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Current Opinion in Virology

The mucosal immune system of the respiratory tract

Shintaro Sato and Hiroshi Kiyono

Most viruses use host mucosal surfaces as their initial portals of infection. The respiratory tract has the body's second-largest mucosal surface area after the digestive tract. An understanding of the unique nature of the mucosal immune system of respiratory organs is therefore extremely important for the development of new-generation vaccines and novel methods of preventing and treating respiratory infectious diseases, including viral infections.

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Introduction

The respiratory, digestive, and reproductive tissues are located inside the body but are continuously exposed to the outside world. Thus they serve as gateways with a surveillance function for the acceptance of beneficial antigens from the outside environment and an immunological function for the rejection of non-beneficial antigens [1]. When the host takes in oxygen via the respiratory tract, essential nutrients via the digestive tract, or (in the case of females) spermatozoa via the reproductive tract, the organs in these tracts are at high risk of the invasion of pathogenic microorganisms. Mucosal epithelial cell layers cover the surfaces of these organs in the same way as skin covers the outside of the body, and they form physical and immunological barriers that prevent the invasion of harmful non-self materials. In addition, most of these mucosa-associated organs, apart from the lower respiratory tract (i.e. the lungs) possess 'resident flora,' which peacefully cohabit with the host and create a mutually beneficial environment. Therefore, the mucosa needs to distinguish not only beneficial and detrimental materials, but also resident and pathogenic bacteria. Beneficial materials or resident bacteria need to be actively taken up, whereas detrimental materials or harmful bacteria need to be selectively excluded. This mechanism is controlled by the mucosal immune system (MIS) [1]. In this review, we focus on the unique nature of mucosal immunity in the respiratory tract and its contribution to the control of viral infection, with an emphasis on the influenza virus as an example of viruses that invade via the respiratory mucosa.

Anatomical uniqueness and physical barrier function of the airways

Mammalian respiratory systems are divided mainly into upper (from the nasal and oral cavities to the throat) and lower (trachea and lung) systems. The two airway compartments are environmentally separated by the glottis, and in healthy people the lower respiratory tract is essentially sterile. Therefore, once microbes colonize the lower tract and grow there, inflammatory responses that can lead to pneumonia are induced. In contrast, the upper respiratory tract is the entrance way for oxygen inhaled in the ambient air, and the mucosal surfaces in this part of the system are consistently exposed not only to resident or opportunistic microorganisms (e.g. *Streptococcus pneumoniae* and *Haemophilus influenzae*) [2], and pathogenic microorganisms (e.g. *Corynebacterium diphtheriae* and influenza virus) [3,4], but also to foreign environmental substances such as various kinds of chemical materials (e.g. tobacco smoke) and allergen particulates (e.g. pollen and house dust).

The luminal side of the respiratory tract is physically protected by layers of epithelial cells that are adhered tightly to each other at tight junctions by occludin and various members of the claudin family, and at adherens junctions by E-cadherin [5,6]. The epithelial cells have well-developed cilia and produce mucus composed primarily of polysaccharides such as mucin (MUC) [7]. Of the different members of the MUC family, MUC1, 4, and 16 are membrane bound and MUC2, 5AC, 5B, and 19 are secreted-type mucins associated with the respiratory tract [8]. Expression of MUC5AC and 5B is increased in respiratory diseases such as asthma and chronic obstructive pulmonary disease via IL-13 and STAT6 signaling pathways [9]. At the bronchi, mucus and movement of cilia act cooperatively to prevent large foreign bodies (>5 μm) from drifting into the alveoli. Foreign bodies that do become trapped are carried toward the mouth by ciliary movement and expelled by coughing. This mechanism is called mucociliary clearance and is important in phylaxis [10]. However, in general, small foreign bodies, including most pathogens, can easily escape the physical barrier system and arrive at the pulmonary alveoli by

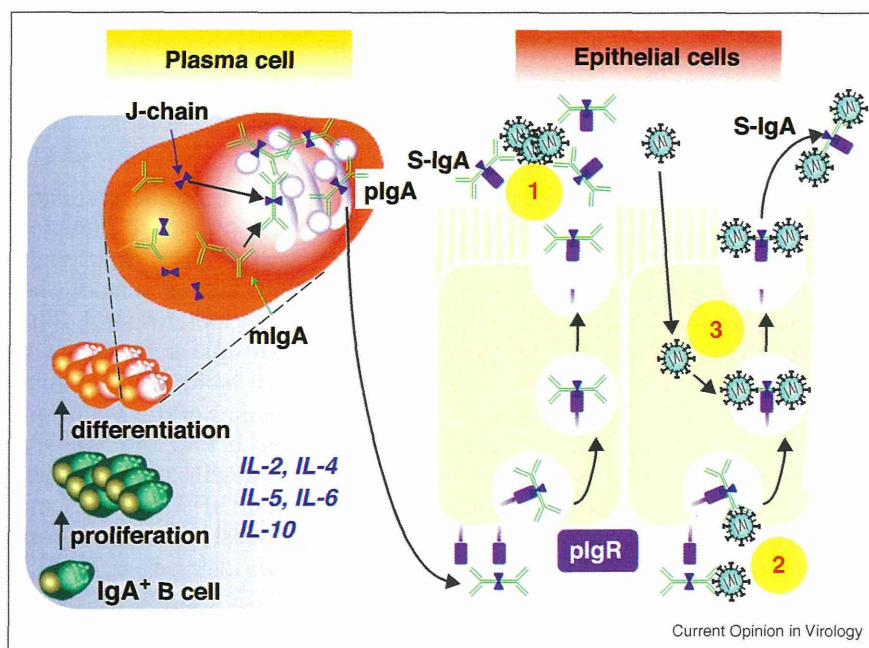
inhalation. A highly developed and orchestrated immunological defense system is therefore needed in the airways.

Induction and regulation of acquired immunity via the MIS

In general, the MIS consists of inductive and effector sites [11]. The inductive tissue consists of secondary mucosa-associated lymphoid tissue (MALT), represented by Peyer's patches (PPs) in the small intestine and nasopharynx-associated lymphoid tissue (NALT) in the rodents nasal cavity [11,12]. In the inductive tissue, mucosally introduced antigens are taken up from the luminal to the basolateral side of the epithelium by antigen-sampling cells. They are then processed and presented by antigen-presenting cells (APCs) such as dendritic cells (DCs) to immunocompetent cells including naïve lymphocytes. The DCs migrate to the T-cell region of the MALT and then present the peptide form of the uptaken antigen to the naïve T cells. In the B-cell region, germinal center formation and antibody class switching occur [13]. A class switch to IgA predominantly occurs in the MALT by the action of the IgA-associated cytokine family of TGFβ, IL-2, IL-4, IL-5, IL-6, and IL-10 [14,15]. Post-switched IgA$^+$ B cells egress from the MALT through efferent lymph vessels under the control of the sphingosine-1 phosphate system, a lipid mediator; they then enter the body circulatory system [13,16]. Finally, these cells migrate to the mucosal layer of the effector tissue and form the necessary cellular network among Th1, Th2, Th17, Treg, and cytotoxic T cells, B cells, and DCs, together with epithelial cells, to provide the appropriate defensive responses.

The MIS thus consists of a unique lymphocyte migration system. For example, IgA$^+$ B cells class-switched in the MALT usually express CCR10 on their surfaces [17], and these cells can migrate independently along antigen administration routes such as peroral and transnasal to various tissues, such as distant mucosal epithelia, in which the specific ligand molecule for CCR10, the chemokine CCL28, is produced. Thus mucosal immunization can induce similar immune responses at both local sites of antigen deposition and distal sites [18–20]. Nasal immunization can therefore effectively induce an antigen-specific mucosal immune response in the reproductive tissue in addition to the respiratory tract [21,22]. Recently, Cha *et al.* have demonstrated that intranasal immunization with adjuvant or virus itself accelerates CCL28 expression in both the uterus and the nasal cavity [23•]. This immunological interconnected circulation pathway of lymphocytes is often called the common mucosal immune system (CMIS); it is peculiar to the

Figure 1



Assembly of IgA in IgA plasma cells, and epithelial transcytosis of pIgA by pIgR. IgA$^+$ B cells, which arrive at the effector tissue, proliferate and differentiate into IgA plasma cells in response to the indicated cytokines. In the IgA plasma cells, mIgA and a J-chain are assembled to form pIgA just before its externalization. The J-chain is required for the interaction between pIgA and pIgR, which are expressed on the basolateral surfaces of adjacent epithelial cells. The pIgA–pIgR complexes move to the apical surfaces of the epithelial cells by transcytosis and are then released to the luminal side by digestion of part of pIgR. There are at least three mechanisms of virus neutralization: (1) S-IgA recognizes the viral epitope and inhibits attachment to the epithelial cells; (2) pIgA can sense and then eliminate viruses that invade the lamina propria; or (3) Viruses that have invaded the cell can be recognized by pIgA–pIgR complexes during their transcytosis.

MIS and is not found in other immune systems [24]. An understanding of the CMIS that bridges the respiratory and reproductive immune systems is very important for the development of mucosal vaccines against sexually transmitted infectious diseases, including papilloma virus and HIV.

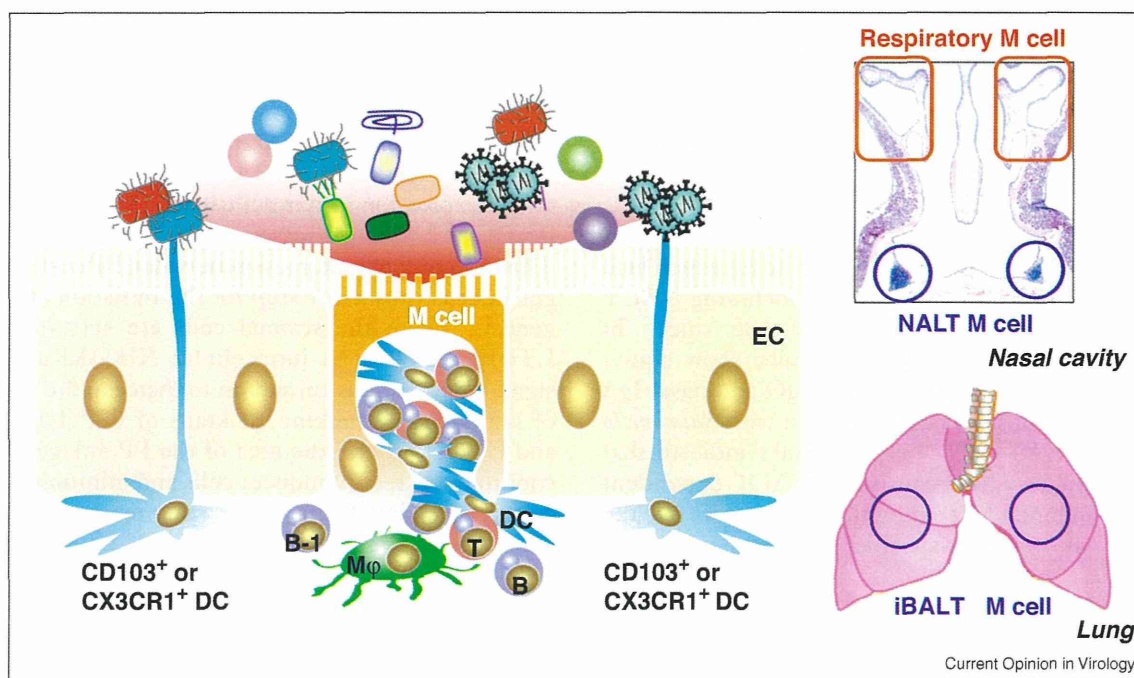
When the IgA^+ B cells arrive at the effector tissue, they are finally differentiated into IgA-producing plasma cells [14]. In the IgA plasma cells, monomeric forms of IgA are tied together by J-chains to form polymeric (pIgA) IgA (Figure 1) [1,25]. pIgA is transported to the luminal side of the epithelial cells by making a complex with poly Ig receptors (pIgR) expressed on the basal membrane side of the epithelial cells [26]. Part of the pIgR is digested on the luminal side, leading to the formation of secretory IgA (S-IgA), which is then released into the lumen (Figure 1) [26]. S-IgA binds to critical viral epitopes that have infected the mucous membrane and then neutralizes their biological activity; this leads to strong inhibition of viral growth both *in vitro* and *in vivo* [27]. For example, S-IgA and pIgA to hemagglutinin, which is a major component of the viral surface including the influenza virus, inhibit cellular attachment and internalization, and intracellular replication of virus, respectively. An *in vitro* experiment has suggested that, in addition to the extracellular virus neutralization activity of secreted pIgA, pIgA can neutralize intracellular viruses, including influenza virus, during pIgR-mediated transport (Figure 1) [27].

The airway MIS: uniqueness of the secondary MALT and antigen-uptake system

Rodent NALT is located on both sides of the nasopharyngeal duct, dorsal to the cartilaginous soft palate. NALT is considered to be a counterpart of the human Waldeyer's ring, which includes the palatal tonsils and adenoids, and it has an important role in the induction and modulation of mucosal immunity in the upper respiratory tract [11,28].

Unlike the peripheral lymph nodes, MALT, including NALT, does not have afferent vessels [29]. Instead, antigens are usually taken in directly from the luminal side of the aerodigestive tract and are instantly captured by DCs waiting immediately beneath the epithelial layers. Although intestinal and lung alveolar DCs, which express CX3CR1 and CD103, respectively, on their surfaces, can directly recognize antigens by extending their dendrites through the tight junctions between the

Figure 2



Microfold cells (M cells) specialize in antigen uptake and their presence in the respiratory mucosal immune system. The cilia of M cells are shorter than those of conventional epithelium cells. On its basal side, the M cell develops a pocket-like structure that can hold immunocompetent cells. M cells, like macrophages, function in active antigen uptake. Because lysosome development in M cells is poor, in most cases the incorporated antigens are just passed through the M cells unmodified and then taken up by DCs. In addition to M cells, DCs that are located in the pulmonary alveoli and express CD103, or in the intestine and express CX3CR1, can directly take up luminal antigens by extending their dendrites. M cells can be found in three different locations in the respiratory tract namely the NALT FAE, the respiratory epithelium, and the iBALT FAE.

epithelial cells to reach the lumen [30,31], antigen is taken up mainly via specialized antigen-sampling cells called microfold cells (M cells) that use a transcytosis apparatus (Figure 2) [32,33]. M cells are found in the follicle-associated epithelium (FAE) covering the MALT and have short cilia that are less well developed than those of neighboring ciliated epithelial cells [34]. In addition, on their basolateral side, M cells have a pocket structure that holds DCs and/or lymphocytes, making this antigen-sampling system able to speedily and effectively deliver antigens to DCs without any antigen modifications [34]. M cells located in the NALT FAE can serve as invasion and sampling sites for not only respiratory pathogens (e.g. group A *Streptococcus* (GAS) [33]), but also intestinal pathogens (e.g. reovirus [35]).

Recently, we discovered cells in the murine nasal cavity epithelium that resembled M cells morphologically and functionally [36•]. Because these cells have the hallmark characteristics of classical M cells but are anatomically located away from the NALT epithelium (e.g. in the turbinate epithelium of the nasal cavity), we named the cells 'respiratory M cells'. Unlike the NALT M cells the respiratory M cells have no pocket structure, but $CD11c^{+}$ DCs, which are distributed throughout the nasal passages in the resting state, migrate to the area underneath the respiratory M cells to receive antigens after intranasal GAS infection [36•].

The number of respiratory M cells per individual nasal cavity is 5–10 times the number of NALT M cells, the average number of which is 200–300 per mouse. This difference might just be a reflection of the large surface area of the nasal turbinates and the relatively small area of the NALT FAE.

Interestingly, the same numbers of respiratory M cells as in wild-type mice exist in Id2-deficient mice, which lack almost all secondary lymphoid tissues, including NALT [36•,37], when compared with wild-type mice. In addition, Id2-deficient and wild-type mice show equivalent levels of antigen-specific serum IgG and nasal IgA production in response to nasal infection with *Salmonella typhimurium* or GAS [36•]. These findings indicate that the upper respiratory MIS consists of NALT-dependent and NALT-independent induction pathways for the generation of antigen-specific mucosal immune responses.

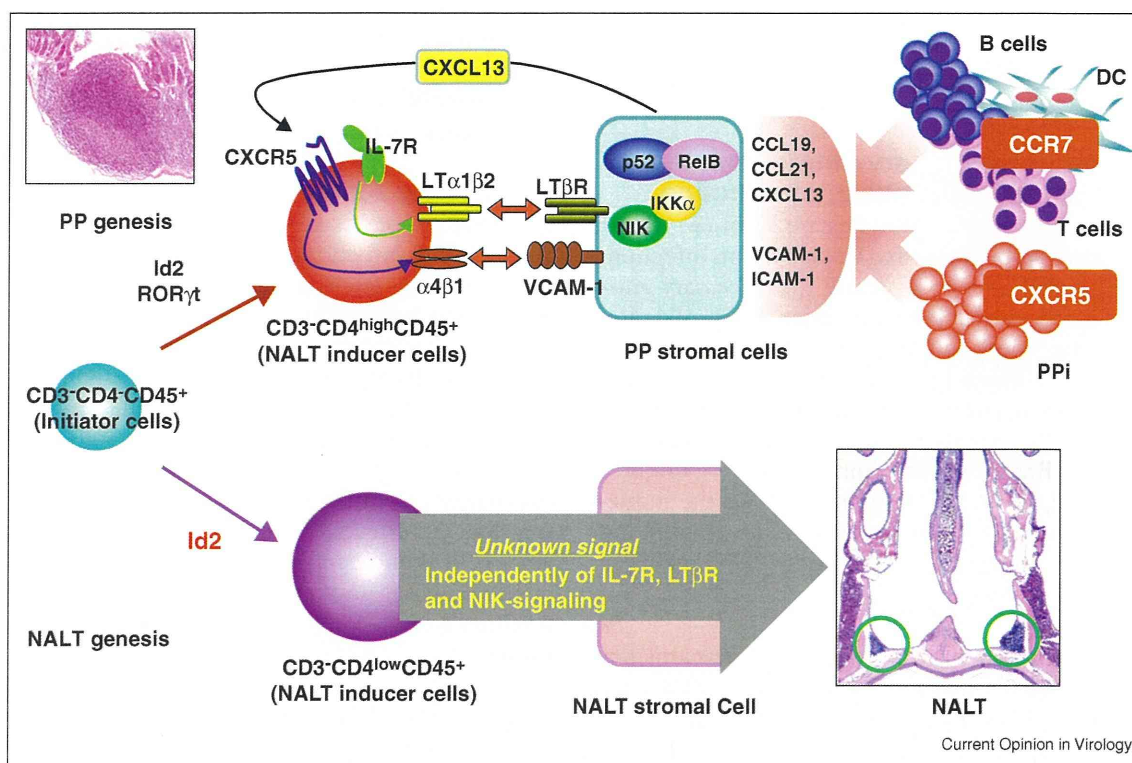
In addition to the presence of M cells in the upper respiratory tract, there have been a few reports of the existence of pulmonary M cells. Teitelbaum *et al.* have shown that *Mycobacterium tuberculosis* rapidly enters through M cells, which might be present on inducible bronchus-associated lymphoid tissue (iBALT: see later section) [38]. The respiratory epithelium therefore has at least three distinct pathogen-invasion and/or antigen-sampling sites: respiratory and NALT M cells in the upper respiratory tract, and pulmonary M cells in the lower respiratory tract. It may be possible that various respiratory pathogens have their own preferred entry sites: for example, the upper respiratory tract M cells for GAS and the pulmonary M cells for *M. tuberculosis*. In support of this speculation, one human study found that a human influenza virus receptor that possesses sialic acid linked to galactose by an α-2,6 linkage (SAα2,6Gal) is dominantly expressed on nasal epithelial cells, whereas a receptor for avian influenza viruses, including H5N1, is found only on alveolar type-II cells in the lower respiratory tract [39]. It is an important future task to extensively characterize the use of the various influenza virus receptors among the different M cells located in the upper and lower respiratory tracts.

Unique characteristics of NALT organogenesis

The organogenesis mechanisms for peripheral lymph nodes and PPs, which are the representatives of MALT in the intestinal tract, are well studied [40]. Organogenesis of these tissues starts during the fetal period with the inflow of lymphoid tissue inducer (LTi) cells to the respective lymph node anlagen [41,42]. LTi cells, the development of which depends on two key transcription factors, Id2 and RORγt, have a common phenotype represented by $CD3^{-}CD4^{+}CD45^{+}$. PP inducer (PPi) cells initially develop in the fetal liver with the expression of IL-7R, α4β1 integrin, and CXCR5 [43,44]. Because the stromal cells around PP anlagen express CXCL13, which is a ligand for CXCR5, PPi cells specifically migrate to the anlagen site and then interact with the stromal cells [45,46]. The PPi cells are then stimulated by IL-7 produced by intestinal stromal and/or epithelial cells, leading to the expression of lymphotoxin (LT)-α1β2, which is a ligand for LTβR expressed on the stromal cells [47–49]. The bidirectional interaction between PPi cells and anlagen cells is thus a key step for the initiation of PP tissue genesis. When the stromal cells are activated via the LTα1β2–LTβR, an intracellular NIK-IKKα–p52/RelB signaling cascade is turned on to increase the production of lymphoid chemokine cocktails of CXCL13, CCL19, and CCL21 around the area of the PP anlagen and thus continuously recruit inducer cells and immunocompetent cells expressing CCR7—the receptor for CCL19 and CCL21—for the development of PPs [50].

NALT organogenesis has several developmental features distinct from those of PP tissue genesis (Figure 3) [11]. First, the NALT organogenesis program starts after birth, whereas PP tissue genesis is initiated and completed during the embryonic period. Second, in the case of PP genesis, the interaction of LTα1β2 and LTβR is the most important step, but the NALT structure is found in both LTα-deficient and NIK-deficient mice, indicating that NALT is developed by a mechanism independent of

Figure 3



Distinct differences in organogenesis programs between PPs and NALT. Peyer's patch (PP) genesis is initiated at the embryonic stage by migration of a few $CD3^-CD4^{high}CD45^+CXCR5^+$ PP inducer cells (PPi) to organizable stromal cells. This migration is controlled by the CXCR5-CXCL13 axis. CXCR5 signals induce a structural change in α4β1 integrin, enabling it to interact strongly with VCAM-1 on the stromal cells. The PPi are then activated by IL-7, leading to expression of the membrane type of lymphotoxin (LT)-α1β2. This LTα1β2 activates a non-canonical NF-κB pathway in the stromal cells via the LTβ-receptor (LTβR), NIK, and IKKα. The activation of NF-κB induces the expression of lymphoid chemokines such as CCL19, CCL21, and CXCL13 and of adhesion molecules such as VCAM-1 and ICAM-1. In response to the expression of these chemokines, immunocompetent cells, including T and B cells, and DCs, which express CCR7 (a receptor for CCL19 and CCL21), and additional PPi are accumulated at the site of PP formation. Therefore, all of the above-mentioned molecules are essential for PP organogenesis. In contrast to PP genesis, NALT genesis is initiated postnatally and is completely independent of IL-7R, LT, LTβR, and NIK-signaling. Whereas PPi require Id2 and RORγt transcription factors for their development from $CD3^-CD4^-CD45^+$ initiator cells, differentiation of $CD3^-CD4^{low}CD45^+$ NALT inducer cells does not require RORγt.

LTα1β2–LTβR signaling. In addition, NALT genesis is found in RORγt-deficient [51], but not in Id2-deficient mice [37]. These observations suggest that the uniqueness of NALT organogenesis stems from the uniqueness of NALT inducer cells.

Inducible bronchus-associated lymphoid tissue for protective immunity

Bronchus-associated lymphoid tissue (BALT) was initially described as a family member of MALT located in the lower respiratory tract [52]. Well-developed BALT is found in a rabbit and feline lung as a region covered by the FAE including M cells similar to PP and NALT; however, BALT is not usually found in the lower respiratory tract of humans and mice [52–54]. In these species, the BALT structure is induced by inflammatory stimulation resulting from influenza virus infection or pneumonia. Therefore, this lymphoid structure belonging to the tertiary lymphoid tissues is often called inducible BALT (iBALT) in humans and mice [55]. The functional and structural characteristics of iBALT resemble those of conventional BALT after influenza infection [55]. In addition, influenza-induced iBALT has the potential to prime influenza-specific T and B cells and to clear virus with the aid of $CD8^+$ T cells, without support from other lymphoid tissues [55]. Although the mechanism of iBALT formation is poorly understood, these studies have also indicated that LTi cells and LTα1β2–LTβR signaling are not required for iBALT formation, because both LTα-deficient and RORγt-deficient mice develop iBALT after influenza infection. It was recently reported that LPS-induced IL-17 produced by $CD4^+$ T cells induces the production of CXCL13 and CCL19 in an LTi cells-independent and lymphotoxin-independent manner, resulting in initiation of the first step for the recruitment of lymphocytes at the anlagen site of iBALT [56•,57]. To develop novel influenza vaccines it might be useful to consider the critical role of iBALT in the

induction of protective immunity. An interesting strategy might be to develop a vaccine that supports the induction of iBALT generation simultaneously with the classical induction of influenza-specific S-IgA and cytotoxic T-lymphocytes.

Contribution of respiratory innate immune responses in the initial phase of infection

Mucosally induced S-IgA antibodies are a critical arm of acquired immunity for protecting the host from infection, but it takes several days to generate the necessary antigen-specific S-IgA at the sites of invasion and replication of an infectious agent. During that time, the innate immune response, which can respond promptly but functions in an antigen non-specific manner, confronts the pathogen by producing inflammatory cytokines and type I IFNs [58,59•]. Because some inflammatory cytokines (e.g. IL-6, IL-12, and TNFα) are needed for the induction of antigen-specific T and B cells, the innate immune response is also extremely important for inducing acquired immunity [58].

In the case of influenza virus infection, the virus attaches to respiratory mucosal epithelial cells and then invades the cytoplasm by endocytosis [60]. Fusion of the endosomal membranes of the epithelial cell with the viral envelope is then observed. This fusion makes a gate pore linking endosome and cytoplasm; thus single-strand RNA (ssRNA) representing the genome of the influenza virus is released into the cytoplasmic region of the epithelial cell [60]. It has been well known that ssRNA viruses including influenza virus are recognized by two kinds of innate immune receptor. Toll-like receptor (TLR)7 (and also TLR8 in humans) is located on the endosome, and its ligand-binding site is turned toward the inside of the endosome [59]. TLR7/8 can sense ssRNA released into the endosome [61,62]. RIG-I recognizes genomic RNA released into the cytoplasmic region of infected cells [63,64]. TLR7/8 and RIG-I activated by ssRNA trigger intracellular signal transductions leading finally to prompt production of type I IFNs and inflammatory cytokines via activation of the transcription factors IRF3/7 and NF-κB [59]. In addition to these above-described receptors that recognize ssRNA, it has recently been reported that NLRP3 inflammasome recognizes the M2 protein of influenza virus and activates caspase-1, leading to the production of IL-1β, IL-18, and IL-33 [65•].

Murine NKp46 (NKp44 in humans), which is a common natural killer (NK) cell-surface marker, can recognize hemagglutinin, which is a major component of the surfaces of viruses, including the influenza virus [66,67]. Therefore, when hemagglutinin is expressed on the surface of the infected host cell, NK cells recognize the infected cells immediately and are activated; the subsequent cytotoxic activity can control the growth of the virus. In the lung, the ratio of NK cells to all lymphocytes (CD45-positive cells) is about 10%—markedly higher than in other organs [68]. In mice depleted in NK cells, the mortality rate from influenza infection of the lower respiratory tract is increased [69,70], indicating that the pulmonary NK cells play an important role in the phylaxis of influenza virus infection.

Concluding remarks

In recent years, anxiety over epidemics of emerging and re-emerging infectious diseases—especially respiratory tract infections such as seasonal and pandemic influenza, SARS, and tuberculosis—has increased worldwide, and effective methods of prevention and therapy need to be developed. In particular, the threat of pandemics of novel strains of highly pathogenic avian influenza has become a major social problem. Although there are some antiviral drugs that target NA and M2 proteins, for maximum prevention of infections with this virus we need to develop an effective vaccine with cross-reactivity. To achieve this, molecular- and cellular-level understanding of the mucosal immunity of the respiratory tract is very important, and continuous analysis will be needed in future.

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