be associated with virus-induced asthma. This work focuses on the immunological mechanisms that may explain why asthma is associated with RSV-and HRV-infection. As the third review article, Kimura et al. (2013) present the molecular mechanisms between various cytokines and innate immunity of viral respiratory infections including virus-induced asthma. The authors also show the signaling pathways with regard to them. In the 4th review article, Tsukagoshi et al. (2013) discuss the genetic characteristics and molecular evolution of respiratory viruses, and epidemiology of asthma. They also show phylogenetic analysis of the detected viruses in the children with respiratory syncytial virus- (RSV) and/or HRV-associated wheezing and asthma. As the 5th review article, Inoue and Shimojo (2013) present epidemiology and pathophysiology of virus-induced asthma in children. They summarize the previous findings and discuss how clinicians can effectively intervene in these viral infections to prevent the development of asthma. Next, Kurai et al. (2013) and Saraya et al. (2014) present pathophysiology of virus-induced COPD and asthma in adults. They summarize current knowledge concerning exacerbation of both COPD and asthma by focusing on the clinical significance of associated respiratory virus infections. Furthermore, influenza A(H1N1)pdm09 virus have suddenly emerged in Mexico in the spring, 2009. The virus can cause influenza pandemy accompanying with pneumonia/wheezing. Obuchi et al. (2013) review essential reports with regard to asthma in patients infected with the virus, and they discuss the utility of influenza vaccines and antivirals. Although HPIV3 is an etiological agent for respiratory disorders such as pneumonia and asthma, there is no prophylactic human vaccine against the virus infection. In the 9th issue as original article, Senchi et al. (2013) present the development of an oligomannose-coated liposome (OML) nasal vaccine against HPIV3 in combination with an effective adjuvant Poly(I:C). They report that their newly-developed vaccine can successfully induce antigen-specific immunity with a small amount of antigen via the nasal route. These results highlight the utility of combining sophisticated systems in the development of a novel vaccine against HPIV3. In the final article, Matsunaga et al. (2014) present the development of monoclonal antibodies (MAbs) against hemagglutinin-neuraminidase (HN) of HPIV3. For synthesizing the antigen protein, they utilized the wheat germ cell-free system. This new cell-free system-based protocol for antigen production enabled to create the MAbs that can be applicable in various immune assays such as flowcytometry and immunoprecipitation analyses. The newly-developed MAbs could thus be a valuable tool for the study of HPIV3 infection as well as the several diagnostic tests of this virus.

In conclusion, we believe that these works are well summarized and informative to glimpse the field of virus-associated asthma/COPD, and may help understanding the basic and clinical aspects of the disease. We would be happy if this collection of papers will offer new stimuli and perspectives for not only researchers but also clinicians working around the exciting and emerging the e-book.

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Pathology of asthma

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Asthma is a serious health and socioeconomic issue all over the world, affecting more than 300 million individuals. The disease is considered as an inflammatory disease in the airway, leading to airway hyperresponsiveness, obstruction, mucus hyper-production and airway wall remodeling. The presence of airway inflammation in asthmatic patients has been found in the nineteenth century. As the information in patients with asthma increase, paradigm change in immunology and molecular biology have resulted in an extensive evaluation of inflammatory cells and mediators involved in the pathophysiology of asthma. Moreover, it is recognized that airway remodeling into detail, characterized by thickening of the airway wall, can be profound consequences on the mechanics of airway narrowing and contribute to the chronic progression of the disease. Epithelial to mesenchymal transition plays an important role in airway remodeling. These epithelial and mesenchymal cells cause persistence of the inflammatory infiltration and induce histological changes in the airway wall, increasing thickness of the basement membrane, collagen deposition and smooth muscle hypertrophy and hyperplasia. Resulting of airway inflammation, airway remodeling leads to the airway wall thickening and induces increased airway smooth muscle mass, which generate asthmatic symptoms. Asthma is classically recognized as the typical Th2 disease, with increased IgE levels and eosinophilic inflammation in the airway. Emerging Th2 cytokines modulates the airway inflammation, which induces airway remodeling. Biological agents, which have specific molecular targets for these Th2 cytokines, are available and clinical trials for asthma are ongoing. However, the relatively simple paradigm has been doubted because of the realization that strategies designed to suppress Th2 function are not effective enough for all patients in the clinical trials. In the future, it is required to understand more details for phenotypes of asthma.

Keywords: asthma, remodeling, epithelial to mesenchymal transition, Th2 cells, cytokines, Th17 cells, Th9 cell

INTRODUCTION

Asthma is characterized by the action of airway leading to reversible airflow obstruction in association with airway hyperresponsiveness (AHR) and airway inflammation (Holgate, 2012). The disease is affecting more than 300 million persons all over the world, with approximately 250,000 annual deaths (Bousquet et al., 2007). In the last couple of decades, as the inhaled corticosteroid has become the major treatment agent for asthma, the mortality of asthma has decreased (Wijesinghe et al., 2009). Meanwhile, allergic diseases, such as asthma, have markedly increased in the past half centuries associated with urbanization (Alfvén et al., 2006). Children have the greatest percentage of asthma compared with other generation groups (Centers for Disease Control and Prevention, 2011). Then, it is expected that the number of the patients will increase by more than 100 million by 2025 (Masoli et al., 2004).

Generally, most asthma starts from childhood in relation to sensitization to common inhaled allergens, such as house dust mites, cockroaches, animal dander, fungi, and pollens. These inhaled allergens stimulate T helper type 2 (Th2) cell proliferation, subsequently Th2 cytokines, interleukin (IL)-4, IL-5 and IL-13 production and release. Many basic and clinical studies suggested that airway inflammation was a central key to the disease pathophysiology. The existence of chronic airway inflammation in asthma has been recognized for over a century. The inflammation is induced by the release of potent chemical mediators from inflammatory cells. Resulted of chronic airway inflammation, airway remodeling, characterized by thickening of all compartments of the airway wall, is occurred and may have profound consequences on the mechanics of airway narrowing in asthma and contribute to the chronicity and progression of the disease.

As allergic sensitization, allergen can be taken up by dendritic cells (DCs), which process antigenic molecules and present them to naïve T helper cells. Consequently the activation of allergen-specific Th2 cells is occurred, the cells play an important role in developing the asthma. Nowadays, it is known that Th17 cells and Th9 cells also modulate the disease. Th17 cells produce IL-17A, IL-17F, and IL-22. These cytokines induce airway inflammation and IL-17A enhance smooth muscle contractility.

Allergic diseases are caused by inappropriate immunological responses to allergens without pathogenesis driven by a Th2-mediated immune response. The hygiene hypothesis has been used to explain the increase in allergic diseases since industrialization and urbanization, and the higher incidence of allergic diseases in more developed countries. The hypothesis has now expanded to include exposure to symbiotic bacteria and parasites

as important modulators of immune system development, along with infectious agents (Grammatikos, 2008). Recently, asthma has not been recognized as a simple Th2 disease, which is characterized by IgE elevation and relatively eosinophilia. Th17 and Th9 cell subtype are known to contribute the inflammation or enhancing smooth muscle contraction or stimulating mast cells.

HISTOPATHOLOGY OF ASTHMATIC AIRWAY

Asthma is considered in terms of its hallmarks of reversible airflow obstruction, non-specific bronchial hyperreactivity and chronic airway inflammation (American Thoracic Society, 1987). Osler (1892) mentioned in the classic textbook, the inflammatory process, affecting the conducting airways with relative sparing of the lung parenchyma. Huber and Koesser (1922) provided a comprehensive perspective of the histopathological features of asthma. That is, the lungs are usually hyperinflated as a consequence of extensive mucous plugging in segmental, subsegmental bronchus and peripheral airways, but the lung parenchyma in general, remains relatively intact in subjects who die in exacerbation, so-called *status asthmatics*. The composition of mucous includes cellular debris from necrotic airway epithelial cells, an inflammatory cells including lymphocytes, eosinophils, and neutrophils, plasma protein exudate, and mucin that is produced by goblet cells (Unger, 1945; Bullen, 1952; Dunnill, 1960; Messer et al., 1960). The airway epithelium typically shows sloughing of ciliated columnar cells, with goblet cell and squamous cell metaplasia as a sign of airway epithelial repair. There is increased thickness of the subepithelial basement membrane, however, some studies have established that the true basal lamina is of normal thickness, and the apparent increase in thickness is related to accumulation of other extracellular matrix components beneath the basal lamina (Roche et al., 1989). The asthmatic airway showed a thickness with inflammatory cell infiltration consisting of an admixture of T lymphocytes and eosinophils, mast cells (Carroll et al., 1997; Hamid et al., 1997). Interestingly, prominent neutrophil infiltrates have been reported to be a specific feature of the clinical entity of sudden onset fatal asthma (Sur et al., 1993).

Nowadays investigators can easily obtain lung tissue and bronchoalveolar lavage (BAL) specimens from the patients with asthma (Salvato, 1968; Djukanovic et al., 1991). Results of studies of BAL (Robinson et al., 1992) and lung tissue specimens (Minshall et al., 1998) have strongly implicated a role for cytokines produced by the Th2 subset of CD4+ T cells in the pathogenesis of asthma. For example, IL-13 plays an important role in regulating the airway inflammation in asthma (Wills-Karp et al., 1998; Zhu et al., 1999).

In recent years, there has been increasing interest in the mechanism of airway wall remodeling in asthma, owing to the increasing realization that airway inflammation alone is not enough to explain the chronicity or progression of asthma (Holgate et al., 1999). The nature of airway remodeling may be considered in terms of extracellular matrix deposition. It is postulated that the injured airway epithelium acts as a continuous stimulus for airway remodeling (Holgate et al., 1999), and this is supported by results of recent cell culture experiments examining interactions of bronchial epithelial cells with myofibroblasts in response to injurious stimuli (Zhang et al., 1999). The remodeling is predicted to have little effect on baseline respiratory mechanics, the physiological effects of extracellular matrix accumulation are predicted to result in an exaggerated degree of narrowing for a given amount of airway smooth muscle (ASM) contraction.

Airway wall thickening is greater in the asthmatic patients than normal subjects, and severe patients have greater (Awadh et al., 1998). This thickness is due to an increase in ASM mass and mucous glands (Johns et al., 2000). The airflow limitation is also compounded by the presence of increased mucous secretion and inflammatory exudate (Chiappara et al., 2001). Thus, the results from many studies have supported that airway remodeling related to airway inflammation. Surprisingly, physical force generated by ASM in bronchoconstriction without additional inflammation induces airway remodeling in patients with asthma (Grainge et al., 2011). Despite these recent advances, further work is necessary to establish a causal relationship between airway remodeling and the severity of asthma (Bento and Hershenson, 1998).

AIRWAY EPITHELIUM

The structural changes in the asthmatic airway result from interdependent inflammatory and remodeling processes (Chiappara et al., 2001). In the processes, inflammation occurs common features, vascular congestion, exudaution, and inflammatory cell recruitment to the interstitial tissue. Furthermore mucus secretion and desquamation of epithelial cells are increased. The chronic inflammatory changes develop epithelium-mesenchymal interactions (Holgate et al., 2000). The number of myofibroblasts, which deposit collagens, increases in the understructure of epithelium, the proximity of the smooth muscle layer and the lamina reticularis in the patients. Subepithelial collagens cause thickening and increasing density of the basement membrane.

The airway inflammation gives damage to the epithelium and damaged epithelial cells will be repaired in the injury-repair cycle. Some studies showed that epithelial cells of untreated asthmatic patients had low level expression of proliferating markers, despite extensive damage, revealing a potential failure in the epithelial injury-repair cycle in response to local inflammation and inhaled agents (Bousquet et al., 2000). Injury to the epithelium results in a localized and persistent increase in epidermal growth factor (EGF) receptor, a mechanism that may cause the epithelium to be locked in a repair phenotype (Puddicombe et al., 2000). Epithelial cells which are in repair phase produced some profibrotic mediators, including transforming growth factor-β (TGF-β), fibroblast growth factor and endothelin, which regulate fibroblast and myofibroblast to release collagen, elastic fiber, proteoglycan, and glycoprotein and these substances induce airway wall thickening (Holgate et al., 2000). Myofibroblast is a rich source of collagen types I, II, and V, fibronectin and tenascin that also accumulate in the airway wall and induce thickening lamina reticularis (Roche et al., 1989; Brewster et al., 1990). This process may contribute phenomena by augmentation of airway narrowing because the inner airway wall volume increases.

Eosinophils seem to contribute to airway remodeling in several ways, including through release of eosinophil-derived TGF-β,

cationic proteins, and cytokines, as well as through interactions with mast cell and epithelial cells. Many of these factors can directly activate epithelium and mesenchymal cells, deeply related to the development of airway remodeling (Kariyawasam and Robinson, 2007; Aceves and Broide, 2008; Venge, 2010). Eosinophil-derived cytokines are in the modulation of Th2 responses that trigger macrophage production of TGF-β1, which serves as a stimulus for extracellular matrix production (Fanta et al., 1999; Holgate, 2001). TGF-β1 induced epithelial to mesenchymal transition (EMT) in alveolar epithelial cells and could contribute to enhance fibrosis in idiopathic lung fibrosis (Wilson and Wynn, 2009). TGF-β1 might also contribute to enhance airway remodeling through EMT. Indeed, anti-TGF-β1 treatment inhibits EMT in airway epithelial cells (Yasukawa et al., 2013).

Airway epithelium is a barrier in the frontline against stimuli from the environment, but in asthmatic epithelium is defective in barrier function with incomplete formation of tight junctions, that prevent allergen from penetrating into the airway tissue (Xiao et al., 2011). The defect would induce that a proportion of the asthma-related had biological properties to infiltrate the epithelial barrier and trigger a danger signal to DCs. Components of house dust mite, cockroach, animal, and fungal can disrupt epithelial tight junctions and activate protease-activated receptors (Jacquet, 2011). The defective epithelial barrier function has also been described in the pathophysiology of other allergic disease. Therefore, healthy barrier function is important to avoid sensitization and development in allergic disease.

AIRWAY SMOOTH MUSCLE

Abnormalities of asthmatic ASM structure and morphology have been described by Huber and Koesser (1922) in the first quarter of twentieth century when they reported that smooth muscle from the patients who died by acute exacerbation was increase much greater than in those who died from another disease. Airflow limitation mainly due to reversible smooth muscle contraction is a most important symptom of the disease. Therefore, ASM plays a material role in asthma. Abnormal accumulation of smooth muscle cells is another mechanism of airway remodeling. Some *in vivo* animal studies confirmed that prolonged allergen exposure increase smooth muscle thickness in the airway (Salmon et al., 1999). It is still unknown whether the phenomenon is occurred by fundamental changes in the phenotype of the smooth muscle cells, is caused by structural or mechanical changes in the non-contractile elements of the airway wall. There are two different ways by which cyclic generation of length and force could influence ASM contracting and airway narrowing. The processes, which are myosin binding and plasticity, have different biochemical and physical mechanisms and consequences. They have the potential to interact and to have a fundamental effect on the contractual capacity of smooth muscle and its potential to cause excessive airway narrowing (King et al., 1999).

Like other muscles, ASM is also provoked to contract with intracellular calcium ions (Ca^{2+}), which comes from the extracellular environment through voltage-dependent calcium channel or from the sarcoplasmic reticulum stores (**Figure 1**). The source

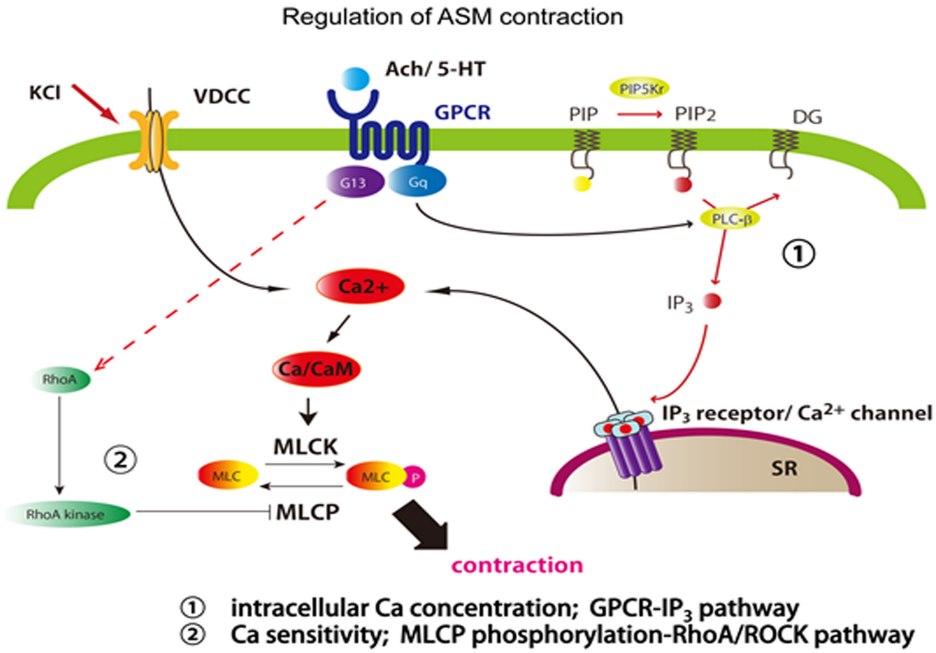

FIGURE 1 | Regulation of ASM contractility. ASM contraction is induced by calcium, regulated two different pathways. First, ASM is evoked by intracellular calcium influx from SR depending on GPCR stimulation or from the extracellular environment through voltage-dependent calcium channel. Second, smooth muscle can be induced calcium sensitivity by RhoA/Rho kinase pathway. RhoA activates Rho-kinase which phosphorylates MLCP. pMLC phosphatase fails to dephosphorylate MLC. KCl, potassium chloride; Ach, acetylcholine; 5-HT, 5-hydroxytryptamine (serotonin); PIP, phosphatidylinositol 4-phosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP5K, 1-phosphatidylinositol-4-phosphate 5-kinase; DG, diacylglycerol; IP3, inositol 1,4,5-trisphosphate.

of Ca^{2+} surge in ASM is mainly from intracellular sarcoplasmic reticulum stores rather than from the extracellular Ca^{2+} seen in cardiac, skeletal, and vascular muscle cells. Ligands to G-ptotein coupled receptor (GPCR), such as acetylcholine and methacholine, induce the activation of phospholipase C (PLC), which in turn leads to the formation of the inositol triphosphate (IP_3; Chen et al., 2012). Then, IP_3 occurs to release Ca^{2+} from sarcoplasmic reticulum (SR) stores, then Ca^{2+} forms a calcium-calmodulin comlex, activates MLC kinase (MLCK) which phosphorylates regulatory MLCs (rMLCs) forming phosphorylated-MLC (p-MLC; Berridge, 2009). Finally, this mechanism occurs to the activation of actin and myosin crossbridges resulting in shortening and contraction (Gunst and Tang, 2000).

And the contraction is also regulated by calcium sensitivity of myosin light chain (MLC; Kudo et al., 2012). The p-MLC is regulated by MLC phosphatase (MLCP) which converts p-MLC back to inactive MLC. MLCP is negatively controlled by Ras homolog gene family, member A (RhoA) and its target Rho Kinase such as Rho-associated, coiled-coil containing protein kinase (ROCK) which phosphorylates myosin phosphatase target subunit 1 (MYPT-1). Upregulation of the RhoA/Rho kinase signaling pathway inducing to inhibition of MLCP would result in increased levels of p-MLC and subsequently increased ASM contraction force. Increased levels of RhoA protein and mRNA were found in airway hyperresponsive animal models and this is probably medicated through inflammatory cytokines, such as IL-13 and IL-17A that themselves directly enhance the contractility of ASM (Chiba et al., 2009; Kudo et al., 2012). For IL-17A, sensitized mouse conditional lacking integrin αvβ8 on DCs shows attenuated reactivity against IL-17A-induce antigen challenge. This is induced by that IL-17A itself enhances the contractile force of ASM, through RhoA/Rho kinase signaling change.

Airway smooth muscle cells also contribute to the inflammatory mechanisms and airway remodeling of asthma. The proactivating signals, including viruses and immunoglobulin E could convert ASM cells into a proliferative and secretory cell in asthma. Naureckas et al. (1999) demonstrated the presence of smooth muscle mitogens in the BAL fluids from asthmatic individuals who underwent allergen challenge. Smooth muscle proliferation is also caused by the production of matrix metalloproteinase (MMP)-2, which has been demonstrated to be an important autocrine factor that is required for proliferation (Johnson and Knox, 1999). Production of MMP-2 from smooth muscle cells suggests that ASM contributes to the extracellular matrix turnover and airway remodeling. These cells may also participate in chronic airway inflammation by interacting with both Th1- and Th2-derived cytokines to modulate chemoattractant activity for eosinophils, activated T lymphocytes, and monocytes/macrophages (Teran et al., 1999).

In addition, recent studies demonstrated that eosinophils can also contribute to airway remodeling during an asthma by enhancing ASM cell proliferation. Halwani et al. (2013) verified that preventing eosinophil contact with ASM cells using specific antibodies or blocking cysteinyl leukotrienes derived from eosinophils was associated with inhibition of ASM proliferation. Moreover, ASM-synthesized cytokines seem to direct the eosinophil differentiation and maturation from progenitor cells, which can promote perpetuation of eosinophilic inflammation and consequently the tissue remodeling in asthma (Fanat et al., 2009). It was also reported that TGF-β alone induces only weak mitogenic effect on ASM cells, however, it synergistically stimulates ASM proliferation with methacholine which is agonist for the muscarinic receptor (Oenema et al., 2013). These smooth muscle cell proliferations related to airway remodeling can be the target to treat asthma.

EPITHELIAL TO MESENCHYMAL TRANSITION ON ASTHMA

As airway remodeling on asthma attracts investigators interested in airway remodeling on asthma, EMTs are recognized to be more important in asthma than before. EMTs are biological processes that epithelial cells lose their polarity and cell adhesion resulted in fragility of tight junction and gain migratory and invasive properties to change their cell formation to mesenchymal cells (Kalluri and Neilson, 2003). It is essential for processes including mesoderm formation and neural tube formation in the development and recently has also been reported to involve in wound healing, in organ fibrosis and in cancer metastasis. First, EMTs were found in the embryogenesis. Epithelial cells are different from mesenchymal cells in their phenotype. Epithelial cells connect each other, forming tight junction. These cells have polarity in cytoskeleton and bound to basal lamina. For mesenchymal cells, the polarity is lost and shaped in spindle. Lately, EMTs are divided into three subtypes, developmental (Type I), fibrosis, tissue regeneration and wound healing (Type II), and cancer progression and metastasis (Type III; Kalluri and Weinberg, 2009).

Type II EMT involves in wound healing, resulted that it contributes airway remodeling in asthma after airway epithelial injury induced by inflammation. Type II EMT indicates that epithelial tissue can be expressed plasticity (Thiery and Sleeman, 2006). It is initiated by extracellular signals, such as connection with extracellular matrix; collagen or hyaluronic acids and by growth factors; TGF-β and EGF. Among those signals, TGF-β is established how it plays important role in airway remodeling and EMT (Phipps et al., 2004; Boxall et al., 2006; Hackett et al., 2009). TGF-β induces the expression of α-smooth muscle actin and vimentin and the downregulation of E-cadherin expression, inducing the dissolution of polarity of the epithelial cell and intercellular adhesion. The such physiological effects of TGF-β signaling in the system have been shown to depend on microenvironment. Bone morphogenesis protein (BMP)-7 fails to attenuate TGF-β-induced EMT, however, one of the family member BMP-4 plays the role of EMT in the airway (Molloy et al., 2008; Hackett et al., 2009). This TGF-β-induced attenuation of intercellular adhesion and wound repair in EMT can be enhanced by the proinflammatory cytokines tumor necrosis factor (TNF)-α (Camara and Jarai, 2010). Furthermore, it was showed that house dust mite, through EGF receptor enhanced TGF-β-induce downregulation of E-cadherin in the bronchial epithelial cells (Heijink et al., 2010). And house dust mite and TGF-β synergistically induced expression of mesenchymal markers vimentin and fibronectin. In chronic house dust mite-exposure model, the airway epithelial cells were shown to elevate TGF-β expression and nuclear phosphorylated Smad3. And in these cells, the tight-junction protein was dissolved, occluding and expressed α-smooth

muscle actin and collagen (Johnson et al., 2011). Inhaled allergens might modify EMT, cooperating with cytokines which also promote asthma.

MAST CELLS AND EOSINOPHILS

Mact cells can induce the activation of mesenchymal cells (Holgate, 2000). The serine protease, tryptase which is released from degranulating mast cells is a potent stimulant of fibroblast and smooth muscle cell proliferation, and is capable of stimulating synthesis of type I collagen by human fibroblasts. A major mechanism involved in the regulation of fibroblast proliferation appears to be cleavage and activation of protease activated receptor-2 on fibroblasts (Akers et al., 2000). Mast cells may also influence the development of airway remodeling in asthma by releasing large amounts of plasminogen activator inhibitor type1. Moreover, Sugimoto et al. (2012) have shown that other mast cell proteases regulate airway hyperreactivity. Mice lacking αvβ6 integrin are protected from exaggerated airway narrowing. Mast cell proteases are differentially expressed, in mouse mast cell protease 1 (mMCP-1) induced by allergen challenge in wild-type (WT) mice and mMCP-4 increased at baseline in β6-deficient mice. MCPs from intraepithelial mast cell and their proteolytic substrates could be regulate airway hyperreactivity.

Eosinophils are circulating granulocytes and at relatively low levels in the bloodstream, upto 3% of white blood cells. These are the major cell types that can be recruited to sites of inflammatory responses (Huang et al., 2009; Isobe et al., 2012; Uhm et al., 2012). The function of eosinophils in asthma is related to their release of toxic granule proteins, reactive oxygen species (ROS), cytokines, and lipid mediators (Liu et al., 2006). The recruit of eosinophils into the epithelium and eosinophilic inflammation is involved in the pathogenesis of asthma. The proinflammatory mediators derived by eosinophil are major contributors to inflammation in asthma, including airway epithelial cell damage and desquamation, airway dysfunction of cholinergic nerve receptors, AHR, mucus hypersecretion, and airway remodeling, characterized by fibrosis and collagen deposition (Kay, 2005; Watt et al., 2005; Kanda et al., 2009; Walsh, 2010). Eosinophils are likely to contribute to airway remodeling with release of eosinophil-derived mediators such as TGF-β, secretion of cationic proteins, and cytokines, as well as having interactions with mast cell and epithelial cells. Those factors can directly activate epithelium and mesenchymal cells (Venge, 2010). Moreover, recent data demonstrated that eosinophils can also contribute to airway remodeling with ASM cell proliferation.

EXTRACELLULAR MATRIX

The airways of asthmatic patients showed excess accumulation of extracellular matrix components, particularly collagen, in the subepithelial connective tissue and adventitia of the airway wall (Kuwano et al., 1993; Gillis and Lutchen, 1999). The cellular interactions in mast cells and fibroblasts through protease activated receptor-2 may contribute an abnormal mesenchymal cell proliferation, and may account for the increased number of fibroblasts and myofibroblasts that are found in the airways of asthmatic subjects. Fibroblasts retain the capacity for growth and regeneration, and may evolve into various cell types, including smooth muscle cells that subsequently become myofibroblasts. Myofibroblasts can contribute to tissue remodeling by releasing extracellular matrix components such as elastin, fibronectin and laminin (Vignola et al., 2000). It was seen that the numbers of myofibroblasts in the airway of asthmatic subjects increased and their number appeared to correlate with the size of the basement reticular membrane (Holgate et al., 2000). Smooth muscle cells also have the potential to alter the composition of the extracellular matrix environment. The reticular basement membrane thickening is a characteristic typical feature of the asthmatic airways. It appears to consist of a plexiform deposition of immunoglobulins, collagen types I and III, tenascin and fibronectin (Jeffery et al., 2000), but not of laminin.

Remodeling processes of the extracellular matrix are less known than the thickening of the lamina reticularis. Most asthmatic subjects present with an abnormal superficial elastic fiber network, with fragmented fibers (Bousquet et al., 2000). In the deeper layer of elastic fibers is also abnormal, the fibers often being often patchy, tangled, and thickened. Some studies using transmission electron microscopy have shown that an elastolytic process occurs in asthmatic patients, and in some patients disruption of fibers has been observed. In the case of fatal asthma, fragmentation of elastic fiber has also been found in central airways, and was associated with marked elastolysis (Mauad et al., 1999). These bundles are seen to be hypertrophied as a result of an increased amount of collagen and myofibroblast matrix deposition occurring during exaggerated elastic fiber deposition (Carroll et al., 1997). Loss of lung elastic recoil force has been shown in adults with persistent asthma and irreversible expiratory airflow obstruction. Persistent asthmatic patients have severe abnormal flow-volume curves in expiration at both high and low lung volumes, and hyperinflation can be seen by residual volume, at forced residual capacity and total lung capacity (Gelb and Zamel, 2000). The increased elastolysis is part of a more complex process that regulates the size of a submucosal network formed by elastic fibers dispersed in a collagen and myofibroblast matrix (Chiappara et al., 2001). These features induce changes in airway, as demonstrated by airway compliance, particularly in those patients who are suffering from asthma for long period, supporting the concept that chronic inflammation and remodeling of the airway wall may result in stiffer dynamic elastic properties of the asthmatic airway (Brackel et al., 2000). Furthermore, disruption of elastic fibers may contribute to a reduction in the preload and afterload for smooth muscle contraction. Though it is difficult to associate aspects of remodeling with disease severity or degree of airways obstruction and hyperresponsiveness (Mauad et al., 2007), some investigators indicated that smooth muscle remodeling is related to the severity of asthma (James et al., 2009). It has shown that the clinical expression of asthma (Brightling et al., 2002), AHR (Siddiqui et al., 2008) and impaired airway relaxation (Slats et al., 2007) are associated with mast cell counts in the ASM layer in asthma. The deposition of extracellular matrix inside and outside the smooth muscle layer in asthma also seems to be related to its clinical severity and is altered as compared to healthy controls (Araujo et al., 2008; Klagas et al., 2009). Yick et al. (2012) have shown that extracellular matrix in ASM was related to the dynamics of airway function in asthma.

IMMUNE RESPONSE

ALLERGIC SENSITIZATION

Regarding to the immune system against allergy, it seems that hygiene hypothesis would provide the reason why the number of the patients with asthma is increasing, in relation with urbanization. The hypothesis is that the Th1 cells polarized response is not induced early in life leaving the body more susceptible to developing Th2 induced disease (Strachan, 2000). First, Strachan (1989) mentioned that the hypothesis was proposed to explain the observation that hay fever and eczema were less common in children from larger families, which were presumably exposed to more infectious agents through their siblings, than in children from small families, especially without siblings. Many bacteria and viruses derive a Th1-mediated immune response, which downregulates Th2 responses. The urban-rural gradient in prevalence has been demonstrated most strongly in children who grew up in environments with a wide range of microbial exposures, who are protected from childhood asthma and atopy (the predisposition to develop IgE against common environmental allergens) in proportion to their level of exposure to bacterial and fungal microbes (Ege et al., 2011).

In association with the airway epithelium and underling mucosa is a specialized population of antigen-presenting cells (APCs) called DCs (Holgate, 2012). As allergen sensitization, DCs take up the allergens and present small peptide from them. DCs express receptors of the innate immune system and process allergens into small peptides and then present them through the major histocompatibility complexes, MHC class I and MHC class II for recognition by T cell receptors. In allergic individuals, it is promoted by interaction of the allergen with IgE attached to FcεRI, the high-affinity receptor for IgE (Sallmann et al., 2011). When individual is born, there is no DCs in the airway. Damage to and activation of the respiratory epithelium are the major stimuli that initiate the ingression of immature DCs from the bone marrow (McWilliam et al., 1994) and cause the release of C–C chemokines which direct DCs migration toward the epithelium and underlying mucosa (Hammad et al., 2010). GM-CSF, which is released from epithelial cells and immune cells in the presence of IL-4 and TNF-α, leads to DCs maturation to a fully competent as APCs. During initial allergen entering to airways to sensitize, Th2 lymphocyte differentiation from naïve T cells requires IL-4 release. The cellular source of the IL-4 is still unclear. There are some hypotheses to explain that (Holgate, 2012). Polarization to Th2 cells subtype is also under epigenetic regulation. From the study with mouse, microRNA-21 has been shown to exert a pivotal role in setting a balance between Th1 and Th2 responses. It works through binding the promoter of the gene encoding IL-12 p35 and inhibiting its activation in favor of a Th2 profile. Conversely, reduced microRNA levels lead DCs to produce more IL-12, and allergen-stimulated T cells to produce more interferon-γ (IFN-γ) and less IL-4, enhancing Th1 delayed-type hypersensitivity (Lu et al., 2011).

DENDRITIC CELL ACTIVATION

As described above, DCs present small peptide from antigens through MHC class I and II/ T cell receptors. Once sensitized, T cells drive the allergic response in progress through interactions with DCs (Veres et al., 2011). DCs spread their processes into the lumen between airway epithelial cells and can detect allergen by forming tight junctions, keeping the epithelial barrier (Blank et al., 2011). In mouse, two distinct DC subsets have been described in accordance with their expression of the CD11c as myeloid [conventional DCs (cDCs), $CD11c^+$] or plasmacytoid DCs (pDCs, $CD11c^-$; Lambrecht and Hammad, 2009). Similarly, human DCs are subdivided into $CD11c^-$ pDCs and $CD11c^+$ myeloid DCs. Induced sputum from asthmatic airways and peripheral blood contain increased numbers of both pDCs and cDCs, which further increase in number upon allergen challenge (Dua et al., 2010). Proteolytic activities of allergens initiate to mature DCs. In a few hours after contact with allergen, pattern-recognition receptors activation, such as Toll-like receptors (TLRs) on DCs augments their homing capacity by upregulating chemokine receptors. It is cDC subtypes that are predominantly responsible for antigen presentation. Mature DCs shape an immunological synapse with the allergen-specific T lymphocytes to initiate a Th response (Holgate, 2012). Whereas some of the Th cells make their way to the B-cell follicle to facilitate immunoglobulin class switching from IgM to IgE, others move back to the airway mucosa to elicit the classical Th2 response through the secretion of the proallergic cytokines. Pattern-recognition receptors have a crucial adjuvant role in directing allergen sensitization. TLRs are key components of the innate immune system that mediate recognition and response to pathogen-associated molecular patterns (PAMPs) in the form of microbial, fungal and viral products and their ligands, including endotoxin which is recognized by TLR4, lipoproteins (TLR2 and TLR6), viral double- and single-stranded RNA (TLR3 and TLR7/8) and bacterial CpG-containing DNA (TLR9) (Akira et al., 2006). Other pattern recognition receptors respond to endogenously generated damage-associated molecular pattern molecules (DAMPs) produced during tissue damage. Inflammatory DCs have been suggested to be necessary and sufficient for the development of Th2 immunity to house dust mite allergen when the first exposure occurs by inhalation. For inhaled allergens, it is proposed that DCs amplify the Th2 immunity through basophiles and, in part, influenced by innate signaling through TLR4 and C-type lectin signaling on epithelial cells and DCs (Trompette et al., 2009). A cooperation of airway epithelium and DCs controls asthma development Th2 activation requires DCs-mediated antigen-presentation. Then, allergic sensitization fails to develop in the absence of DCs (Hammad et al., 2010), while DCs remain inactive in the absence of TLR ligation (Perros et al., 2009). That is, TLRs activation on epithelial cells enhances DCs motility and antigen sampling through the production of Th2-promoting chemokines and cytokines (IL-25, IL-33, GM-CSF).

VIRAL INFECTION TO PREDISPOSITION

The fact that early-in-life sensitization to multiple allergens carries the greatest risk for developing asthma (Simpson et al., 2010) brings the question of what factors result in a predisposition to this phenotype. Although infection with rhinovirus is the major cause of acute exacerbation, in those genetically at risk of asthma, rhinovirus-induced wheezing in the first three years in the life is

also the greatest risk factor for developing asthma at 6 years of age (Jackson et al., 2008). Impaired TLR3-mediated IFN-β and -λ production by asthmatic epithelial cells would make susceptible to both viral infection and allergic sensitization (Wark et al., 2005; Contoli et al., 2006; Bosco et al., 2010; Jartti and Korppi, 2011). Reduced primary IFN production by lower-airway epithelial cells enables some viruses to replicate, leading to cytotoxic cell, release of inflammatory products and enhanced viral shedding. Such events provide a strong stimulus for recruitment of immature DCs and their priming for allergen sensitization (McWilliam et al., 1994, 1996). When asthmatic epithelial cells are received to damage by rhinovirus infection, the cells generate increased amounts of the pro-Th2 cytokine thymic stromal lympoietin (Uller et al., 2010), which stimulates DCs and increases allergic inflammation, whereas exogenous IFN-b applied to asthmatic epithelium exerts anti-Th2 as well as antiviral properties (Cakebread et al., 2011).

CELLULAR IMMUNITY

Asthma is classically considered Th2 disease, with increased IgE and eosinophilic inflammation caused by increased levels of Th2-type cytokines. However, this paradigm has been challenged because of the realization that strategies designed to suppress Th2 function are not effective for all patients. The clinical phenotype of asthma is notoriously heterogeneous. It is shown that cellular immune process in the asthmatic airways in **Figure 2**. Th2 cells activation requires antigen-presentation by DCs. DCs play a role both in the initiation and maintenance of allergic airway inflammation and asthma, and control many aspects of the disease, including bronchial hyperresponsiveness and goblet cell metaplasia, by controlling the recruitment and activation of Th2 cells (Lambrecht and Hammad, 2009; Schuijs et al., 2013). Researches in both mouse and human, mentioned the expression of Th2-type cytokines, such as IL-4, IL-5, and IL-13, in the allergic lung. Experimental asthma models indicate that these cytokines, IL-13 in particular, are critical in driving key pathologic features of the allergic response. Moreover, Th2 blockade is very effective in suppressing these features of allergic disease in mice (Finkelman et al., 2010). The classical asthmatic phenotype is one of eosinophilia concomitant with high IgE levels. However, a proportion of patients are not atopic and do not have eosinophilic inflammation. In fact, it is estimated that as many as 50% of adult patients are encompassed by this non-atopic, non-eosinophilic, non-IgE-dependent subgroup (Lloyd and Saglani, 2013). Molecular therapy data support an overall Th2 association with phenotypes, such that they might satisfy a definition of Th2-associated asthma. However, even these distinctions are too simple, especially when disease severity is considered. Although children with severe asthma have eosinophilic inflammation, high-dose steroids effectively suppress Th2-type cytokines, such as IL-13 and IL-5, but symptoms remain with persistent eosinophilia (Bossley et al., 2012), thus raising the importance of identifying other less steroidsensitive, non-Th2 mediators driving disease. Then, it is apparent that asthma can no longer be considered simply a Th2-mediated disease.

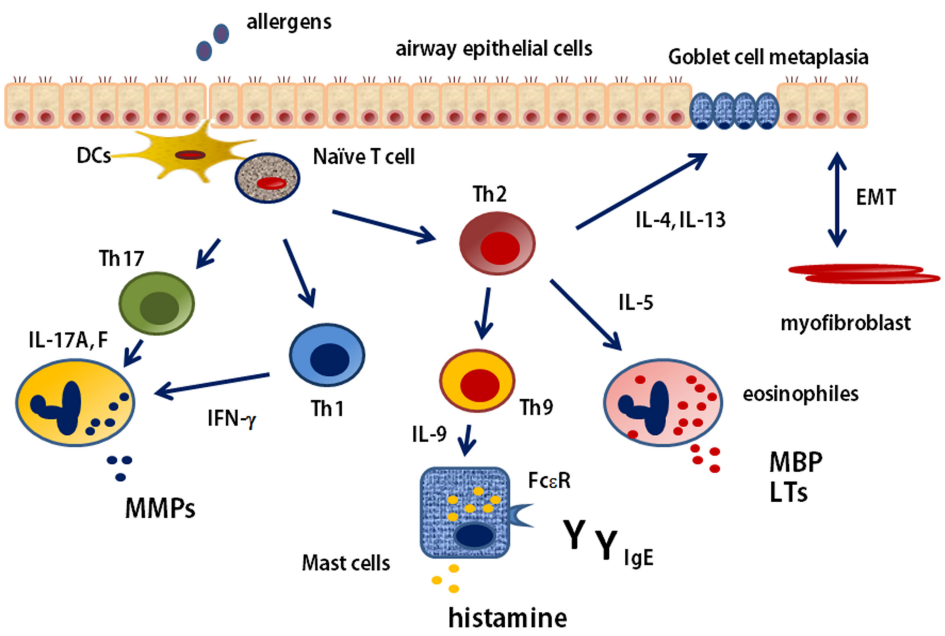

FIGURE 2 | T cell immune response in the asthmatic airways. Naïve T cell is received allergen presentation by DCs. The pathway begins with the development of Th2 cells and their production of the cytokines IL-4, IL-5, and IL-13. These cytokines stimulate allergic and eosinophilic inflammation as well as epithelial and smooth-muscle changes that contribute to asthma pathobiology. Th9 cell can be induced and stimulate mast cells by IL-9. Naïve T cell is also differentiated to Th1 or Th17 cells depending on the existence of cytokines in the microenvironment. Th1 cell and Th17 cell stimulate and induce neutrophilic inflammation. EMT, epithelial-mesenchymal-myofibroblast transition; MMP, matrix metalloproteinase; MBP, major basic protein; LT, leukotriene.

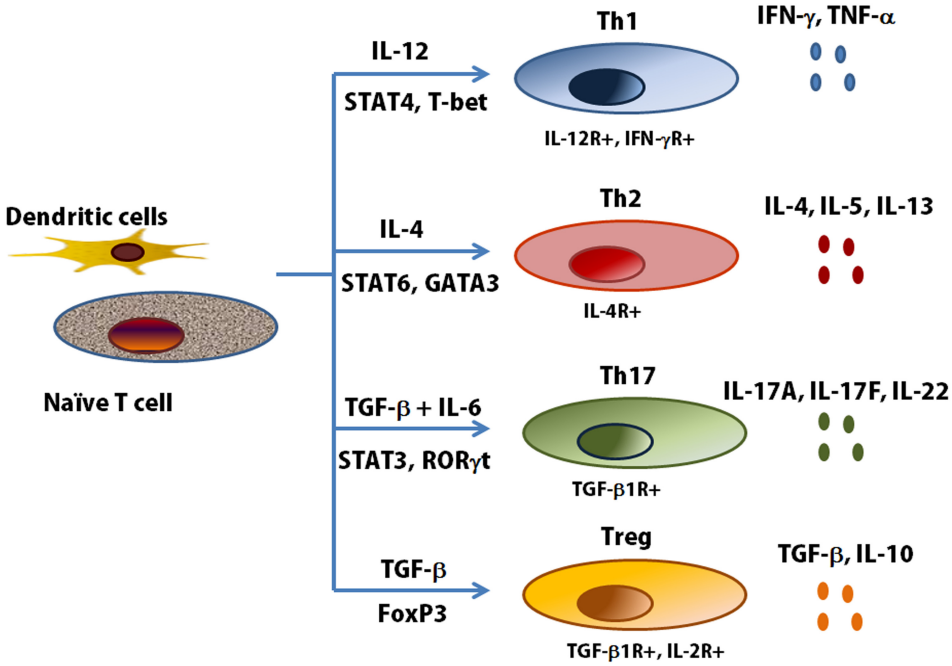

FIGURE 3 | T helper cell subsets and cytokine profiles. Th1, Th2 and Th17 cells are a separate lineage of CD4+ T cells, distinct from other T cell subsets. Every specific T helper cells produce its specific cytokines (Lazarevic and Glimcher, 2011). T-bet, T-box expressed in T cells; FoxP3, forkhead box P3; ROR, retinoid-related orphan receptor.

Effector CD4 cells expressing IL-17A, IL-17F were first described in 2005 (Harrington et al., 2005; Park et al., 2005) and were thought to represent a distinct T-cell lineage that promoted the first revision of the Th1/Th2 paradigm of immunity. Differentiation of naive effector T cells in the presence of IL-6 and TGF-β, leading to the expression of the transcription factor RORγt, results in IL-17 expression through the transcription factors Smad 2/3, signal transducer and activation of transcription (STAT) 3, and nuclear factor κB. Naïve T cells can differentiate several cell types and have specific immune response through the release of cell-type specific cytokines (**Figure 3**). Th17 cells have a role in regulating both neutrophilic and macrophage inflammation in autoimmune disease, and more recently they have been suggested to be involved in asthma and corticosteroid insensitivity (Nembrini et al., 2009). Conversely, their differentiation is restricted by both Th1 and Th2 cytokines including IFN-γ, IL-4, and IL-13 (Park et al., 2005). Specifically, the induction of CXCL8, a potent neutrophil chemokine whose expression is elevated in airway secretions in severe asthma, has directly implicated Th17 cells in neutrophilic airway inflammation. IL-17A itself, but not IL-17F or IL-22, enhances the contractile force of ASM. Sensitized mice lacking the integrin αvβ8 on DCs show reduced activation of this IL-17A-linked pathway with antigen challenge. This reduction in smooth muscle contraction in the airways is reversible by IL-17A, indicating involvement of this cytokine on allergen-induced AHR by acting directly on ASM (Kudo et al., 2012). Allergic induces a strong Th17 response in association with airway neutrophilia and hyperresponsiveness, and this response is abrogated in IL-17F knockout mice (Yang et al., 2008). However, although a good case can be made for IL-17A and IL-17F in mouse models of neutrophilic and corticosteroid-refractory lung responses to allergens, evidence for IL-17 involvement in human asthma is less robust, despite some emerging genetic evidence and a potential role for IL-17A and IL-17F in moderate-to-severe disease (Chakir et al., 2003; Doe et al., 2010). In humans, a subset of Th2 memory and effector cells has been recognized expressing both GATA3 and RORγt and, as a consequence, producing both Th17 and Th2 cytokines (Cosmi et al., 2010). Studies have reported that the number of circulating Th17 cells as well as plasma concentrations of IL-17 and IL-22 increase in proportion to disease severity. In a bronchial biopsy in asthma vs. normal controls, there was no correlation between IL-17A or IL-17F expression and the extent of neutrophilia, nor any link to asthma severity (Doe et al., 2010). The contribution of Th17 cells in human asthma has not been established enough. It is required to clear association of Th17 cells and subphenotype in human asthma.

CYTOKINE TARGETS

IL-4/IL-13

The key cytokines involved in Th2-type immunoreaction are those encoded in the IL-4 gene cluster on chromosome 5q31, containing the genes encoding IL-3, IL-4, IL-5, IL-9, IL-13, and GM-CSF (Bowen et al., 2008). The fact that the Th2 pathway is crucial to asthma pathophysiology has been the driving force for a range of biologics targeting the specific cytokines. The signals of Th2-cell-associated cytokines, IL-4 and IL-13, transmit through the IL-4Ra/IL-13Ra1 complex. IL-4 promotes B-cell isotype switching, the upregulation of adhesion molecules, eotaxin production,

and the development of AHR and goblet cell metaplasia. In animal model, IL-4 deficient mice were shown to be protected from developing asthma (Brusselle et al., 1994). IL-13 can have most of these functions (Wills-Karp et al., 1998; Webb et al., 2000). Furthermore, those cytokines have the potential to induce TSLP, GM-CSF, and CCL20 production by the airway epithelium (Reibman et al., 2003; Kato et al., 2007). Furthermore, IL-13 was shown to have direct effect to enhance ASM, upregulating RhoA protein which stimulates Rho-kinase inducing calcium sensitivity (Chiba et al., 2009). Therefore, a good example is the IL-4 and IL-13 pathway for anti cytokine treatment against asthma.

Given the clear evidence for IL-4 and/or IL-13 in mouse models of disease were launched and a humanized anti-IL-4 neutralizing antibody (pascolizumab) was introduced and showed promising results in human-derived cell lines and monkeys (Hart et al., 2002). However, IL-4-specific antagonists used in clinical trials have failed (Wenzel et al., 2007). More recently, a human monoclonal anti-IL-4Ra antibody (AMG317) has been developed but did not show clinical efficacy (Corren et al., 2010). For IL-13, several neutralizing antibodies have been developed, but trials are still in their infancy. The latter IL-13-antibody (CAT-354) has recently been shown to be safe for use in humans in a phase I clinical trial but its real clinical efficacy remains to be proven (Singh et al., 2010). Attempts to validate importance of IL-13 in human asthma revealed that only 50% of individuals with asthma had elevated IL-13 levels in sputum, irrespective of the severity of the disease (Berry et al., 2004). And Woodruff et al. (2009) have also shown that only 50% of patients express IL-13-responsive genes in the airway epithelial cells, and this is linked to a strong Th2 response in bronchial biopsies, as opposed to in other asthmatics, whose IL-13-responsive gene expression was almost same level from that of normal subjects. Th2-high subjects had greater expression of IL-13 in bronchial biopsies along with greater AHR and higher serum IgE, blood and airway eosinophilia. It was suggested that one IL-13 biomarker was periostin (Woodruff et al., 2009). In a recently published trial, the monoclonal antibody (mAb) to IL-13, lebrikizumab, when administered to patients with chronic moderate-to-severe asthma for 12 weeks, significantly increased baseline spirometry (5.5%). This result was enhanced in those with elevated serum periostin (high periostin 8.2% vs. low periostin 1.2%; Corren et al., 2011).

IL-5

IL-5 is a key cytokine crucial to eosinophil growth, maturation, activation, and survival whose blockade in various animal models has a strong effect on acute and more sustained pulmonary eosinophilia and attendant changes in lung function. It is mainly produced by Th2-lymphocytes, mast cells and eosinophils. Interestingly, IL-5 regulates its own receptor expression during eosinophil ontogeny consisting of an IL-5- specific receptor α-chain, and common β-chain. Because of its restriction to the eosinophil/basophil lineage in humans, IL-5 therapy may attenuate key characteristics of allergic airway inflammation, such as airway eosinophilia, airway remodeling, and AHR, without affecting the function of other immune cells (Trifilieff et al., 2001; Flood-Page et al., 2003; Humbles et al., 2004). It has also been implicated in the induction of AHR, as IL-5 inhalation by asthmatic patients induces eosinophil influx and AHR (Leckie et al., 2000). However, despite markedly reducing both circulating and sputum eosinophilia, two humanized mAbs, mepolizumab and reslizumab, when administered to patients with moderate-to-severe asthma, had no overall effect on any asthma outcome measures. Nonetheless, the studies of mepolizumab for patients with severe asthma requiring oral corticosteroids and persistent sputum eosinophilia showed a good clinical response (Haldar et al., 2009; Nair et al., 2009), as also found in Churg-Strauss and other hypereosinophilic syndromes (Abonia and Putnam, 2011). Similar results have also been obtained with reslizumab (Castro et al., 2011; Spergel et al., 2012). Efficacy of mepolizumab has also been described in severe eosinophilic nasal polyposis in proportion to nasal lavage IL-5 levels (Gevaert et al., 2006). A further development of this approach has been the introduction of a highly active mAb targeting IL-5Rα (benralizumab), which has been defucosylated to enhance its antibody-dependent cell-mediated cytotoxicity potential (Kolbeck et al., 2010). The studies demonstrate that anti-IL-5 therapy is effective in reducing exacerbation frequency in severe asthma, with highest efficacy in subgroups of patients where eosinophils have a pathogenic role. A phase 1 study in mild asthma has shown a strong dose-related reduction of circulating eosinophils lasting 8–12 weeks after a single injection (Busse et al., 2010). It seems, however, that for the majority of asthmatic patients the anti-IL-5 treatment will need to be administered in combination with other therapies that suppress asthma features through other mechanisms. Results of clinical trials targeting the IL5Rα subunit to obtain long-term depletion of eosinophils and basophils are eagerly awaited.

IL-17/IL-22

The rapid emergence and characterization of the Th17 lineage (CD4 T cells producing IL-17 family; IL-17A, IL-17F, IL-22) refines the existing model and provides a more unified perspective of allergic inflammation by CD4+ T cell subsets. Interestingly, some asthmatic individuals, especially those poorly responding to steroid treatment, show airway infiltrations primarily composed of neutrophils. These cells are probably recruited to the airways by IL-17-producing cells that also produce IL-4 (Wang et al., 2010a). In mice, allergic sensitization followed by challenge of the airways induces a strong Th17 response and IL-17 controls bronchial hyperresponsiveness and airway remodeling, and some of these effects are mediated directly on bronchial smooth muscle cells (Pichavant et al., 2008; Wang et al., 2010b; Bellini et al., 2012; Kudo et al., 2012). Moreover, IL-17 can also induce steroid insensitivity in bronchial epithelial cells (Zijlstra et al., 2012). IL-22 can also be produced by Th17 cells. In mouse asthma models, IL-22 seems to exert a dual role. Indeed, IL-22 blockade in Th2 sensitization dramatically reduced eosinophil recruitment, Th2 cytokine and chemokine production, AHR, and mucus production. In contrast, IL-22 inhibition in allergen challenge induced lung inflammation and increased Th2 cytokine production. On epithelial cells, IL-22 has the potential to induce the production of antimicrobial peptides and to promote epithelial repair as well as suppressing the production of proinflammatory chemokines and cytokines (Pennino et al., 2012). Despite these studies, our knowledge of IL-22 in asthma pathophysiology is still limited.

Table 1 | Monoclonal antibodies against IL-17 pathway clinical trials.

mAbs	Description	Phase	Indications
Brodalumab	Full human IgG2/anti IL-17RA	II	Asthma, Ps, PsA, RA
Secukinumab	Full human IgG1K/ anti IL-17A	III	Ps, PsA, RA, AS
		II	veitis
Ixekizumab	Humanized, hinge-modified IgG4/anti IL-17A	III	Ps
		II	RA
		I	PsA
Ustekinumab	Full human IgG1/anti p40 of IL-12/23	III	Crohn's, PsA
		II	AS, sarcoidosis, cirrhosis
		Approved	Ps
CNTO 1959	Full human mAb/anti p19 of IL-23	II	PsA
MK-3222	Humanized mAb/ anti p19 of IL-23	II	Ps
AMG 139	Full human mAb/ anti IL-23	I	Crohn's, Ps
RG4934	Humanized mAb/ anti IL-17A	I	
NI 1401	Full human IgG1 mAb/ IL-17A/F	I	
SCH 900117	Humanized mAb/ IL-17A	I	

Ps: psoriasis; PsA: psoriatic arthritis, RA: rheumatoid arthritis; AS: ankylosing; spondylitis.

IL-17A has been considered as one of most important player in asthma, however, clinical attempts for anti-IL-17A therapy to asthma has just begun (**Table 1**). Any data in anti-IL-17A trials for asthma are not available so far. Some clinical trials targeted at IL-17A have conducted and substantiated importance of IL-17A in autoimmune disorders. Phase II data on secukinumab, ixekizumab, and brodalumab in psoriasis indicate rapid and pronounced effects on measures of disease activity (Hueber et al., 2010). Early clinical trials in psoriatic arthritis, rheumatoid arthritis, and ankylosing spondylitis also support the therapeutic utility of IL-17A inhibition.

In addition, whereas secukinumab and ixekizumab selectively target and neutralize IL-17A, brodalumab binds the IL-17RA chain of the heteromeric IL-17 receptor, which is shared with multiple members of the IL-17 cytokine family and is therefore expected to inhibit the biological activity of IL-17A and IL-17F as well as IL-17C (Ramirez-Carrozzi et al., 2011), IL-17E (IL-25) and potentially other not yet discovered IL-17 family members that utilize IL-17RA (Papp et al., 2012). Considering with these data from clinical trials for autoimmune disease, this hypothetical advantage for IL-17A inhibitors against asthma can be expected to have clinical benefits. We have to wait that data from asthma studies becomes available.

IL-9

Interleukin-9 produced from CD4+ T cell (Th9) has been identified as a subset definite from the classical Th2 cells, requiring the transcription factors IRF4, PU1, STAT6, Smad3, and Notch signaling for development. The cells differentiate in response to IL-4 and TGF-β and are described to promote T cell proliferation, IgE and IgG production by B-cells, survival and maturation of eosinophils, increasing the number of mast cell (Veldhoen et al., 2008; Staudt et al., 2010; Kearley et al., 2011; Elyaman et al., 2012; Goswami et al., 2012). Studies in human have also shown that IL-9 expression increased markedly in response to allergen challenge (Erpenbeck et al., 2003) and IL-9 is highly expressed and localized to tissue lymphocytes during intestinal parasite infection (Faulkner et al., 1998) and to CD3+ cells in bronchial submucosa and BAL (Shimbara et al., 2000). In studies using IL-9 transgenic and knockout mice, direct IL-9 instillation into the lungs and blocking mAbs, it has been shown that IL-9 drives mucus production, both by a direct effect on airway epithelia (Bryce, 2011) and also by interacting with IL-13 (Steenwinckel et al., 2007). Mice with IL-9 overexpression in lung have increased airway inflammation and AHR (Bisgaard et al., 2007; Gern, 2011). IL-9 is also made by ILC2s and boosts production of IL-5 and IL-13 (Rabinovitch et al., 2005). Along with IL-4 and stem cell factor, IL-9 is also a potent stimulus for mast-cell development (Kearley et al., 2011). As IL-9 has been implicated in both inflammatory and remodeling components in mouse models of allergic airway disease, it seems an attractive therapeutic target. Currently, clinical data on anti-IL-9 therapeutics are modest and larger clinical trials are eagerly awaited to conclude whether this form of therapy can be used in the treatment of asthma (Shalev et al., 2011). Two first-in-human, open-label dose-escalation trials of a monoclonal antibody against IL-9, MEDI-528, in normal subjects and subjects with mild asthma have been successfully completed, showing some evidence of efficacy (Parker et al., 2011).

TNF-α

Tumor necrosis factor α, a multifunctional cytokine that exerts a variety of effects, such as growth promotion, apoptosis,

angiogenesis, cytotoxicity, inflammation, and immunomodulation, has been implicated in several inflammatory conditions. This cytokine is not only produced predominantly by activated macrophages but also by other immune (lymphocytes, natural killer cells, mast cells) as well as stromal (endothelial cells, fibroblasts, microglial cells) cells and presents in increased concentrations in bronchoalveolar fluid from the airways of patients with asthma (Broide et al., 1992). Some studies mentioned a relationship between TNF-α and severity of asthma.

The rates of death and complications are high among patients with refractory asthma and account for a disproportionate amount of the health resource burden attributed to asthma (Serra-Batlles et al., 1998). The airway abnormality in severe asthma is different from that in more mild asthma in having a more heterogeneous pattern of inflammatory response (Wenzel et al., 1999), with greater involvement of neutrophilic inflammation and the distal lung (Berry et al., 2005) and increased airway remodeling (Busse et al., 1999). Interest in the role of TNF-α in refractory asthma has been increased by a study showing increased concentrations of TNF-α in BAL from patients with more severe asthma and by an uncontrolled study showing that treatment with the recombinant soluble TNF-α receptor etanercept markedly improved AHR in patients with refractory asthma (Howarth et al., 2005). On the other hand, targeting TNF-α in severe asthma with golimumab yields responders and non-responders (Wenzel et al., 2009). And administration with infliximab for severe asthma also does responders and non-responders (Taillé et al., 2013). Therefore, controlled studies have shown controversial results and the risk-benefit profile of TNF-blocking agents is still debated (Cox, 2009).

The studies suggest that anti-TNF-α agents might improve the condition of a subgroup of patients severe steroid-dependent asthma, who have life-threatening exacerbations and complications of long-term steroid therapy. In the studies, the identification of more neutrophilic asthma that is less dependent upon Th2 mechanisms and, as a consequence, less responsive to corticosteroids might help identify a responsive target subpopulation. Such patients have been shown to have high circulating TNF-α and CXCL-8 as biomarkers (Silvestri et al., 2006). A transcriptomic analysis applied to induced sputum has identified a unique signature with prominence of TNF-α and nuclear factor-κB pathways (Baines et al., 2010). This stratification of asthma into pathway-selective phenotypes is likely to be a key driver for future drug development, as is proving so successful for cancer treatments (Holgate, 2012).

CONCLUSION

Bronchial asthma is a world-wide common disease and characterized by reversible airflow limitation, with non-specific AHR related to airway inflammation. Airway inflammation induces not only asthmatic symptoms which are the reversible airway obstruction and ASM contraction but also airway remodeling. Lately, the information for airway remodeling is increasing, the number of myofibroblasts increases in the understructure of epithelium, the proximity of the smooth muscle layer and the lamina reticularis. And it is more understood what EMT is. EMT can play a important role in airway remodeling. These epithelial and mesenchymal cells cause persistence of the inflammatory infiltrate and induce histological changes in the airway wall, increasing thickness of the basement membrane, collagen deposition and smooth muscle hypertrophy and hyperplasia. Subepithelial collagens cause thickening and increasing density of the basement membrane.

Classically, asthma is considered as Th2 disease, relating to increased IgE and eosinophilic inflammation in the airway. Recent results have shown that not only Th2, but Th17 and Th9 cells subset also contributed the disease, releasing their specific cytokines. These different cytokine give different biological effect. These can be targeted as an anti-cytokine treatment in asthma and some monoclonal antibodies against specific cytokines or their receptors are available. The results of those clinical trials have said that trials failed to control disease, despite it was clearly confirmed that those cytokines contributed the disease in animal model studies. It is required that more information for subphenotype of human asthma and its mechanism in more detail.

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Cellular and humoral immunity of virus-induced asthma

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Asthma inception is associated with respiratory viral infection, especially infection with respiratory syncytial virus (RSV) and/or human rhinovirus (HRV), in the vast majority of cases. However, the reason why RSV and HRV induce the majority of bronchiolitis cases during early childhood and why only a small percentage of children with RSV- and HRV-induced bronchiolitis later develop asthma remains unclear. A genetic association study has revealed the important interaction between viral illness and genetic variants in patients with asthma. Severe RSV- and HRV-induced bronchiolitis may be associated with a deficiency in the innate immune response to RSV and HRV. RSV and HRV infections in infants with deficient innate immune response and the dysfunction of regulatory T cells are considered to be a risk factor for the development of asthma. Sensitization to aeroallergens, beginning in the first year of life, consistently predisposes children to HRV-induced wheezing illnesses, but the converse is not true. Some evidence of virus specificity exists, in that allergic sensitization specifically increased the risk of wheezing in individuals infected with HRV, but not RSV. Administration of Palivizumab, a humanized monoclonal antibody that targets the A antigenic site of the Fusion-protein of RSV, decreases the risk of hospitalization in high-risk infants and the risk of recurrent of wheezing. However, palivizumab did not have any effect on subsequent recurrent wheezing in children with a family history of atopy. These findings suggest that infection with RSV and infection with HRV might predispose individuals to recurrent wheezing through an atopy-independent and an atopy-dependent mechanism, respectively. Respiratory virus-induced wheezing illnesses may encompass multiple sub-phenotypes that relate to asthma in different ways.

Keywords: respiratory syncytial virus, human rhinovirus, virus-induced asthma, cellular immunity, humoral immunity

INTRODUCTION

Infection with respiratory syncytial virus (RSV) and/or human rhinovirus (HRV) is the most important cause of lower respiratory tract disease in infants and young children (Heilman, 1990) and is the major cause of bronchiolitis in infants (Henderson et al., 1979; Rakes et al., 1999). RSV- and/or HRV-induced bronchiolitis during early childhood is strongly linked to the subsequent development of allergies and asthma (Sigurs et al., 2000; Schauer et al., 2002; Lemanske et al., 2005; Kusel et al., 2007; Jackson et al., 2008, 2013; Sly et al., 2008; Stern et al., 2008). However, the reasons why RSV and HRV induce the majority of bronchiolitis cases during early childhood and why only a small percentage of children with RSV- and/or HRV-induced bronchiolitis later develop atopy or asthma remain unclear. Accumulating evidence suggests that both genetic and environmental factors determine the type of immune response to infection with RSV and/or HRV and that the type of immune response may, in turn, affect the development of asthma (Martinez, 2003; Caliskan et al., 2013). This chapter focuses on the immunological mechanisms that may explain why asthma inception is associated with RSV- and HRV-infection.

INTERACTION BETWEEN VIRAL ILLNESS AND GENETIC VARIANTS IN PATIENTS WITH ASTHMA

Asthma inception and exacerbation are associated with respiratory viral infection in the vast majority of cases (Kusel et al., 2007; Jackson et al., 2008; Sly et al., 2008; Stern et al., 2008). Severe bronchitis following infection with RSV can occur in a small subset of infected infants (Sly et al., 2008). Viral infections of the lower respiratory tract are likely to result in wheezing if they induce inflammation and edema of the airway epithelium, decreasing the airway diameter (Sly et al., 2008). The frequency of lower respiratory viral infection accompanied by wheezing influences the risk of asthma inception (Sly et al., 2008). A genetic association study involving 470 children hospitalized for RSV-induced bronchiolitis, their parents, and 1008 randomly selected population controls showed that single-nucleotide polymorphisms (SNPs) of *VDR* (vitamin D receptor), *JUN*, *IFNA5*, *NOS2A*, and *FCER1A* were significantly associated with severe RSV-induced bronchiolitis at both the allele and genotype levels (Janssen et al., 2007). Genetic polymorphisms of *SFTPA* (surfactant protein A), *SFTPD* (surfactant protein D), *TLR* (toll like receptor) 4, *TNF* (tumor necrosis factor), IL4 (interleukin 4), *IL9, IL10, IL8, IL13, IL4RA*, and *CCL5* have been reportedly associated with a susceptibility to RSV-induced bronchiolitis (Huckabee and Peebles, 2009). Very recently, a significant interaction between the 17q21 genotype and human RSV-induced wheezing illness was demonstrated in two birth cohorts of children: the childhood origins of asthma (COAST), and the Copenhagen Prospective Study on Asthma in Childhood (COPSAC; Caliskan et al., 2013). The effects of 17q21 variants on an increased susceptibility to asthma are restricted

to patients with a history of HRV-induced wheezing illness during early life (Caliskan et al., 2013). These studies emphasize the important interaction between virus-induced illness and genetic variants in patients with asthma.

CELLULAR IMMUNITY OF VIRUS-INDUCED ASTHMA

(1) Is RSV- and/or HRV-induced severe bronchiolitis in children associated with a T helper type 2 (Th2)-predominant immune response?

RSV-induced severe bronchiolitis in children is associated with a Th2-predominant immune response (Renzi et al., 1997; Roman et al., 1997; Bendelja et al., 2000; Pala et al., 2002; van der Sande et al., 2002; Joshi et al., 2003; Legge and Braciale, 2003) or a decreased Th1 immune response (Joshi et al., 2003; Legge and Braciale, 2003). The concentration of Th2 cytokines was higher than that of interferon (IFN)-γ in nasopharyngeal secretions (NSP; Bermejo-Martin et al., 2007), particularly in infants less than three months old (Kristjansson et al., 2005). On the other hand, undetectable or very low Th2 cytokine concentrations have also been reported (Bont et al., 2001; Garofalo et al., 2001; Bennett et al., 2007; Garcia et al., 2012).

The majority of virus-infected mouse studies have been performed using RSV because the major group of HRV (88% of known HRV serotypes) uses human intercellular adhesion molecule-1 (ICAM-1) as a cellular receptor and cannot bind to mouse ICAM-1. Regarding HRV-induced asthma mouse models, three novel mouse models of HRV infection have been recently developed: infection with a minor-group HRV (the receptor is the low-density lipoprotein receptor family) in BALB/c mice, infection with a major-group HRV in transgenic BALB/c expressing a mouse-human ICAM-1 chimera, and HRV-induced exacerbation of allergic airway inflammation (Bartlett et al., 2008). These models are likely to be useful for the future development of therapies for asthma exacerbation.

In the majority of RSV-infected mouse studies, the induction of Th2 cytokines (including IL-4 and IL-5) is not observed in bronchial alveolar lavage (BAL) fluid or the lung tissues of RSV-infected mice (Peebles et al., 2001; Chavez-Bueno et al., 2005; Lee et al., 2008). A comparison of mouse strains showed that BALB/c and DBA/2 mice had a significantly higher airway hyper-reactivity over almost the entire time course up to 20 days after RSV exposure, compared with C57BL/6 mice (Tekkanat et al., 2001). However, even the BALB/c mice required a very high intranasal or intratracheal inoculum (10^5 or 10^6 PFUs) to elicit airway hyperresponsiveness (AHR; Tekkanat et al., 2001; Wang et al., 2004). Therefore, both the mouse strains and the amounts of virus that were used should be taken into consideration when comparing different mouse experiments.

(2) Is severe RSV- and/or HRV-induced bronchiolitis associated with deficient IFN production?

The concentrations of IFN-γ in samples of blood mononuclear cells or nasopharyngeal aspirates from RSV- and/or HRV-infected infants were inversely correlated with disease severity (Aberle et al., 1999; Bont et al., 1999, 2001; Renzi et al., 1999; Legg et al., 2003; Bennett et al., 2007; Garcia et al., 2012). These clinical studies suggest that IFN-γ plays an important role in determining the severity of RSV- and/or HRV-induced bronchiolitis.

STAT1-knockout ($STAT1^{-/-}$) BALB/c mice, which are incapable of responding to type II IFN (IFN-γ) or to type I IFN (IFN-α and β), showed markedly increased levels of inflammation, compared with wild-type (WT) mice, after infection with RSV, despite similar virus titers in the lung and similar rates of viral clearance (Durbin et al., 2002). $STAT1^{-/-}$ mice, but not WT or IFN-$\gamma^{-/-}$-infected mice, exhibited eosinophilic and neutrophilic pulmonary infiltrates. Although IFN-γ had been induced in infected lung tissues from both $STAT1^{-/-}$ and WT mice, preferential IL-4, IL-5, and IL-13 induction was only seen in the $STAT1^{-/-}$ mice. These findings suggest that both type I and type II IFNs play important roles in the Th1 antigen-specific immune response to RSV infection (Durbin et al., 2002).

(3) Is infection with RSV and/or HRV during infancy a risk factor for the development of asthma and possible allergic sensitization?

Among the viral wheezing illnesses that occur in outpatients during infancy and early childhood, those caused by HRV infections are the most significant predictors of the subsequent development of asthma at an age of 6 years, with an odds ratio (OR) of 9.8 for a high-risk birth cohort (Jackson et al., 2008). From birth to 3 years of age, wheezing accompanied by RSV infection alone was associated with an increased asthma risk at an age of 6 years, with an OR of 2.6 (Jackson et al., 2008). Two important observations must be considered. First, most severe RSV infections occur between the 8th and 24th postnatal week. 80% of infants experience an HRV infection by the age of 1 year. Second, repeated infection is common at all ages, indicating the possibility of important interactions and outcomes in RSV- and/or HRV-re-infected hosts.

Culley et al. (2002) compared the consequences of re-infection with RSV between neonatal mice (during the 1 week of life) and weanling mice (at 3 weeks of age) that had been initially infected with RSV. The primary infection of neonatal BALB/c mice with RSV was associated with a reduced and delayed IFN-γ response (Culley et al., 2002). Upon re-infection with RSV, a severer weight loss with the increased inflammatory cell recruitment of Th2 cells and eosinophils was induced in the neonatal mice, compared with the weanling mice that had been initially infected with RSV (Culley et al., 2002). These results show the crucial importance of age at the time of the first infection in determining the outcome of re-infection and suggest that the environment of the neonatal lung is a major determinant of cytokine production and disease patterns in later life. Neonatal RSV infection is thought to lead to a predisposition to the development of airway eosinophilia and enhanced AHR via an IL-13-dependent mechanism during re-infection, whereas infection at a later age protects against the development of these altered airway responses after re-infection (Dakhama et al., 2005).

Severe RSV bronchiolitis has been associated with deficient IFN-γ production in humans (Bont et al., 2001; Bennett et al., 2007; Garcia et al., 2012), but the role of this cytokine in determining the outcome of re-infection is unknown. To define the role of IFN-γ in the development of RSV-induced AHR and lung histopathology in mice, WT and IFN-$\gamma^{-/-}$ mice were infected with RSV at a newborn or weaning stage and were re-infected 5 weeks later. (Lee et al., 2008). Both WT and IFN-$\gamma^{-/-}$ mice

developed similar levels of AHR and airway inflammation after the primary infection (Lee et al., 2008). After re-infection, the IFN-$\gamma^{-/-}$ mice, but not the WT mice, developed AHR, airway eosinophilia, and mucus hyperproduction. The intranasal administration of IFN-γ during the primary infection, but not during the re-infection, prevented the development of these altered airway responses upon re-infection in the IFN-$\gamma^{-/-}$ mice. IFN-γ production during primary RSV infection is critical to the development of protection against AHR and airway eosinophilia as well as mucus hyperproduction during subsequent re-infection (Lee et al., 2008).

To define the mechanism underlying the enhanced responsiveness in neonatally infected mice that were re-infected with RSV, the differences between dendritic cells (DCs) from neonatal mice and those from 5-week-old mice were examined. Neonatal lung MHCII-positive ($^+$) CD11b$^+$ DCs expressed higher baseline levels of OX40 ligand (OX40L) and lower cytoplasmic levels of IL-12 than lung DCs from 5-week-old mice (Han et al., 2012). Following RSV infection, OX40L expression was increased in neonatal DCs. The administration of anti-OX40L neutralizing antibody during primary RSV infection in neonatal mice prevented the subsequent enhancement of AHR and the development of airway eosinophilia and mucus hyperproduction upon re-infection. The basal expression levels of thymic stromal lymphopoietin (TSLP) in the lungs were higher in the neonates than in the 5-week-old mice (Han et al., 2012). RSV infection upregulates TSLP production in epithelial cells (Han et al., 2012). The administration of anti-TSLP neutralizing antibody before neonatal RSV infection reduced the accumulation of lung DCs, decreased OX40L expression on lung DCs, and attenuated the enhancement of the airway responses after re-infection (Han et al., 2012).

Regulatory T (T_{reg}) cells have an important role in immune tolerance as early as the embryonic stage (Mold et al., 2008, 2010). T_{reg} cell-mediated protection from asthma is initiated at the neonatal stage. Airborne antigens and ovalbumin (OVA) are efficiently transferred from mother mice to neonatal mice through transforming growth factor (TGF)-β in lactated milk (Polte and Hansen, 2008; Verhasselt et al., 2008). The repeated RSV infection of infant mice impaired T_{reg} cell function, leading to a malfunction in tolerance to OVA contained in lactated milk. As a result, RSV increased allergic airway inflammation in response to OVA sensitization and subsequent challenges, compared with inflammation in uninfected, tolerant control mice (Krishnamoorthy et al., 2012). Virus infection induced GATA-3 expression and Th2 cytokine production in forkhead box P3 (FOXP3)$^+$ T_{reg} cells and compromised the suppressive function of pulmonary T_{reg} cells. These findings highlight a mechanism by which viral infection targets a host-protective mechanism during early life and increases susceptibility to allergic disease (Krishnamoorthy et al. 2012).

HUMORAL IMMUNITY OF VIRUS-INDUCED ASTHMA

Most children experience primary RSV infection when they are infants. The immune system is still immature, and maternally derived antibodies are still present at relatively high levels during this period of life (Brandenburg et al., 1997). The titers of maternally derived neutralizing antibodies are reportedly inversely associated with RSV infection overall (Roca et al., 2002; Ochola et al., 2009) and with the severity of RSV illness (Glezen et al., 1981; Holberg et al., 1991; Piedra et al., 2003; Stensballe et al., 2009). Prophylaxis by Palivizumab, a humanized monoclonal anti-body that targets the A antigenic site of the F-protein of RSV, substantially reduces the risk of hospitalization in high-risk infants (Feltes et al., 2003). However, maternally derived neutralizing antibodies have a relatively weak ability to mount the antibody responses observed in infants (Murphy et al., 1986). Significant increases in serum or nasal wash concentrations of RSV glycoprotein (G protein) or fusion (F) protein-specific IgG and IgA are not seen in all infants after primary RSV infection (Brandenburg et al., 1997; De Alarcon et al., 2001). The response of human neonatal B cells to RSV uses a biased antibody variable gene repertoire that lacks somatic mutations (Weitkamp et al., 2005; Williams et al., 2009). Because of the poor Toll-like receptor stimulation in B cells, antibody affinity maturation is not sufficient to elicit protection against RSV re-infection (Delgado et al., 2009).

Palivizumab prophylaxis decreases the risk of hospitalization in high-risk infants (Feltes et al., 2003) and the risk of recurrent wheezing (Simoes et al., 2007). However, no effect of palivizumab on subsequent recurrent wheezing was seen in children with a family history of atopy or food allergies, compared with untreated infants with an atopic family history (Simoes et al., 2010). This finding suggests that RSV infection predisposes an individual to recurrent wheezing in an atopy-independent manner and that RSV infections of the lower respiratory tract may have differential effects on the development of recurrent wheezing, depending on the genetic predisposition.

Welliver et al. reported the presence of IgE antibodies recognizing whole RSV and purified F and G protein in a high proportion of NPS samples from infants with RSV-induced bronchiolitis, but rarely in samples from infants with other RSV-induced illnesses (Welliver et al., 1981, 1989). Although others have confirmed the presence of RSV-specific IgE serum and NPS from some RSV-infected infants, such findings are generally seen in a small proportion of patients (Bui et al., 1987; Russi et al., 1993; Wilczynski et al., 1994; Rabatic et al., 1997; Aberle et al., 1999) or not at all (De Alarcon et al., 2001).

In a mouse model, primary infection with RSV was capable of leading to the production of RSV-specific IgE, which may contribute to the development of exaggerated Th2-based airway responses upon reinfection in mice initially infected as neonates (Dakhama et al., 2009).

RELATIONSHIP BETWEEN INNATE IMMUNE RESPONSE TO INFECTION WITH RSV- AND/OR HRV-INDUCED ASTHMA

Wark et al. (2005) examined virus replication and innate responses to RV-16 infection using primary bronchial epithelial cells from asthmatic and healthy control subjects. Viral RNA expression and late virus release into the supernatant was increased by 50- and 7-fold, respectively, in cells from asthmatic individuals, compared with the levels in cells from healthy controls. The profound impairment of virus-induced IFN-β production in cultures of cells from asthmatic individuals was observed. In infected asthmatic cells, exogenous IFN-β induced apoptosis and reduced virus replication, demonstrating a causal link between deficient IFN-β, impaired apoptosis, and increased virus replication. Message

et al. (2008) reported that HRV inoculation induced significantly greater lower respiratory symptoms and lung function impairment and increases in AHR and eosinophilic lower airway inflammation in asthmatic individuals, compared with normal volunteers. The virologic and clinical outcomes were strongly related to deficient IFN-γ and IL-10 responses and to augmented IL-4, IL-5, and IL-13 responses. HRV was detected in the lower airway tissue of patients with asthma significantly more often than in non-asthmatic subjects, and its presence was associated with the clinical features of severer disease (Wos et al., 2008).

CONCLUSION

A prospective, repeated characterization of a birth cohort demonstrated that sensitization to aeroallergens, beginning in the first year of life, consistently predisposes children to viral wheezing illnesses and that the converse is not true (Jackson et al., 2013; **Figure 1**). Some evidence suggests virus specificity, in that allergic sensitization specifically increased the risk of wheezing in individuals with HRV infections, but not those with RSV infections. This sequential relationship and the plausible mechanisms by which allergic sensitization can lead to severer HRV-induced lower respiratory illnesses support a causal role for allergic sensitization in this developmental pathway (Jackson et al., 2013). Subrata et al. (2009) suggested that interactions between innate antiviral and allergic inflammatory pathways may lead to severer viral illnesses in atopic children. Ongoing allergic inflammation in the airways may directly lead to impairment of the epithelial cell barrier and the antiviral response (**Figure 1**). Accordingly, respiratory viral infection in atopic children may initiate an atopy-dependent cascade that amplifies and sustains airway inflammation initiated by innate antiviral immunity via harnessing the underlying atopy-associated mechanisms (Subrata et al., 2009). Allergic asthmatic children had higher surface expression of FcεRIα on plasmacytoid (p) DCs and myeloid (m) DCs when compared with that seen in nonallergic, nonasthmatic children. The percentage of FcεRIα$^+$ pDCs and mDCs in allergic asthmatic children was inversely correlated with HRV-induced IFN-α and IFN-λ1, and IFN-α production levels, respectively (**Figure 1**). Following aggregation of FcεRI, HRV-induced IFN-α and IFN-λ1 production from peripheral blood mononuclear cells (PBMCs) was significantly reduced

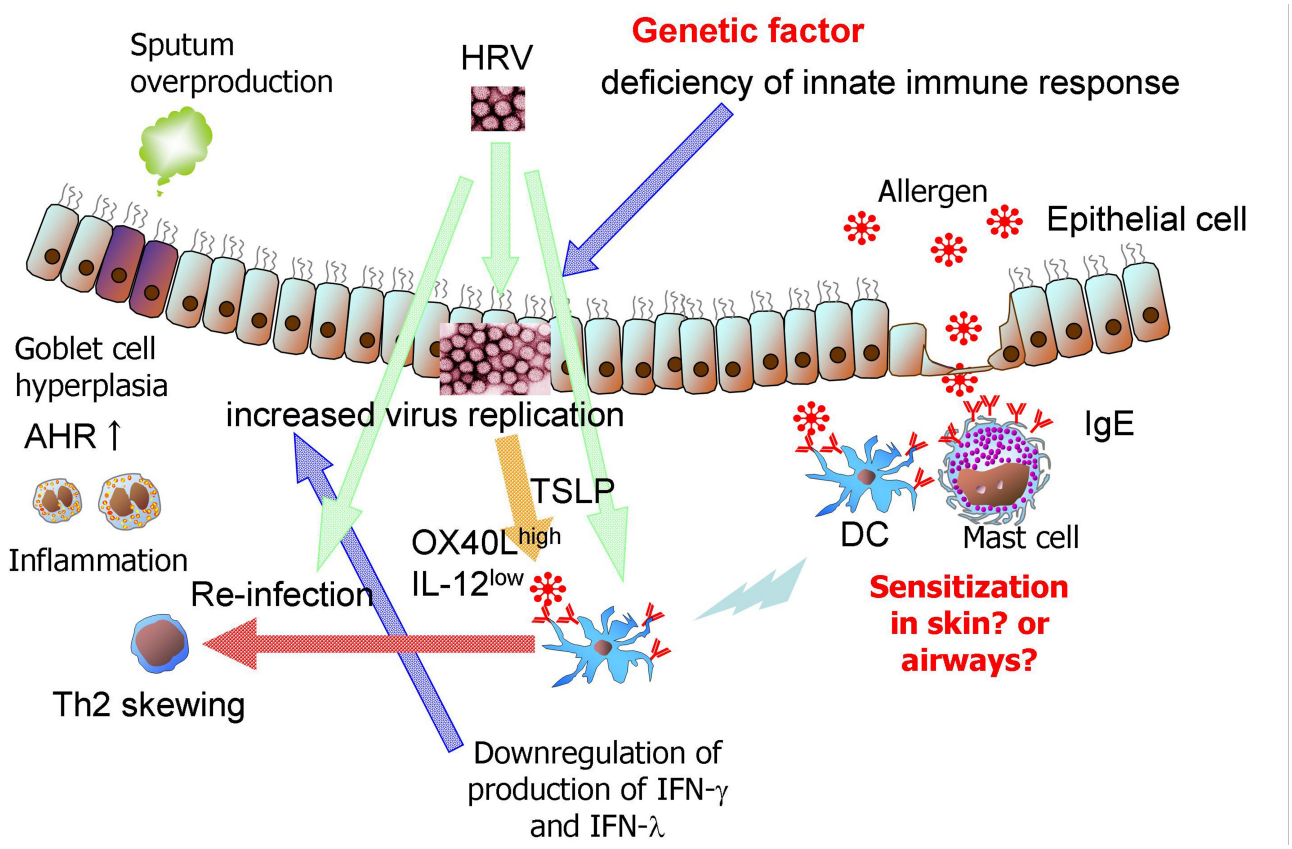

FIGURE 1 | Possible immunological mechanisms of HRV-induced bronchial asthma in infants. Sensitization to aeroallergens, beginning in the first year of life, consistently predisposes children to HRV-induced wheezing illnesses. Ongoing allergic inflammation in the airways may directly lead to impairment of the epithelial cell barrier and antiviral response. Following aggregation of FcεRI, HRV-induced IFN-α and IFN-λ1 production from PBMCs and DCs is significantly reduced compared to that by HRV stimulation alone. Furthermore, impairment of virus-induced IFNs from epithelial cells due to genetic variants increases virus replication. HRV infection upregulates TSLP production in epithelial cells (Kato et al., 2007). Neonatal lung DCs express higher baseline levels of OX40 ligand (OX40L) and lower cytoplasmic levels of IL-12 than lung DCs from adult lung DCs. TSLP upregulates the expression of OX40L on DCs. Airway inflammation and AHR may be enhanced after re-infection

compared to that by HRV stimulation alone (Durrani et al., 2012). These effects may explain why children with allergic asthma have more frequent and severe HRV-induced wheezing and asthma exacerbations. In contrast, the administration of palivizumab prophylaxis had no effect on subsequent recurrent wheezing in children with a family history of atopy (Simoes et al., 2010). This finding suggests that RSV infection predisposes individuals to recurrent wheezing through an atopy-independent mechanism. Several asthmatic or wheezing phenotypes exist in children (Fitzpatrick et al., 2011). Therefore, respiratory virus-induced wheezing illnesses can encompass multiple sub-phenotypes that relate to asthma in different ways.

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Cytokine production and signaling pathways in respiratory virus infection

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It has been confirmed that respiratory virus infections can induce abberant cytokine production in the host. These cytokines may be associated with both elimination of the virus and complications in the host, such as virus-induced asthma. Representative host defense mechanisms against pathogens, including bacteria and viruses, are mediated by the innate immune system. Cells of the innate immune system express essential molecules, namely pattern recognition receptors (PRRs), such as Toll-like receptors, nucleotide-binding oligomerization domain-like receptors, and retinoic acid-inducible gene-I-like receptors. These PRRs can recognize components of pathogens such as bacterial lipopolysaccharide, viral antigens, and their genomes (DNA and RNA). Furthermore, PRRs activate various signaling pathways resulting in cytokine production against pathogen infection. However, the exact mechanisms remain unknown. In this review, we mainly focus on the representative mechanisms of cytokine production through PRRs and signaling pathways due to virus infections, including respiratory virus infections. In addition, we describe the relationships between respiratory infections and virus-induced asthma.

Keywords: cytokine, signaling pathway, respiratory virus, innate immunity, virus-induced asthma

INTRODUCTION

Coordination between innate and adaptive immunity against pathogens is indispensable in higher organisms including humans (Medzhitov, 2007). In particular, innate immunity plays a critical role during primary infection with various bacteria and viruses (Barbalat et al., 2011; Jarchum and Pamer, 2011; Kumar et al., 2011). The specific recognition of microorganisms may represent the basis of innate immunity (Barbalat et al., 2011; Jarchum and Pamer, 2011; Kumar et al., 2011). Specific recognition systems have gradually been clarified and the common platforms are Toll-like receptors (TLRs), the NLR family (nucleotide-binding oligomerization domain-like receptors), and the RLR family [RIG (retinoic acid-inducible gene)-I-like receptors] (Kumar et al., 2011; Yu and Levine, 2011). These molecules are called pattern recognition receptors (PRRs). PRRs can recognize lipopolysaccharides (LPS), viral antigens, and bacterial/viral genomes, leading to the activation of intrinsic signaling pathways (e.g., myeloid differentiation factor 88; MyD88) and the production of various cytokines (Barbalat et al., 2011; Jarchum and Pamer, 2011; Kumar et al., 2011; Ting Tan et al., 2013). The production of such cytokines may activate leukocytes and eliminate the infective agents (Chehadeh and Alkhabbaz, 2013; Ting Tan et al., 2013).

At present, over 50 cytokines have been discovered. They form networks and play pivotal roles in infectious and allergic diseases (Barnes, 2008; Desai and Brightling, 2012; Holgate, 2012). These cytokines are mainly produced by blood cells, lymphoid tissues, and epithelial cells. For example, interferons (IFNs), which are anti-viral cytokines produced by lymphocytes and epithelial cells, are dramatically induced by various viral infections such as influenza (Qin et al., 2011b; Högner et al., 2013; Lopušná et al., 2013). This induction may contribute to the elimination of viruses *in vivo*. Indeed, we use recombinant IFNs to treat chronic viral infections such as hepatitis C (Nagao et al., 2012; Slim and Afridi, 2012). On the other hand, aberrant induction of other cytokines such as interleukin (IL)-4 may induce various allergic diseases, such as virus-induced asthma (Baraldo et al., 2012; Krishnamoorthy et al., 2012). In addition, aberrant induction and an imbalance of various proinflammatory cytokines, for example, IL-1β, IL-6, and tumor necrosis factor (TNF), may induce severe systemic inflammatory response syndrome (Watanabe et al., 2003; Xu et al., 2012). Thus, various cytokines may be associated with the pathophysiology of inflammation and remodeling of the airways post-infection.

Acute respiratory illnesses (ARI) are the most common diseases in humans. Accumulating evidence suggests that around 80% of the causative agents of ARI may be respiratory viruses (Heymann et al., 2004; Fujitsuka et al., 2011). The prognosis is good in most patients with viral ARI; however, viruses causing ARI may be responsible for more severe diseases like bronchitis, bronchiolitis, and pneumonia (Domachowske and Rosenberg, 1999; Sigurs, 2002; Kusel et al., 2007). Furthermore, representative respiratory viruses such as respiratory syncytial virus (RSV) may induce bronchiolitis or pneumonia with wheezing in infants (Stein et al., 1999; Sigurs et al., 2000).

To better understand host defense mechanisms against viruses, it is important to clarify these molecular mechanisms. In this review, we focus on cytokine production and signaling pathways during viral infection. We also discuss the relationships between

cytokine profiles and virus-induced asthma under the main theme "virus-induced asthma."

INFECTION AND INNATE IMMUNITY

Host defense mechanisms against microbial infections constitute the main purpose of innate immunity (an archaic term meaning natural resistance; Jarchum and Pamer, 2011; Kumar et al., 2011). The main platforms of the molecular groups against the pathogens include TLRs, the NLR family (nucleotide-binding oligomerization domain-like receptors), and the RLR family (RIG-I-like receptors). These molecules/receptors can recognize various components including LPS derived from bacteria, viruses, and fungi, viral antigens, and the pathogen genomes (Barbalat et al., 2011; Jarchum and Pamer, 2011; Kumar et al., 2011; Yu and Levine, 2011). Subsequent events activate innate immunity involved in cytokine production in the host (Barbalat et al., 2011; Kumar et al., 2011; Yu and Levine, 2011; Ting Tan et al., 2013). The innate immune system initiates a different mechanism against each pathogen (Chehadeh and Alkhabbaz, 2013; Kemp et al., 2013). Thus, these pathogen-associated receptors are called "PRRs" (Kawai and Akira, 2007; Pang and Iwasaki, 2012). Schematic illustrations of these families are shown in **Figure 1**.

TOLL-LIKE RECEPTORS

As already mentioned, virus infections can induce the production of various cytokines (Yoshizumi et al., 2010; Ishioka et al., 2011; Kato et al., 2011). TLRs may be responsible for cytokine production in bacteria- or virus-infected epithelial cells and immune cells (Rudd et al., 2005; Barbalat et al., 2009). In general, it is thought that TLRs play pivotal roles in innate immunity against viral and bacterial infections (Kawai and Akira, 2011; McIsaac et al., 2012). In humans, 10 types of TLRs have been identified (Akira et al., 2006; Takeuchi and Akira, 2009; Kumar et al., 2011). TLRs possess an extracellular domain containing leucine-rich repeats and a Toll/IL-1 receptor-like structure as the intercellular domain (TIR domain; Janssens and Beyaert, 2003; Akira et al., 2006). TLRs can be classified into three types: lipid ligands (TLR1, 2, 4, 6, and 10), protein ligands (TLR5), and nucleic acid ligands (TLR3 and 7–9; Janssens and Beyaert, 2003; Akira et al., 2006; Vandevenne et al., 2010). Thus, the TLR family can recognize various biological components derived from microorganisms (Janssens and Beyaert, 2003; Akira et al., 2006; Vandevenne et al., 2010). TLR1, 2, 4, 5, and 6 are transmembrane proteins (Janssens and Beyaert, 2003; Akira et al., 2006; Vandevenne et al., 2010), which mainly bind to bacterial components such as bacterial triacylpolypeptides (TLR1), ribopeptides (TLR2), LPS (TLR4), and the bacterial protein

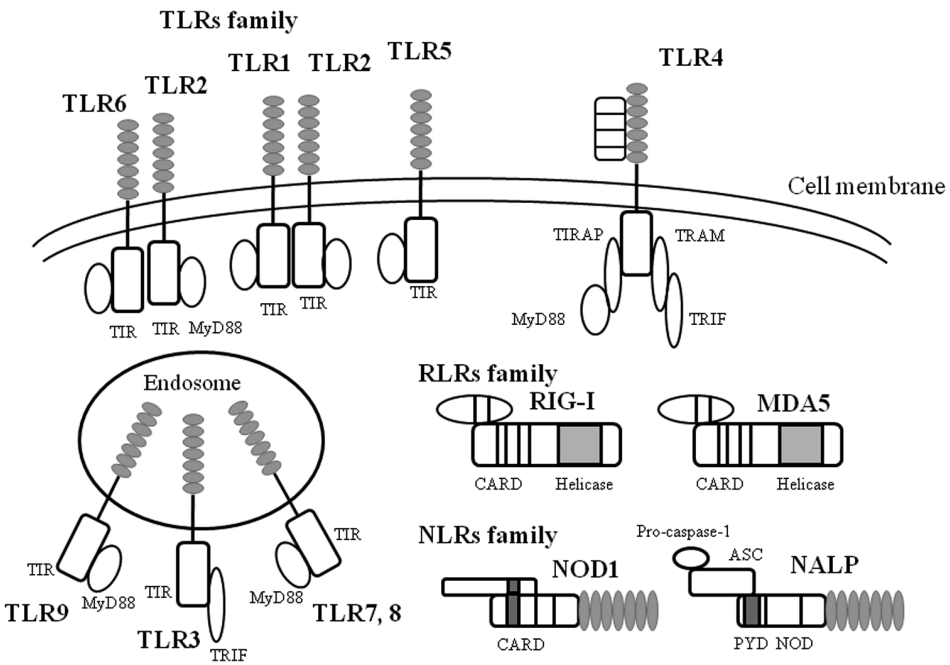

FIGURE 1 | A schematic illustration of microbial infection and innate immunity-related molecules. TLR, RLR, NLR are all expressed on and in a variety of cells in host tissues that recognize a diverse range of microbial signatures. The ectodomain of the TLR consists of leucine-rich repeats. Upon ligand stimulation, all TLRs except TLR3 recruit the adaptor MyD88. TLR3 is activated via the adaptor protein TRIF (Toll/IL-1 receptor domain containing adaptor protein inducing interferon-1β). RLRs consist of a group of cytosolic RNA helicases. RIG-I and MDA5 are composed of two amino-terminal CARD domains and a central helicase domain. In contrast to RIG-I, MDA5 lacks the C-terminal repressor domain. NLR are composed of a central nucleotide-oligomerization domain and a C-terminal leucine-rich repeat domain involved in the recognition of pathogens. The N-terminal domain can be either a CARD or a PYD domain. The NLR subfamily is characterized by an N-terminal PYD domain. ASC, apoptosis-associated speck-like protein containing caspase recruitment domain; CARD, caspase recruit domains; MDA, melanoma differentiation-associated protein; MyD88, myeloid differentiation factor 88; NALP, NACHT, LRR and PYD domains-containing protein; NOD, nucleotide-binding oligomerization domain; PYD, pyrin domain; RIG, retinoic acid-inducible gene; TIR, Toll/IL-1 receptor-like structure as intercellular domain; TIRAP, TIR domain-containing adaptor protein; TLR, Toll like receptor; TRAM, TRIF-related adaptor molecule; TRIF, TIR domain-containing adaptor including IFN-1β.

flagellin (TLR5; Janssens and Beyaert, 2003; Akira et al., 2006; Vandevenne et al., 2010). Interestingly, TLR4 proteins also bind to a major viral antigen of RSV F (fusion) protein (See and Wark, 2008; Klein Klouwenberg et al., 2009). In addition, TLR3, 7, 8, and 9 reside in the endosomes in cells (Janssens and Beyaert, 2003; Akira et al., 2006; Vandevenne et al., 2010). TLR7 and 8 can recognize single strand viral RNA molecules, while TLR3 can also recognize double strand RNA and poly I:C (polyinosinic polycytidylic acid; Janssens and Beyaert, 2003; Akira et al., 2006; Vandevenne et al., 2010). Thus, TLR3, 7, and 8 are essential receptors for many types of RNA viruses including paramyxoviruses (Sendaivirus) and orthomyxovirus (influenza viruses; Melchjorsen et al., 2005; Hammerbeck et al., 2007; Klein Klouwenberg et al., 2009). Moreover, TLR9 recognizes CpG DNA (a phosphodiester bond within cytosine and guanine; Janssens and Beyaert, 2003; Akira et al., 2006; Vandevenne et al., 2010). Thus, TLRs can bind to various components of microorganisms including viruses, leading to cytokine production through activation of signaling pathways in pathogen-infected cells (Janssens and Beyaert, 2003; Akira et al., 2006; Vandevenne et al., 2010). An illustrated summary is shown in **Figure 2**.

TLRs-ASSOCIATED SIGNALING PATHWAYS AND CYTOKINE PRODUCTION

A summarized illustration is shown in **Figure 3**. TLRs possess a common TIR domain (Janssens and Beyaert, 2003; Akira et al., 2006). The TIR domain can bind an adaptor molecule, MyD88 (Picard et al., 2011). MyD88 triggers downstream signaling pathways such as IRAK (IL-1 receptor-associated kinase)-1/4, TRAF6 (TNF receptor associated factor 6), IRF (interferon regulatory factor), and/or NF-κB (Akira, 2003; Takeda and Akira, 2004). These signals may lead to the production of various cytokines such as type I IFN (IFN-α and -1β) and proinflammatory cytokines (TNF-α, IL-1, IL-6, and IL-8; Akira, 2003; McGettrick and O'Neill, 2004; Takeda and Akira, 2004). Thus, the pathways are called "MyD88-dependent pathways" (Akira, 2003; McGettrick and O'Neill, 2004; Takeda and Akira, 2004). Signaling pathways fromTLR1, 2, 5–10 may be dependent on MyD88 (Akira, 2003; McGettrick and O'Neill, 2004; Takeda and Akira, 2004). However, TLR3 signaling pathways appear independent of MyD88 (Akira, 2003; McGettrick and O'Neill, 2004; Takeda and Akira, 2004). TLR4-mediated pathway may involve both MyD88-dependent and -independent pathways. With the exception of MyD88, four types of molecules in the cells have been confirmed as TIR domain-containing molecules, including TIRAP (TIR domain-containing adaptor protein), TRIF (TIR domain-containing adaptor including IFN-1β)/TICAM-1 (TIR domain containing adaptor molecule-1), TRAM (TIRF-related adaptor molecule), and SARM (sterile alpha motif and Armadillo motif domain-containing protein). Of these, TIRAP may be associated with MyD88, while IFN production by TRIF/TICAM-1 of TLR4 is independent of MyD88 (Akira, 2003; McGettrick and O'Neill, 2004; Takeda and Akira, 2004). These results suggest that the signaling pathways of each TLR are unique and complicated (Janssens and Beyaert, 2003; Akira et al., 2006). Nucleic acids (DNA or RNA) derived from pathogens induce the production of cytokines (Akira, 2003; Barbalat et al., 2011). Thus, TLRs can induce various cytokines against infections through activation of the signaling pathways. For example, TLR4

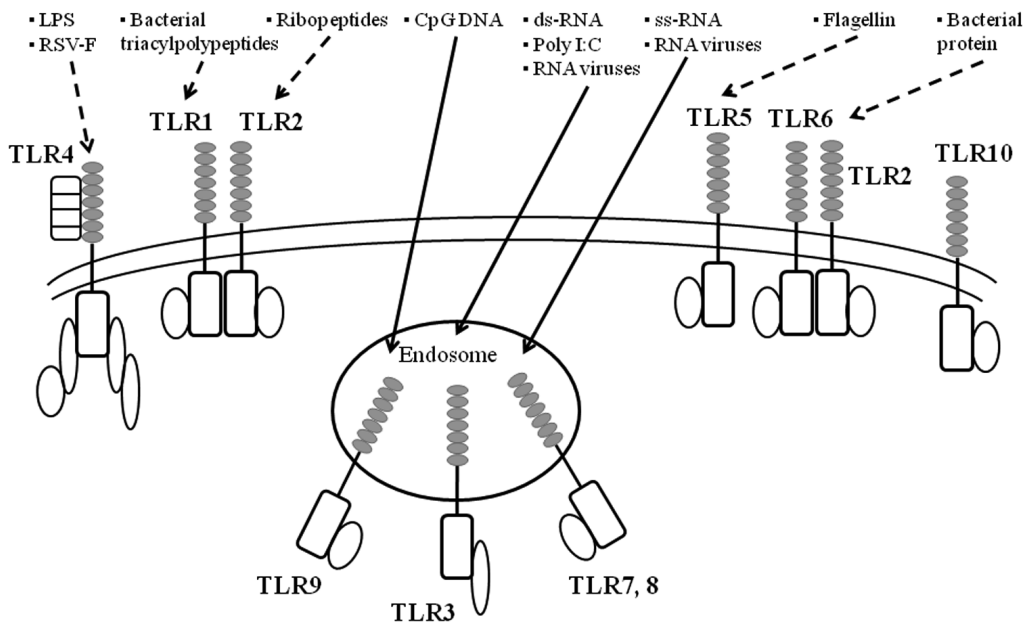

FIGURE 2 | A summarized illustration of TLRs and their ligands. TLR are key initiators of inflammation during host defense. Acting as dimers, the ten different TLRs display differential patterns of recognition of microbial products. TLR4 detects LPS, which is specific to Gram-negative species. TLR1/2 and TLR2/6 recognize triacylated and diacylated polypeptides, respectively, from mycobacteria. TLR5 responds to flagellin from the flagella of multiple bacteria. TLR7 and TLR8 detect single-stranded RNA (ss-RNA) from viruses, and TLR9 detects CpG DNA, which is common in bacterial and viral pathogens. TLR3 recognizes double-stranded RNA (ds-RNA) or synthetic compounds such as Poly I:C.

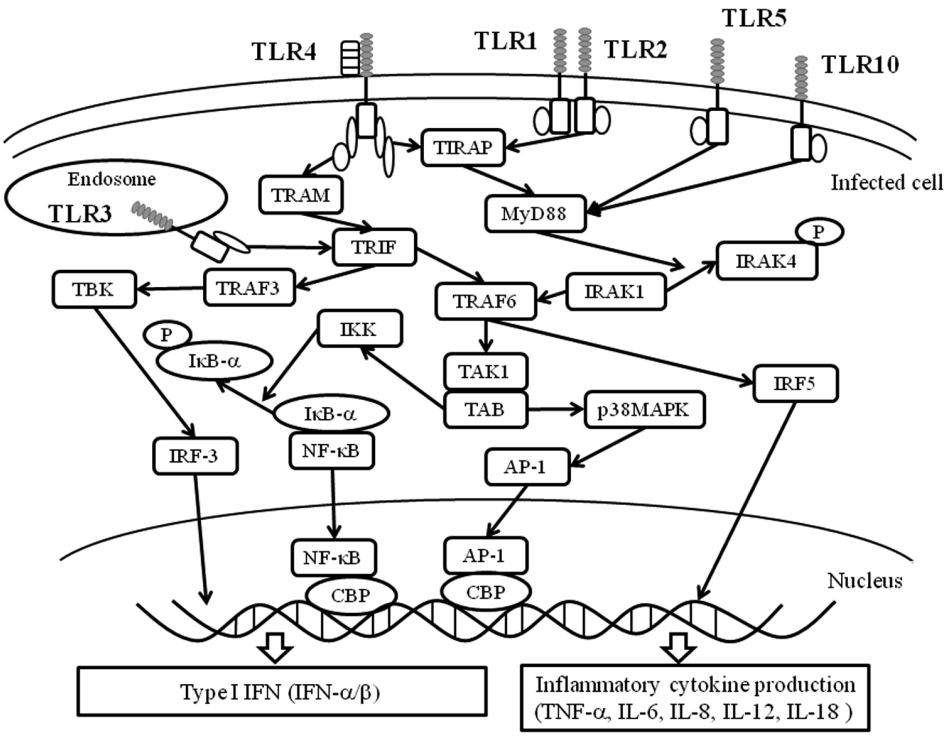

FIGURE 3 | Essential TLR-associated signaling pathways and cytokine production. Upon ligand stimulation, all TLRs, except TLR3, recruit the adaptor MyD88. In turn, MyD88 binds to a protein complex composed of IRAK4, IRAK1, and TRAF6. TRAF6 undergoes self-polyubiquitination resulting in the phosphorylation of TAK1. In turn, TAK1 activates IKK complex that leads to the phosphorylation, ubiquitination, and degradation of IκBα. This allows NF-κB to translocate into the nucleus. Simultaneously, the TAK1-containing complex activates the p38 MAP kinase pathway triggering the activation of AP-1. Together, NF-κB and AP-1 induce the expression of pro-inflammatory cytokines. TLR4 and TLR2, in combination with TLR1 or TLR6, recruit TIRAP that serves as a link adaptor for the recruitment of MyD88. Moreover, TLR4 recruits a second link adaptor named TRAM that allows interaction with the adaptor TRIF. Upon stimulation with an agonist, TLR3 recruits TRIF. TRIF-mediated activation of NF-κB and AP-1 also occurs through a TRAF6-dependent pathway. Upon stimulation, TRIF also binds TRAF3, which activates TBK. TBK phosphorylates IRF3 and permits its homodimerization and nuclear translocation. IRF-3, along with NF-κB and AP-1, cooperate to induce the expression of type I IFNs. AP-1, activator protein-1; CBP, cyclic AMP response element binding factor binding protein; IκB-α, inhibitor of NF-κB -α; IKK, IκB kinase complex; IRF, interferon regulatory factor; IRAK, IL-1 receptor-associated kinase; NF-κB, nuclear factor kappa light chain enhancer of activated B cells; p38MAPK, p38 mitogen activated protein kinase; TAB, TAK1 binding protein; TBK, TANK(TRAF family member NF-κB activator) -binding kinase; TAK1, TGF-1β activated kinase 1; TRAF, TNF receptor associated factor.

a ligand of F protein of RSV can activate MyD88-dependent signaling pathways resulting in the production of Th1 cytokines such as TNF-α, IL-6, IL-8, IL-12, and IL-18 (See and Wark, 2008; Klein Klouwenberg et al., 2009). On the other hand, TLR-3 activates MyD88 independent pathways leading to the production of IFN-1β (Akira, 2003; McGettrick and O'Neill, 2004; Takeda and Akira, 2004).

NUCLEOTIDE-BINDING OLIGOMERIZATION DOMAIN FAMILY AND CYTOKINE PRODUCTION

In macrophages and epithelial cells, NLRs play a pivotal role in the recognition of bacteria and viruses as PRR molecules (**Figure 1**; Wells et al., 2011). At present, about 20 types of NLRs have been confirmed in humans (Schroder and Tschopp, 2010). The representative pathogen PRR-related NLRs are NLRP1, NLRP3 (cryopyrin), and NLRPC4 (Schroder and Tschopp, 2010). These molecules have both signal transduction domains in the N-terminal and leucine-rich repeats in the C-terminal (Schroder and Tschopp, 2010). Thus, NLRs show the properties of both PRR molecules and signaling molecules (Schroder and Tschopp, 2010). In addition, the N-terminal of NLRs acts as a caspase recruitment domain (CARD; Schroder and Tschopp, 2010). For example, NLRP3 binds pro-caspase-1 through activation of TLRs (TLR4) and forms "inflammasome" (Bauernfeind and Hornung, 2013). Activated NLRP3-pro-caspase-1 complex releases active caspase-1 (Schroder and Tschopp, 2010; Bauernfeind and Hornung, 2013). Active type caspase-1 activates pro-IL-1β and pro-IL-18, leading to their production in the cells (Schroder and Tschopp, 2010; Bauernfeind and Hornung, 2013).

RETINOIC ACID-INDUCIBLE GENE-I LIKE RECEPTORS FAMILY

Retinoic acid-inducible gene-I and MDA5 (melanoma differentiation-associated protein 5) are localized in the cytosol and may be able to bind to some ssRNA viruses such as RSV, influenza virus, dengue fever viruses (DFV), and hepatitis C virus, leading to the production of type I IFN (IFN-α/β) in fibroblasts (Breiman et al., 2005; Loo et al., 2008; Jamaluddin et al., 2009; Bustos-Arriaga et al., 2011). In particular, it is known that RIG-I binds to ssRNA (5′-triphosphate RNA) derived from influenza virus and induces type I IFN (Loo et al., 2008). Furthermore, both RIG-I and MDA5 can

bind to DFV type 2 genome and induce the production of type I IFN (Qin et al., 2011a). However, the roles of these molecules in innate immunity are not known at present.

INFLAMMASOME, RLR-ASSOCIATED SIGNALING PATHWAYS, AND CYTOKINE PRODUCTION

Inflammasome as a PRR is a concept of the inflammatory reaction-associated protein complex (Schroder and Tschopp, 2010). It is suggested that both RIG-I and MDA5 can bind to an adaptor molecule, IPS-1(interferon-1β promoter stimulator 1), and activate NF-κB, resulting in the production of type I IFN (Schroder and Tschopp, 2010; Bauernfeind and Hornung, 2013). Inflammasome is composed of some protein complexes such as Apaf-1(apoptotic protease-activating factor 1), ASC (apoptosis-associated speck-like protein containing caspase recruitment domain), NOD (nucleotide-binding domain), and NALP (NACHT, LRR and PYD domain-containing protein; Schroder and Tschopp, 2010; Bauernfeind and Hornung, 2013). The complex recognizes various components of pathogens and uric acid as "danger signals" (Schroder and Tschopp, 2010; Bauernfeind and Hornung, 2013). After recognition of the signals, these signals activate ASC, leading to the conversion of procaspase-1 to caspase-1 (Schroder and Tschopp, 2010; Bauernfeind and Hornung, 2013). The protease caspase-1 activates proinflammatory cytokine precursors such as pro-IL-1β and pro-IL-18, leading to conversion to active forms of IL-1β and IL-18 (Schroder and Tschopp, 2010; Bauernfeind and Hornung, 2013). Interestingly, very recent studies suggest that various inflammatory diseases such as atherosclerosis and rheumatoid arthritis are associated with inflammasome, although the precise mechanisms are not known.

RELATIONSHIPS BETWEEN PRRs, SIGNALING PATHWAYS, AND CYTOKINE PRODUCTION IN RESPIRATORY VIRUS-INFECTED CELLS

In general, cytokine production in immunological cells such as lymphocytes may be induced through each cytokine receptor on the cells (Salek-Ardakani and Croft, 2010; Rossol et al., 2011). Certainly, this process may occur in virus-infected cells (He and Greenberg, 2002). As mentioned previously, cytokine production may trigger innate immunity through PRRs including TLRs, RLRs, and inflammasomes (NLRPs-pro-caspase-1 complex; **Figure 1**; Yu and Levine, 2011). These receptors and/or intracellular protein complexes induce phosphorylation of the signaling molecules. Although the precise mechanisms are not known, the phosphorylation cascades of the molecules lead to cytokine production in virus-infected cells (Yu and Levine, 2011). The representative data of virus infection-associated signaling pathways is shown in **Figure 4**. Briefly, a previous report showed that RSV infection in human fetal lung fibroblasts (MRC-5 cells) induces various cytokines through the activation (phosphorylation) of Akt (murine thymoma viral oncogene homolog/protein kinase B), p38MAPK (mitogen activated protein kinase), ERK1/2 (extracellular signal-regulated kinase), and IκB-α (Seki et al., 2013). Human rhinovirus (HRV) infection in human bronchial epithelium cells (BEAS-2B cells) activated p38MAPK, ERK1/2, and NF-κB (nuclear factor kappa B protein). Human parainfluenza virus (HPIV) infection in MRC-5 cells activated p38MAPK and IκB-α (Yoshizumi et al., 2010). However, it is not currently known how PRRs are associated with the production of these cytokines.

RESPIRATORY VIRUS INFECTION-ASSOCIATED CYTOKINE PRODUCTION

The summarized data of the cytokine production profiles in some respiratory virus-infected cells are shown in **Table 1**. Numerous reports show that most respiratory virus infections can induce the production of various types of cytokines *in vitro* and *in vivo* (Khaitov et al., 2009; Koetzler et al., 2009; Martínez et al., 2009; Sharma et al., 2009; Ishioka et al., 2011; Lewis et al., 2012; Seki et al., 2013). The findings of previous *in vitro* studies suggest that influenza virus type A [subtype A(H1N1) virus]-infected human airway epithelial cells produces significant amounts of IL-1, IL-6, and IL-8 (Hofmann et al., 1997). Production is associated with inflammasome (NLRP3-pro-caspase-1 complex; Pothlichet et al., 2013). HRV-infected airway epithelial cells produced IL-1, IL-6, IL-8, RANTES (regulated on activation normal T cell expressed and secreted), eotaxin, interferon-inducible protein (IP)-10, IL-11, TNF-α, granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-1β, and IFN-λ (Yamaya, 2012). RSV-infected airway epithelial cells produced IL-1, IL-4, PIV-3, IL-6, RANTES, IL-8, IL-11, GM-CSF, andTNF-α (Yamaya, 2012). HPIV-3 infected human lung fibroblasts induced excessive expression of IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-10, G-CSF, GM-CSF, IFN-γ, TNF-α, IL-8 IP-10, eotaxin, and RANTES (Yoshizumi et al., 2010).

Previous *in vitro* studies have demonstrated that elevated IL-6, IL-8, and RANTES are found in sputum and serum in influenza virus infection (Yamaya, 2012). IL-6 and IL-8 were elevated in sputum and serum in HRV infection (Yamaya, 2012). Systemic avian influenza virus [subtype A(H5N1) virus] infection induced excessive production of proinflammatory cytokine, namely a cytokine storm (Ramos and Fernandez-Sesma, 2012). These results imply that cytokine production profiles may vary. Although the detailed information of the signaling pathways is not yet known, these differences may be associated with the pathophysiology of each respiratory virus infection (Schwarze and Mackenzie, 2013).

RELATIONSHIP BETWEEN CYTOKINE PRODUCTION DUE TO RESPIRATORY VIRUS INFECTION AND THE PATHOPHYSIOLOGY OF VIRUS-INDUCED ASTHMA

Viral infections clearly induce inflammation at infected sites. A variety of complicated pathophysiological events occur at these sites. In broad terms, these events may constitute converged cell death and regeneration (Rennard and von Wachenfeldt, 2011). The process of events has been named "remodeling" (Al-Muhsen et al., 2011). Cytokines derived from respiratory virus infections may be associated with airway remodeling (Kuo et al., 2011). It is suggested that the major production sources of cytokines are airway epithelium, fibroblasts, myofibroblasts, and leukocytes within infected regions (Westergren-Thorsson et al., 2010). These cytokines may be associated with remodeling processes following respiratory virus infections (Holtzman et al., 2002).

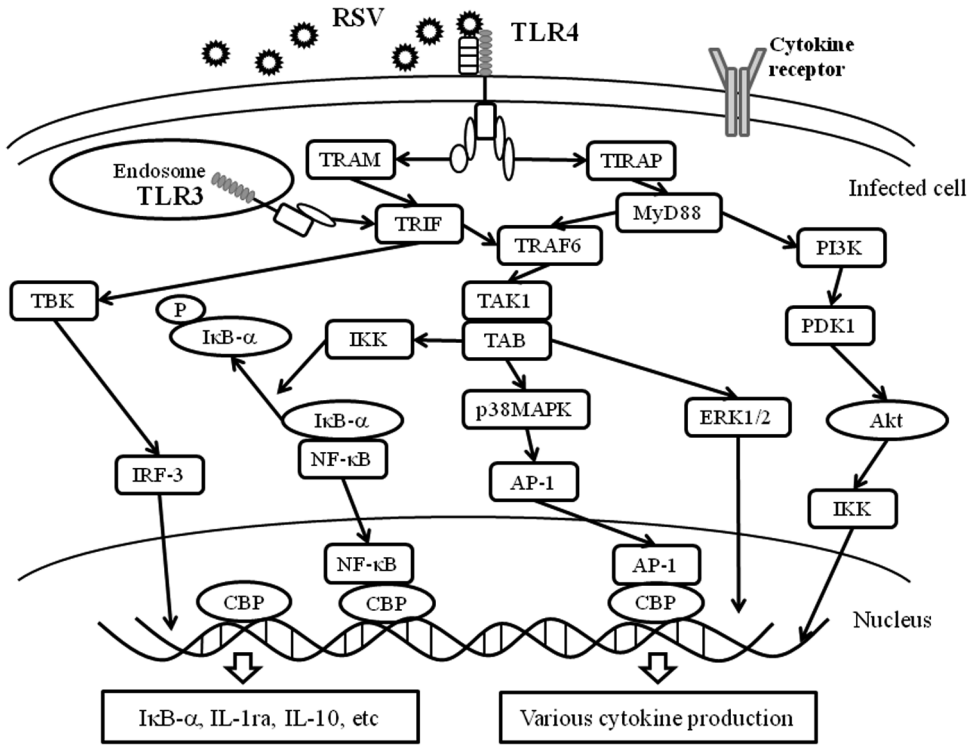

FIGURE 4 | PRRs and virus infection-associated signaling pathways. The innate immune response to the fusion protein of an important respiratory pathogen of humans, RSV, is mediated by TLR4. The RSV F protein induces translocation of TLR4 to the endosome together with TRAM. TLR3 is expressed in intracellular endosomes and responds to the presence of double-stranded RNA (dsRNA), which forms as a product of the replication of the majority of RNA viruses such as RSV. TLR3 and TLR4 activate TRIF-dependent signaling, which activates NF-κB and IRF-3, and results in the induction of proinflammatory cytokines and type I IFNs. Moreover, TLR4 is able to signal via both MyD88-dependent and -independent pathways and is able to activate a response via IRF-3, NF-κB, AP-1, ERK and IKK. These receptors recognize RSV and induce an appropriate antiviral innate immune response in the infected cells. Akt, protein kinase B; ERK1/2, extracellular signal regulating kinase1/2; PDK1, phosphoinositide-dependent kinase 1; PI3K: phosphatidylinositol-3 kinase.

Table 1 | Cytokines and chemokines induced by virus infection.

Viruses	Samples and specimens	Cytokines and chemokines	Reference
RSV	A549	GM-CSF	Ishioka et al. (2011)
	MRC-5	IL-1β, IL-6, TNF-α, IL-1ra, IFN-γ, IFN-λ1a, IL-2, IL-12, IL-4, IL-5, IL-10, IL-13, G-CSF, GM-CSF, eotaxin, RANTES, IL-8, IP-10, MCP-1, MIP-1α, PDGF-bb, VEGF, FGF-basic	Seki et al. (2013)
	HEp-2	IL-1β, MCP-1, MIP-1α, RANTES	Martínez et al. (2009)
	Primary BECs	IFN-β, IFN-λ1	Khaitov et al. (2009)
HRV	Nasal aspirates	IFN-γ, IL-6, IL-8, IP-10, eotaxin, RANTES	Lewis et al. (2012)
	BEAS-2B	IL-6, TNF-α, IL-8, IP-10	Koetzler et al. (2009), Sharma et al. (2009)
PIV	MRC-5	IL-1β, IL-6, TNF-α, IL-1ra, IFN-γ, IL-2, IL-4, IL-5, IL-10, G-CSF, GM-CSF, eotaxin, RANTES, IL-8, IP-10, PDGF, VEGF	Yoshizumi et al. (2010)

RSV, respiratory syncytial virus; HRV, human rhinovirus; PIV, parainfluenza virus.

CONCLUSION

Since the discovery of PRRs, remarkable progress has been made toward understanding the role of innate immunity against pathogens. However, the precise roles of PRRs, the mechanisms of intrinsic signaling pathways, and cytokine production with regard to PRRs are not fully understood. In addition, recent studies suggest that PRRs may be associated with various inflammatory diseases such as gout, rheumatoid arthritis, and atherosclerosis. It would be beneficial to clarify the functional relevancy of infectious diseases and other inflammatory diseases in the near future.

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Molecular epidemiology of respiratory viruses in virus-induced asthma

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Acute respiratory illness (ARI) due to various viruses is not only the most common cause of upper respiratory infection in humans but is also a major cause of morbidity and mortality, leading to diseases such as bronchiolitis and pneumonia. Previous studies have shown that respiratory syncytial virus (RSV), human rhinovirus (HRV), human metapneumovirus (HMPV), human parainfluenza virus (HPIV), and human enterovirus infections may be associated with virus-induced asthma. For example, it has been suggested that HRV infection is detected in the acute exacerbation of asthma and infection is prolonged. Thus it is believed that the main etiological cause of asthma is ARI viruses. Furthermore, the number of asthma patients in most industrial countries has greatly increased, resulting in a morbidity rate of around 10–15% of the population. However, the relationships between viral infections, host immune response, and host factors in the pathophysiology of asthma remain unclear. To gain a better understanding of the epidemiology of virus-induced asthma, it is important to assess both the characteristics of the viruses and the host defense mechanisms. Molecular epidemiology enables us to understand the pathogenesis of microorganisms by identifying specific pathways, molecules, and genes that influence the risk of developing a disease. However, the epidemiology of various respiratory viruses associated with virus-induced asthma is not fully understood. Therefore, in this article, we review molecular epidemiological studies of RSV, HRV, HPIV, and HMPV infection associated with virus-induced asthma.

Keywords: molecular epidemiology, virus-induced asthma, respiratory syncytial virus, human rhinovirus, human metapneumovirus, respiratory viruses

INTRODUCTION

Acute respiratory illness (ARI) is a major cause of morbidity and mortality worldwide (Williams et al., 2002; Sloots et al., 2008). ARI imposes a large burden on health, particularly in children. For community-based care, ARI has been estimated at a cost of over US$100 per case (Ehlken et al., 2005). The disease burden for ARI is estimated at 94,037,000 disability-adjusted life years and 3.9 million deaths (World Health Organization, 2002). Thus, ARI has a huge impact on health and society.

Although severe lower respiratory tract infections have been observed, ARI is most often associated with mild upper respiratory infection (URI). Most ARI cases in early childhood are confirmed as URI, leading to symptoms of the common cold with coryza and cough. In contrast, around one-third of infants with ARI develop lower respiratory tract symptoms such as tachypnea, wheezing, severe cough, breathlessness, and respiratory distress (Tregoning and Schwarze, 2010). In general, viruses are the most common causative agents of ARI. More than 200 different types of viruses are known to cause ARI, with respiratory syncytial virus (RSV), human rhinovirus (HRV), human metapneumovirus (HMPV), and human parainfluenza virus (HPIV) most commonly identified in ARI patients. Indeed, together with these respiratory viruses, human enterovirus (HEV), influenza virus (InfV), human coronavirus (HCoV), adenovirus (AdV), and human bocavirus (HBoV) account for around 70% of ARIs detected (Kusel et al., 2006). Respiratory viral infections can have severe adverse outcomes in patients with established asthma and are associated with nearly 80% of asthma exacerbation episodes (Nicholson et al., 1993; Johnston et al., 1995; Wark et al., 2002; Heymann et al., 2004; Grissell et al., 2005). Accumulating evidence indicates that the etiology of most cases of asthma, namely virus-induced asthma, is linked to such respiratory virus infections. In addition, RSV and HRV are the most frequently detected pathogens and may play an important role in viral induction and exacerbation of asthma.

Molecular biology techniques have developed rapidly over recent years. The application of molecular techniques to the study of virus-induced asthma enhances epidemiologic studies by improving our ability to classify these pathogens into meaningful groups (Foxman and Riley, 2001). In this review, we focus on molecular epidemiological studies of respiratory viruses, including RSV, HRV, HMPV, and HPIV infections, associated with virus-induced asthma.

VIRAL INFECTION AND ASTHMA

In infancy, illnesses such as bronchiolitis share many clinical features with acute asthma, including wheezing, rapid breathing, prolonged expiratory phase inflammation, and respiratory

compromise. Respiratory viruses are detected in the majority of asthma exacerbations in both children (80–85%) and adults (75–80%; Johnston et al., 1995; Grissell et al., 2005). In addition, wheezing illnesses are also closely associated with respiratory viral infections in all age groups (Gern, 2010). Fujitsuka et al. (2011) attempted to detect various respiratory viruses in Japanese children with acute wheezing using PCR technology and found viruses in samples from 86.1% patients: RSV or HRV alone were detected in 40.9 and 31.3% patients, respectively and both RSV and HRV were detected in 12.2% patients. Other previous reports suggested that the prevalence of RSV and HRV is similar (36 and 42%, respectively) in children less than 2 years of age, but differs (27 and 66%) in older children (Johnston et al., 1995; Grissell et al., 2005). In addition, Fujitsuka et al. (2011) suggested that RSV was the dominant species detected in patients with no history of wheezing and/or asthma, while HRV was dominant in patients with such a history. Thus, the main causative viral agent of asthma depends on previous illness and age.

Around one-third of infants who have an acute wheezing illness go on to develop recurrent wheezing, indicating that viral respiratory illnesses in early life promote asthma. Recently, the "two-hit" hypothesis has been proposed, whereby viral infections promote asthma mainly in predisposed children (Gern, 2010). Infants who develop virus-induced wheezing episodes are at increased risk for subsequent asthma, but most acute wheezing illnesses in infancy resolve with no long-term sequelae. It has been recognized for years that RSV infections often produce the first episode of wheezing in children who go on to develop chronic asthma (Lemanske, 2004). Indicators of heightened risk for developing asthma include wheezing episodes caused by HRV infections and the development of atopic features such as atopic dermatitis, allergen-specific IgE for foods or aeroallergens (e.g., house dust, mites, or cat or dog dander), and blood eosinophilia (**Figure 1**). Once asthma has been established, HRV infections are the most common cause of acute exacerbations, especially in children. As in infancy, atopy is an important risk factor for acute episodes of virus-induced wheezing (Kusel et al., 2007). Many previous reports have suggested that such respiratory virus infections are deeply associated with virus-induced asthma (Kusel et al., 2007; Pierangeli et al., 2007; Kuehni et al., 2009; Fujitsuka et al., 2011; Kato et al., 2011). Thus, it is entirely plausible that viral infections induction and/or exacerbation asthma in children.

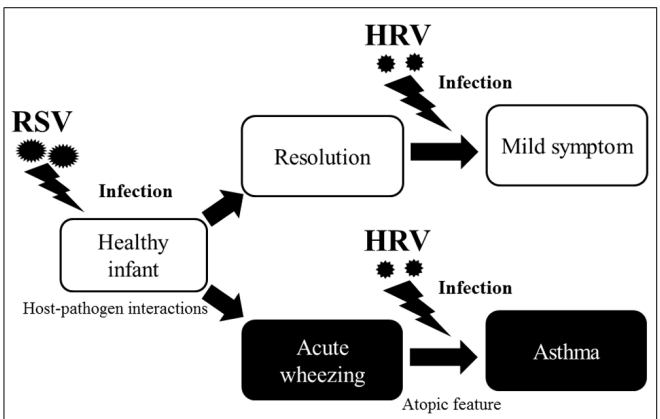

FIGURE 1 | Relationship between respiratory viral infections and development of asthma. Host-pathogen interactions that determine the severity of respiratory illnesses, and risk for subsequent asthma was increased by respiratory virus infection, especially due to RSV, in infants. Although most acute wheezing resolves within a relatively short time, a history of wheezing and host immunological conditions (e.g., atopic features) heightens the risk for asthma. Once asthma is established, HRV infections are the most common causative agents of asthma in children.

MOLECULAR EPIDEMIOLOGY OF RSV

Respiratory syncytial virus of genus *Pneumovirus* and family *Paramyxoviridae* causes ARI in children (Vardas et al., 1999; Peter and James, 2006). RSV infection may cause major problems in infants less than 1 year of age and can lead to life-threatening ARIs such as bronchiolitis and bronchopneumonia (Shay et al., 1999; Leung et al., 2005; Yorita et al., 2007). Epidemiological studies suggest that around 70% of infants have experienced an RSV infection by the age of 1 year, and 100% by the age of 2 years; host response to the virus varies greatly, but includes upper respiratory tract infections, typical bronchiolitis, and RSV-induced wheezy bronchitis (Cane, 2001; Kuehni et al., 2009). Long-term prospective case-control and cohort studies have also linked RSV bronchiolitis to the development of wheezing and asthma later in childhood (Sigurs et al., 1995, 2005, 2010; Henderson et al., 2005). Thus, RSV infections may be associated with the initiation and/or exacerbation of asthma.

The RSV genome encodes 11 proteins (Peter and James, 2006). Among these, the attachment glycoprotein (G) is a major structural protein that may be associated with both infectivity and antigenicity (Johnson et al., 1987; Rueda et al., 1991). Molecular epidemiological studies have shown that RSV can be classified into two phylogenetic subgroups, RSV-A and RSV-B (Mufson et al., 1985). The strains of subgroup A can be subclassified into eight genotypes (GA1–GA7 and SAA1), as can those of subgroup B (BA, GB1–GB4, and SAB1–3; Parveen et al., 2006). From phylogenetic analysis of the *G* gene of RSV, Martinello et al. (2002) showed that RSV belonging to GA3 genotype may be associated with greater severity of illness in, for example, bronchiolitis and pneumonia. Although GA3 genotype has been detected in the United Kingdom, Spain, and New Zealand, it is not the most prevalent strain (Cane et al., 1991; Garcia et al., 1997; Matheson et al., 2006). Martinello et al. (2002) therefore suggested that the association between greater severity of illness and GA3 genotype may be solely due to a transient shift in genotype-specific immune status within the community. In addition, correlations between certain strains and/or genotypes of RSV and slight differences in disease severity have been described previously (Hall et al., 1990; Walsh et al., 1997). Some genotypes such as subgroup A genotypes GA1, GA2, GA5, GA7, and NA1 and subgroup B genotype BA have been detected throughout the world in recent years (Zlateva et al., 2004; Parveen et al., 2006; Zhang et al., 2007; Nakamura et al., 2009; Rebuffo-Scheer et al., 2011). Of these, NA1 is a novel genotype known to be genetically close to GA2 genotype, while GA2 genotype and BA genotype are the most common genotypes of RSV subgroups A and B around the world and have persisted for many years (Tran et al., 2013). Furthermore, a new genotype belonging to RSV-A, ON1, has been detected in some countries, including

Canada, Korea, Malaysia, South Africa, and Japan (Eshaghi et al., 2012; Lee et al., 2012; Khor et al., 2013; Tsukagoshi et al., 2013; Valley-Omar et al., 2013). This genotype contains a unique tandem repeat (72nt sequence duplication) in the C-terminal 3rd hypervariable region of the *G* gene, and may be classified as a subdivision of NA1 (Eshaghi et al., 2012). Some reports have suggested that the severity of illness is not linked to subgroups or genotypes, but is associated with the quantity of RSV in nasopharyngeal aspirate (Sullender, 2000; Campanini et al., 2007). A larger population study is needed to identify the different RSV genotypes circulating in different areas to gain a better understanding of the relationship between disease severity and RSV genotype.

The G protein is a major antigen of RSV and amino acid substitutions may be related to changes in antigenicity. There are some reports of amino acid substitutions, and some positively selected sites in the C-terminal 3rd hypervariable region of the *G* gene have been estimated (Botosso et al., 2009; Yoshida et al., 2012; Kushibuchi et al., 2013). For example, Yoshida et al. (2012) estimated some sites under positive selection in the region (Asn250Ser, Met262Glu, Arg297Lys,and Arg297Glu substitutions in RSV-A strains were estimated by the REL method, and Asn273Tyr and Leu274Pro substitutions of RSV-A, as well as Leu237Pro substitution of RSV-B, were estimated by the IFEL method). Botosso et al. (2009) found 29 and 23 amino acid sites under putative positive selection in RSV-A and RSV-B, respectively. In addition, some unique positively selected sites were found in the *G* gene (Kushibuchi et al., 2013). These amino acid variations at these sites might play a key role in severe respiratory infection, such as bronchiolitis (Goto-Sugai et al., 2010). Furthermore, the rate of molecular evolution of the region might be high. For example, Kushibuchi et al. (2013) estimated the evolutionary rate of RSV-A at 3.63×10^{-3} substitutions/site/year, while that of RSV-B was estimated at 4.56×10^{-3} substitutions/site/year. Thus, it is suggested that this C-terminal 3rd hypervariable region in the *G* gene of RSV-A and -B evolved rapidly (Kushibuchi et al., 2013). Based on host immunological conditions, it is suggested that host immunity such as TLR4 polymorphism is linked to symptomatic RSV infection (Delgado et al., 2009). Thus, both the antigenicity of the viruses and host immune conditions may play important roles in the pathophysiology of severe respiratory infections such as bronchiolitis, pneumonia, and virus-induced asthma (Awomoyi et al., 2007).

MOLECULAR EPIDEMIOLOGY OF HRV

Human rhinovirus are a group of positive-sense ssRNA viruses belonging to genus *Enterovirus* in the family *Picornaviridae* (Turner and Couch, 2007). Although HRVs were previously thought to be mainly associated with the common cold causing mild respiratory symptoms, recent reports strongly suggest that HRVs may induce and/or exacerbate asthma (virus-induced asthma; Chung et al., 2007; Turner and Couch, 2007; Busse et al., 2010; Gern, 2010; Khadadah et al., 2010). One report suggested that HRV wheezing illness within the first three years of life is significantly associated with the development of asthma at age 6 years (Jackson et al., 2008). Another report suggested that HRVs are major agents in the induction of wheezing and exacerbation of asthma (Khadadah et al., 2010). Thus, HRVs are being re-evaluated as important agents of ARI in humans (Imakita et al., 2000; Papadopoulos et al., 2002; Wos et al., 2008). The basis for these lower respiratory symptoms has been a source of controversy in terms of the mechanisms of HRV pathogenesis. There are a variety of potential barriers to HRV infection of the lungs, including temperature-sensitive replication of the virus. For this reason, it is thought that the optimum propagation temperature of HRVs may be 32–35°C *in vitro* (Papadopoulos et al., 1999; Schroth et al., 1999). However, a recent study suggested that HRVs can propagate in lower airway tissues and this may be an important factor in the development of airway obstruction, coughing, and wheezing that can lead to bronchiolitis and pneumonia (Mosser et al., 2005). HRV has been concomitantly isolated with bacterial pathogens in 24–54% of children and 10–18% of adults with pneumonia (Juven et al., 2004; Templeton et al., 2005; Jennings et al., 2008). Thus, it is not clear whether HRV is ever the causative agent for the disease.

Human rhinovirus were previously classified into two species, HRV species A (HRV-A) and species B (HRV-B), containing over 100 serotypes (Turner and Couch, 2007). However, a genetically heterogeneous third species, HRV species C (HRV-C), was discovered recently (Lamson et al., 2006; McErlean et al., 2007). Recent reports suggest that HRV-A, B, and C have a unique and wide genetic diversity (McIntyre et al., 2010; Simmonds et al., 2010; Arakawa et al., 2012). HRV-A and -C appear to be mainly associated with ARIs and virus-induced asthma, while HRV-B has been detected in a relatively small number of patients with ARIs (Linsuwanon et al., 2009; Wisdom et al., 2009; Smuts et al., 2011). Our previous findings obtained from samples from children with ARIs in Japan indicated that HRV-A and -C can be classified into many clusters in the phylogenetic tree, with 30% nucleotide divergence of the *VP4/VP2* coding region (Mizuta et al., 2010a; Arakawa et al., 2012; Kiyota et al., 2013). In addition, Kiyota et al. (2013) estimated that the rate of molecular evolution of the *VP4/VP2* coding region was rapid (3.07×10^{-3} substitutions/site/year) in HRV-C. These results suggest that HRV-A and -C detected in ARI cases are the predominant strains and have varied genetic properties (Wisdom et al., 2009; Mizuta et al., 2010a; Arakawa et al., 2012). Thus, the association between HRV type and disease severity is not fully understood. There may be important differences in the susceptibility of individuals to the replication of HRV in lower airway tissues.

Parry et al. (2000) and Gern et al. (2000) found that weak peripheral blood mononuclear cell (PBMC) Th1 (IFN-γ) response to HRV infection is associated with increased viral shedding, and decreased proliferative response of PBMCs to HRV is associated with increased severity of symptoms. In addition, it was found that weak Th1 responses (IFN-γ/IL-5 mRNA ratio) in sputum are also associated with greater severity of illness (Gern et al., 2000). Furthermore, weak Th1 responses to viral infection in adults with asthma have been associated with decreased lung function and greater airway responsiveness (Brooks et al., 2003). These results indicate that individuals with a weak Th1 response to viruses, and perhaps individuals with asthma in general, may be more susceptible to HRV illnesses, and this association may be strongest in those with more severe disease (Parry et al., 2000; Gern et al., 2000; Brooks et al., 2003). Other epidemiological and biological factors, such as allergy, atopic dermatitis,

or a family history of allergy, may be related to virus-induced asthma (Green et al., 2002; Singh et al., 2007). Recently it is suggested that variants at the 17q21 locus were associated with HRV induced asthma in children who had a history wheezing illnesses, although associations of 17q21 variants with asthma were restricted to children who had a history of HRV wheezing illnesses (Calışkan et al., 2013).

MOLECULAR EPIDEMIOLOGY OF HMPV

Human metapneumovirus is a recently identified RNA virus belonging to the *Paramyxoviridae* family, of genus *Metapneumovirus* (Collins and Crowe, 2007). HMPV is a major pathogen that causes ARI in all ages (Collins and Crowe, 2007). The first HMPV infection appears to take place within the first six months of life, after which infections may occur repeatedly and frequently (Schildgen et al., 2011). The nosocomial impact of HMPV is estimated to be as high as that for RSV. In an HMPV outbreak in Japan, 34.8% of elderly patients who shared the same day care room in a hospital were infected with HMPV (Honda et al., 2006). Higher morbidity is observed in young children, the elderly, and immunocompromised adults (Boivin et al., 2002; Falsey et al., 2003; van den Hoogen et al., 2003; Sumino et al., 2005; Williams et al., 2005; O'Gorman et al., 2006). HMPV is classified into two genotypes (A and B) and four subgroups (A1, A2, B1, and B2) by phylogenetic analysis, using the *F* and *G* genes (Biacchesi et al., 2003; van den Hoogen et al., 2004). Subgroup A2 has been subdivided into two lineages, subgroup A2a and A2b (Huck et al., 2006). It has been suggested that these genotypes circulate in variable proportions in some areas (Gerna et al., 2005; Mackay et al., 2006). Although the molecular epidemiological information on HMPV has gradually accumulated, the detailed epidemiology remains unclear (Mizuta et al., 2010b; Pitoiset et al., 2010; Omura et al., 2011). HMPV infections can occur throughout the year, but seasonality has been described in several studies, with the epidemiological peak occurring several months later than that observed for RSV epidemics (Robinson et al., 2005; Wilkesmann et al., 2006; Madhi et al., 2007; Aberle et al., 2008, 2010; Heininger et al., 2009). It remains unclear whether different HMPV subgroups are associated with differences in the clinical course of disease. Several groups have suggested that HMPV subgroup A might be associated with more severe clinical disease (Martinello et al., 2002; Kaida et al., 2006; Vicente et al., 2006; Arnott et al., 2013), while others have reported that subgroup B may cause more severe illness (Esper et al., 2004; Pitoiset et al., 2010), and still other groups have found no evidence for differential severity caused by different HMPV lineages (Agapov et al., 2006; Manoha et al., 2007; Larcher et al., 2008; Xiao et al., 2010). Previous reports suggested that the substitution rates for the *G* gene (3.5×10^{-3} substitution/site/year) and the *F* gene (7.1×10^{-4} to 8.5×10^{-4} substitution/site/year) are high, and some positively selected sites have been found in the latter (de Graaf et al., 2008; Yang et al., 2009). It may be that there is a correlation between some positively selected epitopes and disease severity. Thus, the association between HMPV subgroup and disease severity is controversial. To gain a better understanding of host responses that may contribute to differences in clinical severity between HMPV subgroups, a more detailed analysis that includes host immunological status is needed.

MOLECULAR EPIDEMIOLOGY OF HPIV

Human parainfluenza virus belong to the *Paramyxoviridae* family. There are two genera of HPIV, *Respirovirus* (HPIV-1 and HPIV-3) and *Rubulavirus* (HPIV-2 and HPIV-4; Karron and Collins, 2007). HPIV is classified into four serotypes (HPIV1–4), all of which can cause various ARI in humans such as URI, croup, bronchitis, asthma, and pneumonia (Henrickson, 2003; Karron and Collins, 2007). Although HPIV type 4 (HPIV4) is rarely reported, HPIV1-3 are important causes of various ARI, including the common cold, croup, bronchitis, bronchiolitis, and pneumonia in children, and they commonly re-infect both children and adults. While such infections are generally mild in healthy persons, they may cause serious diseases in children, such as asthma (Henrickson, 2003; Karron and Collins, 2007). Although fewer HPIV strains have been detected compared with other respiratory viruses such as RSV, HRV, and HMPV, previous reports suggest that HPIV1 and 3 are the dominant viruses in children with ARI (Reed et al., 1997). Indeed, serological surveys indicate that at least 60% of children have been infected with HPIV3 by 2 years of age, approximately 80% have been infected by age 4, and at least 75% have been infected with HPIV1 by 5 years of age (Parrott et al., 1959, 1962). HPIV1 and 3 show high prevalence and are associated with up to 12% of acute lower respiratory tract infections in adults (Azevedo et al., 2003; Matsuse et al., 2005). HPIV1 and HPIV3, may be major agents of ARI throughout the world, along with other viruses such as RSV, HRV, and HMPV (Laurichesse et al., 1999; Iwane et al., 2004; Monto, 2004; Do et al., 2011). In addition, it is suggested that HPIV is a major causative agent of virus-induced asthma (Henrickson and Savatski, 1997). Several previous studies have reported that HPIV1 infections demonstrate clear outbreaks in autumn, mostly in September and November, every 2 years (Knott et al., 1994; Hall, 2001; Counihan et al., 2001). Other studies have reported that HPIV3 causes yearly outbreaks around the globe, mainly in the spring-summer season (Knott et al., 1994; Counihan et al., 2001; Hall, 2001; Mizuta et al., 2013). A recent study suggested that four different types of HPIV cause similar clinical manifestations in patients, and the clinical presentation of HPIV infection may differ depending on patient age (Liu et al., 2013).

Henrickson and Savatski (1996) analyzed the longitudinal evolution of the HN coding region in 13 strains of HPIV1 isolated in the USA. These results showed that the antigenic and genetic subgroups are very stable. In addition, Mizuta et al. (2011) suggested that the evolution of the *HN* gene in the present HPIV1 isolates was relatively slow and that the gene is highly conserved. Only a few reports on the molecular epidemiology of HPIV1 are available and it appears that the molecular epidemiology of HPIV is poorly understood. Larger and more detailed studies on the association of HPIV with asthma are needed.

MOLECULAR EPIDEMIOLOGY OF OTHER VIRUSES

HEV68 was recently detected in asthmatic patients (Hasegawa et al., 2011). HEV68 was found to be relatively acid resistant and thus could be distinguished from acid-sensitive HRV87 (Schieble et al., 1967; Kapikian et al., 1971). HRV87 was recently reclassified

as HEV68 based on phylogenetic analysis and neutralization test, and some laboratories have confirmed its acid sensitivity (Blomqvist et al., 2002; Ishiko et al., 2002; Savolainen et al., 2002). Distinguishing between HRV and HEV based on the acid sensitivity of isolates is therefore not appropriate for HEV68. The number of reports of an association between respiratory disease and HEV68 infection has recently increased. One report of the phylogenetic analysis of HEV68 based on partial VP1 gene sequences indicates wide genetic diversity (Linsuwanon et al., 2012). In addition, Tokarz et al. (2012) showed the presence of multiple clades among the circulating strains, and that all strains are spreading rapidly worldwide and contributing to the prevalence rates of respiratory diseases. In addition, asthmatic individuals infected with HEV68 also have the propensity to develop unstable asthma or an acute attack (Hasegawa et al., 2011).

Influenza virus is also a major causative agent of ARI in both children and adults. Furthermore, asthmatic patients were found among children and adults hospitalized with seasonal InfV (Dao et al., 2010; Dawood et al., 2010). Although it is recognized that viral infections such as RSV or HRV may induce and/or exacerbate asthma, the effect of InfV on asthma remains arguable (Johnston et al., 1995). Although one study suggested that A(H1N1)pdm09 viruses impose greater risk factors on children than seasonal InfV (Tran et al., 2012), InfV vaccine was available before the influenza season since InfV causes more severe illness than other respiratory viruses. Therefore, it is suggested that InfV vaccine be recommended for children with asthma (Kloepfer et al., 2012).

Although the level of detection of HCoV, HBoV, or AdV is relatively low, these infections are also detected in children with acute wheezing (Chung et al., 2007; Jartti et al., 2007). Further studies are needed to clarify the clinical roles of HCoV, HBoV, or AdV infections and those of other respiratory viruses. In particular, the prevalence of HCoV, HBoV, or AdV infection in healthy control subjects, assessment of disease severity by other clinical variables, and the immunological effects should be investigated.

MOLECULAR EPIDEMIOLOGY OF CO-INFECTION

Infants with severe bronchiolitis have an increased risk of developing recurrent wheezing later in life (Chung et al., 2007). HRV may be detected concurrently with other viruses such as RSV, HMPV, InfV, or HCoV (Richard et al., 2008; Fujitsuka et al., 2011). Considering their ubiquity, it is interesting that the number of respiratory viruses detected concurrently with HRV strains is relatively low (Lambert et al., 2007; Mackay, 2007), supporting the concept that HRVs have a direct role in the clinical outcome of infection (Miller et al., 2007). In fact, HRV strains are co-detected with other pathogens in reproducible, but clinically undefined, patterns (Brunstein et al., 2008). The HRV partnership with host immunity may be a mutualistic one, inadvertently imparting an advantage to the host by protecting against more cytopathic respiratory viral pathogens while the host provides a vessel for HRV replication and transmission.

Respiratory viruses other than RSV and multiple viral infections may contribute to the severity of bronchiolitis and asthma. Indeed, it was reported that dual infections of HMPV and RSV or HRV and RSV confer a 5- to 10-fold increase of severe disease in children admitted to pediatric intensive care units (Papadopoulos et al., 2002; Semple et al., 2005). In contrast, other studies reported that co-infection with two respiratory viruses was not significantly associated with disease severity (van Woensel et al., 2006; Wolf et al., 2006). Thus, there is no consensus on the effects of co-infection on disease severity. The effect of dual infection may depend upon which viruses co-infect together. For example, although there was no increase in severity when HRV and/or AdV were detected during RSV infection, co-infection with both HMPV and RSV increased the rate of intensive care unit admissions (Aberle et al., 2005; Semple et al., 2005). Thus, although dual infections and reinfections have been well documented in children, chronic infection with the development of quasispecies cannot be ruled out without obtaining more complete data using high performance detection methods (Hall and McCarthy, 1996).

CONCLUSION

Respiratory viral infections are a major cause of virus-induced asthma in early life. Although antiviral therapy is not yet available for patients infected with respiratory viruses, the detection and identification of these viruses could help to explain serious respiratory illness, provide guidance for medical care, and prevent unnecessary treatment with antibiotics. Based on the results of many related studies, we propose a two-step hypothesis of asthma development in children. The first step is mainly due to RSV infection: when RSV infects bronchial cells, the bronchial cells produce various cytokines and chemokines. These responses cause hyperresponsiveness in the bronchial cells. In other words, RSV infection might create a preparatory step as the first step in the development of asthma. HRV infection might then bring about the second step in the development of asthma. An infant with a history of wheezing caused by RSV infection may develop the heavy wheezing of asthma due to HRV infection followed by RSV infection. To understand the cause of asthma, we need to examine the larger complex picture of genetic susceptibility, immune components, environmental exposures, and the interactions between these elements.

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Epidemiology of virus-induced wheezing/asthma in children

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Wheezing is a lower respiratory tract symptom induced by various viral respiratory infections. Epidemiological studies have revealed the phenotypes of wheezing in early childhood which have different risk factors for the development of asthma among school age children. The major viral species causing wheezing in children include respiratory syncytial virus, rhinovirus, human metapneumovirus and influenza viruses. It has been shown that the impact on the development of asthma is different between those virus species. Moreover, recent studies have also focused on the interaction between virus infection and other risk factors in the development of asthma, such as genetic factors or allergic sensitization. In this review, we summarize the previous findings and discuss how clinicians can effectively intervene in these viral infections to prevent the development of asthma.

Keywords: wheezing, viral respiratory infection, cohort study, interferon, respiratory syncytial virus, rhinovirus

INTRODUCTION

Wheezing is a lower respiratory tract symptom induced by various viral respiratory infections. It is in common in children, with approximately one-third of the children having at least one wheezing episode by age nine. However, about 1 to 2% of affected infants need to be hospitalized due to respiratory distress. Moreover, although the condition is transient in the majority of cases, some children develop recurrent wheezing and are diagnosed to have asthma when they reach school age. In those children, the virus-induced wheezing in early childhood may be associated with the subsequent development of recurrent wheezing and/or asthma in connection to the pathology of asthma, including chronic airway inflammation, thus leading to airway hyperresponsiveness and airway remodeling.

Epidemiological studies are therefore considered to be important for clarifying which populations are at risk for developing virus-induced wheezing accompanied with other severe symptoms, recurrent wheezing and especially asthma. Moreover, recent studies have also focused on the interaction between virus infection and other risk factors for the development of asthma, such as genetic factors or allergic sensitization.

In this review, we summarize the previous findings and discuss how clinicians can effectively intervene in these viral infections to prevent the development of asthma.

PHENOTYPES OF VIRUS-INDUCED WHEEZING

Birth cohort studies have been conducted to clarify the natural history of wheezing in early childhood and to assess the risk factors for the development of wheezing and subsequent asthma. The first large prospective study focusing on the wheezing history was performed by the Tucson Children's Respiratory Study group (Halonen et al., 1999; Sherrill et al., 1999; Stein et al., 1999; Taussig et al., 2003; Morgan et al., 2005). They followed 828 infants until the age of 6 years and identified four different patterns of wheezing in early childhood (never wheeze, transient early wheeze, late-onset wheeze, and persistent wheeze) on the basis of clinical observations. The "never wheeze" phenotype (51.5% of the cohort) was defined as children with no episodes of wheezing during the first 6 years of the life. The "transient early wheeze" phenotype (19.9% of the cohort) was defined as children having at least one lower respiratory tract illness with wheezing during the first 3 years of the life, but no wheezing at 6 years of age. The children with this phenotype had a diminished airway function both before the age of 1 year and at the age of 6 years, were more likely than the other children to have mothers who smoked but not mothers with asthma, and did not have elevated serum IgE levels or skin-test reactivity. The "late-onset wheeze" phenotype (15.0% of the cohort) was defined as children having no wheezing before the age of the 3 years, but having wheezing at 6 years of age. The "persistent wheeze" phenotype (13.7% of the cohort) was defined as children having wheezing both before 3 years of age and at 6 years of age. The children of this phenotype were more likely than the "never wheeze" children to have mothers with a history of asthma, to have elevated serum IgE levels and normal lung function in the first year of life, and to have elevated serum IgE levels and diminished airway function at 6 years of age. Interestingly, these phenotypes have been shown to be associated with different risk factors for the number of encountered viral infections in early childhood (Kusel et al., 2007) and the development of asthma (Taussig et al., 2003; Stein and Martinez, 2004).

Birth cohort studies from Europe using latent class analysis identified more complicated wheezing phenotypes, including an intermediate-onset wheezing phenotype. A population-based birth cohort study of 6265 children in the United Kingdom (the ALSPAC study) identified six wheezing phenotypes in childhood, from birth to age 7 years, and demonstrated that these phenotypes differed in the atopy prevalence and lung function levels at 7–8 years of age (Henderson et al., 2008). Another multicenter birth cohort study of 2810 children in the Netherlands (the PIAMA

study) also identified five wheezing phenotypes in childhood from birth to age 8 years. Interestingly, the wheezing phenotypes identified by the two birth cohort studies were comparable (Savenije et al., 2011).

VIRAL SPECIES CAUSING WHEEZING

The major viral species causing wheezing in children include respiratory syncytial virus (RSV), rhinovirus (HRV), human metapneumovirus (hMPV) and influenza viruses.

RSV

Respiratory syncytial virus is a medium-sized negative-stranded RNA virus of the family *Paramyxoviridae*, which causes respiratory infections mainly in children. Interestingly, the clinical symptoms of RSV infection in infancy and early childhood are extremely variable. Most infants experience an RSV infection before 3 years of age (Ruuskanen and Ogra, 1993), normally escaping with only upper respiratory diseases, whereas approximately 1–2% of them require hospitalization because of severe RSV bronchiolitis (Green et al., 1989; Stretton et al., 1992). This is particularly common in those who are premature or who have chronic lung disease or congenital heart disease. Recently, a humanized monoclonal antibody designed to provide passive immunity against an epitope in the A antigenic site of the F protein of RSV has been widely used for the prophylaxis of severe RSV lower respiratory infection in those children.

HRV

Rhinovirus is a small-sized positive-strand RNA virus of the family *Picornaviridae*, which is well known as the predominant cause of the common cold. Because of the development of PCR techniques, it has been recognized that HRVs cause not only upper respiratory infections, but also lower respiratory infections or asthma exacerbation. HRVs consist of over 100 types classified into one of three species (A, B, and C) according to the phylogenetic sequence criteria. HRV C (HRV-C) is a recently classified group and has been shown to be associated with severe asthma attacks more frequently than other groups of HRV. The prevalence of HRV-associated wheezing increases by age, and it is significantly more common in children with recurrent wheezing episodes than in first-time wheezers in age categories of <24 and <36 months (Jartti et al., 2009).

HMPV

The hMPV is a medium-sized negative-stranded RNA virus of the family *Paramyxoviridae*, which was recently discovered (van den Hoogen et al., 2001), the clinical course of which resembles RSV infection. Similar to RSV, it has been reported that hMPV infection was associated with wheezing among children younger than 3 years, especially during the winter, while hMPV was not significantly associated with wheezing requiring hospitalization among children 3 years of age and older (Williams et al., 2005).

INFLUENZA VIRUSES

Influenza viruses are a medium-sized negative-stranded RNA virus of the family of *Orthomyxoviridae*. Influenza viruses cause severe lower respiratory tract complications, such as bronchitis or pneumonia. In addition, influenza is significantly associated with wheezing during the winter among children younger than 3 years of age although the detection percentage of the influenza virus is lower than that of RSV (Heymann et al., 2004).

RISK FACTORS FOR THE DEVELOPMENT OF VIRUS-INDUCED WHEEZING

BEHAVIORAL OR ENVIRONMENTAL FACTORS

In the Tucson study, it was reported that breast-feeding at early infancy for at least 1 month was associated with lower rates of virus-induced wheezing during the first 4 months of the life (Wright et al., 1989). However, the results of the subsequent studies have been conflicting. A meta-analysis study finally showed that there was no association between any or exclusive breast feeding and wheezing illness (Brew et al., 2011).

Infants exposed to more children at home or day care experienced more frequent wheezing when they were 2 years old, but less frequent wheezing from years eight through year thirteen. Therefore, although exposure to children at home or in day care during infancy increased wheezing in early life, it appears to be protective against the development of frequent wheezing in school age children (Ball et al., 2000).

HOST IMMUNOLOGICAL FEATURES

Interferon (IFN) secretion is important in the clearance of viral pathogens. Therefore, IFN deficiency has been supposed to lead to lower respiratory viral infections. There are three types of interferons: Type I (IFN-α/β), Type II (IFN-γ) and Type III (IFN-λ). It was shown that low IFN-γ production in cord bloods (Copenhaver et al., 2004) or PBMCs in the first year of life (Stern et al., 2007) was a risk factor for wheezing during childhood, in addition to a risk factor for the development of asthma and allergies (Tang et al., 1994). It has recently been clarified that the deficiency of IFN production is related to atopy. It was reported that allergic asthmatic children had an impaired HRV-induced IFN-α and IFN-λ1 production that correlated with an increased FcεRI expression on plasmacytoid dendritic cells in PBMCs, which were reduced by FcεRI cross-linking (Durrani et al., 2012). In addition, it was reported that bronchial epithelial cells from asthmatic individuals produced less IFN-β in response to HRV, leading to impaired apoptosis and increased HRV replication (Wark et al., 2005). Interestingly, it was revealed that allergic sensitization precedes HRV-induced wheezing, but the converse is not true (Jackson et al., 2012). The results of that study suggested that allergic sensitization can lead to more severe HRV-induced lower respiratory illnesses, which is considered to be a risk factor for the subsequent development of asthma.

GENETIC FACTORS

There have been many genetic risk factors reported to be associated with the development of RSV bronchiolitis. Two large scale genetic association studies were performed using a candidate gene approach (Janssen et al., 2007; Siezen et al., 2009). They analyzed 384 single-nucleotide polymorphisms (SNPs) in 220 candidate genes involved in the airway mucosal responses, innate immunity, chemotaxis, adaptive immunity, and allergic asthma. They found that SNPs in genes of the innate immune responses (the transcriptional regulator Jun, alpha interferon, IFN-α, nitric oxide synthase

and the vitamin D receptor) are important for determining the susceptibility to RSV bronchiolitis in term children. As RSV is recognized by Toll-like receptor (TLR) 4, SNPs in the genes of molecules related to TLR4 signaling have also been studied (Tal et al., 2004; Inoue et al., 2007).

In contrast, the genetic factors related to the development of HRV-induced wheezing are less well known (Helminen et al., 2008; Caliskan et al., 2013). However, the 17q21 variants, which were found to be related to childhood-onset asthma in a genomewide association study (Moffatt et al., 2007), were associated with HRV wheezing illnesses in early life, but not with RSV wheezing illnesses (Caliskan et al., 2013).

ASSOCIATION BETWEEN VIRUS-INDUCED WHEEZING AND THE DEVELOPMENT OF ASTHMA, AND EFFECTIVE TYPES OF INTERVENTION TO PREVENT THE SUBSEQUENT DEVELOPMENT OF ASTHMA

It is still unclear whether lower respiratory viral infections are causal factors, or instead serve as indicators, of a predisposition to asthma. Moreover, recent studies have indicated that the impact on the development of subsequent recurrent wheezing or asthma is different between virus species.

It was reported that infant birth approximately 4 months before the winter virus peak, which is the peak of bronchiolitis hospitalizations for that winter season, carried the highest risk for the development of asthma, thus suggesting that a lower respiratory infection with winter viruses, including RSV, in early childhood may be an important factor in the development of asthma (Wu et al., 2008). In a birth cohort study, Sigurs et al. (2010) followed 47 children aged <1 year hospitalized with RSV lower respiratory infection (RSV group) and 93 age- and gender-matched controls (Control group) for 18 years. They found that the RSV group had an increased prevalence of asthma/recurrent wheezing, clinical allergy and sensitization to perennial allergens, compared to the Control group (Sigurs et al., 2010). Meanwhile, it was shown that RSV prophylaxis using Palivizumab, a humanized monoclonal antibody against the RSV fusion protein that prevents severe RSV lower respiratory infection, in non-atopic children decreased the relative risk of recurrent wheezing by 80%, but did not have any effect in infants with an atopic family history (Simoes et al., 2010). These results suggest that RSV predisposes to recurrent wheezing via an atopy-independent mechanism.

Rhinovirus has been implicated as an important pathogen in asthma pathogenesis due to the improvement of PCR for HRV detection. In the Childhood Origins of ASThma (COAST) cohort, HRV in nasal lavage samples were evaluated by PCR. They found that, by age 3 years, wheezing in those with HRV-positive samples (OR, 25.6) was more strongly associated with asthma at age 6 years than aeroallergen sensitization (OR, 3.4; Jackson et al., 2008). As IFN deficiency is related to both atopy and the susceptibility to HRV infection, the inhalation of IFN by HRV-infected children with risk factors for asthma might thus help to prevent the development of asthma.

Recently, pandemic H1N1 influenza virus has been reported to increase the risk of lower respiratory tract complications including asthma attack, pneumonia, and atelectasis even in atopic children without any history of either an asthma attack or asthma treatment, compared to the seasonal influenza virus (Hasegawa et al., 2011). This observation suggests that the pandemic H1N1 influenza virus may be a strong risk factor contributing to the development or exacerbation of asthma.

LIMITATIONS OF EPIDEMIOLOGY STUDIES

The correct diagnosis of individual viral infections is necessary for assessing which virus infection is important for the development of wheezing or the subsequent development of asthma. The principal diagnostic methods for respiratory viruses are virus culture, serology, immunofluorescence/antigen detection, and nucleic acid/PCR-based tests (Tregoning and Schwarze, 2010).

Although virus culture proves that the virus detected in clinically obtained samples is able to infect human cells, viral culture is time-consuming and is not appropriate for analyzing many samples in epidemiological studies. Viral serologic testing is also time-consuming, and generally requires at least two rounds of blood sampling, because viral serological testing can diagnose infections by an increase of a virus-specific antibody in the blood, which usually takes 2 weeks to develop. Most previous epidemiological studies thus evaluated viral infections by immunofluorescence/antigen detection or nucleic acid/PCR-based tests. Antigen detection is based on the use of virus-specific monoclonal antibodies. There are a variety of diagnostic test kits that use nasopharyngeal aspirate, nasopharyngeal wash or nasal swab specimens as the test material, and detect viral antigen by using either a conjugated enzyme or fluorescence. Immunofluorescence/antigen detection is appropriate for epidemiological studies because it is convenient, cheap and possible to use when handling for many samples. However, there is a limitation to the species of target viruses. Nucleic acid tests are significantly more sensitive than the other methods, and are now being multiplexed, allowing for the rapid detection of many viruses concurrently. The PCR method has greatly increased the recovery rates of viruses (Johnston et al., 1995; Rakes et al., 1999). However, the PCR-based diagnosis of viruses, especially HRV, may not necessarily indicate that the virus is causing the observed disease, because virus RNAs can be detected by PCR for several weeks after the onset of clinical symptoms (van Benten et al., 2003; Jartti et al., 2004; Wright et al., 2007).

Another limitation in epidemiological studies assessing wheezing/wheezes in early childhood is the difficulty of diagnosing these conditions in young children based on the clinical assessment of symptoms by both guardians and clinicians. It was reported that the parents of children aged 4 months to 15 years and clinicians agreed on only 45% of occasions that the patient was wheezy or had asthma (Cane et al., 2000), thus suggesting that epidemiological studies using symptom records kept by guardians may sometimes lead to a wrong conclusion. Moreover, it has been shown that even specialists might not always correctly recognize wheezing (Bisgaard and Bonnelykke, 2010).

FUTURE PROSPECTIVE

To our knowledge, previous epidemiological studies regarding the association between viral infections and wheezing/asthma did not evaluate all other risk factors for the development

of wheezing/asthma in childhood, including behavioral factors, environmental factors, host immunological features and genetic factors. Large birth cohort studies evaluating viral infections and these factors in childhood could thus better elucidate the impact of viral infections on the development of wheezing/asthma. However, epidemiological studies only reveal an association, but not a causal relationship between some viral infections and the development of wheezing or asthma. Therefore, in the future, intervention trials with preventative intervention or therapies on a specific virus would be needed to clearly identify when and how clinicians should intervene in such viral infections to thereby prevent the development of wheezing or asthma.

CONCLUSION

Virus-induced wheezing is not only a burden in early childhood, but also may be one of causes or signs of childhood asthma. Therefore, clarifying the risk factors for virus-induced wheezing in epidemiological studies can and have provided clues about the pathogenesis of asthma. Further studies are needed to clarify which virus(es) in which population should be the major target of early intervention for preventing the subsequent development of asthma.

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Virus-induced exacerbations in asthma and COPD

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Chronic obstructive pulmonary disease (COPD) is characterized by chronic airway inflammation and/or airflow limitation due to pulmonary emphysema. Chronic bronchitis, pulmonary emphysema, and bronchial asthma may all be associated with airflow limitation; therefore, exacerbation of asthma may be associated with the pathophysiology of COPD. Furthermore, recent studies have suggested that the exacerbation of asthma, namely virus-induced asthma, may be associated with a wide variety of respiratory viruses. COPD and asthma have different underlying pathophysiological processes and thus require individual therapies. Exacerbation of both COPD and asthma, which are basically defined and diagnosed by clinical symptoms, is associated with a rapid decline in lung function and increased mortality. Similar pathogens, including human rhinovirus, respiratory syncytial virus, influenza virus, parainfluenza virus, and coronavirus, are also frequently detected during exacerbation of asthma and/or COPD. Immune response to respiratory viral infections, which may be related to the severity of exacerbation in each disease, varies in patients with both COPD and asthma. In this regard, it is crucial to recognize and understand both the similarities and differences of clinical features in patients with COPD and/or asthma associated with respiratory viral infections, especially in the exacerbative stage. In relation to definition, epidemiology, and pathophysiology, this review aims to summarize current knowledge concerning exacerbation of both COPD and asthma by focusing on the clinical significance of associated respiratory virus infections.

Keywords: asthma, COPD, respiratory virus, exacerbation, overlap syndrome, human rhinovirus, respiratory syncytial virus

INTRODUCTION

Asthma and chronic obstructive pulmonary disease (COPD) are very common inflammatory diseases of the airways. The World Health Organization (WHO) estimates that asthma accounts for 1 in every 250 deaths worldwide (O'Sullivan, 2005). The prevalence of asthma in developed countries is approximately 10% in adults and even higher in children, while in developing countries, the prevalence is lower but increasing rapidly (Barnes, 2008). In the case of COPD, WHO consensus reports forecast that this disorder will be ranked the third cause of mortality in the world by 2020 (Global initiative for chronic obstructive lung disease [GOLD], 2013[1]). Acute deterioration of symptoms and lung function, which often results in respiratory failure, is a so-called "exacerbation," and it is an important and severe social and medical burden in both diseases.

Respiratory viral infections are common and usually self-limiting illnesses in healthy adults and a major cause of exacerbations in patients with asthma (**Figure 1**) and/or COPD (**Figure 2**).

This review aims to summarize the clinical aspects of exacerbations in asthma and COPD from the perspective of the definition of exacerbations, epidemiology, and pathophysiology, with a special focus on the clinical significance of the presence of respiratory viruses.

PATHOPHYSIOLOGY OF ASTHMA AND COPD

Asthma and COPD are prevalent chronic pulmonary diseases characterized by chronic airway inflammation and airflow limitation. The differences between the two diseases are mainly the cellular and molecular features of airway inflammation and the degree of reversibility of airway flow limitation. Generally, reversibility of airflow limitation is incomplete in COPD, while that in asthma can be complete. Airway inflammation in asthma is characterized by allergic phenotypes, such as dense infiltration of eosinophils and T helper type 2 lymphocytes, associated with atopic status, while that of COPD is mainly accumulation of neutrophils, CD8-positive cytotoxic T cells, and activated macrophages, which are caused by inhalation of harmful substances, such as smoking. With respect to the site of inflammation, asthma involves predominantly larger airways, while in COPD, inflammation affects predominantly small airways and the lung parenchyma, characterized as irreversible airway narrowing because of fibrosis around the small

Abbreviations: ABPA, allergic bronchopulmonary aspergillosis; AdVs, adenoviruses; BAL, bronchoalveolar lavage; COPD, chronic obstructive lung disease; CRP, C-reactive protein; $FEV_{1.0}$, forced expiratory volume in 1 second; GOLD, Global initiative for chronic obstructive lung disease; HMPV, human metapneumovirus; HRV, human rhinovirus; ICS, inhaled corticosteroid; IFN, interferon; IL, interleukin; LABA, long-acting β-agonist combination; LAMA, long-acting muscarinic antagonist; LRT, lower respiratory tract; NPS, nasopharyngeal swabs; NPW, nasopharyngeal washings; OPS, oropharyngeal swabs; PEFR, peak expiratory flow rate; PIV, parainfluenza virus; RSV, respiratory syncytial virus; RTI, respiratory tract infection; RT-PCR, reverse-transcriptase polymerase chain reaction; SABA, short-acting β-agonist inhaler; TLR, toll-like receptor; TLSP, thymic stromal lymphopoietin; URT, upper respiratory tract.

[1]http://www.goldcopd.org/uploads/users/files/GOLD_Report_2013_Feb20.pdf

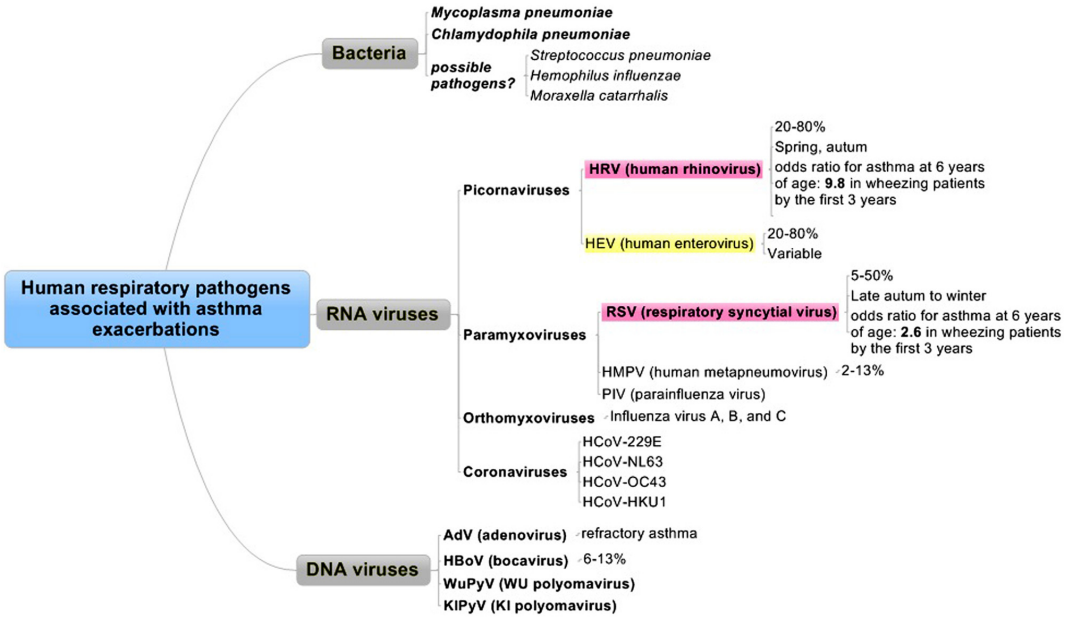

FIGURE 1 | Human respiratory pathogens associated with asthma exacerbations. Pathogens are divided into three categories as bacteria, RNA viruses, or DNA viruses. Red and yellow columns are the most relevant pathogens in order.

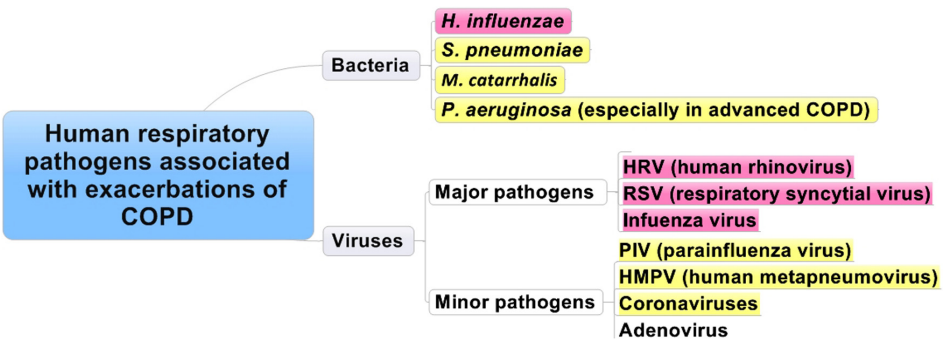

FIGURE 2 | Human respiratory pathogens associated with exacerbations of COPD. Red and yellow columns are the most relevant pathogens in order.

airways or destruction of alveolar walls with protease-mediated degradation (Barnes, 2008). Of note, neutrophilic infiltration could be recognized in bronchial biopsied specimens as well as eosinophils in severe refractory asthma (Wenzel et al., 1999).

DIFFERENCES AND SIMILARITIES BETWEEN ASTHMA AND COPD

As described above, asthma is typically characterized by chronic allergic inflammatory airway inflammation associated with airway hyperresponsiveness that leads to recurrent episodes of bronchial obstruction. In contrast, COPD is characterized by persistent airflow limitation that is usually progressive and ultimately results in respiratory failure. Therefore, it is not difficult to differentiate clinically between the two disorders. However, determining whether a patient has asthma or an exacerbation of COPD is often difficult, because of their clinical similarity. The **Table 1** summarizes the differences between these two diseases, and Tokuda and Miyagi (2007) provided an excellent review of rapid physical diagnosis for COPD patients that focused on inspection, palpation, percussion, auscultation, special maneuvers, and vital signs.

On physical examination, the sound of an expiratory wheeze is identical in asthma, COPD, congestive heart failure, and pneumonia, and it cannot be used to distinguish among these conditions (Kaplan et al., 2009). Thus, physical examination is relatively insensitive for the diagnosis of asthma, but COPD has its characteristic physical findings (Tokuda and Miyagi, 2007) that could be useful in rapid differentiation from those of asthma.

Recent understanding of the innate immune system suggests that it may function independently of the adaptive immune system in some cases or synergistically in others, and the relative contributions of the two systems may explain the disease heterogeneity among asthmatic patients, which might occur in patients with COPD (Holtzman, 2012). It has long been argued that asthma, chronic bronchitis, and emphysema could be considered

Table 1 | Differences between asthma and COPD.

	Asthma	COPD
Age at onset	At any age (usually <40 years)	Usually >40 years
Smoking history	Possible	Usually >10 pack-years
Cough at exacerbation	Usually between 2 and 6 am	Gradual increase
Sputum production	Infrequent	Common
Allergy(eczema or allergic rhinitis)	Common	Infrequent
Airway inflammation		
Main portion	Large airways	Small airways
Pathophysiology	Basement-membrane thickening	Fibrosis of small airways
	Increased airway smooth muscle	Destruction of alveolar walls
Bronchial biopsies	Th2-dominant T cells	Th1-dominant T cells and type1 CTL
	Eosinophils, activated mast cells	Neutrophils and Mφ
Reversibility (peak flow results)	Normalize with time	May improve, but do not normalize
Family history	Common	Uncommon

CTL, cytotoxic T cell; Mφ, macrophage.

as different expressions of one disease entity. This view is called the "Dutch hypothesis" (Kraft, 2006), and it is still under debate, with no consensus about it. There are many similarities in asthma and COPD (Bleecker, 2004), and previous studies suggested that asthma may be a risk factor for the development of COPD (Silva et al., 2004), while the coexistence of asthma and COPD, so-called "overlap syndrome," has recently been attracting attention. Overlap syndrome accounts for about 15–25% of obstructive airway disease (Louie et al., 2013) and shows more frequent or severe exacerbations and higher mortality than COPD alone (Hospers et al., 2000; Hardin et al., 2011). Furthermore, previous reports noted that exacerbations of asthma or COPD are associated with accelerated loss of lung function and quality of life and increased healthcare costs and mortality.

Thus, it is crucial to recognize and understand the clinical features of asthma and COPD patients, not only in the stable phase, but also in exacerbated phases associated with respiratory viral infections. Johnston and Sears (2006) reported that exacerbations of asthma and COPD appear to have a seasonal predilection in a similar fashion.

VIRUS-INDUCED EXACERBATIONS IN ASTHMA AND COPD

VIRUS-INDUCED EXACERBATIONS IN ASTHMA

In bronchial asthma, acute exacerbation involves several issues (**Figure 3**), such as the definition of acute exacerbation of asthma, recognition of the clinical symptoms of respiratory tract infection (RTI), assessment of the risk factors for acute exacerbation, considering the possibility of other diseases (differential diagnosis), diagnostic methods, appropriate sample collection, and treatment or prevention. An older study showed that asthmatic patients had a 6.2 times greater chance of having viral RTIs than a control group (Abramson et al., 1994), while Corne et al. (2002) found that the detection rates of human rhinovirus (HRV) in asthmatic (10.1%) and healthy participants (8.5%) were similar. The term virus-induced exacerbation of asthma is not uncommon, but only a small number of such studies were prospective (Nicholson et al., 1993; Johnston et al., 1995). Furthermore, RTIs do not always lead to an exacerbation, and there is little evidence that treating or preventing the infection may cure or prevent an exacerbation. In this regard, we mainly discuss the details of "viral infection and exacerbation of asthma," focusing on the accumulation of useful expertise for understanding this unfavorable condition in adult asthmatic patients.

Definitions of acute exacerbation of asthma

The diagnosis of asthma is usually defined based on history and variability of the peak expiratory flow rate (PEFR) and/or of forced expiratory volume in 1 s ($FEV_{1.0}$) of at least 20%, either with therapy or spontaneously. There is no clear consensus definition for asthma exacerbation; clinical trials usually define a severe exacerbation as the need for treatment with systemic corticosteroids, hospital admission, or emergency treatment for worsening asthma, or a decrease in morning peak flow >25% baseline on two consecutive days (O'Byrne et al., 2001). According to the latest NIH National Asthma Education and Prevention Guidelines, asthma exacerbations are acute or subacute episodes of progressively worsening shortness of breath, cough, wheezing, and chest tightness, or some combination of these symptoms, characterized by decreases in expiratory airflow and objective measures of lung function (spirometry and peak flow) (National Asthma Education and Prevention Program, 2007), identical to the definition of the Global Initiative for Asthma guidelines (2012)[2] (**Figure 3**). However, a joint task force of the American Thoracic Society and European Respiratory Society has recently defined asthma exacerbations as events characterized by a change from the patient's previous status (Reddel et al., 2009). Severe exacerbations were defined as events that require urgent action to prevent hospitalization or death, whereas moderate exacerbations were defined as the status of an asthmatic patient who required a prompt change in treatment due to being outside the patient's usual range of day-to-day asthma variation. Mild exacerbations are not defined because such events can be

[2]http://www.ginasthma.org/local/uploads/files/GINA_Report_March13.pdf

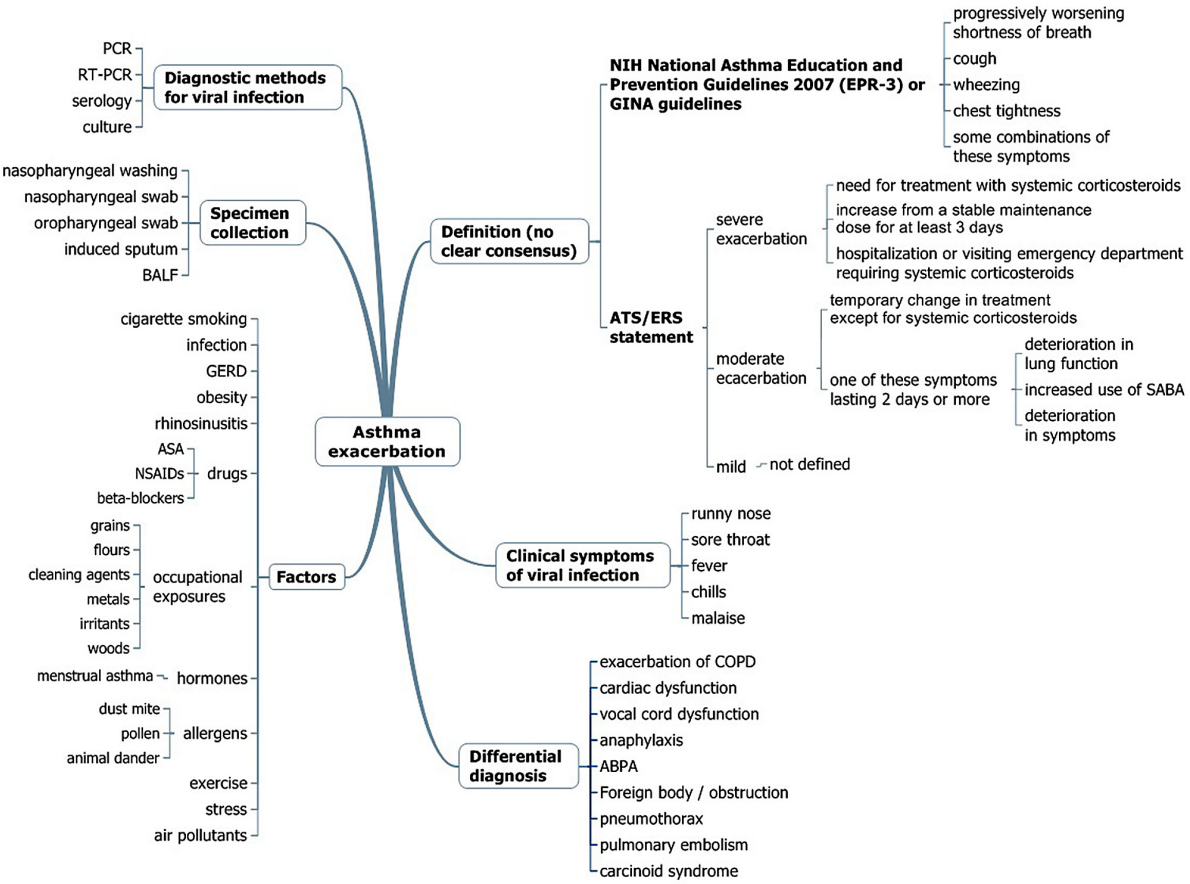

FIGURE 3 | Multidisciplinary assessment for asthma exacerbation. PCR, polymerase-chain reaction; RT-PCR, real-time PCR; BALF, bronchoalveolar lavage fluid; GERD, gastroesophageal reflux disease; ASA, acetylsalicylic acid; NSAIDS, non-steroidal anti-inflammatory drugs; SABA, short-acting β-agonist inhalers; GINA, Global Initiative for Asthma; ABPA, allergic bronchopulmonary aspergillosis; EPR, expert panel report.

indistinguishable from loss of asthma control (Reddel et al., 2009; **Figure 3**).

Epidemiology

Asthma exacerbations are more common in female than in male patients, and the higher prevalence of asthma in adult women contrasts with the higher prevalence of asthma in male children (Bjornson and Mitchell, 2000). Between 14 and 45% of acute asthma exacerbations in children is thought to be related to viral RTIs. Although the incidence in adults is less clear, previous reports showed that RTIs associated with asthma exacerbation in adults ranged from 10–21% (Teichtahl et al., 1997) to 45–80% (Johnston et al., 1995; Atmar et al., 1998), of which 60% have HRV (Johnston et al., 1995; Atmar et al., 1998; Tan, 2005; **Figure 1**). Despite their widely differing designs, these studies suggest that viral infections are involved in about 50% of asthma exacerbations among adults and in probably substantially more childhood asthma exacerbations. Another report also showed that the virus most commonly implicated in asthma exacerbations appears to be HRV (Murray et al., 2004). In addition to HRV, other respiratory tract viruses, such as respiratory syncytial virus (RSV), influenza viruses, coronaviruses, human metapneumoviruses (HMPVs), parainfluenza viruses (PIVs), adenoviruses (AdVs), and bocaviruses, have all been detected in subjects with asthma exacerbations (Jackson and Johnston, 2010). In adults requiring hospital admission for an acute severe asthma exacerbation in a 1-year period, virus was identified in 29% of the subjects, with HRV and influenza A the most commonly identified infectious agents (Teichtahl et al., 1997).

Diagnosis of viral infection: diagnostic methods and sample collection

Molecular methods of viral detection have superior sensitivity and specificity compared to cell culture-based methods (McErlean et al., 2010). In the setting of acute exacerbation, the reverse-transcriptase polymerase chain reaction (RT-PCR) method can detect viruses in approximately 80% of wheezing episodes in school-aged children and in approximately one-half to three-quarters of acute wheezing episodes in adults (Jackson and Johnston, 2010). With respect to sample collections for viral detection, Xiang et al. (2002) reported that nasopharyngeal secretions and induced sputum during acute exacerbations of asthma in adult patients were equal, while Falsey et al. (2012) found that the diagnostic yields using RT-PCR for detection of any virus

in adults hospitalized with respiratory illness were superior in sputum samples (36%) than in nose and throat swabs (23%). However, the study had some limitations in that no test for HRV or AdVs was used. Another report showed that the sensitivity rates for oropharyngeal swabs (OPS), nasopharyngeal swabs (NPS), and nasopharyngeal washings (NPW) obtained from hospitalized patients with acute febrile lower respiratory tract (LRT) infections were 54.2, 73.3, and 84.9%, respectively (for OPS vs. NPS and NPW, $p < 0.00001$; for NPS vs. NPW, $p < 0.003$; Lieberman et al., 2009). Taken together, these studies appear to suggest that induced sputum and NPS/NPW are better methods for identifying respiratory viruses. Regarding HRV, bronchoalveolar lavage (BAL) cells were positive for HRV RNA during infection in 80% of samples, whereas nasal lavage fluid was positive in the same patients in 100%, and BAL fluid was positive in only 37%. This suggests that HRV is able to infect the lower airways, and that HRV RNA is largely cell-associated (Murray et al., 2004).

Causes of acute asthma exacerbations

Eczema and a family history of asthma are the dominant non-infectious risk factors for pediatric asthma, while the triggers of adult-onset disease are less well defined. The causes for asthma exacerbation have been described and categorized. Of note, clinicians should recognize the seasonal trends for exacerbations of wheezing or asthma in adults, which occur 1–2 weeks later than in children, suggesting household transmission of the same strain (Johnston et al., 1996). HRV can be documented throughout the year, with a predilection for late spring and fall (Nagel et al., 2008), whereas RSV can be detected in late autumn to winter (**Figure 1**).

Viruses. The most important viruses relevant to asthma development are RSV and HRV. Jackson et al. (2008) demonstrated that, in a large, high-risk cohort, children had an increased risk of asthma at 6 years of age if they wheezed in the first 3 years of life with RSV [odds ratio (OR) 2.6], HRV (OR 9.8), or both HRV and RSV (OR 10).

Respiratory syncytial virus. By 1 year of age, 50–65% of children will have been infected with this virus, and by 2 years of age, nearly 100% has been infected (Openshaw, 1995). The exact mechanisms by which respiratory viral infection causes asthma exacerbation remains to be determined, but the respiratory viruses implicated in exacerbations have themselves been largely identified (**Figure 1**). The role of severe RSV infection as a risk factor for asthma in adulthood is less certain, but it is still under study. RSV is an important pathogen of young children and accounts for ∼70% of severe infantile viral bronchiolitis and/or pneumonia cases, most of whom have wheezing, and it is the most common cause of hospital admission in the winter season during the first year of life (Blanken et al., 2013). Furthermore, this study showed the strongest evidence that human RSV-mediated bronchiolitis has long-term effects using palivizumab (a humanized monoclonal antibody against RSV F protein that prevents infection by RSV in infancy). In children under 5 years, RSV and PIV are the most common pathogens, whereas in older children, rhinovirus and influenza A virus are more prevalent (Beasley et al., 1988). Even in elderly persons, RSV causes pneumonia (Falsey et al., 2006), exacerbations of COPD, and acute deterioration in those with cardiac disease, and it contributes substantially to excess deaths in the winter season (Olszewska and Openshaw, 2009).

Human rhinovirus. Recent studies have identified infection with HRV as a predominant respiratory pathogen associated with asthma later in life (Kusel et al., 2007). HRV is the most important virus type associated with exacerbations of asthma leading to hospital admission in both adults and children (Johnston et al., 1996). Tan et al. (2003) reported that picornaviruses (rhinovirus/enterovirus) and AdV were most commonly identified in near-fatal asthma, whereas influenza virus predominated in COPD. Corne et al. (2002) found that the detection rates of HRV in asthmatic (10.1%) and healthy participants (8.5%) were similar, but the LRT symptoms were significantly more severe and longer-lasting in the asthmatic group than in the healthy group. Thus, HRV is the most common and important cause of exacerbation in both children and adults (Johnston et al., 1996; Rakes et al., 1999; Copenhaver et al., 2004; Message et al., 2008; Dougherty and Fahy, 2009; Olenec et al., 2010; Jackson et al., 2012). HRV can now be classified into three species (HRV-A, B, and C) based on their genetic properties (http://www.ictvonline.org/), while over 100 serotypes have been identified. Molecular epidemiological studies suggest that HRV-A and -C are the major prevalent species, with wide genetic divergence (Fujitsuka et al., 2011).

Adenoviruses. Adenoviruses are well known as a primary cause of acute respiratory infection, particularly in young children. AdVs are associated with up to 7% of virus-related asthma exacerbations (McErlean et al., 2010), and they cause a wide variety of clinical syndromes, such as diarrhea, keratoconjunctivitis, and hemorrhagic cystitis (Brodzinski and Ruddy, 2009). It has been demonstrated that 94% of children with refractory asthma has detectable AdV antigens, compared with 0% of controls (Macek et al., 1994; **Figure 1**).

Parainfluenza virus. As previously noted, PIV is one of the most common pathogens for asthma exacerbation in children under 5 years. In adults with asthma, PIV infections have also been commonly demonstrated in several longitudinal studies of RTIs, but they have been identified less commonly in studies of patients seen in the hospital or emergency department (Atmar et al., 1998).

Other viruses. Most asthma studies describe relatively low levels of influenza viruses in asthmatic patients with exacerbations, approximately 1–9% of all virus-related asthma exacerbations. Several studies indicated that human bocaviruses (Gendrel et al., 2007; Vallet et al., 2009) and HMPV (Williams et al., 2005; Ong et al., 2007) are associated with exacerbations of asthma, especially in children.

Bacteria. *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* are found more frequently in the airways of patients with asthma than in healthy patients (Nisar et al., 2007), but their role in exacerbations is less clear (Xepapadaki et al., 2008). In previous studies, some have reported mycoplasmal infection in up to 25% of children with wheezing (Henderson et al., 1979) or identified it in 20% of exacerbations in asthmatic children requiring hospitalization and in 50% of children experiencing their first asthmatic

attack (Biscardi et al., 2004). However, others have not been able to confirm these observations (Cunningham et al., 1998).

Other specific pathogens, including *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Moraxella catarrhalis*, HRV, and RSV, have been shown to increase mucus secretion, which is recognized in asthma or COPD by characteristic goblet cell hyperplasia or enhanced mucus secretion (Fahy, 2002; Bisgaard et al., 2007; Kraft et al., 2008).

Fungus. Allergic bronchopulmonary aspergillosis (ABPA) is a unique form of asthma caused by colonization of the LRT (Vlahakis and Aksamit, 2001). ABPA is caused by an exaggerated T helper type 2 response to the ubiquitous mold *Aspergillus* spp., by which colonization leads to allergic and/or asthma symptoms (Edwards et al., 2012).

Other factors. The diverse etiologies for asthma exacerbation are well known, including viruses, allergens (dust mite, pollen, animal dander), smoking, gastroesophageal reflux disease, obesity, rhinosinusitis, stress, occupational exposures, hormones (menstrual asthma), drugs (acetylsalicylic acid, non-steroidal anti-inflammatory drugs, beta-blockers), exercise, and air pollutants. Physicians should be aware of these risk factors for asthma exacerbation (Dougherty and Fahy, 2009).

Mechanisms of viral-induced asthma exacerbations

Respiratory virus infection affects the pathogenesis of asthma. Bronchial epithelial cells are at the site of respiratory virus infection and replication. Respiratory virus infection induces production of various cytokines or chemokines and causes injury to epithelial cells or disruption of tight junctions. This inflammatory process may be amplified by intrinsic factors (susceptibility gene, family history of atopy, lung development) or environmental factors (respiratory virus infection, allergen exposure, smoking, and air pollutants, etc.; Hashimoto et al., 2008; Dougherty and Fahy, 2009). Some studies showing a deficiency in interferon (IFN)-β and IFN-λ production in response to HRV inoculation in airway epithelial cells cultured from asthmatic versus normal subjects (Holtzman, 2012) suggested that asthmatic patients have deficient IFN-β, IFN-λ, and perhaps some of the IFN-αs, but the precise mechanism or mechanisms behind deficient IFN production in these patients remain unknown.

Virus-associated clinical symptoms and exacerbations of asthma

In general, upper respiratory tract (URT) symptoms include rhinorrhea, sneezing, blocked nose, sore throat, hoarse voice, head or face ache, chill, and fever, while LRT symptoms include symptoms such as wheeze, cough, shortness of breath, and chest tightness (Corne et al., 2002). Tan et al. (2003) reported that virus-positive patients had a significantly increased frequency of URT symptoms of rhinorrhea, sore throat, fever, chills, and malaise. Nicholson et al. (1993) reported that, in adults with asthma, about a quarter of laboratory-confirmed viral and chlamydial acute upper respiratory infections was associated with mean decreases in peak expiratory flow of > 50 L/min, and half was associated with mean decreases of >25 L/min. The report also noted that respiratory pathogens were implicated in almost half of the most severe asthma exacerbations with a > 50 L/min mean decrease in peak expiratory flow. Viral infections have been shown to enhance both the reactivity of the lower airway and the magnitude of bronchoconstriction in response to inhaled contractile substances in asthma. The latter effect can persist for several weeks after infection, presenting as LRT symptoms (Cheung et al., 1995) accompanied by a decrease in peak expiratory flow. Thus, physicians should be aware of decreased peak expiratory flow, URT, or LRT symptoms associated with viral infections.

Treatment

The term "virus-induced exacerbation" is not uncommon, but only a small number of prospective studies have been conducted so far (Nicholson et al., 1993; Johnston et al., 1995). Importantly, respiratory infections do not always result in an exacerbation, and there is little evidence that treating or preventing the infection may cure or prevent an exacerbation (Xepapadaki and Papadopoulos, 2010). However, another study found that URT infections were strongly associated with exacerbations of asthma leading to hospital admission, in both adults and children (Johnston et al., 1996), and they may have contributed to asthma mortality, especially in the setting of hospital admission. Specific anti-viral therapies have not been established except for influenza viral infection, which have been recommended for persons with asthma or COPD. Furthermore, regarding preventive therapy for RSV, palivizumab as described above is now commercially available, and it might be appropriate for infants and young children with congenital heart disease, bronchopulmonary dysplasia, and prematurity before 35 weeks of gestation (Dawson-Caswell and Muncie, 2011). Blanken et al. (2013) stated that palivizumab treatment in healthy preterm infants born at a gestational age of 33–35 weeks reduced the number of wheezing days during the first year of life.

In this regard, several therapeutic strategies would need to be taken early in the course of infection to maximize the effects of treatments such as systemic corticosteroids, antibiotics if necessary, and short-acting β-agonist inhalers (SABAs), followed by inhaled corticosteroid (ICS) and long-acting β-agonist combination (LABA) therapy. Kerstjens et al. (2012) reported that additive long-acting muscarinic antagonist (LAMA) therapy with tiotropium (known as a cornerstone of COPD treatment) significantly increased the time to the first exacerbation and improved $FEV_{1.0}$ in poorly controlled asthmatic patients with standard therapy (ICS and LABA). Similarly, tiotropium improved lung function and reduced the chance of rescue inhaler (SABA) in patients with overlap syndrome (Magnussen et al., 2008).

VIRUS-INDUCED EXACERBATIONS IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

DEFINITION OF EXACERBATION IN COPD

Exacerbation of COPD is an event characterized by an acute increase in respiratory symptoms beyond normal day-to-day variation (Vestbo et al., 2013). Clinicians and researchers should always keep in mind that exacerbations of COPD are neither defined consistently nor matched in individual studies. Definitions of exacerbations are roughly divided into two groups, event-based exacerbations and symptom-based exacerbations, depending on

the patients' symptoms or clinical events, respectively. Symptoms were defined and include dyspnea, cough, and sputum volume or purulence. Clinical events were defined as a status requiring additional treatments such as systemic antimicrobials or steroids with or without admission. Diseases such as pneumonia, congestive heart failure, and pulmonary embolism that mimic and/or aggravate exacerbations were generally excluded from exacerbations of COPD.

CLINICAL IMPORTANCE OF EXACERBATION

The clinical course of COPD, as well as that of asthma, is punctuated by exacerbations, which are characterized by sudden symptom worsening beyond the expected daily variation. Exacerbations are important events in the clinical course of COPD, because they are associated with significant mortality. The in-hospital mortality rate of patients admitted to the hospital with exacerbations of COPD was 8%, increasing to 23% after 1 year of follow-up (Groenewegen et al., 2003). Exacerbations are correlated with accelerated loss of lung function and quality of life and increased healthcare costs (Seemungal et al., 1998; Donaldson et al., 2002; Miravitlles et al., 2002).

FREQUENCY OF EXACERBATIONS

Previous studies showed that the annual rate of event-based exacerbations of COPD was 0.85–1.30 per patient per year (Calverley et al., 2007; Tashkin et al., 2008; Wedzicha et al., 2008; Seemungal et al., 2009; Hurst et al., 2010). The INSPIRE study showed that the rate of symptom-based exacerbations was about two times as high as that of event-based exacerbations (Seemungal et al., 2009). In the ECLIPSE study, the exacerbation rates were 0.85 per person for patients with moderate disease (GOLD stage 2) and 2.00 for those with very severe disease (GOLD stage 4; Hurst et al., 2010). Thus, the rate of exacerbation seems to depend on the disease severity (GOLD stage). However, it is particularly worth noting that the ECLIPSE study showed a subgroup of COPD patients that appeared to be susceptible to exacerbations, irrespective of GOLD stage. Other factors for exacerbations were several environmental factors, such as seasons or inhalation of harmful substances, and epidemic peaks in exacerbations of COPD were noted in both late fall and winter in the same fashion as in adult asthma (Johnston and Sears, 2006).

CAUSES OF EXACERBATIONS

It has been reported that exacerbations are predominantly caused by bacterial and viral respiratory infections (**Figure 2**), and air pollution has a minor contribution. Previous studies showed that bacteria (*H. influenzae*, *S. pneumoniae*, *Moraxella catarrhalis,* and *P. aeruginosa*) and respiratory viruses (HRV, RSV, influenza virus, HMPV, coronavirus, and AdVs) were recognized during exacerbations. Bacteria, such as *H. influenzae*, *S. pneumoniae*, *Moraxella catarrhalis,* and *P. aeruginosa* were also detected in stable patients (Sapey and Stockley, 2006; Sethi and Murphy, 2008). When strains of bacteria are changed among the same species or there is emergence of other bacteria, this might cause inflammation in the lung in COPD patients and result in exacerbation (Sethi et al., 2002). The role of atypical respiratory pathogens, such as *Mycoplasma pneumoniae* and *C. pneumoniae*, in exacerbations of COPD is poorly recognized (Sapey and Stockley, 2006; Sethi and Murphy, 2008; Perotin et al., 2013). On the other hand, Blasi et al. (2002) showed that *C. pneumoniae* may be associated with exacerbation of COPD. Viruses such as HRV, RSV, and influenza virus have a higher prevalence in patients with exacerbations of COPD than in stable patients (Rohde et al., 2003; Wilkinson et al., 2006a).

ROLES OF RESPIRATORY VIRAL INFECTION IN COPD EXACERBATIONS

A few decades ago, it was considered that the role of respiratory viral infections was not a major cause in exacerbations of COPD because of the low sensitivity for viral detection, which depended on conventional technical methods such as viral culture or serological tests. However, recent studies have used new diagnostic technologies such as PCR or RT-PCR methods, which have a higher sensitivity for viral detection than conventional methods. Viral detections accounted for 22–57% of exacerbations of COPD in recent studies(**Figure 4**) using PCR or RT-PCR with observational periods of at least 1 year. The major viruses associated with exacerbations were HRV (3.1–26.6%), RSV (0.7–40.5%), and influenza virus (2.0–22.4%; Seemungal et al., 2001; Rohde et al., 2003; Tan et al., 2003; Beckham et al., 2005; Papi et al., 2006; Hutchinson et al., 2007; Ko et al., 2007; McManus et al., 2008; Kherad et al., 2010; Dimopoulos et al., 2012; Perotin et al., 2013).

Major respiratory viruses detected during exacerbations of COPD were HRV, RSV, and influenza virus, similar to those of

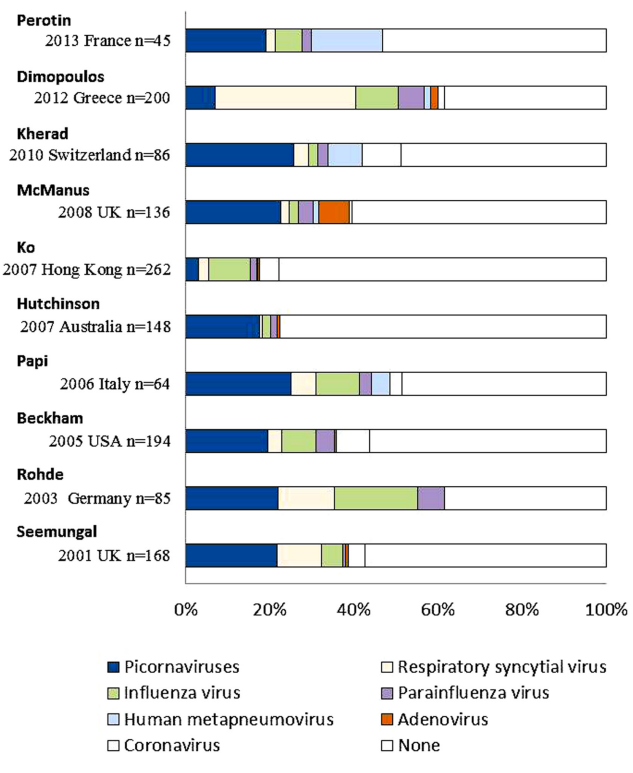

FIGURE 4 | Viral detection in exacerbations of COPD using PCR or RT-PCR methods in recent studies. Picornaviruses include human rhinovirus and human enterovirus. Multiple viruses were detected in individual patients per one episode.

asthma (Tan et al., 2003). Other viruses, such as PIV, human metapneumovirus, AdV, and coronavirus, were also noted in patients with COPD exacerbations. The proportion of viral-related exacerbations seemed to be similar among the various GOLD stages, while that of bacteria-related exacerbations increased with higher stage or a decrease in lung function (Dimopoulos et al., 2012).

HRV

Human rhinovirus was the most detected virus during exacerbations of COPD, but not only HRV infection alone but also co-infection with HRV and bacteria may cause exacerbations (Wilkinson et al., 2006b). Mallia et al. (2011) reported that experimental HRV infection showed more severe and prolonged lower respiratory symptoms, airway obstruction, and neutrophilic airway inflammation in COPD patients than in control subjects. They stated that rhinovirus infection led to elevation of neutrophil elastase, which is associated with reduction of antimicrobial peptides, such as secretory leukoprotease inhibitor and elafin. This reduction of antimicrobial peptides predisposes to secondary bacterial infection (Mallia et al., 2011, 2012).

Previous reports showed that COPD exacerbations may be associated with an impaired host response to HRV. For example, reduced IFN production was observed in COPD patients compared to control subjects, which may be associated with the mechanism of viral and subsequent bacterial infection related to severe exacerbations. Impaired humoral immunity was also related to exacerbation of COPD. Patients hospitalized with COPD exacerbations had lower serum levels of rhinovirus-specific antibodies than those not hospitalized with COPD exacerbations (Yerkovich et al., 2012). Thymic stromal lymphopoietin (TSLP) is a key pro-allergic cytokine that has recently been linked to asthma(Redhu and Gounni, 2012). In addition, genome-wide association studies showed that polymorphisms near or within the *TSLP* gene were associated with various allergic diseases, including bronchial asthma (Hirota et al., 2011; Ober and Yao, 2011). TSLP may contribute to exacerbations of the pathogenic effects of HRV infection via a Toll-like receptor (TLR)3-dependent pathway (Calven et al., 2012).

RSV

Respiratory syncytial virus has been detected in both stable and exacerbated cases of COPD. RSV detection in stable COPD patients might be associated with insidious airway inflammation and accelerated decline in $FEV_{1.0}$ (Wilkinson et al., 2006a). However, this was not confirmed by another study (Falsey et al., 2006). RSV increases the expression of TLR3 on the surface of airway epithelial cells, which is associated with increased sensitization to double-stranded RNA and its related infections (Groskreutz et al., 2006).

Influenza virus

Influenza virus has been associated with mortality and morbidity in chronic lung diseases (Harper et al., 2009). A meta-analysis showed that influenza vaccination prevented exacerbations in COPD patients (Poole et al., 2000) and reduced the mortality and morbidity in elderly persons (Nichol et al., 2007). Anti-viral treatment such as neuraminidase inhibitors may reduce the severity of disease (Kaiser et al., 2003). Thus, treatment to prevent influenza has been recommended for COPD patients (Harper et al., 2009).

ARE PATIENTS WITH COPD SUSCEPTIBLE TO RESPIRATORY VIRAL INFECTIONS?

Greenberg et al. (2000) investigated viral infections with or without COPD in a longitudinal cohort study. They demonstrated that annual symptomatic documented respiratory viral infection rates were similar for COPD and age-matched controls (0.45/year and 0.54/year, respectively). However, the COPD patients had more office visits and hospitalization than controls.

Of note, Mallia et al. (2011, 2012) confirmed these findings in a human experimental study; they found no significant differences in the frequencies of successful HRV infection between COPD and control groups when these groups were experimentally inoculated with HRV. Respiratory symptoms and a decline in lung function were more severe in the COPD group than in the control group.

DIFFERENCES IN VIRAL AND NON-VIRAL EXACERBATIONS

Several studies have suggested that respiratory virus-associated exacerbations are more critical events than those due to other causes, in that viral-detected exacerbations showed a larger decline in lung function and longer recovery time than non-viral exacerbations (Seemungal et al., 2001; Bafadhel et al., 2011). As described in the chapter on HRV, respiratory viral infections themselves exacerbated COPD patients and tended to provoke secondary bacterial infections. Viral and sequential bacterial infections may be associated with severe respiratory symptoms (Wilkinson et al., 2006b; Harper et al., 2009; Mallia et al., 2012).

MECHANISMS OF VIRUS-INDUCED COPD EXACERBATIONS

As shown in the **Table 1**, the pathological features of COPD are fibrosis around small airways involving several different cells (neutrophils, macrophage, CD8 lymphocytes) and destruction of lung parenchyma. Neutrophils have been found to be associated with both stable and exacerbated COPD (Hogg et al., 2004; Papi et al., 2006).

Changes in neutrophil counts during exacerbations in both sputum and peripheral blood have been found to be related to the $FEV_{1.0}$ value. Levels of tumor necrosis factor-alpha and interleukin (IL)-8 in sputum were associated with neutrophilic inflammation (Keatings et al., 1996). Especially in patients who suffered from frequent exacerbations, they had persistently higher systemic IL-6 and C-reactive protein (CRP) levels, which may explain the greater decline in lung function (Perera et al., 2007).

Inflammatory cytokines in sputum during exacerbations have been found to be elevated regardless of whether the infection is viral or bacterial (Aaron et al., 2001), and their levels were higher with exacerbations than when stable. Eosinophils are considered characteristic cells in asthma, but they are also detected with exacerbations of COPD (Saetta et al., 1994). Indeed, Papi et al. (2006) demonstrated that virus-associated exacerbations in COPD patients were related to increased eosinophil counts and the level of eosinophil cationic protein. Furthermore, Bafadhel et al. (2011) showed that serum C-X-C motif chemokine 10 (CXCL10) is implicated as a more potent predictive maker for

virus-associated exacerbations, and it is known as IFN-λ-induced protein 10.

TREATMENT OF STABLE AND EXACERBATION STATES

Inhaled bronchodilators, LAMA and LABA, are the main pharmacological therapies in stable COPD patients (Tashkin et al., 2008; Wedzicha et al., 2008; Vestbo et al., 2013). Although Vogelmeier et al. (2011) reported that the tiotropium (LAMA)-treated group had a lower exacerbation rate than the salmeterol (LABA)-treated group in their head-to-head study, both LAMA and LABA treatments decreased exacerbation rates and improved lung function or health-related quality of life. Tashkin et al. (2009) found that combination LAMA/LABA therapy improved pulmonary function ($FEV_{1.0}$) and respiratory symptoms better than LAMA therapy alone. ICS, the main treatment for asthma, is also prescribed in COPD patients and may reduce airway inflammation and decrease exacerbation rates only in moderate and severe COPD patients (Calverley et al., 2007). Treatment with macrolide antibiotics has been reported to prevent COPD exacerbations and improve patient quality of life and symptoms, especially in patients who have frequent exacerbations (Albert et al., 2011; Yamaya et al., 2012a), although this intervention could lead to unfavorable events such as increasing the prevalence of macrolide-resistant pathogens or cardiac toxicity.

It has been estimated that most exacerbations of COPD are due to respiratory viral and/or bacterial infections. Thus, the major pharmacological components of managing exacerbations of COPD include SABAs, short-course systemic glucocorticoids, and antibiotics (Vestbo et al., 2013). However, anti-viral therapies are rarely prescribed, because specific anti-viral therapies do not exist, except for influenza virus and RSV. Treatment for influenza appears appropriate in patients with COPD (Harper et al., 2009), while the utility of treatment for RSV has not been confirmed in adults. It is doubtful that systemic corticosteroid treatment affects the clinical course of respiratory viral infections. Lee et al. (2011) showed that short-course systemic steroid treatment did not affect viral load or shedding, and humoral immunity may be diminished by steroid treatment.

Some research has shown that LAMA may affect viral infections. Tiotropium, one of the LAMAs, may inhibit HRV and RSV infections by reducing the levels of intercellular adhesion molecule-1, which is the binding site for most HRVs (Iesato et al., 2008; Yamaya et al., 2012b).

SUMMARY AT A GLANCE

The associations between virus infections and asthma and/or COPD were reviewed, and the significance of viral infections, as well as their effect on the clinical course, was discussed.

(1) HRV and RSV are major causes of exacerbations both in asthma and COPD patients.
(2) The frequency of viral detection in both asthma and COPD patients appears to be similar to that of healthy subjects, but the effect on their clinical course is different; asthma and COPD patients tend to have more severe or persistent respiratory symptoms or decreases in pulmonary function, and mortality may be increased.
(3) Since discrimination between asthma and COPD is difficult, especially during exacerbations, whenever clinicians encounter patients in whom obstructive airway disease is suspected, multidisciplinary assessment is required for diagnosis.
(4) The clinical findings of both asthma and COPD, so-called "overlap syndrome," are commonly recognized in general practice, and virus-associated exacerbations in this disease may lead to a poor prognosis.

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Epidemiology of virus-induced asthma exacerbations: with special reference to the role of human rhinovirus

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Viral respiratory infections may be associated with the virus-induced asthma in adults as well as children. Particularly, human rhinovirus is strongly suggested a major candidate for the associations of the virus-induced asthma. Thus, in this review, we reviewed and focused on the epidemiology, pathophysiology, and treatment of virus-induced asthma with special reference on human rhinovirus. Furthermore, we added our preliminary data regarding the clinical and virological findings in the present review.

Keywords: epidemiology, pathophysiology, treatment, human rhinovirus, asthma exacerbation

INTRODUCTION

More than 200 different types of viruses, such as human rhinovirus (HRV), human metapneumovirus (HMPV), respiratory syncytial virus (RSV), and human parainfluenza virus (HPIV), are known to cause acute respiratory illness (ARI; Tsukagoshi et al., 2013). We recently reported the issue of "virus-induced exacerbation in asthma and chronic obstructive pulmonary disease" (Kurai et al., 2013a), however, among these causative viruses, HRV is now recognized to have a major impact on asthma pathogenesis (Fujitsuka et al., 2011). From this perspective, we reviewed the literature regarding the epidemiology of HRV-induced asthma in adults, together with preliminary epidemiological data obtained at our institution.

CLINICAL VIROLOGY OF HUMAN RHINOVIRUS

HRV belongs to the genus Enterovirus and family Picornaviridae (Turner and Couch, 2007). HRV possesses a single strand positive-sense RNA (ssRNA) genome of approximately 7.2 kb. The viral capsid is composed of four viral proteins (VP1-4) which are assembled into 60 protomers, resulting in a small icosahedral structure with a diameter of about 28–30 nm (Turner and Couch, 2007). Genetically, HRV is classified into three species; HRV-A, -B, and -C (Simmonds et al., 2010). Furthermore, these species of HRV have more than 150 genotypes (Andries et al., 1990; Arakawa et al., 2012; Kiyota et al., 2013, 2014). Molecular epidemiological studies suggest that the dominant species are HRV-A and -C, while HRV-B is relatively rarely detected (Arakawa et al., 2012; Kiyota et al., 2013). In particular, the VP1 and VP2 proteins have variations in their amino acid sequences, accounting for the large number of viral serotypes (Turner and Couch, 2007). The host receptor for HRV in respiratory epithelial cells is the intracellular adhesion molecule 1 (ICAM-1, CD54) for the 84 major HRV serotypes (HRV-A and -B), or low-density lipoprotein receptor (LDLR) for the other minor HRV serotypes. The receptor for HRV-C is not yet known. It has been suggested that the optimal temperature for replication of HRV is relatively cool (33–35°C), which would limit infections to the upper airway; however, large or medium sized airways lower in the respiratory tract are now also considered cool enough for HRV replication, in spite of the higher temperature of the lung parenchyma (37°C; McFadden et al., 1985). Therefore, HRV is potentially a causative agent of more severe ARI such as bronchiolitis and pneumonia (Turner and Couch, 2007; Watanabe et al., 2010; Smuts et al., 2011; Arakawa et al., 2012), and may be associated with virus-induced asthma (Johnston et al., 1995; Linsuwanon et al., 2009; Fujitsuka et al., 2011; Smuts et al., 2011). HRV might therefore be involved in various ARIs and additional respiratory complications (Kiyota et al., 2013). Lieberman et al. (2009) reported that the detection of any virus include HRV, the sensitivity rates for nasopharyngeal swab (73.3%) was superior than that of oropharyngeal swab (54.2%), respectively.

VIRUS-INDUCED COLDS AND THEIR NATURAL COURSE AMONG THE GENERAL POPULATION

The common cold is the third most common primary diagnosis in office visits (Hsiao et al., 2010), and this disease is generally self-limiting, usually lasting up to 10 days (Fashner et al., 2012). Among the general population, HRV infection causes common colds at a frequency of 25–53% (Makela et al., 1998; van Gageldonk-Lafeber et al., 2005). Tyrrell et al. (1993) reported that intranasal

inoculation with either HRV serotypes 2, 9, and 14, coronavirus type 229E, or RSV in healthy volunteers induced patterns of symptom development which were not substantially different from each other. However, individual signs or symptoms occurred earliest in HRV infections, then in coronavirus, and lastly in RSV, appearing up to 5 days after inoculation, which demonstrated the long incubation periods of RSV in volunteers (Tyrrell et al., 1993).

HRV has been implicated in patients with acute otitis media, exacerbation of chronic obstructive pulmonary disease, common cold, and lower respiratory tract infections in neonates, the elderly and immunocompromised. Arruda et al. (1997) researching the frequency and natural history of HRV infections in adults during autumn, demonstrated that the first symptom noticed most often was sore throat (40%) in HRV culture- or PCR-positive patients, and stuffy nose in HRV-negative patients (27%), using nasal wash specimens. Respiratory symptoms typically develop after 1–2 days after inoculation in studies, and uncomplicated HRV infections usually peak 2–4 days after inoculation. The median duration of HRV colds is 1 week, but up to 25% last more than 2 weeks (Gwaltney et al., 1967; Rotbart and Hayden, 2000). It should be noted that in illness caused by HRV, viral shedding occurs naturally for up to 21 days, but predominantly over a 3–4 days period.

HRV INFECTION AMONG ASTHMATICS: *IN VIVO* OR *IN VITRO* EXPERIMENTAL STUDIES

HRV-A type16 (HRV-16), a major group virus commonly used for experimental human infection, and HRV-A type1 (HRV-1), which has been used in animal models of HRV infection, are closely related. Grunberg et al. (1999) reported that experimental HRV-16 infection via nasal inhalation leads to a transient decrease of $FEV_{1.0}$ in patients with asthma, and this decreased lung function was correlated with enhanced cold symptoms and / or airway hyperresponsiveness. Contoli et al. (2006) demonstrated that type III interferon (IFN-λ) production levels in *ex vivo* cell cultures derived from bronchial epithelial cells (BECs) and macrophages obtained from asthmatic patients, were lower than in those derived from healthy controls. Furthermore, deficient interferon-λ production was correlated with HRV viral load, severity of clinical symptoms and $FEV_{1.0}$. Message et al. (2008) demonstrated that the severity of intranasally inoculated HRV-induced clinical illness in asthmatic subjects was correlated to virus load and lower airway virus-induced inflammation.

On the other hand, DeMore et al. (2009) reported that no difference in clinical symptoms, and patterns of viral shedding, was noted between subjects with persistent allergic asthma and healthy subjects after experimental infection with HRV. These different results after experimental HRV infection in individual studies in asthmatic patients and healthy subjects might be dependent on the severity of the asthma of those subjects who enrolled in the studies. Indeed, in several reports, neither defective IFN induction by HRV, nor increased HRV replication was observed in primary human BECs derived from subjects with well controlled asthma (Lopez-Souza et al., 2009; Bochkov et al., 2010; Sykes et al., 2014). A few animal models for rhinovirus infection have been showed because a major group of HRV (i.e., HRV-16) did not bind mouse ICAM-1. Only a minor group of HRV (i.e., HRV-1B) infected the mouse. In this regard, Bartlett et al. (2008) generated a transgenic BALB/c mouse expressing a mouse-human ICAM-1 chimeric receptor for HRV-16 infection. This study also showed asthma exacerbation model by intraperitoneally sensitized with ovalbumin with aluminum hydroxide followed by intranasal inoculation of HRV-1B or UV-inactivated HRV-1B.

HRV AND ASTHMA EXACERBATIONS: CLINICAL FINDINGS

Although data regarding virus respiratory infections (VRIs) as precipitators of asthma attacks in adults are less clear, Nicholson et al. (1993) reported that VRIs are as commonly linked to exacerbations in adults as they are in children (Johnston et al., 1996; Fujitsuka et al., 2011). This study showed that viruses were detected in 44% of clinical exacerbative episodes with a decrease in peak expiratory flow rate (PEFR) of 50 mL/minute or more, and the most commonly identified virus was HRV, followed by coronaviruses and parainfluenza viruses (Nicholson et al., 1993). Thus, the virus most commonly detected in asthma exacerbations appears to be HRV.

Although HRV is well known as the most frequent cause of the common cold, the implications of HRV infection vary according to respiratory diseases. **Table 1** shows the frequency of HRV infection in various adult respiratory diseases such as exacerbation of asthma (Nicholson et al., 1993; Atmar et al., 1998; Tan et al., 2003), common cold (Makela et al., 1998; van Gageldonk-Lafeber et al., 2005), exacerbation of COPD (Seemungal et al., 2001; Rohde et al., 2003; Tan et al., 2003; Beckham et al., 2005; Papi et al., 2006; Hutchinson et al., 2007; Ko et al., 2007; McManus et al., 2008; Kherad et al., 2010; Dimopoulos et al., 2012; Perotin et al., 2013), community acquired pneumonia (Jennings et al., 2008; Johnstone et al., 2008; Johansson et al., 2010; Lieberman et al., 2010; Fry et al., 2011; Wootton et al., 2011; Luchsinger et al., 2013; Takahashi et al., 2013; Huijskens et al., 2014), exacerbation of idiopathic pulmonary fibrosis (Wootton et al., 2011), and asymptomatic infection (Fry et al., 2011).

The risk of exacerbations of asthma in adults is elevated after children return to school, and around December 25th (the Christmas holiday in westernized countries), and this is likely to be due to social interactions with children at these times. Prospective monitoring studies using reverse transcription polymerase chain reaction (RT-PCR) indicate that as many as 85% of acute asthma exacerbations in children, and about 60% in adults, were associated with the presence of upper respiratory tract (URT) infections. Corne et al. (2002) found that the detection rates of HRV in asthmatic (10.1%) and healthy participants (8.5%) were similar, but the LRT symptoms were significantly more severe and longer lasting in the asthmatic group than in the healthy group based on one definition of URT and LRT symptoms (**Table 2**; Johnston et al., 1995).

There is no common antigen across all strains of HRVs; therefore, no reliable diagnostic method for HRV infection has been established using HRV antigens or HRV-specific antibody. Although viral culture is the conventional method for HRV detection, culture methods are not practical in clinical settings for the detection of HRV, because of its slow growing character and requirement for specific culture conditions. Furthermore, the diagnostic capability of molecular amplification techniques

Table 1 | HRV infection and its frequency in acute and chronic respiratory diseases in adults.

	Frequency (%)	Reference
Exacerbation of asthma	26–36	Nicholson et al. (1993), Tan et al. (2003), Atmar et al. (1998)
Common cold	25–53	Makela et al. (1998), van Gageldonk-Lafeber et al. (2005)
Exacerbation of COPD	3–27	Tan et al. (2003), Perotin et al. (2013), Rohde et al. (2003), Seemungal et al. (2001), Papi et al. (2006), Hutchinson et al. (2007), Ko et al. (2007), McManus et al. (2008), Kherad et al. (2010), Dimopoulos et al. (2012), Beckham et al. (2005)
Community-acquired pneumonia	2–12	Johnstone et al. (2008), Jennings et al. (2008), Lieberman et al. (2010), Johansson et al. (2010), Takahashi et al. (2013), Luchsinger et al. (2013), Huijskens et al. (2014)
Exacerbation of idiopathic pulmonary fibrosis	5	Wootton et al. (2011)
Asymptomatic infection	2	Fry et al. (2011)

Table 2 | Respiratory symptoms.

Upper respiratory symtoms	Lower respiratory symptoms
Runny nose	Cough during the day
Sneezing	Cough during the night
Blocked or stuffy nose	Wheeze during the night
Itchy, sore, or watery eyes	Difficulty breathing shortness of breath
Sore throat	
Hoarse voice	
Fever of shivery	
Headaches or face aches	
Aches or pains elsewhere	

Cited and adapted from Johnston et al. (1995).

such as nucleic acid sequence-based amplification and RT-PCR is superior to those of culture methods (Loens et al., 2006).

PATHOGENESIS OF HRV-ASSOCIATED ASTHMA EXACERBATIONS

Experimental HRV infections have been shown to lead to a long-lasting excessive airway narrowing in volunteer subjects with asthma (Cheung et al., 1995; Grunberg et al., 1999). Of note, rhinovirus, unlike influenza and other viruses, causes minimal cytotoxicity (Fraenkel et al., 1995), and the amount of epithelial damage does not correlate with the severity of the symptoms. HRV infection can cause additive or synergistic effects in exacerbation of asthma via the influx of additional inflammatory cells in the airways with preexisted inflammation, resulting in airway cholinergic hyperresponsiveness (Nagarkar et al., 2010), as an allergic response. The effects of HRV infection such as enhanced contractility of airway smooth muscle (ASM) cell and impaired relaxation to cholinergic or β-adrenaergic agonists are attributed solely to binding of the virus to its host receptor ICAM-1 on the ASM cell surface. This proasthmatic-like effect was recognized even in the situation of complete inhibition of viral replication *in vitro*, but not in the setting of pretreatment of ASM with neutralizing antibody directed against for ICAM-1 (Grunstein et al., 2001). Thus, the HRV attachment to ICAM-1 itself can affects the contractility of ASM cells in the absence of any cytopathic effects, and Chun et al. (2013) reported that A 549 cells infected with HRV *in vitro* produced a higher value of IL-8 and RANTES than those of RSV or adenovirus. In addition, only the combination of HRV with Der f1 (house dust mites antigen) acted synergistically to induce IL-8 production. These findings are the reason why the HRV can be a major pathogen for acute exacerbation of asthma. We present a schema for pathogenesis in HRV associated asthma exacerbations (**Figure 1**), which requires the following steps, (1) HRV attachment to airway epithelial cells, (2) an innate immune response which leads to epithelial damage, (3) infection-related airway remodeling.

ATTACHMENT TO AIRWAY EPITHELIAL CELLS

When RT-PCR is used to either supplement or replace conventional culture techniques, viruses have been found in approximately one half to three quarters of adults experiencing an acute wheezing episode (Jackson and Johnston, 2010), and the majority (59%) of viruses identified were HRVs (Nicholson et al., 1993). However, the evidence is weak, and mechanisms are poorly understood. Initially, HRV-A and -B attach to airway epithelial cells via ICAM-1 or LDLR (Kennedy et al., 2012). The receptor or receptors for the recently identified group HRV-C have yet to be clarified. HRV-infected BECs secrete a wide range of cytokines and chemokines such as IL-1, IL-6, CCL5/RANTES (regulated on activation, normal T cell expressed and secreted), CXCL8/IL-8, GM-CSF, and CXCL10/interferon-inducible protein 10 (IP-10; Jackson and Johnston, 2010; Proud, 2011), which induce neutrophilic, lymphocytic, and eosinophilic inflammation together with airway hyperresponsiveness and airway remodeling (Wark et al., 2002; Proud, 2011).

THE INNATE IMMUNE RESPONSE

Clearance of viral pathogens begins with interferon secretion, and the underproduction of these factors has been postulated to lead to viral-induced exacerbations. There are three types of interferons, based on the receptors they bind: type I (IFN-α/β), type II (IFN-γ),

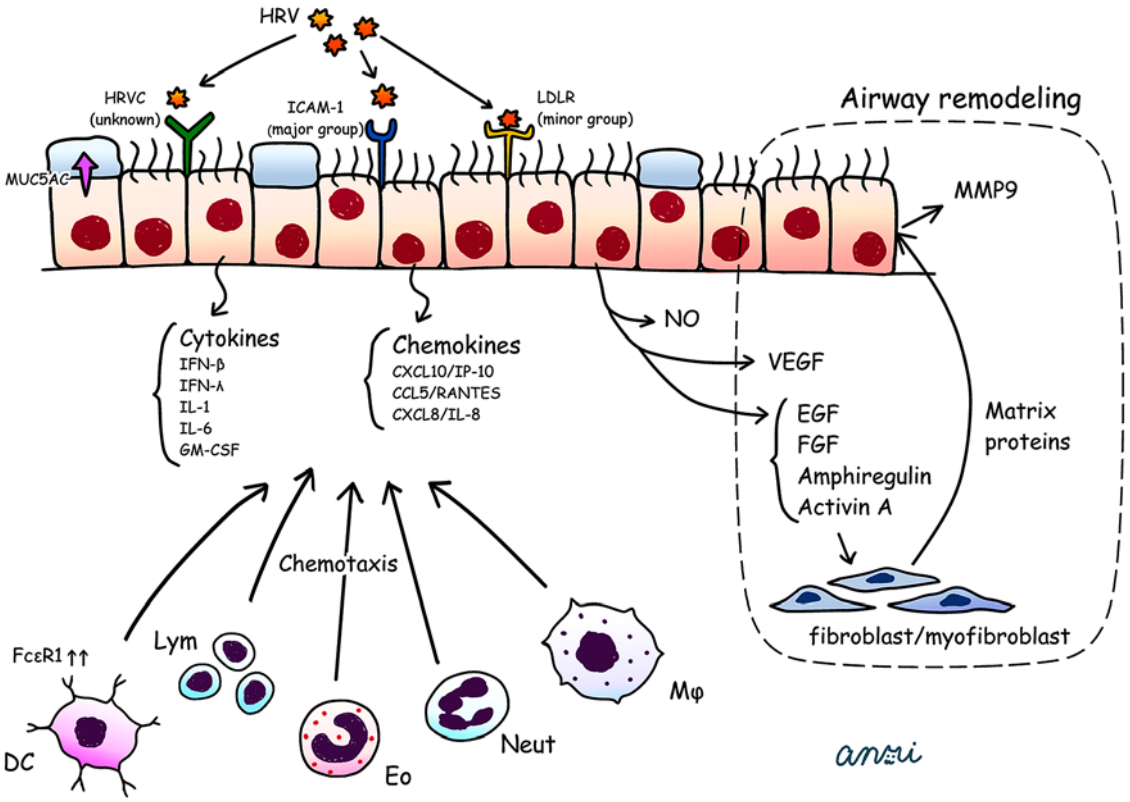

FIGURE 1 | HRV attaches to airway epithelial cells via ICAM-1, LDLR, and unknown receptors for HRV-C. HRV infected bronchial epithelial cells secrete a wide range of cytokines (IFN-β, IFN-λ, IL-1, IL-6, GM-CSF) and chemokines (CXCL10/IP-10, CCL5/RANTES, CXCL8/IL-8) together with NO, VEGF, and EGF, FGF, amphiregulin and activin A. These cytokines and chemokines attract various inflammatory cells such as DCs with upregulation of FcεR1, lym, Eo, Neut, and Mϕ. The VEGF, EGF, FGF, amphiregulin and activin A promote the release of matrix proteins from fibroblasts/myofibroblasts, which enhance the production of MMP9 from airway epithelial cells. These phenomena could lead to airway remodeling (dotted area; i.e., thickening of the lamina reticularis). HRV infection induces secretion of MUC5AC, which impairs mucociliary clearance. DCs, dendritic cells; EGF, epidermal growth factor; Eo, eosinophil; FGF, fibroblast growth factor; GM-CSF, granulocyte macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule 1; LDRL, low-density lipoprotein receptor; Lym, lymphocyte; MMP, matrix metalloproteinase; Mϕ, macrophage; Neut, neutrophil; NO, nitric oxide synthase; RANTES, regulated on activation, normal T cell expressed and secreted; VEGF, vascular endothelial growth factor.

and type III (IFN-λ). HRV infection induced epithelial expression of mRNA for both type I and type III IFNs, and it has been suggested that impaired epithelial production of IFN-β and IFN-λ in asthmatic subjects may contribute to viral exacerbations of asthma (Wark et al., 2005; Contoli et al., 2006). Contoli et al. (2006) showed significant inverse correlations between *ex vivo* production of IFN-λ and severity of symptoms, bronchoalveolar lavage viral load and airway inflammation, and a strong positive correlation with reductions in lung function during *in vivo* infection. Genome-wide association studies showed that single nucleotide polymorphisms involve in various diseases. Interferon-λ polymorphisms may effect on the incidence of HRV infection (Russell et al., 2014).

Message et al. (2008) reported virus load in asthmatic subjects as being related to increased lower airway inflammation, and in turn increased lower airway inflammation being related to increased symptoms, reductions in lung function, and increases in bronchial hyperreactivity. These data suggest a causal role for HRV infection in the pathogenesis of asthma exacerbations.

Investigating virus-allergen interactions, Durrani et al. (2012) demonstrated that another mechanism that increased expression and cross-linking of the high-affinity IgE receptor, FcεRI, on plasmacytoid dendritic cells is associated with reduced HRV-induced IFN-α and IFN-λ1 secretion, and allergic asthmatic children have significantly reduced HRV-induced IFN-α and IFN-λ1 production after cross-linking of FcεRI.

Type 2, or inducible, nitric oxide synthase (iNOS) is the major NOS isoform found in epithelial cells and can generate substantial amounts of nitric oxide (NO). The NO molecules both inhibit the replication of HRV in airway epithelial cells, and suppresses HRV-induced cytokine production (Proud, 2005). Although the measurement of fractional NO concentration in exhaled breath (FENO) may be used to support the diagnosis of asthma (Dweik et al., 2011), however, increasing of FENO seems to be not always correlated with viral load during the period of HRV infection (Sanders et al., 2004).

Other factors such as allergy, allergen exposure, tobacco smoke, particulates, ozone, stress, and infections such as sinusitis commonly contribute to exacerbations of asthma.

HRV INFECTION AND AIRWAY REMODELING

Grainge et al. (2011) reported that repeated bronchoconstriction in asthma promotes airway remodeling, and there is now clear evidence that airway remodeling begins in early childhood, and can be present even before clinical diagnosis of asthma is established (Pohunek et al., 2005). Increasing evidence regarding HRV-induced wheezing or exacerbation of asthma raises the possibility that HRV infections could contribute to the initiation and subsequent progression of airway remodeling, which involves multiple factors such as increased epithelial release of Mucin5AC (MU5AC), activin A, amphiregulin, matrix metalloproteinase 9 (MMP9), epidermal growth factor (EGF), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF).

HRV infection upregulates production of MUC5AC from epithelial cells, which leads to airflow obstruction in asthma (Hewson et al., 2010). Activin A is a member of the TGF-β superfamily and amphiregulin, a member of the EGF family, alters repair processes (Leigh et al., 2008). Both activin A and amphiregulin have been linked to subepithelial basement membrane thickening in asthma. MMP9 appears to have important roles in asthma exacerbation and airway remodeling (Sampsonas et al., 2007). Expression of VEGF and its receptors is increased in asthmatic subjects, and VEGF is the major proangiogenic activator in asthmatic airways (Feltis et al., 2006; Simcock et al., 2007).

IMPACTS OF VIRAL INFECTION ON ASTHMA EXACERBATION: PRELIMINARY DATA FROM THE KYORIN COHORT STUDY

Kuga et al. (2000) reported that 61.5% of adult asthmatic patients with common cold suffered an asthma attack, and common cold was significantly associated with acute exacerbations of asthma. They also stated that HRV infection might be important as the virus was detected by RT-PCR in throat gargles (Kuga et al., 2000). Virus-induced exacerbation of asthma is a critical issue for the general physician. However, among asthmatic patients with exacerbative status, distinguishing between those patients which have VRIs, and those who do not, is difficult. Furthermore, epidemiological data regarding adult asthma exacerbations have been sparsely reported. To investigate the prevalence of VRI in exacerbations of adult asthma in both hospitalized or not-hospitalized patients, characterization of clinical and radiological findings was performed. A prospective observational cohort study was conducted at Kyorin University Hospital, Tokyo, Japan from August 2012 to August 2013 (Kurai et al., 2013b). All patients with respiratory symptoms associated with exacerbation of asthma were included, and samples were collected by nasopharyngeal or oropharyngeal swab, and subjected to a PCR method to detect common respiratory viruses. The 44 patients who were enrolled consisted of hospitalized ($n = 15$) or not-hospitalized patients ($n = 29$; **Table 3**). In these two groups, the subject's backgrounds were similar for age, sex, smoking rates, and duration of illness, however, the measured value of SpO_2 was significantly lower in hospitalized patients ($87 \pm 2.3\%$) than in non-hospitalized patients ($96.2 \pm 0.7\%$). The incidence of VRI was significantly higher in the former group (46.7%, $n = 7$) than in the latter group (6.9%, $n = 2$; $p = 0.002$). In the latter group, influenza virus alone was detected in both patients. Furthermore, all hospitalized patients (100%, $n = 15$) had wheezing or severe exacerbation based on the ATS (American Thoracic Society)/ERS (European Respiratory Society) statement (Reddel et al., 2009), whereas, among non-hospitalized patients, only nine patients (31%) were considered as having a severe exacerbation ($p < 0.001$), and 10 patients (38.4%) had wheezing ($p < 0.001$). These findings suggested that virus infection was certainly associated with the hypoxemia and / or wheezing which resulted in a severe or serious asthma attack, based on the Japanese guidelines (Ohta et al., 2011) or the ATS/ERS statement (Reddel et al., 2009). Previous studies using

Table 3 | Comparison of the clinical characteristics of hospitalized and non-hospitalized asthma attack patients.

	Hospitalized patients	Non-hospitalized patients	
Number of cases	15	29	Total 44
Number of virus positive cases	46.7% ($n = 7$)	6.9% ($n = 2$)	$p = 0.002$**
Age	52 ± 5.8	60 ± 3.2	NS
Sex (M/F)	5/10	10/19	NS
Smoker	33.3% ($n = 5$)	24.1% ($n = 7$)	NS
Duration of illness (years)	20.6 ± 4.7	18.0 ± 4.4	NS
SpO_2(%)	$87.0 \pm .2.3$	96.2 ± 0.7	$p < 0.001$***
Wheezing	100% ($n = 15$)	38.4% ($n = 10$)	$p < 0.001$***
Severe or serious asthma attack on Japanese guideline[†]	80% ($n = 12$)	6.9% ($n = 2$)	$p < 0.001$***
Severe attack on ATS/ERS statement[††]	100% ($n = 15$)	31% ($n = 9$)	$p < 0.001$***
WBC(/μl)	$10{,}028 \pm 1{,}568$	$9{,}850 \pm 2{,}220$	NS
CRP(mg/dL)	4.1 ± 1.5	1.1 ± 0.15	NS
IgE(IU/mL)	687 ± 191	545 ± 191	NS

[†] *Defined by Ohta et al. (2011),* [††] *defined by Reddel et al. (2009). ** p < 0.01, *** p < 0.001. All data are presented as (mean ± SD).*

PCR-based viral diagnostics found that viral respiratory infections were detected in up to 85% of exacerbations of asthma in children and about 50% of exacerbations in adults (Nicholson et al., 1993; Johnston et al., 1995), which is similar to our results. Serum inflammatory or allergic markers are not different between the hospitalized and non-hospitalized patients (**Table 3**).

In hospitalized patients, the viruses identified were HRV ($n = 5$), HMPV ($n = 1$), and RSV ($n = 1$). At the time of admission, the virus-positive group ($n = 7$) had significant lower values of SpO_2 (81.4 ± 3.9%) than those of the virus-negative group ($n = 8$, SpO_2: 91.8 ± 1.3%, $p < 0.007$), and for the patients whose data are available, the frequency of hypercapnea ($PaCO_2 \geqq 45$ Torr) was significantly higher in the virus positive group (66.7%, $n = 4$) than in the virus negative group (0%; $p = 0.014$; **Table 4**). The mechanisms for hypercapnea in virus infected individuals have not been elucidated. However, Cheung et al. (1995) reported that HRV infection causes long lasting excessive airway narrowing in response to methacholine in asthmatic subjects. We speculated that smooth muscle might have a role in exaggerated airway narrowing in virus positive asthmatic patients, as described by King et al. (1999).

Interestingly, the incidence of ground glass opacities (GGO) on high resolution computed tomography seemed to be higher for virus-positive hospitalized patients than for virus-negative patients, but it did not reach statistical significance. For example, **Figure 2A** shows a patchy GGO with thickening of interlobular septa in a 28-year-old woman who was admitted during an asthma attack induced by HRV-A. **Figure 2B** also shows GGO in a 62-year-old man with an asthma attack caused by HRV-C infection. These GGO in both patients could only be detected in HRCT, not in chest X-ray.

These results suggested that HRV was the major cause of virus-induced asthma, and was possibly involved in lower airway or lung parenchyma features, appearing as GGO. Viral infection significantly exaggerated the respiratory status (low SpO_2 and hypercapnea) when compared to that of virus-negative asthma exacerbative patients at the time of admission. Indeed, in recent years, HRV has been recognized as a common cause of hospital admission, both as an agent of bronchopneumonia and through exacerbation of chronic pulmonary conditions, even in the elderly over 65 years of age (Pierangeli et al., 2011).

Curiously, after initiation of treatment with intravenous steroid, both the virus-positive and -negative groups had no significant difference in duration of respiratory failure, wheezing, days in hospital, and even in the time required for steroid treatment.

TREATMENT

No established treatment for prevention of HRV-induced asthma is available, and we describe the exploratory interventions as follows.

INHALED CORTICOSTEROID

Inhaled corticosteroid (ICS) is the main drug for regular asthma therapy. ICS treatment improved airway hyperresponsiveness in asthmatic patients experimentally challenged with HRV, however, ICS treatment did not reduce accumulation of inflammatory cells, except for eosinophils in bronchial epithelium (Grunberg et al., 2001). Double-stranded RNA (dsRNA), a viral product and a ligand for the Toll-like receptor-3 (TLR3), upregulates the expression of inflammatory chemokines in airway epithelial cells. Matsukura et al. (2013) reported that treatment of BEAS-2B cells with fluticasone propionate significantly and dose-dependently inhibited dsRNA-induced expression of CCL5, CXCL8, and CXCL10 protein

Table 4 | Clinical characterization of hospitalized patients with asthma attack based on the presence of virus infection.

	Virus positive	Virus negative	
Number of hospitalized patients	7	8	Total 15
Age	49.4 ± 8.8	54.3 ± 8.2	NS
Sex (M/F)	3/4	3/6	NS
Smoker	28.6% ($n = 2$)	37.5% ($n = 3$)	NS
Duration of illness (years)	28 ± 5.8	11.4 ± 5.1	NS
SpO_2(%)	81.4 ± 3.9	91.8 ± 1.3	$p = 0.007$**
Wheezing	100% ($n = 7$)	100% ($n = 8$)	NS
Severe or serious asthma attack on Japanese guideline†	100% ($n = 7$)	62.5% ($n = 5$)	NS
Severe attack on ATS/ERS statement ††	100%($n = 7$)	100%($n = 8$)	NS
$PaCO_2 \geq 45$ Torr	66.7%(4/6)	0% (0/6)	$p = 0.014$*
$SpO_2 \leq 88\%$	71.4% (5/7)	22.2% (2/8)	NS
Duration of respiratory failure (days)	5.7 ± 2.5	3.7 ± 1.8	NS
Duration of wheezing (days)	6.7 ± 1.1	7.1 ± 1.8	NS
Duration of steroid treatment (days)	13.7 ± 3.8	12.9 ± 3.5	NS
Duration of hospital stays (days)	7.3 ± 2.0	7.3 ± 1.8	NS

*$p < 0.05$, **$p < 0.01$. All data are presented as (mean ± SD).*

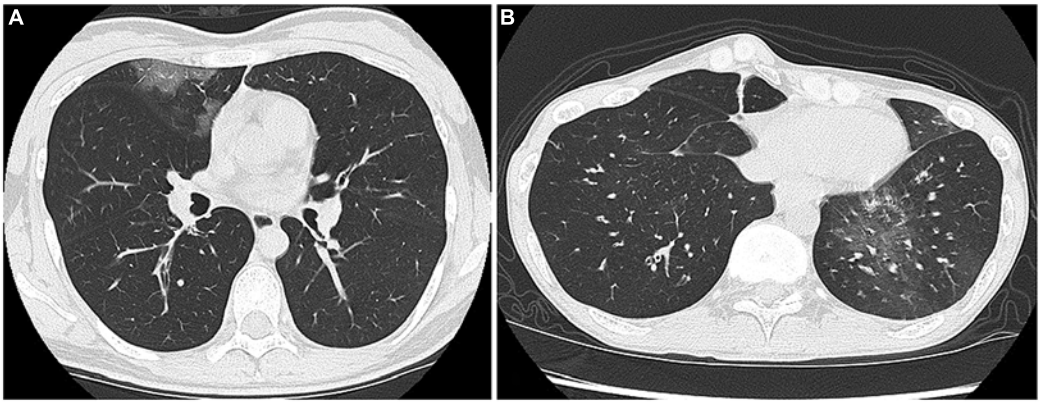

FIGURE 2 | (A) Shows a patchy GGO with thickening of interlobular septa in a 28-year-old woman who was admitted during an asthma attack induced by HRV-A. **(B)** Also shows GGO in a 62-year-old man with an asthma attack caused by HRV-C infection.

and mRNA. To confirm the effect on ssRNA, such as that of HRV, would need further studies.

LEUKOTRIENE RECEPTOR ANTAGONIST

Leukotriene receptor antagonist was prescribed in asthmatic patients with or without ICS. Montelukast treatment did not improve asthma control or cold symptom scores when HRV were experimentally inoculated into mild asthmatics, or healthy subjects (Kloepfer et al., 2011). It is uncertain whether leukotriene receptor antagonist treatment is effective in the reduction of asthma symptoms associated with HRV infection.

ANTI-IgE THERAPY

Zambrano et al. (2003) reported that high serum IgE levels in mildly asthmatic children with experimental HRV infection may be associated with enhanced lower respiratory symptoms and elevation of inflammatory markers, such as nasal eosinophil cationic protein and expired nitric oxide, than those of healthy subjects and/or low IgE asthmatic patients. The prevalence of asthma was closely associated with the serum IgE levels standardized for age and sex (Burrows et al., 1989), and airway hyperresponsiveness appears to be closely linked to the allergic diathesis, as reflected by the serum total IgE level (Sears et al., 1991). Omalizumab, an anti-IgE monoclonal antibody, was indicated in inadequately controlled moderate-to-severe persistent allergic asthma patients who were treated with high dose ICS. Durrani et al. (2012) showed that the IgE receptor FcεRI is inversely associated with IFN-α and IFN-λ1 secretion when plasmacytoid dendritic cells derived from allergic asthmatic children were challenged with HRV. Omalizumab downregulates FcεRI expression on dendritic cells (Prussin et al., 2003), which may reduce exacerbation of asthma associated with increased production of IFNs, through FcεRI.

ANTI-VIRAL TREATMENT

No drugs are clinically used in HRV infection, although several drugs have been tried for treatment and prevention of HRV infection. These drugs are summarized in a review (Jacobs et al., 2013). IFNs had a potential protective role in viral induced asthma (Cakebread et al., 2011; Gaajetaan et al., 2013). Becker et al. (2013) showed that exogenous IFN-α, IFN-β, IFN-λ1, and IFN-λ2 inhibited HRV replication in BECs from healthy donors.

MACROLIDE THERAPY

Macrolides are known to possess anti-inflammatory and immunomodulatory actions extending beyond their antibacterial activity in pulmonary inflammatory disorders (Takizawa et al., 1995; Min and Jang, 2012). Erythromycin inhibits HRV infection by reducing ICAM-1 expression on the surface of human tracheal epithelial cells, and modulates inflammation by suppressing the production of proinflammatory cytokines (Suzuki et al., 2002).

OTHER AGENTS

Yamaya et al. (2014) reported that the mucolytic drug ambroxol hydrochloride, antibiotic drug of levofloxacin (Yamaya et al., 2012b), and bronchodilators (Tiotropium, Tulobuterol, and Procaterol) for asthma or COPD (Yamaya et al., 2011, 2012a, 2013) may have a beneficial effect in HRV infection, by inhibiting HRV replication and partly reducing ICAM-1 expression and acidic endosome production, via the inhibition of NF-kappaB activation (Yamaya, 2012).

SUMMARY

We reviewed the previous reports regarding HRV-induced asthma exacerbations, together with our results from an institutional prospective study. HRV is a major pathogen for asthma exacerbations, and certainly associated with more serious clinical conditions such as hypoxemia or hypercapnea in hospitalized patients. Further accumulation of evidence of virus-induced asthma for multidisciplinary assessment would be helpful for physicians in recognizing the condition or understanding the pathogenic mechanisms.

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Influenza A(H1N1)pdm09 virus and asthma

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Respiratory viral infection is a major cause of asthma exacerbations in both children and adults. Among the respiratory viruses, influenza virus is a particularly important pathogen due to its enormous morbidity and mortality in annual epidemics. The swine-origin influenza A virus, designated as A(H1N1)pdm09, emerged in the spring of 2009 and caused the first influenza pandemic in the 21st century. With the emergence of the novel A(H1N1)pdm09 virus, numerous epidemiologic studies detected asthma as a frequent comorbid condition in patients infected with this virus. Here we review recent reports regarding asthma in patients infected with influenza A(H1N1)pdm09 virus, and we discuss the utility of influenza vaccines and antivirals.

Keywords: asthma, pandemic influenza, influenza A(H1N1)pdm09 virus, vaccine, antiviral drug

INTRODUCTION

Asthma is a chronic airway disease with the symptoms of repetitive cough, wheezing and dyspnea, with reversible airway narrowing accompanied by airway hyper-responsiveness (Ohta et al., 2011). It is estimated that worldwide, approximately 300 million people including both children and adults have asthma (Masoli et al., 2004). Inhaled irritants, inhaled allergens, and microorganism infections of the respiratory tract are common causes of asthma exacerbations. Respiratory viral infection is closely associated with asthma (Jacoby, 2002; Papadopoulos et al., 2011). Human rhinovirus (HRV) is the most common virus in asthmatics of all ages (Papadopoulos et al., 2011). Respiratory syncytial virus and enterovirus are also frequently detected in infants, whereas influenza virus seems to induce severe exacerbations, mostly in adults (Papadopoulos et al., 2011).

Influenza virus causes influenza characterized by a sudden onset of high fever and respiratory symptoms such as cough, sore throat and coryza, as well as systemic symptoms such as headache, muscle ache and fatigue. Influenza epidemics occur yearly during the autumn and winter in temperate regions, whereas the disease patterns in tropical and subtropical regions are less well established (World Health Organization, 2009). Annual epidemics result in approximately three to five million cases of severe illness and approximately 250,000 to 500,000 deaths, which occur mostly among people age 65 or older (World Health Organization, 2009).

INFLUENZA A(H1N1)pdm09 VIRUS

Influenza A viruses can be subtyped according to their two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Currently, there are 16 subtypes of HA (H1–H16) and nine subtypes of NA (N1–N9), and all have been found in wild aquatic birds, which are the natural reservoir of influenza A viruses. Only two subtypes of these viruses (H1N1 and H3N2) are currently circulating in humans, as seasonal influenza. Influenza A viruses have negative-sense, single-stranded, and eight-segmented RNAs as the genome (Lamb and Choppin, 1983). It is known that simultaneous infection of a single cell by two distinct influenza A viruses can lead to gene reassortment (Hause et al., 2012), which results in the generation of a novel influenza virus strain. It is believed that most human pandemic influenza A viruses arose in this manner.

In March and early April 2009, a novel swine-origin influenza A(H1N1) virus, designated as A(H1N1)pdm09, emerged in Mexico and the United States (Centers for Disease Control and Prevention, 2009) and rapidly spread worldwide. Genetic and evolutionary analyses revealed that this pandemic virus contains a combination of gene segments which had not been reported previously in swine or human influenza viruses in any part of the world. In the late 1990s, reassortment among North American avian (unknown subtype), human A(H3N2), and classical swine A(H1N1) viruses resulted in triple reassortant swine A(H3N2) and A(H1N2) viruses.

A triple reassortant swine A(H1N2) virus then reassorted with a Eurasian avian-like swine A(H1N1) virus, resulting in A(H1N1)pdm09 virus (Garten et al., 2009; Smith et al., 2009; **Figure 1**). The polymerase basic 2 (PB2) and polymerase acidic (PA) gene segments were derived from the avian virus lineage, whereas the polymerase basic 1 (PB1) gene segment was from human A(H3N2) virus. The HA, nucleoprotein (NP), and nonstructural protein (NS) gene segments were from classical swine A(H1N1) virus. The NA and matrix (M) gene segments were from the Eurasian avian-like swine A(H1N1) virus.

A(H1N1)pdm09 virus has none of the known hallmarks of virulent influenza viruses such as highly pathogenic avian A(H5) and A(H7) viruses, except for an amino acid substitution of aspartic acid by glycine at position 222 (D222G) in the HA, which was observed in severe and fatal cases with high frequency. The D222G substitution changes the receptor binding specificity of the virus from α2–6 (mammalian type) to α2–3 (avian type) sialylated

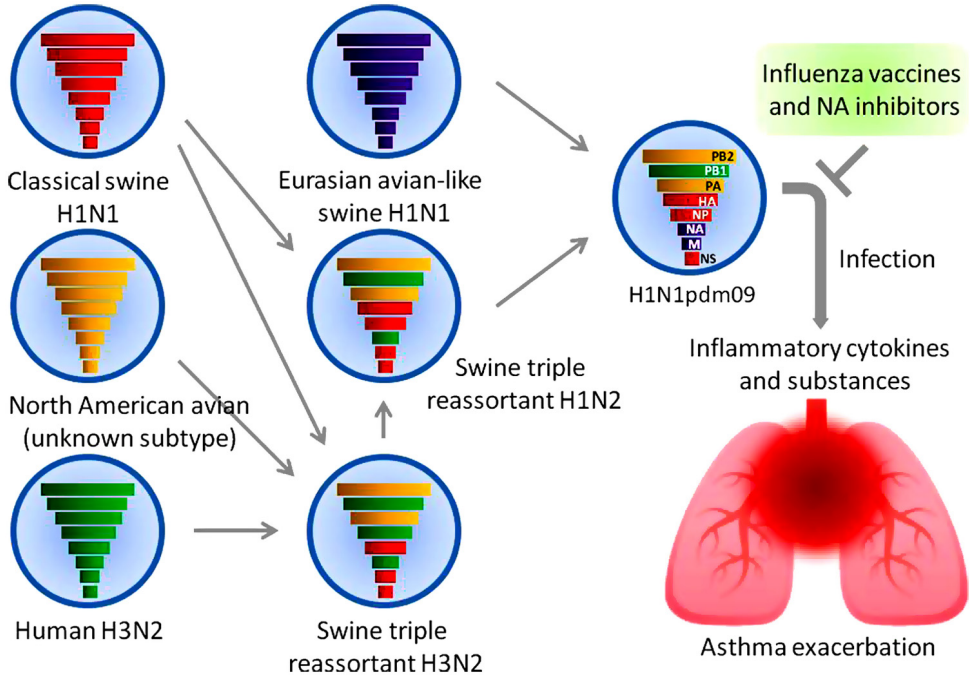

FIGURE 1 | A(H1N1)pdm09 virus and asthma. The reassortment of a triple reassortant swine A(H1N2) virus with a Eurasian avian-like swine A(H1N1) virus resulted in the pandemic A(H1N1) 2009 virus. The colored solid rods represent the gene segments as follows. Classical swine A(H1N1) virus: red. North American avian virus: yellow. Human A(H3N2) virus: green. Eurasian avian-like swine A(H1N1) virus: purple. Airway inflammation induced by the viral infection causes an exacerbation of asthma. Early treatment with antiviral drugs and vaccination represents the mainstay of management.

glycans (Puzelli et al., 2010; World Health Organization, 2010; Belser et al., 2011). This amino acid substitution may result in a more efficient infection of human alveolar type II pneumocytes, which express avian type receptors, reducing the availability of progenitor cells for essential lung functions and thus leading to severe pulmonary impairment.

We recently reported that A(H1N1)pdm09 viral isolates derived from fatal cases manifested sporadic amino acid changes in the PB2 and PA proteins (which are subunits of viral RNA polymerase) more frequently than those derived from mild cases (Obuchi et al., 2012). More recently, reassortant viruses generated by reverse genetics have shown that lysine or isoleucine at position 340 or 649 of the PB2, respectively, and threonine at position 667 of the PB2 also contribute to virulence in a mouse model (Uraki et al., 2013). Further studies are needed to elucidate the role of the viral RNA polymerase of A(H1N1)pdm09 virus as a virulence factor.

A(H1N1)pdm09 VIRAL INFECTION AND ASTHMA

Widespread activity of pandemic A(H1N1) 2009 occurred and reached its peak a couple of months earlier than the usual seasonal influenza in the northern hemisphere, from April 2009 to January 2010 (Amato-Gauci et al., 2011; Jhung et al., 2011). The A(H1N1)pdm09 viral infection was considered a mild disease, similar to seasonal influenza. However, many severe and fatal cases were observed not only in the high-risk groups, but also among healthy children and young adults during the pandemic waves (Athanasiou et al., 2010; Reichert et al., 2010).

Asthma was one of the most common underlying medical conditions among patients hospitalized with A(H1N1)pdm09 viral infection in 2009 worldwide (Jain et al., 2009; Van Kerkhove et al., 2011). Kloepfer et al. (2012) reported that children with asthma had increased susceptibility to A(H1N1)pdm09 viral infection. They collected weekly nasal samples from 161 children (95 with asthma and 66 without asthma) between September 5 and October 24, 2009, and a total of 346 viral infections were detected. The majority were HRV (62%), followed by enterovirus (12%), A(H1N1)pdm09 virus (10%), adenovirus (2%), and others. Overall, 34% of the children were infected with A(H1N1)pdm09 virus during the study period. The incidence of A(H1N1)pdm09 viral infection was significantly higher in the children with asthma (41%) than in the children without asthma (24%), whereas the incidences of HRV (95% each) and the other viral infections (47% vs. 41%) were similar.

A Canadian group reported that children admitted to a hospital with A(H1N1)pdm09 viral infection tended to have pre-existing asthma to a greater extent compared to the children with seasonal influenza A viral infection (15% vs. 5%), although there was no significant difference in the severity of pre-existing asthma between the groups of children with these infections (Morris et al., 2012). An age-matched control study in Hong Kong demonstrated that hospitalized children with A(H1N1)pdm09 viral infection were more susceptible to asthma exacerbations compared to seasonal A(H1N1) (8.1 vs. 1.0%) or A(H3N2) (8.1 vs. 1.0%) viral infection (Chiu et al., 2011). A Japanese group reported similar findings (Hasegawa et al., 2011). It seems likely that

A(H1N1)pdm09 viral infection rather than A(H1N1) or A(H3N2) viral infection may enhance the already elevated inflammatory response and worsen the symptoms in asthma. The underlying mechanisms of increased susceptibility to A(H1N1)pdm09 viral infection and the asthma exacerbation remain to be explored.

In contrast, it is not clear whether A(H1N1)pdm09 viral infection can frequently cause the development of asthma compared to seasonal A(H1N1) or A(H3N3) viral infection. The study by Hasegawa et al. (2011) mentioned above showed that 7 (31.8%) of 22 asthmatic children with A(H1N1)pdm09 viral infection admitted to a hospital between October and December 2009 were not previously diagnosed with asthma. The sample size of that study is small, and thus a larger patient population must be studied.

Influenza A viral infection induces the production of interleukin 1 beta (IL-1β), IL-6, IL-8, tumor necrosis factor-alpha (TNF)-α, histamine, protease, interferon (IFN)-α, and IFN-gamma (IFN-γ) from airway epithelial cells and other cells including peripheral blood basophils (Yamaya, 2012). These proinflammatory cytokines, monokines, and inflammatory substances may contribute to the development of airway inflammation, damaging the barrier function and leading to a subsequent asthma attack (**Figure 1**).

Camp et al. (2013) examined the phenotypic differences in virulence and immune response in A(H1N1)pdm09 virus isolates obtained from hospitalized patients with severe pneumonia. In that study, all viral isolates showed high similarity in nucleic acid sequences in viral gene and replication levels in nasal turbinates and lung, but the isolates' virulence and host responses in mice varied. Proinflammatory cytokines such as IL-1β, TNF-α and a keratinocyte-derived chemokine (KC) were expressed early in mice infected with virulent isolates compared to avirulent isolates, including a vaccine strain of A(H1N1) virus in the 2008–2009 season, A/Brisbane/59/2007. In vitro experiments demonstrated that a virulent isolate – but not an avirulent isolate – was able to replicate productively in macrophages, suggesting that viral susceptibility to macrophages may be one of the key determinants of their pathogenicity (Camp et al., 2013).

UTILITY OF INFLUENZA VACCINES AND ANTIVIRAL DRUGS IN PATIENTS WITH ASTHMA

Many respiratory viruses are associated with asthma exacerbations, among which the influenza virus is the only virus for which both vaccines and antiviral drugs are available (**Figure 1**). Two types of influenza vaccines are currently available; inactivated vaccine and live, attenuated vaccine. The live, attenuated nasal-spray influenza vaccine has been approved for use in the United States since 2003. However, it has not been recommended in high-risk groups including asthmatics because its safety is not fully demonstrated. The widespread use of inactivated influenza vaccines contain a trivalent mixture of strains of A(H1N1)pdm09, A(H3N2), and type B viruses likely to circulate during the next influenza season. Many studies indicated that no increase in asthma exacerbations was reported for both vaccinated children and adults (American Lung Association Asthma Clinical Research Centers, 2001; Kramarz et al., 2001; Hak et al., 2005).

A randomized, open-label study to investigate the safety and immunogenicity of two administrations of an unadjuvanted, inactivated A(H1N1)pdm09 virus vaccine was conducted in the United States (Busse et al., 2011). The results indicated that both the 15-μg (standard dose) and 30-μg vaccine doses generally provided excellent seroprotection against viral antigen 21 days after a single immunization in patients (12 to 79 years of age) with mild-to-moderate asthma. In patients with severe asthma, the response to the 15-μg dose was lower than that to the 30-μg dose. The authors of that study did not identify any safety concerns with the A(H1N1)pdm09 vaccine. Collectively, the findings described above indicate that inactivated influenza vaccines are well tolerated in patients with asthma.

Specific antiviral drugs against influenza viruses could be used for the treatment and prophylaxis for influenza. Based on their chemical properties and spectra of activity against influenza viruses, the drugs can be classified into two categories: the M2 ion channel inhibitors, i.e., adamantanes (amantadine and rimantadine) and the NA inhibitors, i.e., zanamivir, oseltamivir, peramivir, and laninamivir. Currently, the NA inhibitors are exclusively used for the treatment and prophylaxis of influenza because the circulating strains of A(H1N1)pdm09 and A(H3N2) viruses have a known amino acid substitution of serine by asparagines at position 31 in the M2 protein, which confers resistance to the adamantanes.

Few studies have examined the safety of NA inhibitors in patients with asthma. A double-blind, placebo-controlled crossover study indicated that zanamivir inhaled as a dry powder did not significantly affect the pulmonary function and airway responsiveness of subjects (19 to 49 years of age) with mild or moderate asthma (Cass et al., 2000). However, a number of studies suggested that the use of NA inhibitors was beneficial in hospitalized patients with A(H1N1)pdm09 viral infection, particularly when they are started within 48 h after the onset of illness (Domínguez-Cherit et al., 2009; Jain et al., 2009; Louie et al., 2010). Accordingly, WHO recommended that for patients at increased risk for severe or complicated illness, treatment with oseltamivir or zanamivir should be started as soon as possible after the onset of illness (Writing Committee of the WHO Consultation on Clinical Aspects of Pandemic (H1N1) 2009 influenza, 2010). Although A(H1N1)pdm09 virus resistance to NA inhibitors has been detected at very low frequency among circulating viral strains (World Health Organization, 2013b), there is concern about the recent report that oseltamivir-resistant A(H1N1)pdm09 viral mutants were detected in untreated patients and from a few clusters in some countries (Samson et al., 2013).

CLOSING REMARKS

Epidemiological studies as described above demonstrated that A(H1N1)pdm09 viral infection is closely associated with asthma in both children and adults. Although A(H1N1)pdm09 virus has not shown a high mortality rate similar to that of the highly pathogenic avian influenza virus of the H5N1 subtype, patients with A(H1N1)pdm09 viral infection were more susceptible to asthma exacerbation compared to A(H1N1) or A(H3N2) viral infection. Detailed analyses of virus-host interactions are needed to elucidate the mechanism underlying A(H1N1)pdm09 viral infection-induced asthma.

Since March 31, 2013 when the public health authorities of China reported three cases of human infection with an avian influenza A(H7N9) virus, a total of 135 human cases including 44 fatal cases have been reported in China and Taiwan as of August 12, 2013 (World Health Organization, 2013a). The current avian influenza viral infections in humans present considerable pathogenic potential with high mortality rates, suggesting that the pandemic viruses, if they emerge in human beings, could also present high pathogenicity and result in an excessive number of deaths in high-risk groups, including asthmatics. It will therefore be important to make preparations for drugs and vaccines for anti-influenza treatments and the prophylaxis of influenza.

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Development of oligomannose-coated liposome-based nasal vaccine against human parainfluenza virus type 3

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Human parainfluenza viruses (HPIVs) are the etiologic agents of lower respiratory infections and pneumonia in infants, young children and immunocompromised hosts. The overarching goal for the prevention of HPIV infection is the development of an effective vaccine against HPIVs. In the present study, we investigated the effectiveness of oligomannose-coated liposomes (OMLs) as an antigen-delivery system in combination with a synthetic double-stranded RNA analog for the induction of mucosal and systematic immunity against HPIV3. Full-length hemagglutinin-neuraminidase (HN) protein was synthesized using the wheat germ cell-free protein production system and then encapsulated into OML to serve as the antigen. Intranasal administration of the HN-filling OML (OML-HN) with the synthetic double-stranded RNA adjuvant, polyriboinosinic-polyribocytidylic acid [poly(I:C)] generated significant viral-specific systemic and mucosal immune responses as evidenced by the prominent induction of serum IgG and nasal wash IgA, respectively. On the other hand, no significant immune responses were observed in mice immunized with OML-HN without the adjuvant. Furthermore, serum from mice immunized with OML-HN plus poly(I:C) significantly suppressed viral infection in cell culture model. Our results provide the first evidence that intranasal co-administration of OML-encapsulated HN with the poly(I:C) adjuvant augments the viral-specific immunity against HPIV3.

Keywords: HPIV3, HN, vaccine, oligomannose-coated liposome, adjuvant

INTRODUCTION

Human parainfluenza viruses (HPIVs) belong to the Paramyxoviridae family and are one of the major causes of acute respiratory infections (ARIs) and asthma in infants and young children (<5 years old). HPIVs were classified into four serotypes including HPIV1-4 (Henrickson, 2003; Mizuta et al., 2011). In particular, human parainfluenza virus type 3 (HPIV3) is an important infectious agent, second only to respiratory syncytial virus (RSV), that causes bronchiolitis and pneumonia in infants (Glezen et al., 1984; Counihan et al., 2001; Belshe et al., 2004; Schmidt, 2011). Therefore, the development of a practical vaccine that can prohibit HPIV3 infection in infants is urgently needed.

Currently, there is no prophylactic human vaccine against HPIV3 infection. Several previous studies employed attenuated viruses or recombinant viruses for vaccination by intranasal administration (Haller et al., 2000; Karron et al., 2011; Schmidt et al., 2011; Mason et al., 2013). The HPIV3 cp45 is a practical nasal vaccine that is derived from the JS wild-type strain of HPIV3 through 45 passages in African green monkey cells at a low temperature. This vaccine has been evaluated in clinical human trials and is known to induce the hemagglutination-inhibiting (HAI) antibody in seronegative children (Skiadopoulos et al., 1999; Karron et al., 2003; Belshe et al., 2004). The rB/HPIV3b vaccine is a cDNA-derived chimeric HPIV3 in which the genomic cDNA is partially recombined with bovine PIV3 (BPIV3); the hemagglutinin-neuraminidase (HN) and F genes from HPIV3 fused with BPIV3 whole genome (Schmidt et al., 2001; Karron et al., 2012). The rB/HPIV3 vaccine was shown to induce significantly higher titers of HAI antibodies against HPIV3 in seronegative children. A major limitation of these vaccines is their potential to cause actual infection diseases in children or immunocompromised hosts because they are live attenuated vaccines. Therefore, it is necessary to develop a safer HPIV3 vaccine with lower risks for infection that will be useful for infants and young children in clinics. In this regard, component vaccines are desirable because they use non-infectious viral subunit proteins as antigens. A previous report demonstrated the efficacy of subunit vaccines that target the HPIV3 HN and F proteins in an animal model (Ray et al., 1988). Other reports also demonstrated the induction of protective antibodies that prohibit HPIV3 infection in response to subunit vaccines that target HPIV3 antigens (Morein et al., 1983; Ray et al., 1985; Ambrose et al., 1991; Brideau et al., 1993). A caveat of subunit-based vaccination strategies is their requirement for large amounts of antigens, thus rendering them costly to produce. Therefore, it is important to develop an effective subunit vaccine that utilizes lower quantities of antigen.

To circumvent the aforementioned problems, oligomannose-coated liposome (OML) was used as a natural and non-toxic antigen-delivery system. OML efficiently targets proteins to antigen presenting cells (APCs), such as macrophages or dendritic cells (Shimizu et al., 2007; Nishimura et al., 2013). Furthermore, previous reports showed that antigens incorporated into OML were efficiently delivered to APCs by intranasal administration (de Haan

et al., 1995; Ishii and Kojima, 2010; Giddam et al., 2012). The effect of OML was shown to be relatively ineffective at inducing humoral immunity, while it preferentially activated cell-mediated immunity via cytotoxic T lymphocytes (CTLs). Therefore, for optimal induction of both humoral and mucosal immunity it is necessary to use vaccination strategies that combine OML with other adjuvant systems.

Herein, we sought to utilize OML in combination with an adjuvant double-stranded RNA polyinosinic-polycytidylic acid [Poly(I:C)] for the induction of effective humoral and mucosal immunity against HPIV3. The overarching goal was to establish a vaccination strategy that required a small amount of antigen and a few doses. Poly(I:C) is an effective adjuvant for antibody and multi-functional CD4+ T cell responses against viral infection. Poly(I:C) was shown to be an effective mucosal adjuvant for the development of antigen-specific immunity even when hosts were immunized with a relatively small quantity of antigen (Ichinohe et al., 2005; Hasegawa et al., 2009). In addition, we also took advantage of the wheat germ cell-free protein production system to synthesize our antigen, full-length HPIV3-HN protein (Takai et al., 2010). Our results highlight the utility of combining sophisticated systems in the development of a novel vaccine against HPIV3.

MATERIALS AND METHODS

CONSTRUCTION OF WHEAT CELL-FREE EXPRESSION VECTOR

HPIV3 (C243) cDNA was kindly provided by Dr. Tsukakoshi. The HN fragment was amplified by PCR using the primers BamHI-HN F (5′-GAGAGGATCCCATGGAATACTGGAAGCAT) and NotI-HN R (5′-GAGAGCGGCCGCTTAACTGCAGCTTTTTGGA). The amplified fragment was digested with BamHI and NotI and cloned into either pEU-His or pEU-GST vectors that were previously digested with the same enzymes. GST-tagged HPIV3-HN (GST-HN) construct was mutated using the reagents of a PrimeSTAR Mutagenesis Basal Kit (TakaraBio, Otsu, Japan) according to the manufacturer's instructions.

CELL-FREE PROTEIN SYNTHESIS AND PURIFICATION

In vitro transcription and cell-free protein synthesis were performed as described (Takai et al., 2010). The bilayer translation method was used to synthesize His-tagged HPIV3-HN (His-HN) protein using wheat germ extract that was optimized for Ni-affinity purification (WEPRO 7240H; Cellfree Sciences, Yokohama, Japan) and a robotic synthesizer (Protemist XE; Cellfree Sciences) according to the manufacturer's instructions. The cell-free translation reaction (15 ml) was separated into soluble and insoluble fractions by centrifuged at 15000 rpm for 15 min. The insoluble fraction was lysed using 8M Urea at room temperature for 6 h and then mixed with Ni-sepharose High Permormance beads (GE Helthcare, Hino, Japan) in the presence of 20 mM imidazole. The beads were washed three times with washing buffer (20 mM Tris–HCl, pH 7.5, 500 mM NaCl) containing 40 mM imidazole. The His-HN protein was then eluted using washing buffer containing 8M Urea, 500 mM imidazole. Purified His-HN proteins were concentrated approximately 10–20-fold using Amicon Ultra Centrifugal Filters (Merck Millipore, Billerica, MA, USA). Full-length GST-HN protein and GST-HN deletion mutant proteins synthesized using wheat germ extract optimized for GST-affinity purification (WEPRO 1240G; Cellfree Science) according to the manufacturer's instructions. Quantification of synthesized proteins was performed by densitometric scanning of the Coomassie Brilliant Blue® (CBB)-stained bands.

PREPARATION OF LIPOSOMES

Liposomes were prepared as described previously (Giddam et al., 2012; Nishimura et al., 2013). Briefly, a chloroform:methanol (2:1, *v*/*v*) solution containing 1.5 μmol of DPPC, 1.5 μmol of cholesterol and varying amounts of Man3-DPPE (0.15–0.0015 μmol) was added to a flask and evaporated to prepare a lipid film. PBS containing 1 or 0.5 mg/ml of full-length HPIV3-HN protein was added to the dried lipid film and multi-lamellar vesicles were prepared by intense vortex dispersion. The vesicles were extruded 10 times through a 1 μm pore polycarbonate membrane (Nucleopore, Pleasanton, CA, USA). The amount of entrapped protein was measured using a Modified Lowry Protein Assay Kit (Pierce, Rockford, IL, USA) in the presence of 0.3% (*w*/*v*) sodium dodecyl sulfate using HN as the standard. The particle size of the liposomes was determined using a dynamic light scattering particle size analyzer (BioMedCore Inc., Yokohama, Japan).

IMMUNIZATION OF MICE

Female BALB/c mice (Japan SLC Inc., Hamamatsu, Japan), age 6–8 weeks at the time of immunization, were used in all of the experiments. All animal experiments were carried out in accordance with the Guides for Animal Experiments Performed at Yokohama City University (YCU) and approved by the International Animal Care and Use Committee of YCU. Three to six mice for each experimental group were anesthetized with isoflurane prior to being immunized. For the primary immunization, 13 μl of single-shot mixtures were prepared as containing OML-HN (0.1 or 1.0 μg) and/or poly(I:C) (10 μg), and administered 6.5 μl of mixtures into each nostril. Three weeks later, the secondary immunization was administered in the same manner. Two weeks after the secondary immunization, as tertiary immunization, 16 μl single-shot mixtures were prepared as containing OML-HN (0.2 or 2.0 μg) and/or Poly(I:C) (10 μg), and administered 8.0 μl of mixtures into each nostril.

ENZYME-LINKED IMMUNOSORBENT ASSAY

Serum was collected on days 7 and 14 after the secondary immunization and on days 7, 14, 21, and 28 after the tertiary immunization. On day 28 after the third immunization, all of the mice were sacrificed and nasal wash fluid was collected by washing the nasal cavity of the excised head with 1 ml of PBS(-) containing 0.1% BSA. The levels of IgG and IgA antibodies against HPIV3-HN in the serum and nasal wash fluid were determined by enzyme-linked immunosorbent assay (ELISA) as described previously. Briefly, ELISA was conducted sequentially from the solid phase (Anti-GST coated 96-well plate; Thermo, Waltham, USA) with a ladder of reagents consisting of the following: (1) GST-HN protein and GST protein as a control; (2) serum or nasal wash fluid; (3) either anti-mouse IgG antibody-conjugated HRP (1:10000, Thermo) or anti-mouse IgA antibody-conjugated HRP (1:10000, BETHYL, Montgomery, TX,

USA); (4) TMB substrate buffer (Thermo); and (5) 2M sulfuric acid. The chromogen produced was measured by determining the absorbance at 450 nm with an ELISA reader. The relative levels of IgG and IgA antibodies against HN were determined relative values calculated as follows; Relative values = mean value in immunized vaccine group/mean value in immunized OML-empty group. Each values were normalized with the optic values to GST protein.

IMMUNOBLOTTING

Using standard immunoblotting methods, the presence of HN-specific IgG was detected using pooled serum from each group of mice and incubated with anti-mouse IgG HRP-conjugated secondary Ab (Thrmo) at a dilution of 1:10000 in TBST. Immobilon was used for detection (Merck Millipore).

QUANTITATIVE REAL-TIME PCR

We performed an infection inhibitor assay by mouse serum using immortalized MRC5 cells (pNifty cells). pNifty cells were seeded in 24-well plates at a concentration of 2.5×10^5 cells per well, and after 12 h the cells were infected with pre-incubated HPIV3 virus (10^7TCID$_{50}$) with or without 5 μl mouse serum in 200 μl DMEM supplemented with 5% fetal bovine serum (FBS) and 1% penicillin and streptomycin (PS). At 4 h post-infection, the cells were washed and replaced in 200 μl of DMEM containing 10% FBS and 1% PS. At 48 h after medium change, the cells were washed with PBS and total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized using a cDNA synthesis kit (Toyobo, Osaka, Japan) and subjected to RT-PCR analysis with the SYBR Premix Ex gent Kit TaqII (Takara Bio) using an Applied Biosystems 7300 real-time PCR System. The primer sets used were as follows: HPIV3, 5′-CTCGAGGTTGTCAGGATATAG-3′ and 5′-CTTTGGGAGTTGAACACAGTT-3′; mGAPDH, 5′-CCATGGAGAAGGCTGGGG-3′ and 5′-CAAAGTTGTCATGGATGACC-3′.

RESULTS

GENERATION OF OML VACCINE AGAINST HPIV3-HN

To produce the full-length HPIV3-HN antigen, we subcloned full-length HN cDNA into two different cell-free expression vectors, pEU-His and pEU-GST for the expression of N-terminally His-tagged or GST-tagged fusion proteins, respectively (**Figure 1A**). We found that both His-HN and GST-HN proteins were efficiently synthesized using the wheat germ cell-free system (**Figures 1B,C**). His-HN proteins precipitated into the insoluble fraction (**Figure 1B**) were purified using Ni-sepharose beads in the presence of 8M Urea. GST-HN proteins were purified using glutathione sepharose beads in a regular buffer (**Figure 1C**).

After the large scale preparation of His-HN, the protein was incorporated into OML (**Figure 1D**). The particle diameter of HN-filling OML (OML-HN) and empty OML were 882 and 519 nm, respectively. The amount of carrier HN protein was approximately 32 mg per 1 mg cholesterol (**Figure 1E**) and the molar ratio of enclosed-OML to non-enclosed OML was found to be approximately 7:3 (data not shown).

IMMUNIZATION OF MICE WITH OML-HN

We investigated whether intranasal administration of OML-HN could induce a humoral immune response against the HN. **Figure 2A** depicts the time course for the immunizations and blood collection from the immunized mice. Mice were immunized intranasally with OML-HN (1 or 0.1 μg) with or without Poly(I:C), OML with or without Poly(I:C), Poly(I:C) only, or PBS. One week after the third immunization, HN-specific serum IgG was detected in mice immunized with OML-HN (1 μg) plus Poly(I:C). The serum IgG levels were increased between days 7 and 14 and reached the peak at 21 days after the final immunization. Mice immunized with the lower amount of OML-HN (0.1 μg) plus Poly(I:C) also produced HN-specific serum IgG at 28 days after the last immunization (**Figure 2B**). In contrast, there was no significant HN-specific serum IgG in mice immunized with OML-HN without Poly(I:C) or the other negative control groups (**Figure 2B**).

We next measured the levels of serum IgG and nasal wash IgA in each individual mouse by ELISA and immunoblotting (**Figures 3A–C**). Mice immunized with OML-HN (1 μg) plus Poly(I:C) exhibited prominent induction of HN-specific IgG (**Figures 3A,C**). HN-specific IgA in nasal wash fluid was most prominently induced in mice with OML-HN (1 μg) plus Poly(I:C) compared to other groups (**Figure 3B**). Interestingly, the induction of the HN-specific IgA was higher in mice that were immunized with the lower amount of antigens, OML-HN (0.1 μg) plus Poly(I:C) (**Figure 3B**).

EPITOPE MAPPING OF INDUCED ANTIBODIES

We next determined the region of HN that was recognized by the HN-specific serum IgG produced by the mice that were immunized with OML-HN (1 μg) plus Poly(I:C). Three domain mutants of HPIV3-HN, the N-terminal region (1-190), the middle region (168-408) and C-terminal region (400-572) were synthesized using the wheat germ cell-free system (**Figure 4A** left) and protein production was confirmed by SDS-PAGE (**Figure 4A** right). Based on ELISA analysis, all of the serum samples contained HN-specific antibodies that had high reactivity to the N-terminal region (**Figure 4B**).

EFFECT OF OML-HN VACCINE ON HPIV3 INFECTION *IN VITRO*

We asked whether sera from the immunized mice could inhibit the HPIV3 infection of fibroblast cells. Infectious HPIV3 virions were pre-incubated with mouse serum harvested from three mice that were immunized with OML-HN plus Poly(I:C) and then used to infect MRC5 cells. Cellular HPIV3 mRNA was measured using quantitative reverse transcription PCR (qRT-PCR). The levels of HPIV3 mRNA were significantly reduced in cells pre-incubated with the immunized mouse serum compared to control serum that immunized with OML-empty (**Figure 4C**). These results indicate that the immunization with OML-HN plus Poly(I:C) induced serum antibodies that protect HPIV3 infection.

DISCUSSION

Herein, we developed a novel subunit vaccine against HPIV3-HN using OML and a mucosal adjuvant Poly(I:C). Consequently we

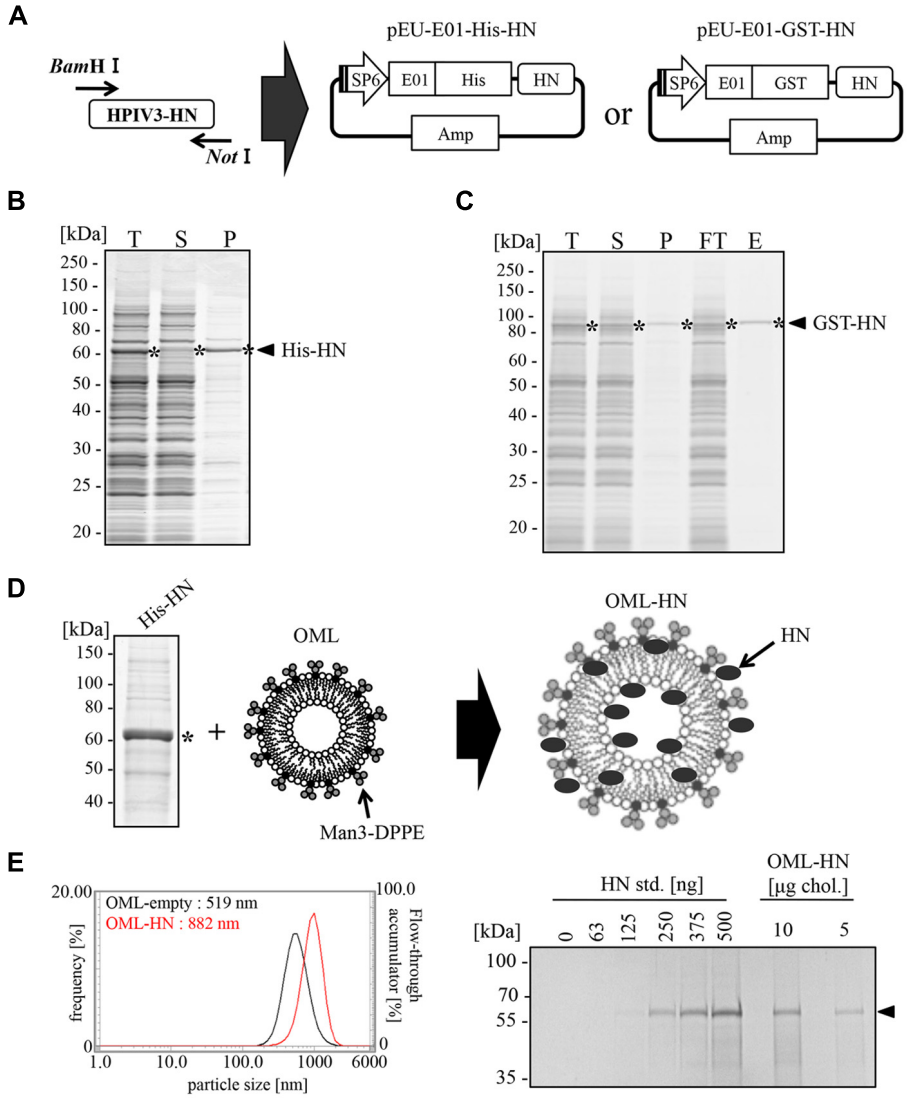
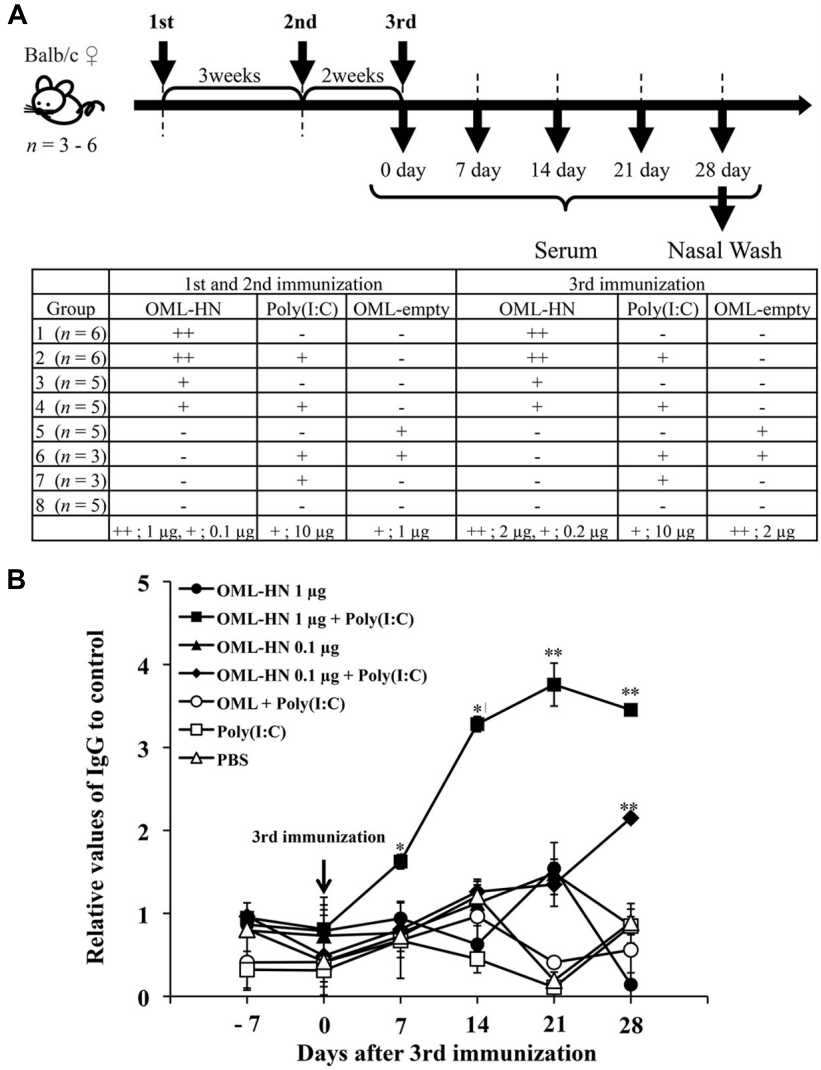


FIGURE 1 | Production of full-length HPIV3-HN and its encapsulation into OML. (A) Schematic representation of the expression vector for the wheat germ cell-free system. Full-length HN cDNA was subcloned into either pEU-His or pEU-GST vectors. **(B)** Expression and solubility of His-HN proteins that were synthesized using the wheat germ cell-free system. The translation reaction mixture (T) was subjected to centrifugation followed by the separation of the soluble supernatant (S) and insoluble precipitate (P) fractions. These samples were subjected to SDS-PAGE and visualized with Coomassie Brilliant Blue (CBB) staining. The single asterisk indicates the band of His-HN. **(C)** Purification of recombinant GST-tagged HPIV3-HN proteins that were synthesized using the wheat germ cell-free system. The translation mixture (T) was subjected to the separation into supernatant (S) and precipitate (P) fractions by centrifugation. GST-HN proteins were purified using glutathione sepharose bead and then resolved by SDS-PAGE and CBB staining. The single asterisk indicates the band of GST-HN. FT, Flow through fraction; E, elute fraction. **(D)** Schematic of the production of OML-encapsulated His-HN. The single asterisk indicates the band of His-HN. **(E)** The amount of encapsulated HN was quantitated by SDS-PAGE (right panel). The diameter of OML-HN or control OML were measured by dynamic light scattering particle size analyzer (left panel). The arrow indicates the band of His-HN.

successfully induced antigen-specific immunoglobulin G and A with the immunization of lower quantities of HN antigen via the nasal route. Furthermore, the immunized mouse serum exhibited the ability to suppress the virus infection in cell culture model. These results indicate that our newly developed OML vaccine could offer a powerful means to protect HPIV3 infection.

In our current study, we used the wheat germ cell-free protein production system to synthesize full-length HN protein as an antigen (Takai et al., 2010). In comparison to cell-mediated procedures such as *Escherichia coli* and baculovirus systems, the wheat germ cell-free system is beneficial for the rapid and efficient preparation of high-quality proteins (Endo and Sawasaki, 2006). Moreover, this cell-free system is suitable for the generation of toxic viral proteins for immunization and beneficial for the purification of naturally folded proteins, as well as scalability. This system, however, may not be cost-effective for preparing large



	1st and 2nd immunization			3rd immunization		
Group	OML-HN	Poly(I:C)	OML-empty	OML-HN	Poly(I:C)	OML-empty
1 ($n = 6$)	++	-	-	++	-	-
2 ($n = 6$)	++	+	-	++	+	-
3 ($n = 5$)	+	-	-	+	-	-
4 ($n = 5$)	+	+	-	+	+	-
5 ($n = 5$)	-	-	+	-	-	+
6 ($n = 3$)	-	+	+	-	+	+
7 ($n = 3$)	-	+	-	-	+	-
8 ($n = 5$)	-	-	-	-	-	-
	++ ; 1 μg, + ; 0.1 μg	+ ; 10 μg	+ ; 1 μg	++ ; 2 μg, + ; 0.2 μg	+ ; 10 μg	++ ; 2 μg

FIGURE 2 | Serum antibody responses in mice immunized with OML-HPIV3-HN. (A) Balb/c mice were immunized with the indicated antigens and/or adjuvant on days 0, 21, 35 as described in the Materials and Methods. Serum was collected from each mouse on days 7, 14, 21, and 28 after the third immunization. The bottom panel summarizes the time course for the immunizations. **(B)** HN-specific IgG in pooled serum from each group was measured by ELISA. Data represents the mean ± SE. (*t* test: $^{**}P < 0.01$, $^{*}P < 0.05$)

amounts of viral antigens for vaccine development. Therefore, efforts were made to reduce the amount of antigen needed vaccination. Herein, we utilized a OML and Poly(I:C) vaccination strategy in an attempt to reduce the amount of antigen required. OML is a lipid vesicle that has mannose on its surface, which aids in efficient targeting to APCs (Shimizu et al., 2007; Nishimura et al., 2013). In a previous report, antigenic proteins incorporated into OML were efficiently delivered to APCs via intranasal administration (Ishii and Kojima, 2010). In that report, intranasal administration of 5 μg ovalbumin (OVA) incorporated into OML four times effectively induced immune responses in mice (Ishii and Kojima, 2010). Poly(I:C) is a synthetic double-stranded RNA (dsRNA) molecule that induces effective mucosal immune responses by stimulating Toll-like receptor 3 (TLR3) as a molecular mimic of dsRNA, which is a byproduct of viral replication (Ichinohe et al., 2005; Hasegawa et al., 2009). The efficacy of nasal vaccines made of subunit proteins in the combination with mucosal adjuvants was demonstrated for influenza virus and RSV (Ichinohe et al., 2005; Hasegawa et al., 2009; Ainai et al., 2010; Kamphuis et al., 2013). We utilized a mucosal adjuvant Poly(I:C) to induce HN-specific antibodies in serum and nasal wash fluid through intranasal immunization with OML-HN. Using our vaccination strategy, we were able to decrease the amount of antigen required to 20% relative to previous reports (Mader et al., 2000; Ishii and Kojima, 2010).

The mucosa of respiratory tracts is the site of defense against virus infection since respiratory viruses attack and infect the respiratory mucosal tissues and cells (Tamura and Kurata, 2004). Mucosa is generally protected by mucin and defensin produced from goblet cells and Paneth cells. The TLR family members,

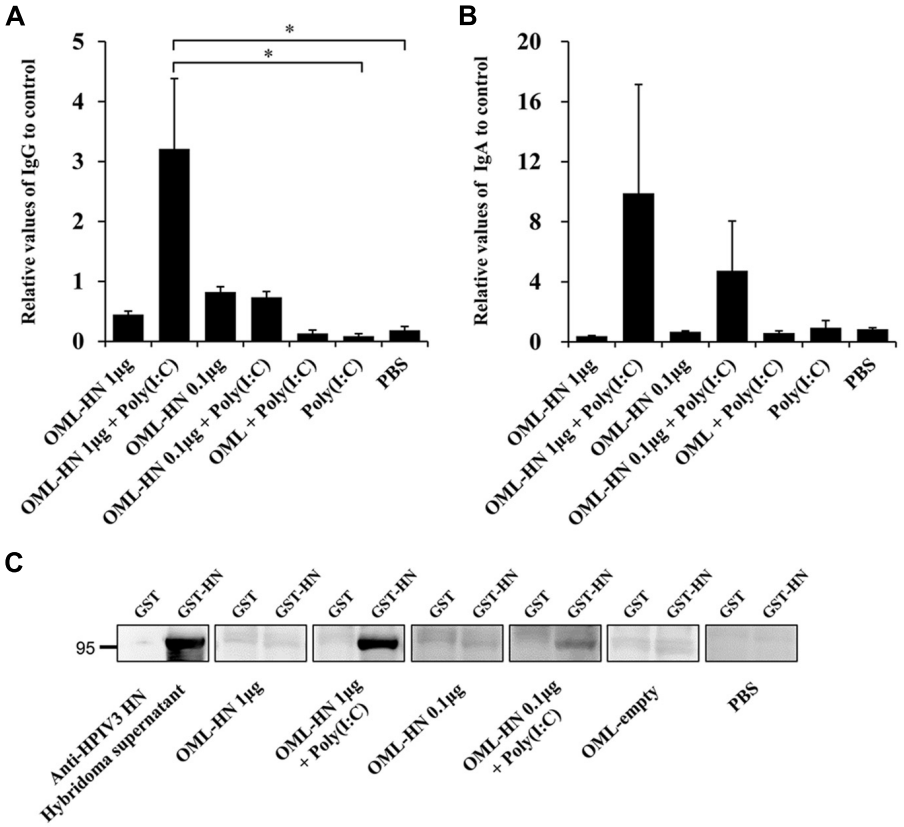


FIGURE 3 | Measurement of HPIV3-HN-specific serum IgG and nasal wash IgA. (A) The levels of HN-specific IgG in the serum of each immunized mouse on day 21 after the third immunization were determined by ELISA. **(B)** The levels of HN-specific IgA in nasal wash fluid on day 28 after the third immunization were determined by ELISA. Each bar represents the mean ± SE (*t* test: $*P < 0.05$). **(C)** Immunoblot analysis of recombinant HN proteins in mouse sera. Recombinant GST or GST-HN proteins were subjected to SDS-PAGE followed by the immunoblotting with the indicated serum.

TLR3, TLR7, TLR8, and TLR9 can recognize viral nucleotides and induces type I interferon (IFN-I) production if viruses intrude into tissues beyond the barrier. IFN-I activates the defense mechanism against virus by promoting the maturation of DCs and the induction of NK cells (Takeda et al., 2003; Akira et al., 2006). On the other hand, it is known that Microfold cells (M cells) promotes adherence and transport of antigens to APCs (Sato and Kiyono, 2012). M cells reside in the follicle-associated epithelium of Peyer's patches in the intestinal tract or nasal lymphoid tissue (NALT) of rodents in the upper respiratory tract, and plays a pivotal role in the induction of antigen-specific immunity (Nochi and Kiyono, 2006). The APCs promote adaptive immune responses by presenting antigens to naïve B cells and activate it to differentiate into antigen specific B cells. In the mucosa, secretory IgA is transported to mucosal surface by polymeric Ig receptor (pIgR) and the secreted IgA plays an important role in the protection of viral infection in the respiratory tract (Mostov and Deitcher, 1986). It is known that the intranasal immunization can activate mucosal immunity thereby enhancing the induction of mucosal IgA in addition to the generation of systemic IgG against viral antigen. Our current study employed OML as an effective tool to deliver the antigen to APCs and M cells in respiratory mucosa. A recent report demonstrated that OML-mediated intranasal immunization can efficiently induce Th2 cytokines such as IL-5 and IL-6 that eventually help produce secretory IgA in mucosal system in mouse model (Ishii and Kojima, 2010). We further combined OML with a mucosal adjuvant Poly(I:C) to facilitate the specific mucosal immunity against HPIV3-HN. Poly(I:C) has been shown to be an effective mucosal adjuvant stimulating TLR3 as a molecular mimic. A previous report indicated that a nasal influenza virus vaccine combined with Poly(I:C) synergistically induced IFN-1 and Th2 cytokine leading to an effective humoral immunity including secretory IgA in mucosa (Ichinohe et al., 2005). Our current study also demonstrated that the combinatory use of nasal vaccine with Poly(I:C) has a profound effect in inducing mucosal immunity against viral antigen.

Our newly developed OML-HN vaccine has several advantages as compared with previously developed vaccine methods including live attenuated vaccines. Although there is no practical prophylactic vaccine against HPIV3 infection, several previous studies have indicated that attenuated vaccines created by reducing the virulence of HPIV3 can indeed effectively induce the mucosal immunity when treated by intranasal administration (Karron et al., 2003). However, a major problem of these vaccines is their potential to cause a live infection in infants and immunocompromised hosts. Furthermore, there is a small risk of reversion to

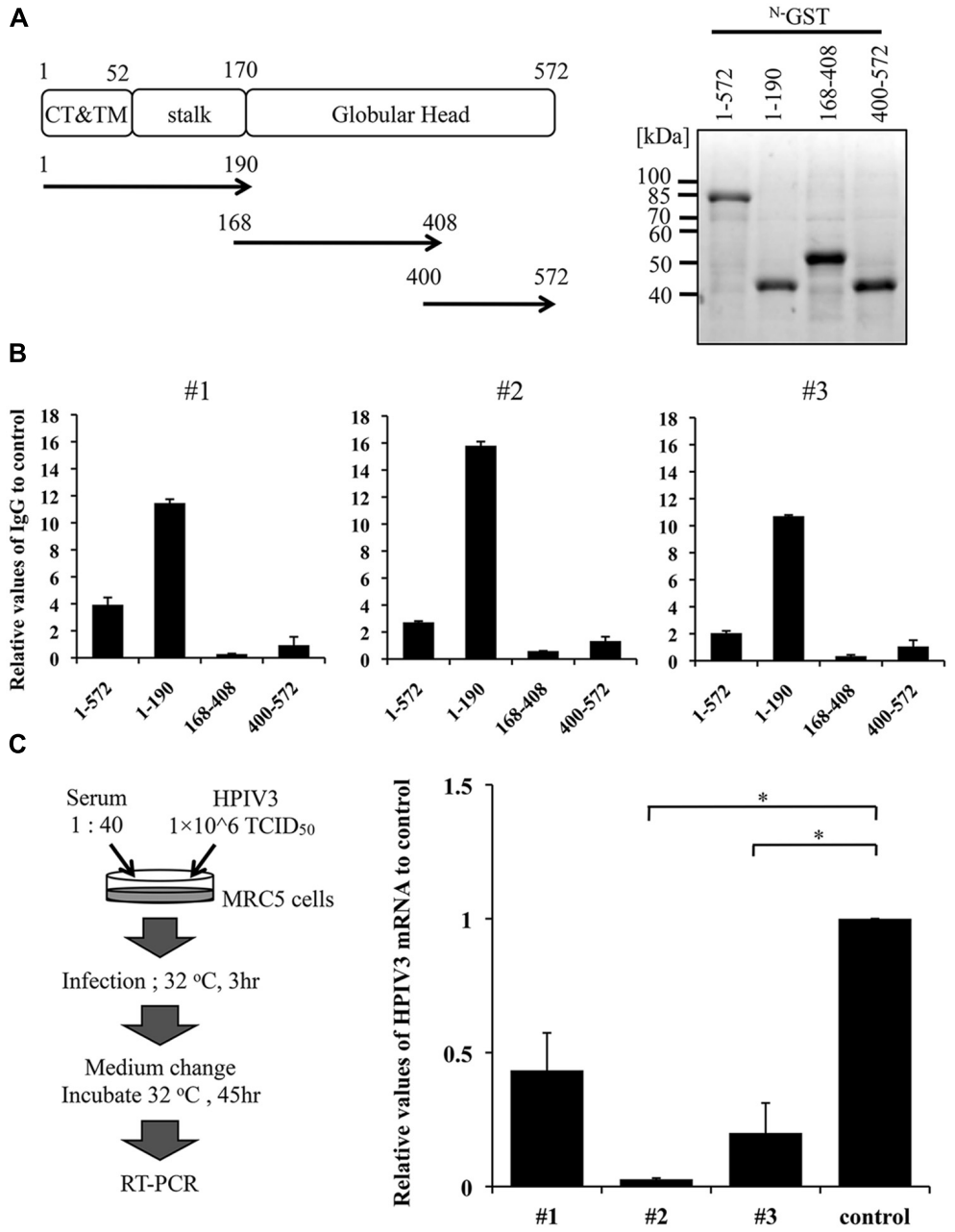


FIGURE 4 | Anti-infectious activity of mouse serum. (A,B) Epitope mapping of antibodies induced in the immunized mouse. We selected arbitrary three representative sera from mice immunized with OML-HN (1 μg) plus Poly(I:C) that exhibited the highest HN-specific IgG induction (#1–#3). The full-length and three deletion mutants of GST-HN were produced using the wheat germ cell-free system. These purified proteins were separated by SDS-PAGE and visualized using CBB staining **(A)**. Using the recombinant HN proteins, the target region of the three sera (#1–#3) was analyzed by ELISA **(B)**. **(C)** Schematic representation of the experimental procedure of infection-inhibitory assay (left panel). Immunized mouse sera (#1–#3) were tested for this assay. An OML-empty-treated mouse serum was used as a control. The anti-infection ability was measured using quantitative real-time PCR for HPIV3-HN mRNA. Each bar represents the mean ± SE of two independent experiments as normalized by control serum (*t* test: $*P < 0.05$).

virulence by genetic mutations that results in the onset of severe disease. Therefore, it is desirable to develop a safer HPIV3 vaccine with lower risks of infection. On the other hand, intranasal subunit vaccines against HPIV3 have been demonstrated to be effective in animal models without the risk of viral replication and live infection. According to a report by Ray et al., the intranasal administration of HN and F proteins extracted from virions could induce significant anti-viral immunity in hamsters (Ray et al., 1988). However a drawback of subunit vaccines is their requirements for large amounts of antigens and concomitant high cost. Therefore, it is important to develop a cost-effective subunit vaccine that dispenses with substantial quantity of antigens. In order

to overcome this problem, we used OML and Poly (I:C) aiming for efficient vaccine delivery and immune response, respectively. Indeed, our current study demonstrated that a combination of OML-HN with poly(I:C) induced antigen-specific IgA and IgG by three times more than the immunization without poly(I:C). The safety in the use of either OML or poly(I:C) has been reported in previous studies. For OML-based vaccine, Fukasawa et al. have reported that OML has indeed no obvious toxicity and immunogenicity by itself (Fukasawa et al., 1998). Furthermore, Poly(I:C) has shown to be non-toxic as compared with conventional vaccine adjuvant such as cholera toxin subunit B (Ichinohe et al., 2005). However, further careful analysis should be necessary to validate the effectiveness and feasibility of our newly-developed vaccine strategy using virus infection models with multiple genotypes of HPIV3.

In our current study, we demonstrated that the OML-based vaccine incorporated with full-length HN protein induced IgG that targets the N-terminal region of HN protein. The N-terminal region of HN contains the stalk region while the C-terminal region contains the globular head domain. The stalk region of HN is known to play a crucial role in virion-host cell fusion via an interaction with F protein while the globular head binds sialic acid and neuraminidase (Moscona, 2005; Porotto et al., 2012). Although effective antigenic epitopes for HPIV vaccine remain elusive (Henrickson, 2003), a monoclonal antibody targeting the stalk region of HPIV2-HN has been shown to have a profound inhibitory activity against viral infection (Yuasa et al., 1995). Based on the observation, it seems that the antibodies induced by our vaccine system could also target the stalk region since they effectively blocked the viral infection in cell culture model. Further careful analysis will be required for the mapping of the epitope affecting virus infection in our current model.

In this study, we did not investigate other routes of antigen administration besides the intranasal route. However, previous studies have indicated that non-nasal immunization of HPIV3 components failed to prohibit the infection of HPIV3 in a cotton rat model. Indeed, the intramuscular immunization with HN and F recombinant proteins could not protect virus infection in upper respiratory tract although it had some effects on the protection of pneumonia and lower respiratory tract infection (Ambrose et al., 1991). It is generally believed that intranasal immunization has a great benefit for protecting virus infection itself by inducing antigen-specific secretory IgA in respiratory mucosa (Hirabayashi et al., 1990; Durrer et al., 2003). Our current study also confirmed this advantage of nasal vaccination where the OML-based nasal vaccine provides high performance for the induction of antigen-specific secretory IgA in nasal wash fluids. Therefore, intranasal administration of OML-based vaccine with poly(I:C) adjuvant could be an effective way of vaccination against respiratory viruses including HPIV3.

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Wheat germ cell-free system-based production of hemagglutinin-neuraminidase glycoprotein of human parainfluenza virus type 3 for generation and characterization of monoclonal antibody

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Human parainfluenza virus 3 (HPIV3) commonly causes respiratory disorders in infants and young children. Monoclonal antibodies (MAbs) have been produced to several components of HPIV3 and commercially available. However, the utility of these antibodies for several immunological and proteomic assays for understanding the nature of HPIV3 infection remain to be characterized. Herein, we report the development and characterization of MAbs against hemagglutinin-neuraminidase (HN) of HPIV3. A recombinant full-length HPIV3-HN was successfully synthesized using the wheat-germ cell-free protein production system. After immunization and cell fusion, 36 mouse hybridomas producing MAbs to HPIV3-HN were established. The MAbs obtained were fully characterized using ELISA, immunoblotting, and immunofluorescent analyses. Of the MAbs tested, single clone was found to be applicable in both flow cytometry and immunoprecipitation procedures. By utilizing the antibody, we identified HPIV3-HN binding host proteins via immunoprecipitation-based mass spectrometry analysis. The newly-developed MAbs could thus be a valuable tool for the study of HPIV3 infection as well as the several diagnostic tests of this virus.

Keywords: human parainfluenza virus 3, monoclonal antibody, cell-free protein synthesis, proteomics

INTRODUCTION

Human parainfluenza viruses (HPIVs) are major causes of lower respiratory infections in infants, young children, the immunocompromised, the chronically ill, and the elderly (Glezen et al., 1984; Counihan et al., 2001; Weinberg et al., 2009). HPIVs belong to the Paramyxoviridae family of medium-sized enveloped viruses and their genomes are organized on a single negative-sense strand of RNA. Of the four predominant serotypes of HPIV, the HPIV3 is the most frequently detected in viral infections in respiratory tracts. In fact, HPIV3 is second only to respiratory syncytial virus (RSV) as a cause of pneumonia and bronchiolitis in infants and young children. The HPIV3 genome contains approximately 15,000 nucleotides encoding at least six common structural proteins (3′-N-P-C-M-F-HN-L-5′; Storey et al., 1984; Tashiro and Homma, 1985; Wechsler et al., 1985). The two envelope glycoproteins, hemagglutinin-neuraminidase (HN) and fusion (F), are necessary for viral entry, cell-fusion and syncytium formation (Horvath et al., 1992; Hu et al., 1992; Takimoto et al., 2002; Porotto et al., 2012).

The HN protein is found on the lipid envelope of HPIVs, where it likely exists as a tetramer. HN is important for HPIV3 infection of host cells because it functions in virus-host cell attachment via sialic acid receptors and in virus release from cells with its neuraminidase activity (Huberman et al., 1995; Porotto et al., 2001; Chu et al., 2013). HN can be recognized by the host immune system and antibodies against epitopes within HN can neutralize its activity through the inhibition of the function and/or activity of either hemagglutinin or neuraminidase. Therefore, characterization of the protein structure and function of HN is of great importance for the understanding of HPIV3 infection and the host immunity against this virus.

Polyclonal antibodies and monoclonal antibodies (MAbs) against HPIV3 antigens have previously been generated, and animal antiserum to HPIV3 HN is also commercially available. However, the antibodies demonstrated cross-reactivity to other HPIV family viruses and exhibited relatively high non-specific background staining in immunoassays (Goswami and Russell, 1983; Waner et al., 1985). MAbs have been also produced to several components of HPIV3 (van Wyke Coelingh et al., 1985; Rydbeck et al., 1986). Although these commercially available MAbs have been shown to be specific, the utility of these antibodies for several immunological and/or proteomic analyses for

understanding the nature of HPIV3 infection have not been fully delineated.

In our current study, we utilized the innovative wheat germ cell-free protein production system to generate the antigen protein. The main advantage of the cell-free protein system is the synthesis of proteins that are properly folded and that possess biological activity because the proteins are expressed in a eukaryotic cell system. Moreover, this system is capable of producing toxic proteins, such as viral antigens, that cause severe cytotoxicity or interference with host cellular physiology. By utilizing HPIV3-HN protein synthesized by the wheat cell-free system, we established multiple hybridoma clones producing MAbs that specifically targeted the viral antigen and were applicable for several immunoassays. Furthermore, we used the MAbs in proteomic analyses for identifying host proteins that potentially act as HN binding partners.

MATERIALS AND METHODS

CONSTRUCTION OF WHEAT GERM CELL-FREE EXPRESSION VECTOR

Amplification of HN fragment from HPIV3 (Strain C243) genome was performed with the following primers: forward (5′-AGGAGTAAAGTTACGCAATCCAA) and reverse (5′-ATATTTCCCTTTTGTCTATTGTCTG). For producing the expression vector of wheat germ cell-free system, The HN open-reading frame was amplified by PCR using the forward primer (5′-GAGAGGATCCCATGGAATACTGGAAGCAT) and reverse primer (5′-GAGAGCGGCCGCTTAACTGCAGCTTTTTGGA). The amplified fragment was subcloned using BamH I and Not I into pEU-His or pEU-bls-S1 (bls; biotin ligase site) vectors. Biotinylated HN mutants were generated using the PrimeSTAR Mutagenesis Basal kit (TakaraBio, Otsu, Japan) according to the manufacturer's instructions.

HN protein fragments comprising CT, TM, and stalk regions were generated by the wheat cell-free system based on the template cDNA amplified from clinical isolates of HPIV1 (GeneBank No. JQ901977; Beck et al., 2012), HPIV2 (GeneBank No. AF533010; Skiadopoulos et al., 2003), and Mumps (GeneBank No. AB699704; Momoki, 2013) using following primer sets. HPIV1: (5′-ATGCTTATACTCTGGAGTCAAGA) and (5′-TCTAGCAAAACRTGAAGTTGAG); HPIV2: (5′-AAAAACCTA-AAATAAGCACGAA); and (5′-CCATTCTGGCCTATATYATAAT), Mumps: (5′-TTACTTATAAGACTGCGGTGC) and (5′-CTTGCA-ATGAGTTCTACTCTGA). Synthetic cDNA encoding the CT, TM and stalk regions of Sendai virus (UniProtKB entry no. P04853; Miura et al., 1985) was generated by Operon Biotechnologies (Huntsville, WI, USA). The amplified fragments were subcloned into pEU-bls-S1 vectors using In-fusion cloning system (TakaraBio) according to the manufacturer's instructions.

CELL-FREE PROTEIN SYNTHESIS AND PURIFICATION

In vitro transcription and cell-free protein synthesis were performed as described (Takai and Endo, 2010; Takai et al., 2010). For cell-free protein synthesis, the ENDEXT Wheat Germ Expression S Kit (CellFree Sciences, Yokohama, Japan) was used according to the manufacturer's instructions for the bilayer translation method. GST fusion and Biotinylated proteins were produced as previously described (Sawasaki et al., 2008; Takahashi et al., 2012). His-HPIV3-HN protein, used for the generation of hybridoma, was synthesized using the robotic synthesizer (Protemist XE; CellFree Sciences) according to the manufacturer's instructions. The cell-free translation reaction mixture (15 ml) was separated into soluble and insoluble fractions by centrifugation at 15,000 rpm for 15 min. The insoluble fraction was lysed by the addition of 8 M Urea at room temperature for 6 h, then mixed with Ni-sepharose High Performance beads (GE Healthcare, Waukesha, WI, USA) in the presence of 20 mM imidazole. The beads were washed three times with washing buffer (20 mM Tris–HCl pH 7.5, 500 mM NaCl) containing 40 mM imidazole. His-HN proteins were then eluted with washing buffer containing 8 M Urea, 500 mM imidazole. Amicon Ultra centrifugal filters (Millipore, Bedford, MA, USA) were used to concentrate the purified His-HN proteins by approximately 10- to 20-fold. The protein concentration was determined using the Bradford method with bovine serum albumin (BSA) as a protein standard.

IMMUNIZATIONS AND GENERATION OF HYBRIDOMAS

Monoclonal antibodies specific for HPIV3-HN were generated using the previously described hybridoma technology (Kimura et al., 1994). In brief, 300 μg of N-terminal, His-tagged full-length HPIV3-HN protein was injected into the footpad of Balb/c mice using keyhole limpet hemocyanin as an adjuvant. Four weeks later, spleen cells were isolated and fused to the myeloma cell line SP2/O using polyethylene glycol 1500 (PEG 1500) as previously described (Kimura et al., 1996). Isotype determination was performed with the mouse MAb isotyping test kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol.

ELISA

Microtiter plates coated with HPIV3-HN were incubated with threefold serial dilutions of each antibody (starting from 1:300 dilution of a hybridoma culture supernatant). After incubation with a peroxidase-conjugated secondary antibody and washing with PBS, the colorimetric signal of tetramethylbenzidine was detected by measuring the absorbance at 405 nm (Abs) using a plate reader.

IMMUNOBLOTTING

Recombinant HPIV3-HN proteins (equivalent to ∼100 ng) or HPIV3-infected HeLa cell lysates were separated by 10% SDS-Gel and transferred onto a PVDF membrane (Millipore). The membrane was then soaked in Tris-buffered saline (TBS) containing 5% (w/v) skim milk for 1 h and incubated with a MAb (hybridoma supernatant, 1:10 dilution) in TBS containing 0.1% (v/v) Tween-20 (TBST) overnight at 4°C. After washing three times with TBST, the membrane was incubated for 1 h in TBST containing goat-anti mouse IgG-HRP antibody (1:10000; GE Healthcare, Buckinghamshire, UK). After washing three times in TBST, the blot was detected with ImmobilonWestern Chemiluminescent HRP Substrate (Millipore) using FluorChem FC2 (Alpha Innotech, Santa Clara, CA, USA) in accordance to the manufacturer's protocol.

IMMUNOPRECIPITATION

HeLa cells were infected with HPIV3 (Strain C243) at multiplicity of infection (MOI) of 100 for 48 h. The cells were lysed with

immunoprecipitation buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5% NP-40, 200 μM PMSF, 50 μM VO_4, 2 μg/ml Aprotinin, 5 μg/ml Leupeptin, 1 μg/ml Pepstatin A). For immunoprecipitation assay, cell lysate or wheat germ cell extract generating full-length HPIV3-HN was incubated with the individual MAb (hybridoma supernatant), preclearing overnight using proteinA/G sepharose beads, for 2 h at 4°C. After washing three times with immunoprecipitation buffer, immunocomplexes were eluted from the beads with 2x SDS sample buffer. Then, the bound protein was analyzed by immnoblotting.

IMMUNOFLUORESCENCE

HeLa cells were grown on coverslips for 24 h and the cells were infected with HPIV3 (MOI = 100); mock-infected cells served as the control. At 48 h post-infection, the cells were washed with PBS before fixation with 3% formalin in PBS at room temperature for 15 min. Cells were washed twice with PBS for 5 min followed by 100% methanol for 10 min at −20°C. Cells were permeabilized with PBS containing 0.05% Triton X-100 for 10 min at room temperature. The cells were incubated with individual primary antibodies for 1 h at room temperature. After being washed twice with PBS, the cells were incubated with secondary antibodies for 1 h at room temperature. The nucleus was counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Microscopic imaging was performed with an FV1000-D confocal laser scanning microscope (Olympus, Tokyo, Japan) equipped with a 60x oil-immersion objective.

FLOW CYTOMETRY

HeLa cells were seeded in 6-well plates at a concentration of 2×10^5 per well 24 h before infection, and the cells were washed with medium containing 0.0001% trypsin and infected with HPIV3 (MOI = 100); mock-infected cells were used as a control. At 4 days post-infection, cells were harvested in PBS containing 5 mM EDTA and washed twice with PBS. The cells were then incubated with either MAbs (hybridoma supernatant; 5X dilution) or non-immunized hybridoma supernatant in PBS containing 2% Blocking One (NACALAI TESQUE, INC., Kyoto, Japan) for 1 h at room temperature. After being washed twice with PBS, the cells were incubated with phycoerythrin (PE)-conjugated anti-mouse IgG antibodies (Beckman Coulter, Fullerton, CA, USA) for 1 h at room temperature. Flow cytometric analysis of the cells (10,000 cells per sample) was performed on a FACScanto II instrument (BD Biosciences, San Jose, CA, USA).

EPITOPE MAPPING AND SPECIFICITY OF MAbs USING ALPHASCREEN ASSAY

The AlphaScreen assay was performed using 384-well ProxiPlates (PerkinElmer, Boston, MA, USA). Biotinylated virus proteins or GST (negative control) were incubated with a 40-fold dilution of MAbs (hybridoma supernatant) in 15 μl of binding mixture containing reaction buffer (100 mM Tris-HCl, pH 7.5, 1 mg/ml BSA, 0.01% Tween-20) at 26°C for 30 min. Then, combine 10 μl of the detection mixture containing 0.1 μl protein G-conjugated acceptor beads and 0.1 μl streptavidin-coated donor beads (AlphaScreen IgG detection kit, PerkinElmer) in reaction buffer were incubated at 26°C for 1 h. Antigen-antibody interactions were analyzed using an Envision microplate reader (PerkinElmer).

PROTEOMIC ANALYSIS

Cell lysate from HPIV3-infected or mock-infected HeLa cells were immunoprecipitated with #21 MAb. The binding proteins were separated by SDS-PAGE and transferred to PVDF membranes. For LC-MS/MS analysis, the membranes digested with trypsin. LC-MS/MS analysis was performed using a TripleTOF MS (TripleTOF 5600 system, AB SCIEX, Foster City, CA, USA) and the Analyst version 1.6 TF (AB SCIEX) coupled to an DiNa-AP (KYA Technologies, Tokyo, Japan). Prior to injection into the mass spectrometer, the tryptic digests were filtered through a Ultrafree-MC, GV 0.22 μm filter (Millipore), then loaded onto a reverse phase pre-column (HiQ sil C18W-3, 500 μm id × 1 mm, KYA Technologies) and resolved on a nanoscale HiQ sil C18W-3 (100 μm id × 10 cm; KYA Technologies) at a flow rate of 200 nL/min with a gradient of acetonitrile/0.1% (v/v) formic acid. Peptides were separated using a 30 min gradient from 5 to 100% solvent B [0.1% (v/v) formic acid/80% (v/v) acetonitrile]. Solvent A was 0.1 % (v/v) formic acid/2% (v/v) acetonitrile. The obtained MS and tandem-MS data were searched against the human protein sequences in the Swiss-Prot database (version Jan 2013, 20233sequences) using the Protein Pilot software 4.5 (AB SCIEX).

RESULTS

PRODUCTION OF MAbs

In our current study, we attempted to produce the full-length HPIV3-HN by the wheat cell-free production system (**Figure 1A**). Complementary DNA encoding HPIV3-HN open-reading frame was sub-cloned into pEU-His, the expression vector designed specifically for the wheat germ cell-free system for expressing His-tagged protein. Consequently, His-tagged HPIV3-HN protein was synthesized by this procedure in a large scale. Since HPIV3-HN exhibited high insolubility in regular buffer, the protein was suspended in the buffer including 8 M urea. This suspended His-tagged HPIV3-HN was further purified using Ni-sepharose followed by the elusion with imidazole. Balb/c mice were then immunized with purified full-length HPIV3-HN protein. After 4 weeks, splenocytes were isolated and hybridomas were created (**Figure 1A**). Finally, 36 stable hybridomas were obtained and designated #1 to #36. The resulting hybridomas were screened by ELISA with plates coated with HPIV3-HN protein conjugated with BSA. Of the 36 hybridoma clones established, seven clones (#4, #5, #7, #10, #14, #21, #23) exhibited relatively high absorbances (**Figure 1B**). Titration analyses with diluted hybridoma supernatants or antigenic HN protein revealed that these seven antibodies had higher specificities and intensities than control hybridoma supernatants (**Figure 1C**). These seven hybridomas were processed for further characterization.

IMMUNOBLOTTING ANALYSES OF MAbs

We next tested the MAbs in immunoblotting analysis. First, recombinant HPIV3-HN protein was separated by SDS-PAGE followed by immunoblotting with MAbs isolated from the seven

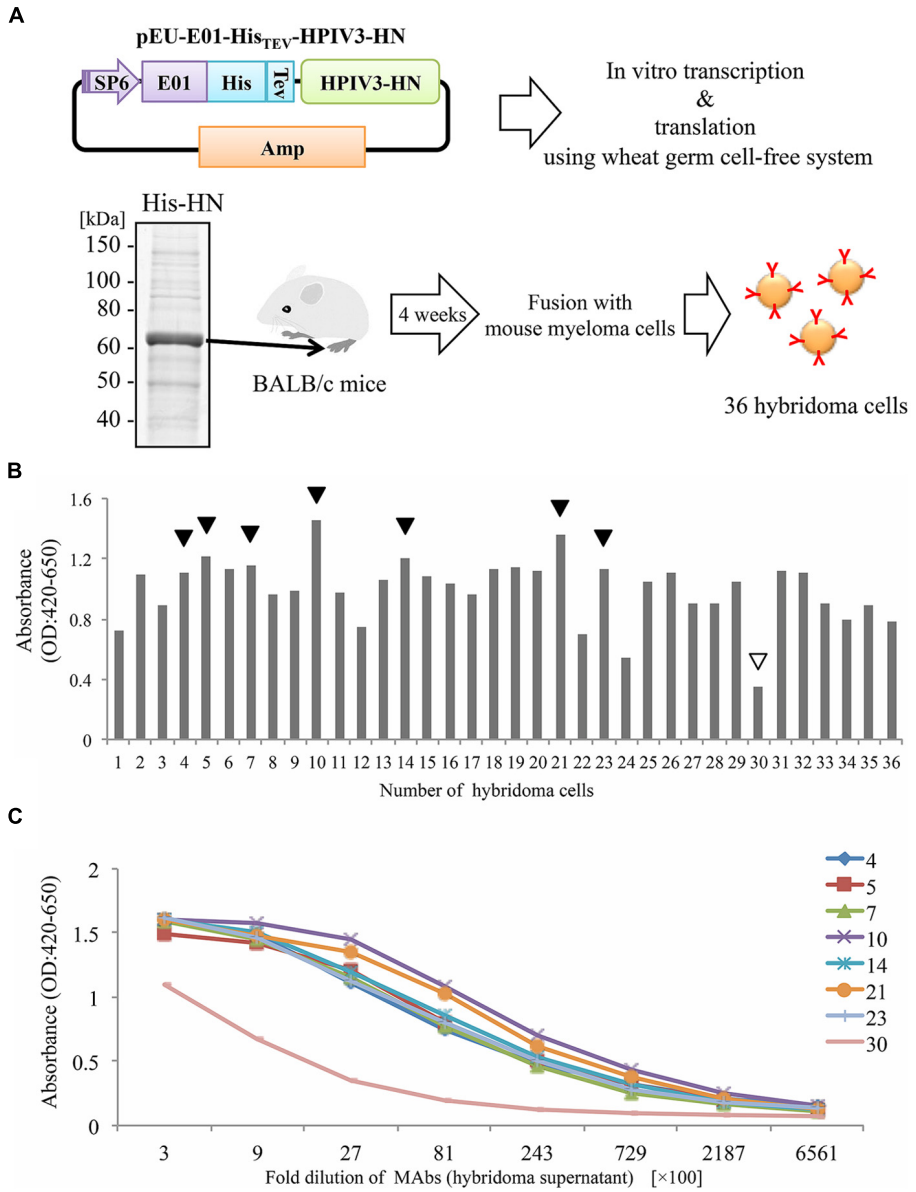


FIGURE 1 | Production of hybridoma cells generating anti-HPIV3-HN antibodies. (A) Schematic diagram of hybridoma cells production generating anti-HPIV3-HN monoclonal antibody (MAb). The recombinant Histidine-tagged recombinant HPIV3-HN (His-HN) protein was produced by wheat germ cell-free system and then purified by nickel-chelated sepharose beads in the presence of 8 M urea. The purified protein was separated by SDS-PAGE and visualized by CBB-staining. Purified His-HN protein was injected into the footpad of Balb/c mice. After 4 weeks, immunized mouse splenocytes were fused with myeloma cells and then 36 hybridoma cells were established. SP6, SP6 promoter sequence; E01, translation enhancer sequence; His, Histidine-tagged sequence; TEV; TEV protease recognized sequence. **(B,C)** The specificity of MAbs (hybridoma supernatant) evaluated by ELISA. The specificity of 36 MAbs in 2700-fold dilution was determined **(B)**. The black arrows indicate the selected MAbs while the white arrow depicts a selected clone as a negative control (clone no. #30). The selected eight MAbs were diluted at serial points and analyzed by ELISA **(C)**.

hybridomas. As shown in **Figure 2A**, all seven MAbs recognized a single band that corresponded to the recombinant protein. Next, immunoblotting analysis was performed with cell lysates from HeLa cells either infected- or mock-infected with HPIV3. The all seven MAbs detected a 63 kDa protein band that was consistent with the molecular mass of the HN protein (**Figure 2B**). No other bands were detected by the MAbs indicating that they specifically recognized HN.

IMMUNOFLUORESCENT ANALYSIS

We next performed an immunofluorescent (IF) analysis of HPIV3-infected HeLa cells with seven MAbs (**Figure 2C**). Three of the MAbs (#7, #21, and #23) exhibited prominent IF staining of the infected cells, while the other four MAbs had no detectable IF staining. The IF staining with the antibodies revealed that HN protein exhibited granular staining pattern throughout cytoplasm and plasma membrane in HPIV3-infected cells (**Figure 2D**), which

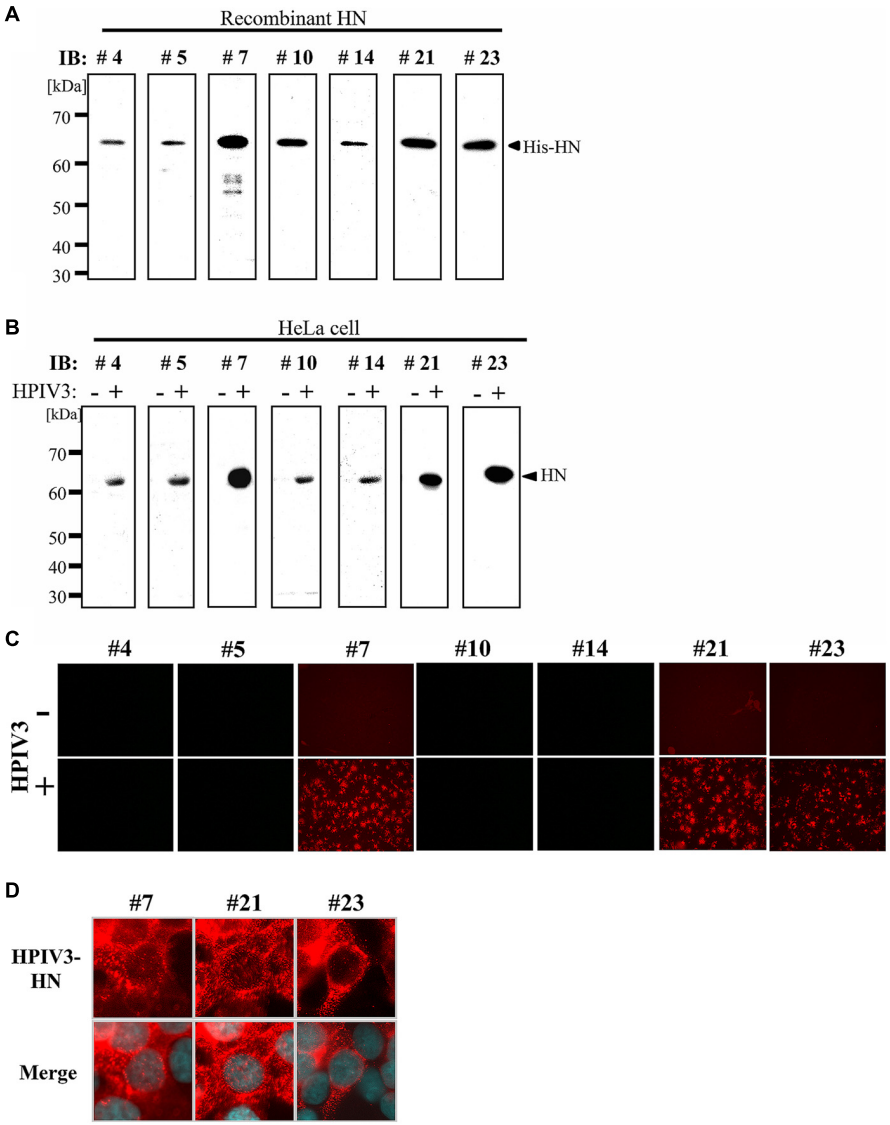


FIGURE 2 | Immunoblotting and immunofluorescent analysis. **(A,B)** Detection sensitivity of the MAbs for recombinant His-HN **(A)** or HPIV3-infected cell lysate **(B)**. Recombinant HPIV3-HN (100 ng) was separated using 12.5% SDS-gel and transferred to a PVDF membrane, followed by incubation with MAbs (hybridoma supernatants) at a 1:10 dilution **(A)**. HeLa cells were infected or mock-infected with HPIV3. After 48 h, cells were lysed with SDS-PAGE loading buffer. The total protein was separated in 12.5% SDS-gel and immunobloted with indicated MAbs **(B)**. **(C,D)** Immunofluoresent analysis of HN (red) in HPIV3-infected HeLa cells. HeLa cells were infected or mock-infected with HPIV3. After 48 h, cells were fixed, and then stained with MAbs (hybridoma supernatant; red) and DAPI (blue). Confocal microscopic analysis was performed at 40× **(C)** and at 600× magnifications **(D)**.

was consistent with previous studies (Ali and Nayak, 2000; Stone and Takimoto, 2013). The control mock-infected cells did not show any signals when stained with the antibodies (**Figure 2C**). We thus selected the three MAbs (#7, #21, and #23) for further characterization.

IMMUNOPRECIPITATION

We next examined whether these selected antibodies were useful in immunoprecipitation analysis. The wheat germ extract containing full-length HPIV3-HN with glutathione-*S*-transferase (GST) tag was incubated with protein A/G-coated sepharose beads (GE Helthcare) together with the three selected antibodies or non-immunized mouse IgG antibody. The precipitated samples were subjected to immunoblotting analysis with anti-GST antibody. The GST tagged HN protein was precipitated by all selected antibody (#7, #21, and #23) but not non-immunized IgG (**Figure 3A**).

FLOW CYTOMETRY ANALYSIS

We next addressed the usability of the antibodies #7, #21, and #23 in flow cytometry analysis. HeLa cells infected with HPIV3 were stained with the antibodies and then subjected to FACS analysis.

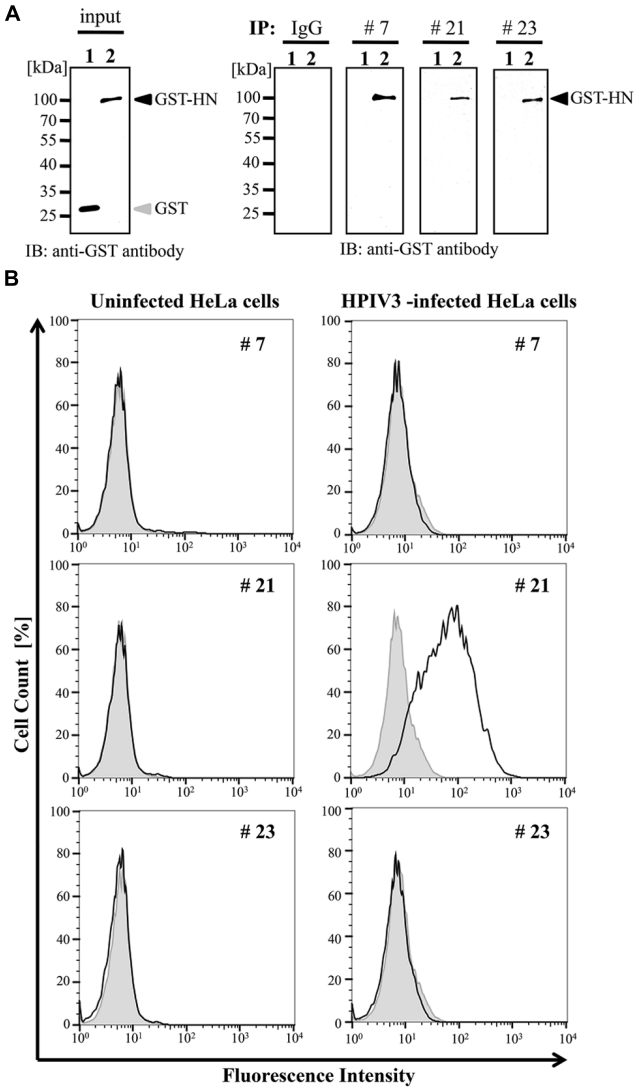


FIGURE 3 | Immunoprecipitation and flow cytometry assay. **(A)** Recombinant GST-HPIV3-HN or GST protein was immnoprecipitated with either #7, #21, #23 MAbs, or IgG (negative control), respectively. Then bound proteins were analyzed with immunoblotting using anti-GST antibody. **(B)** HPIV3-infected or uninfected HeLa cells were harvested at 4 days post-infection, followed by incubation with indicated MAbs. The cells were then fixed and stained with anti-mouse secondary antibody. The population of stained cells was calculated by flow cytometry. The shaded histogram shows negative hybridoma supernatant and the bold line shows specific MAbs.

Our results demonstrated that only #21 MAbs could specifically detect virus-infected cell populations, and distinguished between viral-infected and uninfected cell population with flow cytometory (**Figure 3B**).

EPITOPE MAPPING OF MAbs

To determine the binding domain of the MAbs within the HPIV3-HN protein, we synthesized six different deletion mutants of HPIV3-HN as depicted in **Figure 4A**. All of the deletion mutants contained N-terminal biotin tag were incubated with the antibodies (#7, #21, or #23), followed by the addition of AlphaScreen streptavidin donor and protein A acceptor beads, as depicted in **Figure 4B**. The reactivity was measured and calculated by the level of the AlphaScreen luminescent signal. The results showed that two MAbs (#7 and #23) reacted against the cytoplasmic tail (CT) of HPIV3-HN whereas #21 MAb detected extracellular stalk region of the protein (**Figures 4C–E**). This result is fully consistent with the result of flow cytometry analysis (**Figure 3B**).

SPECIFICITY OF MAb

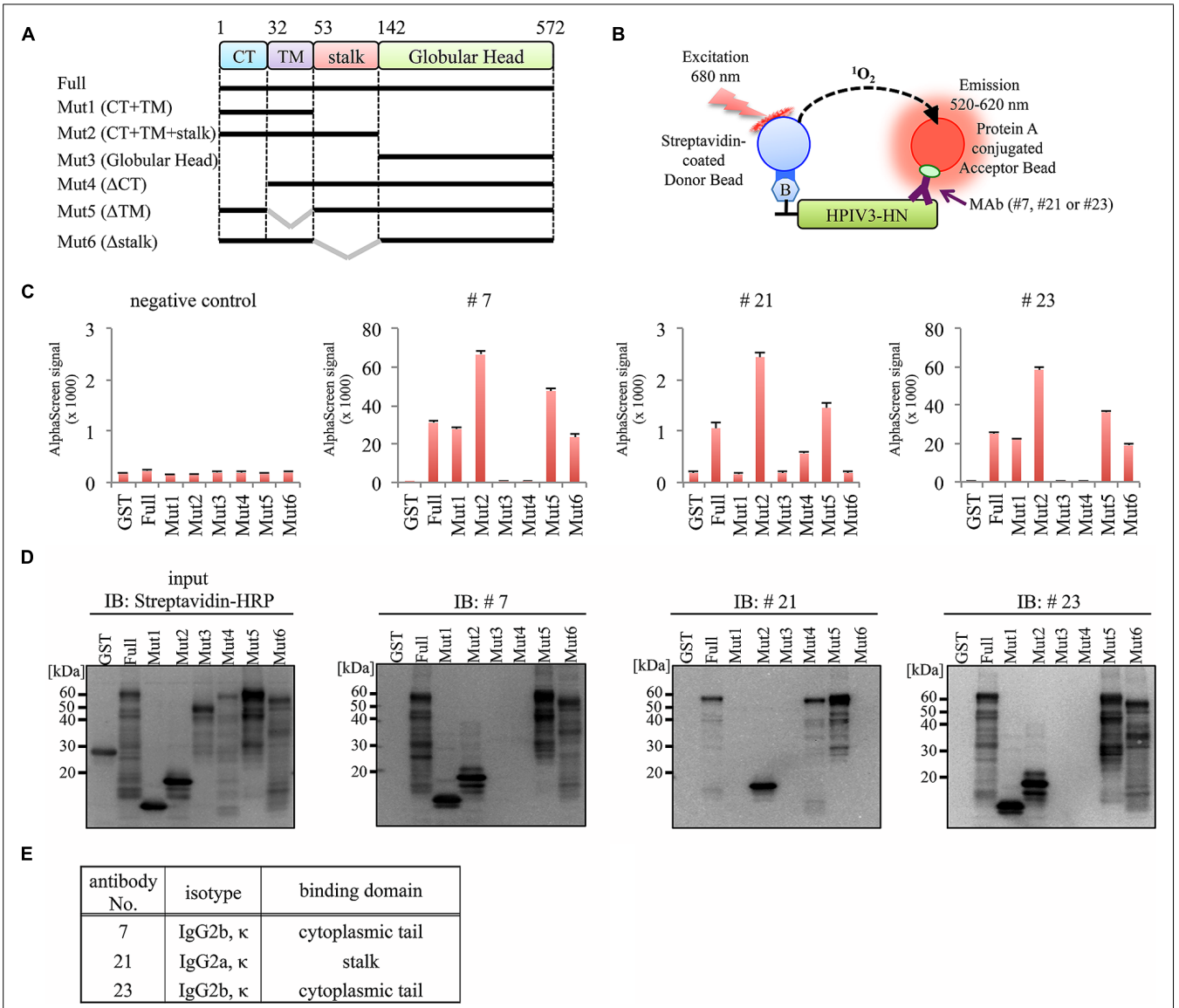
We next investigate the specificity of MAbs using HN protein frangments derived from HPIV1, Sendai virus (SeV), HPIV2, and Mumpus virus (MuV). Partial HN protein fragments containing cytoplasmic tail (CT), transmembrane domain (TM), and stalk region were produced with a N-terminal biotin tag by wheat cell-free system, and then incubated with the antibodies (#7, #21, or #23) followed by the AlphaScreen (**Figure 5A**). The antigen reactivity was measured based on the level of the AlphaScreen luminescent signal (**Figure 5B**). Notably, there was no cross-reactivity to HN proteins derived from other Paramyxoviruses except for HPIV3. This was also confirmed by immunoblotting analysis (**Figure 5C**). These results indicate the specificity of the antibodies for HPIV3-HN.

PROTEOMIC ANALYSIS

We next utilized our newly developed #21 MAb for the identification of host proteins that bind to HPIV3-HN during HPIV3 infection. Cell lysate from HPIV3-infected or mock-infected cells were immunoprecipitated with #21 MAb. Precipitated samples were collected and then digested with trypsin followed by LC-MS/MS analysis (**Figure 6A**). Annotation analysis using the Swiss-Prot database revealed that 10 proteins were putative HN binding proteins (**Figure 6B**). Based on the number of corrected peptides, top four proteins (HSP70, HSP90, tubulin, alpha 1c, and SERPINA3) were selected as the most likely candidates for association with HN and subjected to further binding analysis. The pull-down analysis of the host proteins with recombinant HPIV3-HN was performed. The subsequent immunoblotting analysis demonstrated that HPIV3-HN could indeed interact with these four proteins (**Figure 6C**). These results demonstrate the availability of our newly-developed antibody in comprehensive proteomic analysis.

DISCUSSION

Herein we produced HPIV3-HN proteins by the wheat cell-free system, and created MAbs that selectively target the HPIV3-HN protein. Characterization of the most potent MAb confirmed the antigen-specificity and usability in various applications including immunoblotting, immnofluorescent, flow cytometry, and immunoprecipitation analyses. Furthermore, the MAb could capture the endogenous HN protein from HPIV3-infected cells to identify HN-binding host proteins via mass spectrometry-based proteomic analysis. Our current results demonstrated the generation of useful antibody against HPIV3-HN and also shed new light



antibody No.	isotype	binding domain
7	IgG2b, κ	cytoplasmic tail
21	IgG2a, κ	stalk
23	IgG2b, κ	cytoplasmic tail

FIGURE 4 | Epitope mapping for generated antibodies. (A) Schematic diagram of seven deletion mutants of HPIV3-HN for epitope mapping. These proteins were produced as N-terminal biotinylated protein by wheat germ cell-free system. **(B)** Schematic diagram of the AlphaScreen assay used to detect the binding of MAb to full-length or deletion mutants of HPIV3-HN. The interaction between antibodies and recombinant proteins was monitored by AlphaScreen with protein A-conjugated acceptor beads and streptavidin-coated donor. Upon excitation at 680 nm, singlet oxygen molecules were produced from the donor beads, which reacted with the acceptor beads, resulting in light emission that was measured between 520 and 620 nm. **(C)** In AlphaScreen assay, the binding activity was measured as the level of the AlphaScreen luminescence signal. Error bars represent standard deviations from three independent experiments. **(D)** The biotinylated-full-length HN, its deletion mutants and GST proteins were separated by SDS-PAGE and transferred to PVDF membrane, followed by immunoblotting with Strepavidin-HRP andibody (left panel) and indicated MAb (right panel). **(E)** Summary of properties of selected MAbs. Immunoglobulin isotyping was carried out with mouse monoclonal antibody isotyping test kit.

on the unexplored molecular link between the PIV3-HN and host proteins.

Currently, cell-based production (e.g., *E. coli* system or baculovirus-insect cell system) of recombinant virus proteins has been widely used. However, it is often difficult to produce sufficient quantities of viral antigens in the conventional cell-based system because many viral antigens are usually insoluble, cytotoxic, and are expressed in the inclusion body fraction. In contrast, the cell-fee protein production system enables the synthesis of toxic proteins that are otherwise excluded from production in live cells. Among the cell-free approaches, the wheat germ cell-free system employs a eukaryotic translation system that warrants the synthesis of properly folded and biologically active proteins similar to proteins that are expressed in living mammalian cells (Endo and Sawasaki, 2005, 2006). These advantages underscore the suitability and availability of the wheat germ cell-free system for the generation of antigenic proteins that can be used for animal immunizations to generate

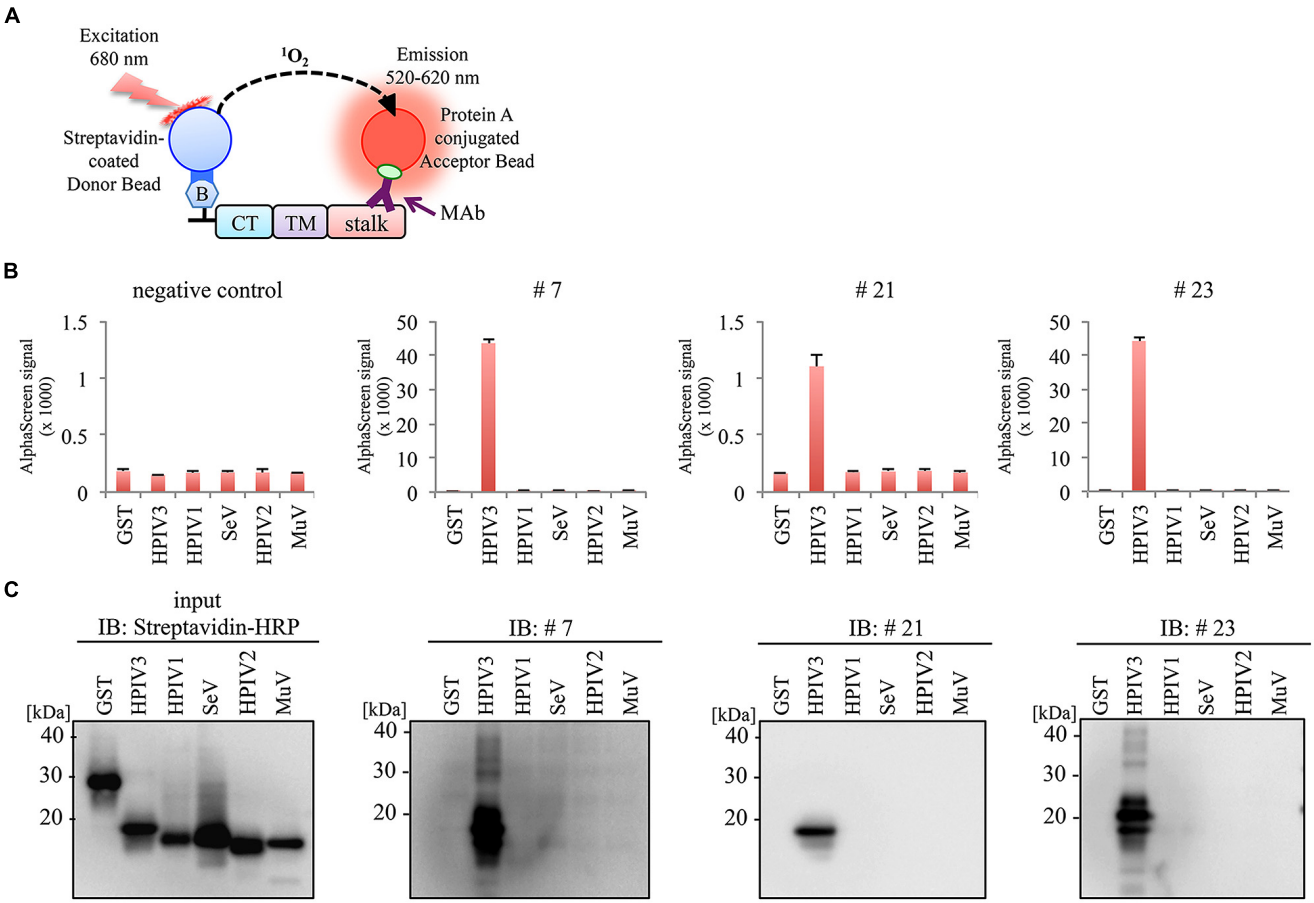


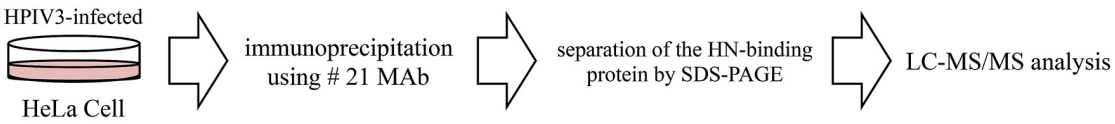
FIGURE 5 | No evident cross-reactivity of selected MAb to other paramyxoviruses. (A) Schematic diagram of AlphaScreen assay. The biotinylated partial HN proteins derived from several paramyxoviruses containing CT, TM and stalk region were produced by wheat germ cell-free system. **(B,C)** Specificity of MAbs was varidated by AlphaScreen assay **(B)** and immunoblotting **(C)**. In AlphaScreen assay, the binding activity was measured as the level of the AlphaScreen luminescence signal **(B)**. Error bars represent standard deviations from three independent experiments. The biotinylated partial HN proteins were separated by SDS-PAGE and transferred to PVDF membrane, followed by immunoblotting with either Strepavidin-HRP andibody (left panel) or anti-HN MAbs (right panel; **C**).

MAbs. Our current study clearly demonstrated the benefit of using viral proteins synthesized by the wheat germ cell-free system to efficiently produce the MAbs against the viral antigen. Using this approach, we have created MAbs against HPIV3-HN that detected both denatured and native forms of the antigen. These MAbs were useful in various immunological assays including ELISA, IF, immunoblotting, and immunoprecipitation. Further careful studies of structural aspects are needed to determine whether the MAb can affect the virus infectivity.

We identified Hsp70 as a putative HN binding protein. Several previous studies demonstrated that Hsp70 was involved in the regulation of other RNA viruses. Hsp70 is known to associate with viral PB1 and PB2 subunits of influenza A virus, and it negatively regulated the expression of viral proteins in infected cells (Li et al., 2011). In another study, Hantavirus infection induced the expression of HSP70 that interacted with nucleocapsid protein and its overexpression suppressed viral infection in Vero E6 cells (Yu et al., 2009). In contrast, Hsp70 was found to positively regulate rabies viral infection. Indeed, rabies infection induced the cellular expression of Hsp70 and accumulation in Negri body-like structures, which are the site of viral transcription and replication. Inhibition of Hsp70 resulted in a significant decrease of viral mRNAs, viral proteins, and virus particles (Lahaye et al., 2009, 2012). Taken together these results indicated a pivotal role of Hsp70 in viral replication and the pathogenicity of viral infection. Hsp70 binds and regulates many cellular proteins, as well as viral proteins (Pratt and Toft, 2003; Mayer, 2005; Silver and Noble, 2012), and the effects of Hsp70 on viral infection are diverse and unique between different viral species or cell systems. Further studies are required to investigate the precise molecular mechanism by which the association of Hsp70 and HN proteins mediate HPIV3 replication.

We also found that PIV3-HN can interact with Serpin3a. Serpin3a, as also known as alpha-1-antichymotrypsin, is a member of serpin proteins involved in the inhibition of serine and other types of proteases (Bauman et al., 2002). In humans, the majority

A



B

Accession number	name	peptide	Da	Molecular function	Subcellular localization
P08107	HSP70	10	70,052	Chaperone	cytoplasm
P08238	HSP90	6	83,264	Chaperone	cytoplasm
Q9BQE3	Tubulin alpha-1C	6	49,895	structural constituent of cytoskeleton	cytoplasm, cytoskeleton, microtubule
P01011	SERPINA3	6	47,651	protease inhibitor	secreted
P07195	LDH-B	5	36,638	oxidoreductase	cytoplasm
P04406	GAPDH	5	36,053	oxidoreductase, transferase	cytoplasm, cytoskeleton, membrane, nucleus
P10809	HSP60	4	61,055	chaperone	mitochondrion
P06733	MBP-1	4	47,169	lyase, repressor	cell membrane, cytoplasm, membrane, nucleus
P14618	PKM	4	57,937	kinase, transferase	cytoplsdm, nucleus
P60842	eIF4A1	3	46,154	helicase, hydrolase, initiation factor	cytoplasm

C

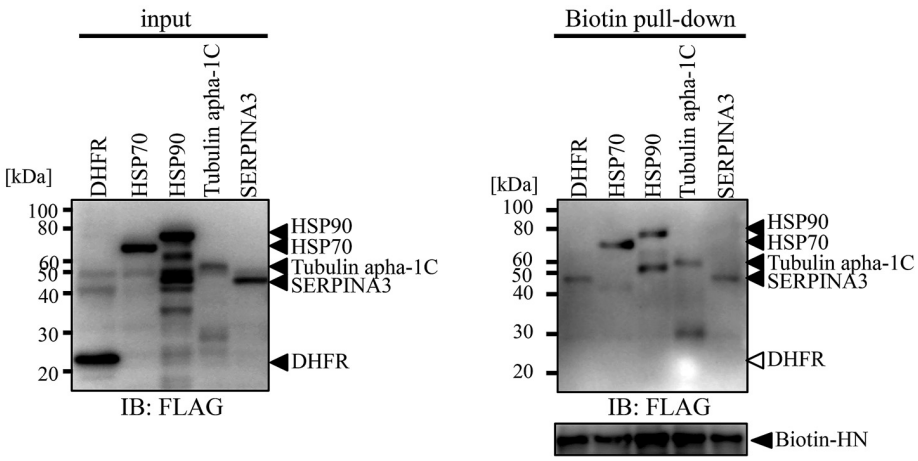


FIGURE 6 | Proteomics analysis using selected MAb. (A) Schematic diagram of proteomic analysis for the identification of PIV3-HN-binding protein. HPIV3-infected HeLa cell lysate was immunoprecipitated with #21 MAb. The bound proteins were separated by SDS-PAGE and analyzed by LC-Ms/Ms. **(B)** The panel shows the list of putative HN-binding proteins identified by mass spectrometry analysis. **(C)** FLAG-tagged HSP70, HSP90, tubulin alpha 1C, or SERPINA3 proteins were mixed with HPIV3-HN. Samples were pull-down with the streptavidin magnetic beads and the collected proteins were separated by SDS-PAGE. The bound protein detected by immunoblotting analysis with anti-FLAG antibody. The right arrows indicated the position of each protein.

of serpins regulates the functions of proteases involved in the response against body's injuries such as coagulation, fibrinolysis, inflammation, wound healing and tissue repair. Serpins have been also implicated in various pathologies in respiratory system such as airway hyperresponsiveness (AHR) and asthma (Sivaprasad et al., 2011). The physical interaction of HPIV3-HN with serpin3a may be involved in hindering the function of Serpin3a toward respiratory disorders. Further analysis may shed new light on understanding the etiology of HPIV3-induced asthma.

In summary, we utilized the wheat cell-free production system to create and characterize MAbs that may be useful in various immunological applications. Our newly-developed MAbs could thus provide a valuable means to explore HPIV3 infection in human cells.

ACKNOWLEDGMENTS

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