

pool and human influenza genes (antigenic shift). The A subtype viruses and B viruses cause an antigenic drift every year, resulting in an annual epidemic or local outbreak of influenza. To recover from influenza or to prevent influenza, both innate and adaptive immune responses must be induced in the respiratory mucosa following viral infection. Thus, the respiratory tract mucosa is not only the site of infection by influenza viruses but also the site of defense against viral infection in the host.

Most influenza viruses are detected and destroyed within a few hours by the innate immune mechanisms, which are not antigen-specific and do not require a prolonged period of induction (2,3). Several components such as mucus, macrophages, interferon (IFN)  $\alpha$ ,  $\beta$  and other cytokines, fever, natural killer (NK) cells and complement are involved in the innate immune system. If influenza viruses can escape these early defense mechanisms, they are detected and eliminated by adaptive immune mechanisms, where T and B cells and their products function as antigen-specific effectors (cytotoxic T lymphocytes [CTLs] and antibodies [Abs]) to target the virus. Also, antigen-specific memory cells (T and B cells) are involved in the prevention of the subsequent viral infection. The effector cells and molecules involved in the defense mechanisms following influenza virus infection are shown in Fig. 1. This review summarizes recent findings on these defense mechanisms induced following influenza virus infection in the respiratory tract mucosa. Furthermore, this review discusses that the development of a mucosal vaccine which is capable of inducing both secretory-IgA (S-IgA) and serum IgG Abs will be strategically important to control influenza. Such a vaccine can provide broad cross-protection against variant viruses including viruses with pandemic potential by the S-IgA Abs and prevent lethal influenza pneumonia by the serum IgG Abs.

## 2. Innate Immunity against influenza

### 2-1. Effectors involved in innate immunity

Influenza virus infection triggers the innate immune responses where the following effector cells, molecules and factors are implicated in the restriction of viral spread. Some

of these effectors work within a few days following infection, not only as helpers to keep infection under control, but as communicators (antigen-presenting cells [APCs]) or activators (cytokines) in the subsequent adaptive immune response. Examples of the action of these effectors are listed in the following text. i) Inhibitory factors in the mucus of the respiratory tract, that are similar to or identical to N-acetylneuraminic acid-containing receptors for HA molecules reduce markedly the ability of the viruses to infect host cells (4-6). ii) Nasal and pulmonary levels of IFN- $\alpha$  and - $\beta$  rise rapidly after infection and correlate directly with the degree of viral replication in ferrets, mice and humans (2,7,8). Alveolar macrophages or lymphocytes from infected lungs have been shown to release interferons in vitro (8). iii) Macrophages secrete IL-1, IL-6, TNF- $\alpha$  and IL-12 which activates NK cells (9). Macrophages recovered from infected lungs mediate lysis of infected cells, probably through apoptosis-dependent phagocytosis (10,11). In addition, cytokines (IL-1, TNF- $\alpha$  and IL-6) produced by macrophages induce fever, and the magnitude of the febrile response correlates strongly with the level of virus shedding in humans and animals (2,12). iv) The NK cells that are detected in pulmonary lymphocytes 48 h after influenza virus infection produce IFN- $\gamma$  and limit viral spread by virus-infected cell lysis, which is mediated probably by pore formation in the infected cells involving perforin (13-16). v) Complement mediates protection; this is highlighted by the increase in mortality in C5-deficient mice infected with a lethal dose of influenza virus (17,18).

### 2-2. Enhancement of innate immunity

Efforts to activate the effectors involved in innate immune responses would lead to the enhancement in the adoptive immune responses, because some of the effectors act in both systems as a bridge. For example, the intranasal administration of cholera toxin (CT) prior to infection with influenza viruses activates macrophages and NK cells via the binding of CT to the receptor (ganglioside GM1) on the cells and results in non-specific replication reduction of the infected viruses in the respiratory tract (19) (Fig. 2). The non-specific activation of macrophages (APCs) and the enhancement of cytokine production by CT cause the subsequent enhance-

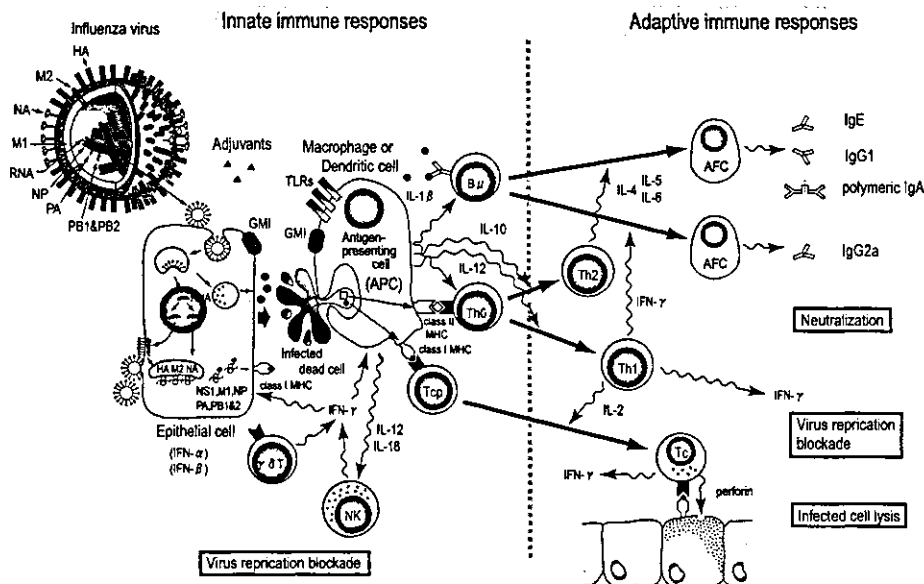


Fig. 1. Defense mechanisms induced by influenza virus infection.

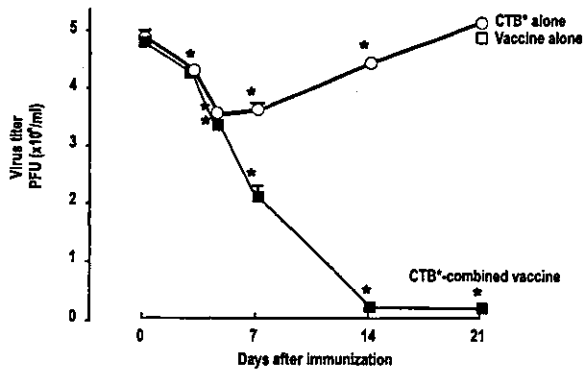


Fig. 2. Non-specific reduction of influenza viral replication by pre-treatment with cholera toxin adjuvant (CTB\*). Mice were immunized intranasally with CTB\* (1  $\mu$ g) alone (○), A/PR8 vaccine alone (1  $\mu$ g) (□) or, CTB\*-combined vaccine (■), and then challenged 3, 5, 7, 14 and 21 days later with A/PR8 viruses. Three days after challenge, the virus titer in the nasal wash (PFU/ml) was measured. Each value represents the mean  $\pm$  SD of the virus titer/ml in the nasal washes of the mice in each group of six mice. The asterisk represents a statistically significant difference between non-immunized and infected mice.

ment of the adaptive immune responses against influenza viruses, which results in complete protection against infection. Similarly, pretreatment of mice with *Propionibacterium acnes* (*Corynebacterium parvum*) or baculovirus before a lethal viral challenge results in lower lung-viral titers and lower mortality (20-22). In these cases, various constituents of the pathogens used for the pretreatment can activate macrophages and dendritic cells (DCs) via members of the Toll-like receptor (TLR) family on these cells to induce protection against infection (23-25). For example, bacterial DNA has stimulatory effects on mammalian immune cells by the presence of unmethylated CpG dinucleotides, which are far more common in bacterial DNA than in vertebrate DNA (26,27). Recognition of CpG DNA by mammalian immune cells is mediated by TLR9, which can distinguish bacterial DNA from self-DNA (23). Intranasal administration of CpG DNA can enhance mucosal Ab responses to co-administered inactivated influenza vaccines (28). Thus, various constituents of the pathogens can be used to enhance innate immune responses via various receptors, such as TLRs and GM1 ganglioside, on macrophages and DCs, so as to induce augmented mucosal immune responses in the respiratory tract (29).

### 3. Adaptive immunity against influenza

#### 3-1. Roles of APCs in adaptive immunity

APCs (macrophages and DCs) are essential in the induction of the adaptive immune responses (25). Exogenous viral antigens, which comprise inactive viral particles, intact viruses and apoptotic, infected cells, are taken up by APCs through endocytosis and provide a potential source of peptides that could bind to MHC class I or II molecules in the APCs (30-34). In addition, influenza-infected macrophages can also act as APCs (35), and APCs secrete IL-12, which contributes to Th1-type helper cell development (9). APCs also secrete IL-1 $\beta$ , one of the most important cytokines in bridging between the innate and adaptive immune systems (19).

#### 3-2. Roles of T and B cells in adaptive immunity

Virus-specific CD4<sup>+</sup> helper T cell precursors, from which

Th1-type and Th2-type cells are produced, and CD8<sup>+</sup> precursor T cells for CTLs recognize MHC class II- and MHC class I-antigenic peptide complexes on APCs, respectively. These cells are subsequently activated by cytokines produced by APCs (36). Th1 cells secrete IFN- $\gamma$  and IL-2, and help IgG2a Ab production by Ab-forming cells (AFCs) in mice, while Th2 cells secrete IL-4 and IL-5, and help IgA, IgG1 and IgE Ab production by AFCs (3). Th1 cells also enhance the proliferation of CD8<sup>+</sup> CTLs by secreting IL-2 (37,38). The Abs produced during these responses contribute to viral neutralization (NT) by binding with viral antigens (4,30). Th1 cells mediate delayed-type hypersensitivity (DTH) reaction by secreting IFN- $\gamma$ , which results in the inhibition of virus replication (40-42). In turn, CTLs recognize MHC class I-antigenic peptide complexes on virus-infected epithelial cells and destruct the virus-infected cells mainly by exocytosis of granules containing perforin and granzymes (43-45). IFN- $\gamma$  secreted by NK and CD8<sup>+</sup> T cells seems not to be essential for the target cell lysis (46,47).

### 4. Mucosal immune system in the respiratory tract

The mucosal immune system can be divided into two sites, inductive sites and effector sites. The inductive sites are mucosal-associated lymphoid tissues (MALT), where initial responses including antigen uptake by APCs and priming of T and B cells for IgA Ab production are induced and the effector sites are the mucosa that covers the internal surface of the whole body, where IgA AFCs are found and where S-IgA Abs play a protective role. Thus, specific IgA AFC precursor populations, induced by antigenic stimulation at one inductive site, migrate not only to the effector site near the original inductive site, but also to other mucosal sites via the homing pathways. This system is collectively referred to as the common mucosal immune system (48-54), and is responsible for the recovery from influenza and for influenza prevention in the respiratory tract.

#### 4-1. Common mucosal immune system

The mucosal immune response in the upper respiratory tract is induced in the nasopharyngeal-associated lymphoid tissues (NALT) in rodents (52,53). The NALT seems to be functionally and anatomically different from Waldeyer's ring, which comprises the nasopharyngeal tonsil (adenoid), the paired tubal tonsils, the paired palatine tonsils and the lingual tonsil in humans (55). Since AFC responses are induced in NALT cell cultures from naïve mice after in vitro culture with influenza virus, it is evident that the NALT comprises inductive tissues from which mucosal AFC precursors originate (56). NALT is also a mucosal inductive site for virus-specific cellular immune responses (57). Exogenous antigens penetrate through highly pinocytotic and phagocytotic M cells present on the NALT and interact with resident T and B cells, resulting in a large number of IgA AFC precursors (47,48,51). The primed T and B cells then leave the NALT and enter the cervical lymph nodes and eventually the general circulation via the thoracic duct. The primed T and B cells then migrate to the lamina propria mucosae of the respiratory tract, intestinal tract, and other sites where IgA AFC precursors differentiate into specific IgA AFCs. Of relevance to this, intranasal immunization is superior to oral immunization in inducing S-IgA Abs in not only the respiratory tract but also the gastrointestinal tract (58). Thus, the NALT appear to be the inductive site that most effectively provides S-IgA Ab in the respiratory tract that is required for protection against

influenza virus infection. In addition, the intranasal route may be the most practical for other vaccines whose protective sites are mucosa, other than the respiratory tract, and that are sensitive to gastrointestinal conditions such as low pH and the presence of proteolytic enzymes (59).

The mucosal immune responses in the lower respiratory tract are induced in the bronchus-associated lymphoid tissue (BALT), which has been well characterized in rats and rabbits, but not in humans and mice (54). Since AFC responses are induced in lung cultures from naïve mice after *in vitro* culture with influenza viruses, it seems probable that the BALT or its equivalent tissue in mice works as the inductive site in the lung from which mucosal AFC precursors originate (60).

#### 4-2. NALT

The NALT, which is composed of paired lymphoid cell aggregates in the noses of rodents, is the only well-organized MALT in the upper respiratory tract (52,53). It is situated in the mucosa of both lateral walls of the nasal cavity, near the nasal floor on the posterior side of the palate (Fig. 3). It can be isolated easily by peeling away the palate from the upper jaw. It consists of a reticular network filled with various types of lymphoid and non-lymphoid cells. The NALT is not only an inductive tissue from which precursors of mucosal AFCs and CTLs originate (56,57), but also an important site in lymphocyte re-circulation, since NALT lymphocytes migrate back to the NALT and cervical lymph nodes (CLNs) in far greater numbers than cells from Peyer's patches, and, reciprocally, cells from the CLNs migrate back more frequently to the NALT than to Peyer's patches (52).

#### 4-3. Secretion of S-IgA Abs

AFCs that disseminate to the lamina propria underneath the mucosal epithelium via the general circulation cause S-

IgA Ab production in the mucosal secretions. The S-IgA Abs are the J-chain-containing dimeric IgA (dIgA) Abs that are produced by IgA AFCs. The dIgA Abs bind to a polymeric Ig receptor (pIgR) on the basolateral surface of the epithelial cells and are carried to the apical surface. They are released as S-IgA Abs by combining with the secretory component (SC), which is the extracellular region of pIgR cleaved by a specific protease (50). Thus, anti-influenza HA-specific polymeric IgA (pIgA) Abs injected intravenously could be transported more efficiently into nasal secretions than monomeric IgA (mIgA) or IgG1 Abs (61). Blocking of the transcytosis of dIgA in pIgR-knockout mice immunized intranasally with an adjuvant-combined influenza vaccine resulted in a marked increase in serum IgA concentration and a decrease in the nasal IgA concentration (62).

### 5. Recovery from influenza following primary viral infection by adaptive immune responses

#### 5-1. Primary Ab responses

##### 5-1-1. Ab responses in humans experimentally infected with influenza virus

In naïve children experimentally infected with influenza A virus, infection with live, attenuated A viruses induces a high-level anti-HA IgA Ab response with lower levels of IgM and IgG Abs observed in the nasal wash within 2 weeks after infection (63). The nasal wash IgA and IgG Ab titers persist for 1 year in about half of the vaccinated subjects. Approximately 85% of seronegative adult volunteers who are infected with a live attenuated virus developed an IgA Ab response. In addition, there is evidence for active secretion of IgA Abs. In individuals infected with wild-type or attenuated viruses, the nasal wash IgA Abs are mainly polymeric and almost

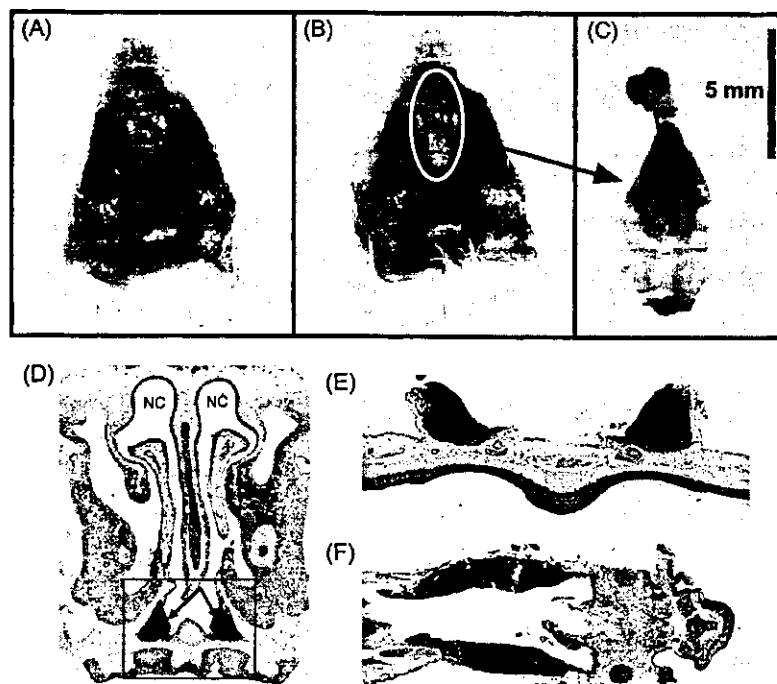


Fig. 3. Isolation method and histology of NALT of BALB/c mice. NALT was isolated from the rest of the nasal tissue by peeling away the palate from the upper jaw (A and B). A palate fragment isolated from the upper jaw, including a pair of NALT on the posterior surface (C). A frontal section of the upper jaw stained by hematoxylin and eosin showing that the NALT are situated in the mucosa of both lateral walls of the nasal cavity, near the nasal floor (D). A cross section of an isolated palate showing that the NALT is found beneath the columnar epithelium of the nasal cavity (E). A horizontal section of an isolated palate showing the longitudinal presence of NALT (F).

completely IgA1. IgA1 is one of two subclasses of human IgA and possesses an elongated proline-rich hinge region (64). It is predominant subclass in secretions (70-95% of total IgA) and in serum (about 90% of total IgA), although IgA2 predominates in the colon (about 60% of total IgA).

### 5-1-2. Ab responses in the upper respiratory tract of naïve mice infected with mouse-adapted virus

Infection with mouse-adapted viral strains results in either non-lethal respiratory disease (influenza model) or lethal viral pneumonia (viral pneumonia model), depending on the volume of virus suspension used for the intranasal administration under anaesthesia (65,66). Nasal virus titers in the influenza model, in which 2  $\mu$ l of a virus suspension (1  $\mu$ l to each nostril) is administered by intranasal dropping, peak within 3-5 days and decline to undetectable levels by 10 days (56) (Fig. 4A). The expression of nucleoprotein (NP) mRNA in the epithelial cells adjacent to the NALT, which shows the presence of the infected cells, changes in parallel with the viral titer (Fig. 4B). This infection also induces a significant accumulation of lymphoid cells (T and B cells) in the NALT, which peaks at approximately day 7 postinfection (Fig. 4C). In parallel with this change, all of virus-specific IgA, IgG and IgM AFC responses, developing on day 5 and peaking on day 7, are found in the lamina propria mucosae adjacent to the NALT (nasal mucosa); IgA and IgG Ab production predominates, followed by IgM Abs (Fig. 4D). On day 7 postinfection, the nasal mucosa adjacent to the NALT

contains the greatest number of IgA AFCs per total cells in the CLNs, nasal mucosa adjacent to the NALT and the other nasal mucosa. The appearance of AFCs is accompanied by the appearance of virus-specific IgA Abs in the nasal wash (Fig. 4D). The appearance of the Abs correlates inversely with decrease of virus titers in the nasal area (Fig. 4A) and implies their involvement in the recovery from primary infection. Thus, the diffuse lining (the lamina propria mucosae) of the nasal passage is the site of virus-specific Ab production in response to influenza virus infection. In addition, it is the site of long-term virus-specific IgA Ab production, persisting for the life of the animal (67, 68).

### 5-1-3. Ab responses in the lung of naïve mice infected with influenza A virus

A study of AFC responses within lung tissue following primary intranasal infection showed that IgM AFCs are first detected at day 5 and then peaked at day 10, whereas IgG and IgA AFCs are detected at day 10 and peaked around day 18 with slightly more IgG than IgA AFCs (60). The AFCs appear earlier in the spleen than in the lung and disappeared more rapidly from splenic tissue. A possible causal relationship between the AFC responses and recovery from influenza or viral pneumonia remains to be examined.

### 5-1-4. Intraepithelial cell prevention of viral assembly by S-IgA Abs in naïve mice infected with mouse-adapted influenza A virus

It has been postulated that the dIgA Abs, which are actively transcytosed across epithelial cells via pIgR, can bind to newly synthesized viral proteins within the epithelial cells to prevent viral assembly (69). This defense mechanism may be involved in either recovery from influenza after primary viral infection or prevention of influenza by re-infection.

### 5-2. T cell-mediated immune responses following primary viral infection

#### 5-2-1. Involvement of CTLs in the recovery from influenza in mice

Mice infected with a sublethal dose of virus usually clear the infective particles from the respiratory tract within 10 days after primary infection (Fig. 4A). Influenza virus-specific CD8<sup>+</sup> T cells appear from day 5 and accumulate in the nasal mucosa, peaking on day 7 after the primary infection (70). The T cell accumulation is detected marginally in the NALT, moderately in the CLNs with a peak on day 7, and most abundantly in the spleen with a peak around day 13. In addition, they are found in the lamina propria and the intraepithelial lymphocyte compartment of the respiratory epithelium in the nasal mucosa. The recruitment of the virus-specific CD8<sup>+</sup> T cells into the nasal mucosa following a primary intranasal infection is analogous to the recruitment of the same effector cells into the lung following a pulmonary infection (71,72). These evidences suggest that influenza virus-specific CTLs, as well as the S-IgA Abs, which are induced in the NALT and recruited into the nasal mucosa, are involved in the recovery from influenza in the upper respiratory tract (56,57). CLNs may serve to amplify mucosal immune responses initiated in NALT.

#### 5-2-2. Involvement of Th1 cells in the recovery from influenza in mice

Low-level DTH responses are induced by Th1 cells on day 7 after infection with live virus in mice (40-42). The involvement of Th1 cells in the recovery from influenza remains to be investigated, although Th1 cells can prevent viral replication by producing IFN- $\gamma$  (42,73).

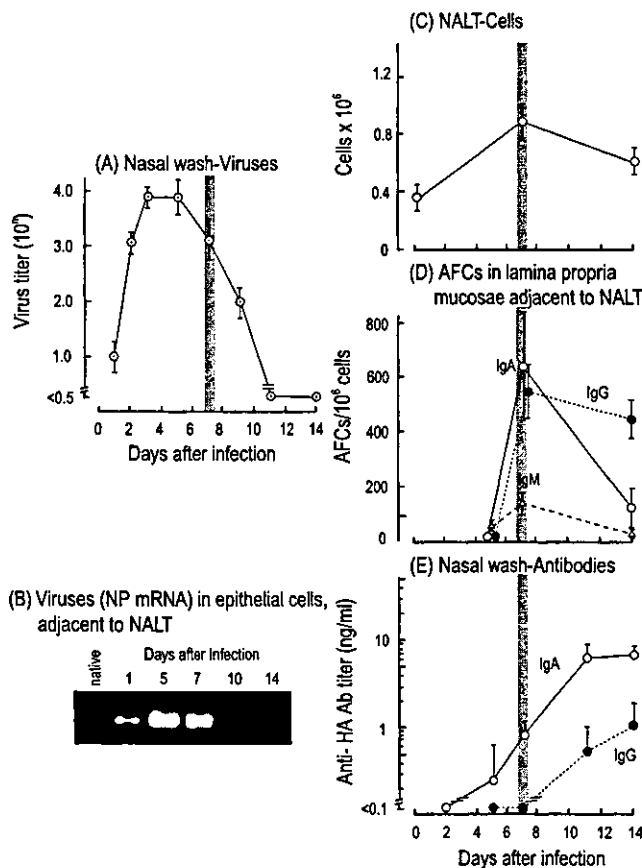


Fig. 4. Kinetics of virus titers in the nasal wash (A), expression of NP mRNA in the epithelial cells (B), number of lymphoid cells in the isolated NALT (C), AFC responses in the isolated NALT, including the mucosa (D), and Ab responses in the nasal wash in mice infected with a small volume of A/PR8 virus suspension (E). The shaded column in each figure indicates day 7 post-infection.

### 5-2-3. Cytokine responses in naïve mice infected with influenza A virus

Strong IL-2, weak IL-4, strong IL-6 and strong IFN- $\gamma$  mRNA expressions are induced in the NALT of infected mice during the early days of infection (74). IFN- $\gamma$  mRNA is expressed by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells at around 7 days postinfection. Both anti-viral IgA Abs in the nasal wash and IgG2a-rich Abs in the serum are also detected at 11 days after the infection. In addition, persistent expression of IL-5 and IL-10 (Th2-type cytokines), together with IFN- $\gamma$ , was detected in the T cell fraction isolated from lung tissue and airways of infected mice (75,76). Two cytokines are involved in IgA responses; TGF- $\beta$  induces switching to IgA production and IL-5 or IL-6 acts on B cells committed to IgA production to differentiate into IgA-secreting cells (77,78). On the other hand, IFN- $\gamma$  released from the Th1 cells participate in isotype switching to IgG2a in the mouse (36,79). Based on these collective results, the cytokine profile in the respiratory tract of infected mice can be classified as a mixed type of Th1 and Th2.

### 5-3. Involvement of both Abs and CTLs in the recovery from influenza

For many years, MHC class I-restricted CTLs that recognize NP and other conserved gene products were thought to be the principal effectors in the recovery from primary influenza virus infection (80,81). However, CD4<sup>+</sup> T-cell-dependent anti-viral Ab responses, as well as CD8<sup>+</sup> CTLs, seem to be indispensable for the recovery of mice from primary influenza virus infection (73,82-84). For example, virus-neutralizing Abs of IgG but not IgM or IgA isotypes can cure influenza virus pneumonia in SCID mice (85). In addition, the CD4<sup>+</sup> T cells and MHC class II<sup>+/+</sup> bone marrow cells in the short term radiation chimeras made with MHC class II<sup>-/-</sup> recipients are able to clear influenza virus from MHC class II<sup>-/-</sup> lung cells (86). This implies that immune CD4<sup>+</sup> T cells in these chimeras function to help the Ig-producing B cells. The recovery process involves two phases: an early phase (days 5-7), characterized by a rapid decrease in virus titer, is T-cell-dependent, while a late phase (day 7 onwards), characterized by a more protracted decrease that ultimately results in clearance, is B-cell-dependent (87,88). These results suggest that influenza viruses after primary infection are eliminated initially via killing of the virus-infected epithelial cells by MHC class I-restricted CD8<sup>+</sup> CTLs, which appear transiently in the respiratory mucosa with a peak on day 7 postinfection. The viruses, which still survive, are then eliminated via NT by mucosal IgA Abs, which are detected on day 5 and reach a plateau at around day 11, and IgG Abs, which diffuse from serum across the mucosa.

## 6. Prevention of influenza following secondary viral infection by adaptive immune responses

### 6-1. Prevention of influenza by preexisting Abs

#### 6-1-1. Direct role of S-IgA Abs in protection against virus infection

Anti-influenza S-IgA Abs purified from the respiratory tracts of mice immunized with influenza viral HA molecules, when administered intranasally, protect non-immune mice from influenza virus infection (89,90). Treatment with anti-IgA Abs, but not with anti-IgG or anti-IgM Abs, of mice immunized with live influenza virus abrogates the protection (91,92). Thus, IgA Abs play a direct role in protection against influenza. In addition, pIgA and S-IgA have several-fold

higher activities than monomeric IgA (mIgA) in hemagglutination inhibition (HI) and virus NT, which are derived from their polymeric nature (93). Thus, S-IgA Abs in the respiratory tract play a causal role in providing cross-protection against infection with variant (drift) viruses within a subtype and different subtype viruses within the A virus.

#### 6-1-2. Cross-protection by S-IgA induced by primary viral infection

Mice previously infected with A/Rec 31 (H3N1) virus are strongly protected against challenge with A/Vic (H3N2) virus in parallel with the presence of cross-reactive S-IgA Abs in the lung (94). Mice previously infected with A/Yamagata (H1N1) viruses are also protected against challenge with PR8 (H1N1) virus in proportion to the amount of cross-reactive S-IgA Abs in the nasal wash (4). Thus, S-IgA Abs in the respiratory tract are directly involved in cross-protection against infection with variant (drift) viruses within a subtype of influenza A viruses.

#### 6-1-3. Virus-Ig complex formation in the upper respiratory tract

Elimination of challenge viruses (A/PR8 virus) from the nasal area occurs earlier in mice immunized 4 weeks previously with A/PR8 (H1N1), A/Yamagata (H1N1) and A/Guizhou-X (H3N2) viruses, in that order, compared to naïve mice (4). The early viral elimination, as assessed by the PFU (infectious virus) reduction, correlates with the level of A/PR8 virus-reactive Abs in the immunized mice and the appearance of viral-Ig complexes shortly after challenge infection. Thus, local Abs present at the time of challenge virus infection are involved in the prevention of influenza by forming virus-Ig complexes shortly after infection.

### 6-2. Prevention of influenza by secondary Ab responses

#### 6-2-1. Prevention of influenza by secondary Ab responses in humans

Children who previously experienced natural infection or who received a live virus vaccine exhibit a marked reduction in both the amount and duration of virus shedding when compared to subjects without prior exposure to influenza A virus infection (39,63). The nasal wash IgA Ab response to the influenza HA correlates with this resistance to challenge infection. Thus, re-infection results in a secondary IgA Ab response, which is provided by memory Th and B cells and characterized by a rapid rise in IgA Ab titer, a higher peak titer and maintenance of detectable levels of Ab over a longer period of time.

#### 6-2-2. Protective roles of local S-IgA Abs and systemic IgG Abs in the respiratory tract

Serum IgG Abs in the immunized mice seem to be important for preventing lethal influenza pneumonia (95). To confirm this hypothesis, the distribution and concentration of specific IgA and IgG Abs in the mucus or serous fluid from different sites of the respiratory tract were examined under conditions of complete protection against challenge infection with a lethal dose of influenza virus in mice immunized intranasally with the vaccine (62,96). The specific S-IgA Abs, which are secreted actively across the mucosal membrane, are present at high levels in the mucus of nose, trachea, bronchi and bronchioli, whereas the specific IgG Abs, which could access the mucosal surfaces by passive diffusion from serum, are found predominantly in the serous fluid of alveolar epithelia (Fig. 5). Thus, S-IgA Abs are involved primarily in the prevention of influenza in the upper respiratory tract, whereas serum IgG Abs predominate in the prevention of lethal influenza pneumonia.

Respiration tract (RT) of mice		Distribution (%)		Concentration ( $\mu\text{g/ml}$ )		Local IgA/serum IgA	
		IgA	IgG	IgA	IgG	(Local IgG/serum IgG)	
Upper RT	Nasal cavity Pharynx	73.6	5.8	22.0	5.7	(3.9)	137 (0.46)
	Mucosal epithelia						
Lower RT	Trachea	5.1	0.3	21.4	3.6	(5.8)	134 (0.29)
	Bronchi Bronchioli	17.4	3.2	5.9	3.6	(1.6)	37 (0.29)
	Respiratory bronchioli Pulmonary alveoli	3.9	90.8	0.15	12.3	(0.01)	0.99 (0.99)
Serum				0.16	12.5	0.01	1 (1)

Fig. 5. Distribution and concentration of A/PR8 HA-specific IgA and IgG Abs in different sites of the respiratory tract (RT) of immunized mice. Mice were immunized intranasally with an adjuvant-combined A/PR8 inactivated vaccine, which provided a minimal dose for complete protection against challenge infection with a lethal dose of the virus. In the immunized mice, anti-HA IgA and IgG Ab titers in nasal wash, tracheal wash, broncho-alveolar wash and serum were measured. The Ab titers were converted to the concentration of mucus (or serous fluid) in different sites of the RT, based on the mucus (or serous fluid) volume in each site, which was calculated from the surface area of each site and the estimated thickness of mucus (or serous fluid). The surface area of each site was estimated using serial tissue sections of nose, trachea and lungs. The distribution (%) shows the ratio of the Ab amount in each site of RT (mucus or serous fluid) to that in the total RT.

### 6-3. Prevention of influenza by secondary T cell-mediated immune responses

#### 6-3-1. Prevention of influenza by secondary CTL responses in humans

Volunteers with a high CTL (class I MHC-restricted CD8<sup>+</sup> cytotoxic T lymphocyte) activity shed fewer viruses than those with a low CTL activity when experimentally administered with wild-type influenza A virus (97). Thus, the CTL activity of memory T cells correlates with resistance to influenza. However, the epidemiologic behavior of influenza viruses in humans suggests that the overall contribution of CTL to disease reduction during re-infection with influenza A virus is small, because repeated infection of humans with influenza A virus bearing internal viral antigens provides little resistance to disease caused by new influenza variants (63,98).

#### 6-3-2. Prevention of influenza by secondary CTL responses in mice

CTL memory cells induced by primary infection are stimulated by re-infection, resulting in an accelerated appearance of CTL activity in mice challenged with different subtype viruses (70-72, 99). Thus, secondary influenza virus specific-CTL responses appear about 2 days earlier, and with higher activities, than primary CTL responses, and are involved in the clearance of different subtype viruses from the lung and nose.

#### 6-3-3. Prevention of influenza by secondary Th responses in mice

The memory Th1 cells have antiviral activity in mice challenged with different subtype viruses (42,100). Specific T cells mediate protection and recovery of JHD<sup>-/-</sup> mice (B cell-deficient mice) immunized with live virus and challenged with a lethal dose of influenza virus (101). CD4<sup>+</sup> and CD8<sup>+</sup> T cells, but not Abs, are involved in cross-protection between viruses (H1N1, H2N2 and H3N2) for primary infection and viruses (H1N1 and H3N2) for challenge infection (heterosubtypic immunity) using mice lacking IgA, all Ig, NKT cells, or  $\gamma\delta$  T cells (102). These results imply the involvement of memory Th cells and CTLs in heterosubtypic immunity. In contrast,  $\beta$ 2-microglobulin-deficient mice, infected with the H3N2 influenza virus and challenged with the H1N1 influenza virus 3-4 weeks later, exhibit increased

survival and enhanced clearance of virus relative to non-immune controls. This suggests that CD4<sup>+</sup> T cells and Abs are involved in heterosubtypic immunity (103). The defense mechanisms are redundant, raising the possibility that one of protective mechanisms that are the primary means of protection in the respiratory tract in wild-type mice may function preferentially in T cell subset, B cell or Ig-deficient mice. Thus, IgA Abs, as well as CTLs, seem to play a major role in heterosubtypic immunity.

### 7. Basis for the development of an effective mucosal influenza vaccine and perspectives

The major adaptive immune responses involved in defense against influenza in the respiratory mucosa are summarized in Fig. 6 and in the following text. (i) In naïve mice, specific S-IgA Abs and CTLs are the major effectors involved in the recovery from influenza following primary virus infection. The S-IgA Abs first appear at day 5, then increase and reach a plateau at around day 11 postinfection. The CTLs also appear transiently in the nasal mucosa with a peak around day 7 postinfection. Sublethal doses of influenza viruses are eliminated from the upper respiratory tract within 10 days after primary viral infection. (ii) In the pre-immunized animals, the preexisting S-IgA and IgG Abs encounter and inactivate the re-infected viruses shortly after infection by forming virus-Ig complexes. The local S-IgA Abs react not only to homologous viruses but also to variant viruses in the same subtype. The strong cross-reactivity of the S-IgA Abs appears to derive from its polymeric nature, resulting in an overall increase in avidity of the Ab for the influenza virus compared to the serum IgG Abs. IgM Abs, as well as IgA Abs, are actively secreted because of the pIgR-mediated active transport of J chain-containing polymeric immunoglobulin molecules, although active secretion was most frequent for IgA Abs in children immunized with live virus vaccines (63). The mechanism underlying the role of IgM Abs in protection against influenza remains to be investigated. IgG Abs, which transude from the serum to the mucus by diffusion, react mainly with homologous virus. (iii) In the pre-immunized animals, CTL memory cells induce an accel-

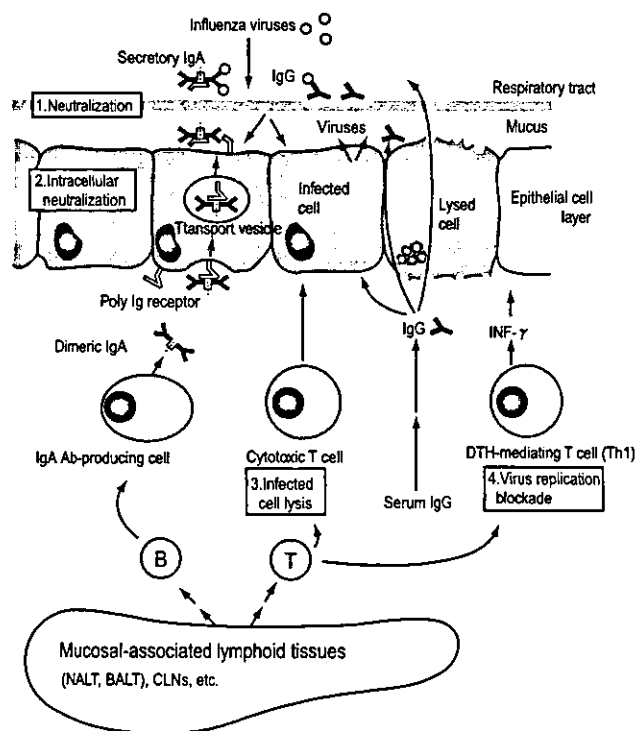


Fig. 6. Major adaptive immune responses involved in defense against influenza in the respiratory tract mucosa.

erated CTL production from day 3 onwards after re-infection and the produced CTLs are involved in the lysis of the epithelial cells infected with different subtype viruses (within the same type) to prevent the spread of infection. In the absence of the preexisting Abs, B memory cells induce an accelerated IgA and IgG Ab production from day 3 onwards after re-infection and the produced Abs are involved mainly in the elimination of both homologous and drift viruses within the same subtype by forming virus-Ig complexes. (iv) The memory Th1 cells that mediate DTH are involved in blocking viral replication by secreting  $\text{INF-}\gamma$  in mice challenged with different subtype viruses, although the Th1 cell induction following live virus infection is lower than that induced by inactivated virus and viral antigens. (v) On the transport of dIgA Abs by pIgR through infected epithelial cells, the dIgA Abs bind to newly synthesized viral proteins within the infected cells and prevent viral assembly thereby preventing the spread of infection.

To control influenza, protective immunity must be induced in advance by the administration of a vaccine. Currently available, inactivated vaccines, which are composed of either entire virions ("whole virus" vaccines), virions subjected to treatment with ether ("split-product" vaccines) or purified glycoproteins ("subunit vaccines"), are injected parenterally (2). As discussed and shown in Fig. 6, the major protective immunity induced by influenza virus infection is provided by S-IgA Abs, IgG Abs and CTLs in the respiratory tract. However, inactivated vaccines induce mainly serum IgG Abs rather than mucosal IgA Abs, which are cross-reactive among drift viruses within a subtype, and CTLs that are cross-reactive among different subtypes. Thus, inactivated vaccines are effective in protecting against an epidemic of homologous viruses but relatively ineffective against an epidemic of heterologous viruses (2,63,98). Therefore, we advocate an intranasal administration of inactivated vaccine to elicit

S-IgA Ab induction to improve the protective efficacy of the inactivated vaccines (104,105). Furthermore, the FDA recently approved a cold-adapted, live-attenuated vaccine (Flumist, MedImmune Vaccines, Inc., USA) for intranasal administration. This vaccine can induce IgA Abs, IgG Abs and CTLs (3,106). However, the live-virus vaccine is only approved for the age group of 5-49 years, thus excluding two major high-risk groups, the infants and the elderly, in addition to immunodeficient patients and pregnant women. The live vaccine seems to cause coryza, sore throat and febrile reactions. Because of these problems, several trials are currently underway to test new mucosal vaccines using inactivated viruses or viral components (28,107-109).

We have demonstrated that intranasal immunization with inactivated vaccines, used in conjunction with CT B subunit (CTB) containing a trace amount of CT (0.1%) (CTB\*) [or *Escherichia coli* heat-labile toxin B subunit (LTB) containing a trace amount of the heat-labile toxin (0.5%) (LTB\*)], provides effective cross-protection in the upper respiratory tract against variants (drift viruses) within the subtype of the influenza A viruses or variants of the B viruses (109-112). The strong cross-protection in the upper respiratory tract is provided mainly by S-IgA Abs, whereas the weak cross-protection in the lower respiratory tract is provided by IgG Abs (111-113). However, the use of heat-labile enterotoxin (LT) or CT as an adjuvant with the nasal influenza vaccine may not be clinically safe, because an intranasal virosomal vaccine adjuvanted with LT (NasalFlu, Berna Biotech, Switzerland), following licensing in 2001, has been linked to several cases of transient Bell's palsy (facial paralysis) (114). Thus, clinically safer and more effective adjuvants are required for the intranasal administration of inactivated influenza vaccine.

The mechanisms by which CT or LT enhances mucosal immune responses against influenza viral antigens involve stimulation of the innate immune system (17). Thus, CT or LT alone can reduce the replication of the viruses non-specifically in the upper respiratory tract when administered intranasally into mice together with infectious viruses. In addition, reduction of viral replication correlates with the activation of APCs (macrophages, DCs and others). Therefore, new and effective adjuvants may arise from a screen of materials that stimulate the function of APCs, including ganglioside GM1 and ligands of several TLRs (18,22-29). One attempt involved development of a nontoxic form of an adjuvant based on LT and CT together (115). Another is the use of ligands for TLR family of receptors on the APCs, such as CpG DNA (28). It is therefore clear that the development of a new and promising adjuvant will help to realize a safer and more effective adjuvant-combined nasal influenza vaccine in humans.

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## The Roles of Two I $\kappa$ B Kinase-related Kinases in Lipopolysaccharide and Double Stranded RNA Signaling and Viral Infection

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### Abstract

Viral infection and stimulation with lipopolysaccharide (LPS) or double stranded RNA (dsRNA) induce phosphorylation of interferon (IFN) regulatory factor (IRF)-3 and its translocation to the nucleus, thereby leading to the IFN- $\beta$  gene induction. Recently, two I $\kappa$ B kinase (IKK)-related kinases, inducible I $\kappa$ B kinase (IKK- $\beta$ ) and TANK-binding kinase 1 (TBK1), were suggested to act as IRF-3 kinases and be involved in IFN- $\beta$  production in Toll-like receptor (TLR) signaling and viral infection. In this work, we investigated the physiological roles of these kinases by gene targeting. TBK1-deficient embryonic fibroblasts (EFs) showed dramatic decrease in induction of IFN- $\beta$  and IFN-inducible genes in response to LPS or dsRNA as well as after viral infection. However, dsRNA-induced expression of these genes was residually detected in TBK1-deficient cells and intact in IKK- $\beta$ -deficient cells, but completely abolished in IKK- $\beta$ /TBK1 doubly deficient cells. IRF-3 activation, in response not only to dsRNA but also to viral infection, was impaired in TBK1-deficient cells. Together, these results demonstrate that TBK1 as well as, albeit to a lesser extent, IKK- $\beta$  play a crucial role in the induction of IFN- $\beta$  and IFN-inducible genes in both TLR-stimulated and virus-infected EFs.

**Key words:** Toll-like receptor • interferon regulatory factor 3 • NF- $\kappa$ B • embryonic fibroblasts • IFN- $\beta$

### Introduction

Toll-like receptors (TLRs) are essential for the recognition of invading pathogens and serve as an important link between innate and adaptive immunity (1, 2). TLRs can discriminate various microbial components, such as triacylated lipopeptides (recognized by TLR1/TLR2 heterodimer), diacylated lipopeptides (TLR2/TLR6 heterodimer), LPS (recognized by TLR4), double stranded RNA (dsRNA; recognized by TLR3), flagellin from bacterial flagella (TLR5), single stranded RNA (recognized by TLR7/8), and bacterial DNA containing the unmethylated CpG motif (TLR9; references 3–12). Recently, it has been also reported that TLR11 potentially functions to prevent infection of uropathogenic bacteria in mice (13). Intracellular signaling

pathways of TLRs are now being studied extensively (1, 2). The cytoplasmic region of TLRs contains a Toll/IL-1 receptor (TIR) domain that is common in both TLR and IL-1 receptor families. The TIR domain triggers TLR signaling by recruiting a cytoplasmic molecule, MyD88, through the homophilic interaction of the TIR domains (1, 2). MyD88 also has the death domain that can associate with other death domain-containing proteins such as IL-1 receptor asso-

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*Abbreviations used in this paper:* dsRNA, double stranded RNA; EF, embryonic fibroblast; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; IKK, I $\kappa$ B kinase; IKK- $\beta$ , inducible IKK; IRF, IFN regulatory factor; ISRE, IFN-stimulated response element; JNK, c-Jun NH<sub>2</sub>-terminal kinase; MAP, mitogen-activated protein; poly(I:C), polyinosine-polycytidylic acid; SeV, Sendai virus; TBK1, TANK-binding kinase 1; TIR, Toll/IL-1 receptor; TLR, Toll-like receptor; TRAF, TNF receptor-associated factor; TRIF, TIR domain-containing adaptor inducing IFN- $\beta$ ; VSV, vesicular stomatitis virus.

ciated kinases 1 and 4 (14). This pathway leads to NF- $\kappa$ B activation through TNF receptor-associated factor (TRAF) 6 and is essential for production of proinflammatory cytokines in response to almost all TLR ligands (15–18).

TLR3 and TLR4 signaling also induce the IFN- $\beta$  gene, which is distinctly regulated from the proinflammatory cytokine genes. Molecular mechanisms for type I IFN (IFN- $\alpha/\beta$ ) induction have been well studied in viral infection (19–21). Induction of IFN- $\beta$  is primarily regulated at transcriptional levels. Previous papers have shown that the IFN- $\beta$  gene expression is regulated by several transcription factors, such as NF- $\kappa$ B, ATF-2/c-Jun, IRF-3, and IRF-7. Gene disruption studies have demonstrated the critical role of IRF-3 and IRF-7 in the transcription of type I IFN genes (22, 23). During viral infection, IRF-3 is mainly responsible for initial activation of the IFN- $\beta$  gene (24), whereas IRF-7 expression depends on IFN- $\beta$ , thereby resulting in robust induction of type I IFNs in an autocrine positive feedback manner (21, 25). IRF-3 is located in the cytoplasm in uninfected cells. After viral infection, IRF-3 is phosphorylated at multiple serine/threonine residues located in the COOH-terminal portion. Phosphorylated IRF-3 forms a homodimer that translocates into the nucleus and activates promoters containing the IRF-3 binding site, termed IFN-stimulated response element (ISRE)/IRF-binding element.

IRF-3 activation is also critical for TLR3- and TLR4-induced up-regulation of the IFN- $\beta$  gene. These events are independent of MyD88, but dependent on another TIR domain-containing adaptor, TIR domain-containing adaptor inducing IFN- $\beta$  (TRIF)/TIR-containing adaptor molecule 1 (26–30). The TRIF-related adaptor molecule is involved in TLR4- but not TLR3-mediated IFN- $\beta$  expression (31, 32). Although the molecular mechanism to induce IRF-3 activation is poorly understood, recent papers suggested that IRF-3 is phosphorylated by two I $\kappa$ B kinase (IKK)-related kinases, inducible IKK (IKK- $i$ ) and TANK-binding kinase 1 (TBK1; references 33, 34). IKK- $i$ , also called IKK- $\epsilon$ , was originally identified as an LPS-inducible kinase (35, 36). TBK1 is also known as NF- $\kappa$ B activating kinase or TRAF2 associated kinase (37–39). These two kinases share homology with IKK- $\alpha$  and IKK- $\beta$ . Both kinases have been reported to interact with TRAF2 and the TRAF-binding protein TANK/I-TRAF, and function upstream of IKK- $\alpha$  and IKK- $\beta$  (37, 40). Deficiency of TBK1 in mice resulted in the embryonic lethality caused by massive liver degeneration, and TBK1-deficient (TBK1 $^{-/-}$ ) embryonic fibroblasts (EFs) showed reduced expression of certain genes regulated by NF- $\kappa$ B (39). Thus, these two IKK-related kinases have been implicated in NF- $\kappa$ B activation. Recently, two groups have shown that IKK- $i$  and TBK1 phosphorylate and activate IRF-3 and IRF-7, leading to the transcriptional activation of genes for IFN- $\beta$ , RANTES, and ISG54 in viral infection as well as in a TRIF-dependent signaling pathway (32, 33). These *in vitro* studies suggest that IKK- $i$  and TBK1 have similar functions; however, it remains unknown how the two kinases act under physiological conditions.

In this work, we have investigated the physiological roles of IKK- $i$  and TBK1 by gene targeting. LPS-induced expression of IFN- $\beta$ , but not proinflammatory cytokines, was markedly reduced in TBK1 $^{-/-}$  cells. Similar defects were also observed in poly(I:C)-stimulated and virus-infected TBK1 $^{-/-}$  cells. Although IKK- $i$  $^{-/-}$  cells showed normal IFN- $\beta$  gene induction, analysis of IKK- $i$ /TBK1 doubly deficient (IKK- $i$  $^{-/-}$ TBK1 $^{-/-}$ ) cells clearly showed that IKK- $i$  is also critically involved in poly(I:C)-induced up-regulation of IFN- $\beta$  and IFN-inducible genes. Poly(I:C)-induced activation of IRF-3, but not NF- $\kappa$ B, was also impaired in IKK- $i$  $^{-/-}$ TBK1 $^{-/-}$  cells, indicating that IKK- $i$  and TBK1 act through IRF-3 to activate IFN- $\beta$ . Thus, IKK- $i$  and TBK1 play important roles in IFN- $\beta$  gene induction by LPS, dsRNA, and during viral infection.

## Materials and Methods

**Cells, Virus, and Reagents.** Thioglycollate-elicited peritoneal cells were collected 3 d after intraperitoneal injection of 2 ml of 4% thioglycollate. EFs were prepared from day 11.5–14.5 embryos as described previously (16). Recombinant vesicular stomatitis virus (VSV) was a gift from T. Abe and Y. Matsuura (Osaka University, Osaka, Japan). Sendai virus (SeV) Z strain was provided by T. Shioda (Osaka University, Osaka, Japan). EFs were infected with  $10^9$  RNA copies/ml of VSV or multiplicity of infection 10 of SeV. LPS from *Salmonella minnesota* Re-595 was purchased from Sigma-Aldrich. Synthetic Pam<sub>3</sub>CSK<sub>4</sub> (bacterial lipopeptide) was obtained from Boehringer. Poly(I:C) was purchased from Amersham Biosciences. Poly(I:C) was complexed with cationic lipids, Lipofectamin 2000 reagents (Invitrogen), and added to EFs. TNF- $\alpha$  and IL-1 $\beta$  were purchased from Genzyme.

**Plasmids.** To construct the expression vectors for wild-type IKK- $i$  and mutant IKK- $i$  derived from the IKK- $i$ -targeted allele, IKK- $i$  cDNA from wild-type and IKK- $i$  $^{-/-}$  EFs was sequenced, cloned, and inserted into pFLAG-CMV2 vector (Sigma-Aldrich). The human IRF-3 expression vector was constructed by ligation of human IRF-3 cDNA into pFLAG-CMV2 vector. The expression vector for human TBK1 was constructed as described previously (30). The mouse IFN- $\beta$  promoter luciferase reporter was generated by PCR as described previously (30).

**Generation of IKK- $i$  $^{-/-}$  and TBK1 $^{-/-}$  Mice.** Genomic DNA fragments encoding IKK- $i$  and TBK1 were screened from the 129/Sv mice genomic library and characterized by restriction enzyme mapping and sequencing analysis. The targeting vectors were designed by replacing both a 1.0-kbp fragment of the IKK- $i$  gene and a 1.5-kbp fragment of the TBK1 gene with a neomycin-resistant gene cassette. A herpes simplex virus thymidine kinase gene driven by MC1 promoter was used for negative selection. The targeting vectors were transfected into E14.1 ES cells. Targeted ES cells were identified among G418 and ganciclovir doubly resistant clones by PCR and Southern blotting and subsequently injected into C57BL/6 blastocysts. Male chimeric mice obtained were mated with C57BL/6 female mice. The F1 progenies were intercrossed to generate homozygotes for each mutated allele.

**Measurement of IL-6 Production.** EFs were stimulated with the indicated stimulants for 18 h. Concentration of IL-6 in the culture supernatants were measured by ELISA. The ELISA kit for IL-6 was purchased from R&D Systems.

**Northern Blot Analysis.** Total RNA was isolated using Sepazol-RNA I (Nacalai Tesque), electrophoresed, and transferred to

nylon membranes. Hybridization was performed with the indicated cDNA probes as described previously (16). All cDNA probes were obtained from subtractive screenings as describe previously (16).

**Electrophoretic Mobility Shift Assay (EMSA).** EFs ( $10^6$  cells) were stimulated with 1.0  $\mu\text{g}/\text{ml}$  or 10  $\mu\text{g}/\text{ml}$  LPS or 10  $\mu\text{g}/\text{ml}$  poly(I:C) for the indicated periods. Nuclear extracts were prepared and incubated with a radio-labeled oligonucleotide probe containing NF- $\kappa\text{B}$  binding sites or ISRE, and visualized by autoradiography as described previously (16).

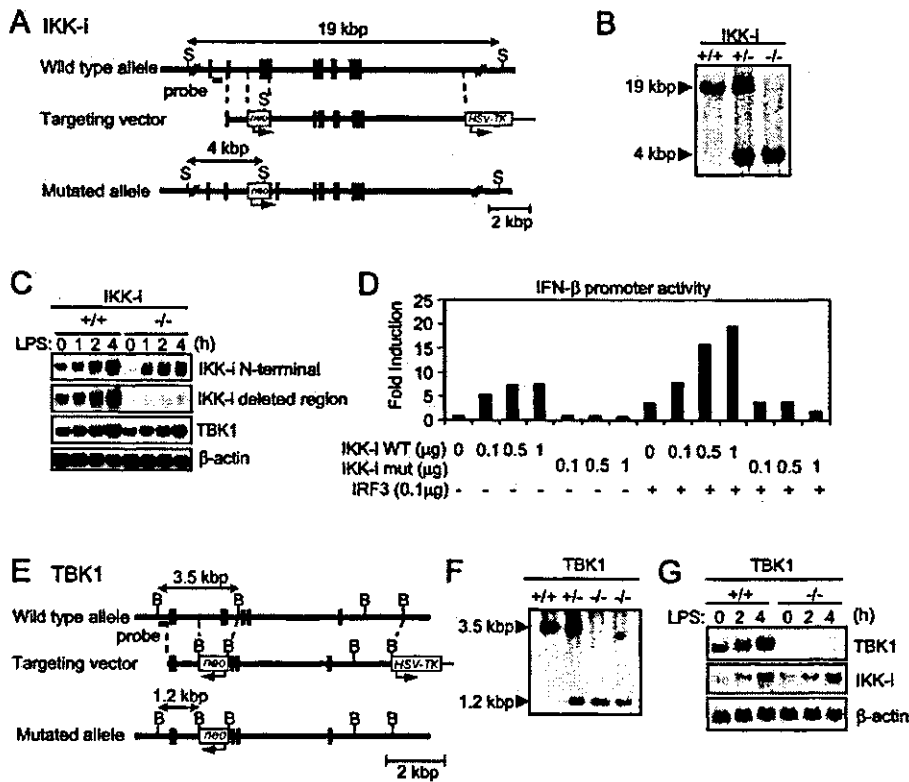
**Western Blot Analysis.** To prepare whole cell extracts, LPS- or poly(I:C)-stimulated cells were lysed in lysis buffer containing 1% Triton X-100, 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 5 mM EDTA, 10% glycerol, and protease inhibitor cocktail (Roche Diagnostics). Nuclear extracts were prepared from virus-infected cells as described previously (16). Whole cell or nuclear extracts were separated on SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was blotted with specific antibodies. The membrane-bound Abs were visualized with horseradish peroxidase-conjugated Ab to rabbit IgG (Amersham Biosciences) using the ECL system (PerkinElmer). Anti-c-Jun NH<sub>2</sub>-terminal kinase (JNK) Ab, anti-extracellular signal-regulated kinase (ERK) Ab, and anti-NF- $\kappa\text{B}$  p65 Ab were obtained from Santa Cruz Biotechnology, Inc. Antibodies against

phospho-JNK and phospho-ERK were purchased from Cell Signaling Technology. Polyclonal anti-IRF-3 antibody was raised as described previously (28).

**Native PAGE Assay.** Native PAGE was performed as described previously (41). In brief, EFs ( $10^6$  cells) were stimulated with 10  $\mu\text{g}/\text{ml}$  poly(I:C) for the indicated periods, and whole cell lysates were prepared. Cell lysates in native PAGE buffer (62.5 mM Tris-HCl, pH 6.8, 15% glycerol) were separated on a 7.5% gel and immunoblotted with anti-IRF-3 Ab.

**Reporter Assay.** Human embryonic kidney (HEK) 293 cells were seeded onto 24-well plates and transiently transfected with the indicated expression vector together with a reporter plasmid using Lipofectoamin 2000 reagents. EFs seeded onto six-well plates were transiently transfected with empty pEF-BOS vector (MOCK) or pEF-BOS-human TBK1 together with the IFN- $\beta$  promoter reporter plasmid using FuGENE 6 transfection reagent (Roche Diagnostics). 24 h after transfection, the cells were stimulated with 10  $\mu\text{g}/\text{ml}$  LPS for an additional 12 h. Luciferase activity of whole cell lysates was measured using a dual-luciferase reporter assay system (Promega). The *Renilla* luciferase reporter gene (Promega) was used as an internal control.

**Microarray Analysis.** Total RNA was extracted from EFs stimulated with or without LPS for 2 or 4 h and subjected to syn-



**Figure 1.** Generation of *IKK-i*<sup>-/-</sup> and *TBK1*<sup>-/-</sup> mice. (A) The structure of the *IKK-i* gene, the targeting vector, and the predicted mutated allele are shown. Closed boxes denote the coding exon. S, SphI. (B) Southern blot analysis of offspring from the heterozygote intercrosses. Genomic DNA was extracted from mouse tails, digested with SphI, electrophoresed, and hybridized with the radio-labeled probe indicated in A. Southern blotting gave a single 19-kbp band for wild type (+/+), a 4-kbp band for homozygous mutants (-/-), and both bands for heterozygous mice (+/-). (C) Northern blot analysis of thioglycollate-elicited peritoneal cells. Thioglycollate-elicited peritoneal cells from wild type and *IKK-i*<sup>-/-</sup> mice were stimulated with 100 ng/ml LPS for the indicated periods. Total RNA (10  $\mu\text{g}$ ) was electrophoresed, transferred to a nylon membrane, and hybridized using the *IKK-i* NH<sub>2</sub>-terminal fragment, the deleted region of *IKK-i* gene in targeting construct, *TBK1*, or  $\beta$ -actin cDNA fragment as a probe. (D) IFN- $\beta$  promoter reporter assay. HEK293 cells were transiently cotransfected with the indicated expression vectors and

the IFN- $\beta$  luciferase reporter vector (0.1  $\mu\text{g}$ ). 36 h after transfection, luciferase activity in whole cell lysates was measured. (E) The structure of the *TBK1* gene, the targeting vector, and the predicted mutated allele are shown. Closed boxes denote the coding exon. B, BamHI. (F) Southern blot analysis of EFs from the embryos of the heterozygote intercrosses. Genomic DNA was extracted from EFs, digested with BamHI, electrophoresed, and hybridized with the radiolabeled probe indicated in E. Southern blotting gave a single 3.5-kbp band for wild-type (+/+), a 1.2-kbp band for homozygous mutants (-/-), and both bands for heterozygous mice (+/-). (G) Northern blot analysis of EFs. Total RNA was extracted from wild-type and *TBK1*<sup>-/-</sup> EFs after stimulation with 1.0  $\mu\text{g}/\text{ml}$  LPS for the indicated periods, electrophoresed, transferred to a nylon membrane, and hybridized using the *IKK-i*, *TBK1*, or  $\beta$ -actin cDNA fragment as a probe.

thesis of cRNA probe. Preparation of cRNA, hybridization, and scanning of the microarray were performed according to the manufacturer's instructions. A microarray (MG U74A version 2; Affymetrix, Inc.) was used for the analysis. Data analysis was performed with Microarray Suite software (version 5.0; Affymetrix, Inc.) and GeneSpring software (Silicon Genetics).

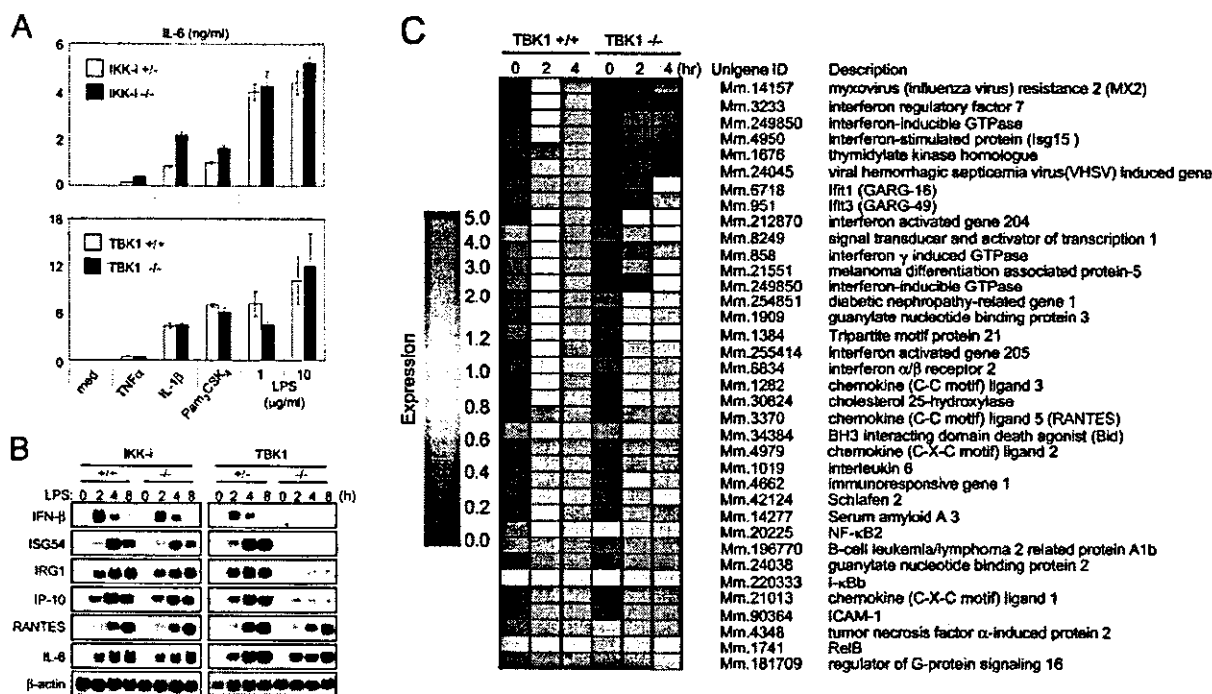
## Results

**Generation of *IKK-i*<sup>-/-</sup> and *TBK1*<sup>-/-</sup> Mice.** To investigate the physiological roles of *IKK-i* and *TBK1*, we generated *IKK-i*<sup>-/-</sup> and *TBK1*<sup>-/-</sup> mice by gene targeting. The targeting vector used to generate *IKK-i*<sup>-/-</sup> mice was constructed to replace exons 7 and 8 of the murine *IKK-i* gene encoding a part of the kinase domain with a neomycin resistant gene cassette (Fig. 1 A). *IKK-i*<sup>-/-</sup> mice were born at the expected Mendelian frequency, were fertile, and appeared to be healthy (Fig. 1 B). Using an NH<sub>2</sub>-terminal fragment of the *IKK-i* gene as a probe, we detected *IKK-i* transcripts in thioglycollate-elicited peritoneal cells from homozygous mutant mice (Fig. 1 C). However, sequencing analysis revealed that the *IKK-i* mRNA from the mutant allele lacked the targeted exons and contained a premature stop codon, generating a truncated protein that

contained only a part of kinase domains (unpublished data). Transient transfection assay was used to assess biological activity of wild-type and the mutant *IKK-i* protein (Fig. 1 D). Wild-type *IKK-i*, but not the mutant, protein induced activation of the IFN- $\beta$  promoter in a dose-dependent manner. In addition, the activity of IRF-3 was enhanced by coexpression of wild-type, but not mutant *IKK-i*. Therefore, we concluded that the mutant *IKK-i* protein does not have an activity that can lead to IFN- $\beta$  induction.

The targeting vector used to generate *TBK1*<sup>-/-</sup> mice was constructed to replace exon 9 of the murine *TBK1* gene with a neomycin-resistant gene cassette (Fig. 1 E). Mice heterozygous for *TBK1* were born and appeared healthy. However, *TBK1*<sup>-/-</sup> mice died at approximately embryonic day 14.5 (E14.5) as reported previously (39 and unpublished data). We obtained fibroblasts from embryos (EFs) at E12.5 (Fig. 1 F) and examined *TBK1* mRNA expression. In wild-type EFs, *TBK1* mRNA was detected and increased after LPS stimulation. However, in LPS-stimulated *TBK1*<sup>-/-</sup> EFs, *TBK1* mRNA was not detected, whereas *IKK-i* gene induction was normal (Fig. 1 G).

***IL-6* Production Was Normal in *IKK-i*<sup>-/-</sup> and *TBK1*<sup>-/-</sup> Cells.** *IKK-i* and *TBK1* have been implicated in NF- $\kappa$ B activation (35–40). Because *IL-6* production is dependent



**Figure 2.** Impaired induction of IFN- $\beta$  and IFN-inducible genes in LPS-stimulated *TBK1*<sup>-/-</sup>, but not *IKK-i*<sup>-/-</sup> EFs. (A) *IL-6* production by EFs. Control (*IKK-i*<sup>+/+</sup> or *TBK1*<sup>+/+</sup>), *IKK-i*<sup>-/-</sup> (top), or *TBK1*<sup>-/-</sup> (bottom) EFs were stimulated with 10 ng/ml TNF- $\alpha$ , 10 ng/ml *IL-1* $\beta$ , 100 ng/ml Pam<sub>3</sub>CSK<sub>4</sub>, or 1.0 or 10  $\mu$ g/ml LPS for 24 h. The concentration of *IL-6* in the culture supernatants was measured by ELISA. Data are shown as mean  $\pm$  SD of triplicate samples of one representative experiment from three independent experiments. (B) Gene induction in LPS-stimulated EFs. Control (*IKK-i*<sup>+/+</sup> or *TBK1*<sup>+/+</sup>), *IKK-i*<sup>-/-</sup> (left), or *TBK1*<sup>-/-</sup> (right) EFs were stimulated with 1.0  $\mu$ g/ml LPS for the indicated periods. Total RNA was extracted and subjected to Northern blot analysis for the indicated genes. (C) Microarray analysis of LPS-stimulated EFs. Wild-type and *TBK1*<sup>-/-</sup> EFs were stimulated with 1.0  $\mu$ g/ml LPS, and RNA was collected at the indicated time points and used to conduct microarray analysis. Absolute expression was displayed using GeneSpring software. The color code for absolute signal strength is indicated on the left.



on NF- $\kappa$ B, we first measured IL-6 production by EFs in response to various stimuli known to activate NF- $\kappa$ B. EFs were stimulated with TNF $\alpha$ , IL-1 $\beta$ , the TLR2 ligand Pam<sub>3</sub>CSK<sub>4</sub> (triacylated lipopeptides), and the TLR4 ligand LPS. As shown in Fig. 2 A, IKK- $i^{-/-}$  and TBK1- $i^{-/-}$  EFs produced similar amounts of IL-6 compared with wild-type cells in response to all stimulants tested. Moreover, EMSA analysis showed that NF- $\kappa$ B DNA binding activity was not affected in response to TNF- $\alpha$ , IL-1 $\beta$ , or LPS in both mutant EFs (unpublished data).

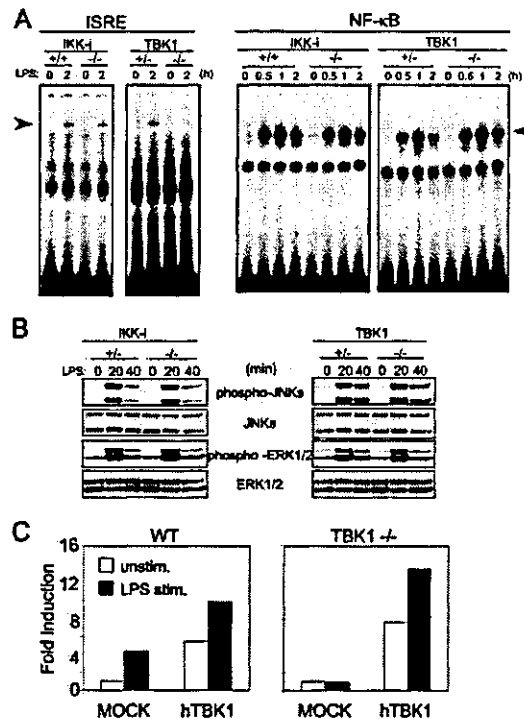
**Induction of a Set of IFN-inducible Genes in Response to LPS Was Impaired in TBK1- $i^{-/-}$  EFs.** LPS stimulates IRF-3 activation, which leads to expression of IFN- $\beta$  and a set of IFN-inducible genes. Therefore, we first performed Northern blot analysis to evaluate the expression of LPS-inducible genes regulated by IRF-3 (Fig. 2 B). Expression of IFN- $\beta$  and IFN-inducible genes such as ISG54, IP-10, IRG1, and RANTES were up-regulated in LPS-stimulated IKK- $i^{-/-}$  EFs. These responses were also intact in bone marrow-derived dendritic cells, and thioglycollate-elicited peritoneal macrophages of IKK- $i^{-/-}$  mice (unpublished data). In contrast, although mRNA induction of RANTES and IL-6 was similar to that of wild-type EFs, mRNA induction of IFN- $\beta$ , ISG54, IP-10, and IRG1 was severely impaired in TBK1- $i^{-/-}$  EFs.

Next, we performed DNA microarray analysis of LPS-stimulated EFs. Wild-type and TBK1- $i^{-/-}$  EFs were stimulated with LPS for 2 and 4 h. Genes induced in wild-type EFs are shown in Fig. 2 C. LPS-stimulated expression of IFN-inducible genes such as Mx2, IRF-7, IFN-inducible GTPase, and ISG15 were not observed in TBK1- $i^{-/-}$  EFs. In contrast, TBK1- $i^{-/-}$  EFs showed normal induction of MyD88-dependent genes such as IL-6, ICAM-1, and I $\kappa$ B- $\beta$  (Fig. 2 C).

Next, we examined activation of intracellular signaling events in LPS-stimulated EFs. EMSA analysis revealed augmentation of NF- $\kappa$ B binding activity in both IKK- $i^{-/-}$  and TBK1- $i^{-/-}$  EFs, comparable to that observed in wild-type EFs (Fig. 3 A). Alternately, ISRE binding activity was not augmented in TBK1- $i^{-/-}$  EFs, but was observed in both wild-type and IKK- $i^{-/-}$  EFs. Next, we examined the activation of mitogen-activated protein (MAP) kinases. Wild-type, IKK- $i^{-/-}$ , and TBK1- $i^{-/-}$  cells showed comparable levels of phosphorylation of JNK and ERK (Fig. 3 B).

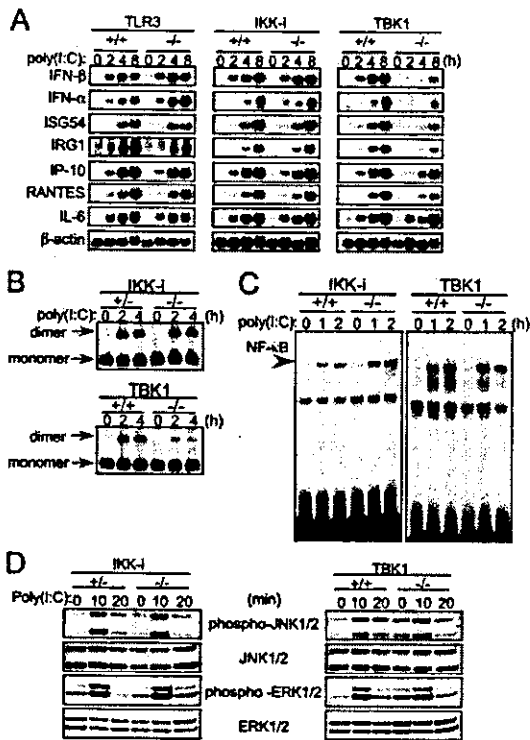
To confirm the direct involvement of TBK1 in induction of IFN- $\beta$ , we investigated whether reconstitution of TBK1 in TBK1- $i^{-/-}$  EFs could restore activation of the IFN- $\beta$  promoter upon LPS stimulation (Fig. 3 C). LPS activated the IFN- $\beta$  promoter in mock-transfected wild-type EFs but not in TBK1- $i^{-/-}$  EFs. In contrast, transient expression of human TBK1 conferred the ability to activate the IFN- $\beta$  promoter in LPS-stimulated TBK1- $i^{-/-}$  EFs. Thus, these results demonstrate that TBK1 is essential for induction of the TLR4-mediated IFN- $\beta$  expression.

**The Response to Poly(I:C) Is Impaired in TBK1- $i^{-/-}$  EFs.** Next, we examined the response to the TLR3 ligand, poly(I:C). Because simple addition of poly(I:C) alone to



**Figure 3.** Requirement of TBK1 in LPS-induced ISRE-binding and activation of the IFN- $\beta$  promoter. (A) Impaired ISRE-binding in TBK1- $i^{-/-}$  EFs. Control (IKK- $i^{+/+}$  or TBK1- $i^{+/+}$ ), IKK- $i^{-/-}$ , or TBK1- $i^{-/-}$  EFs were stimulated with 1.0  $\mu$ g/ml LPS for the indicated periods, and ISRE binding (left) and NF- $\kappa$ B binding activity (right) were determined by EMSA. (B) Activation of MAP kinases in LPS-stimulated EFs. Control (IKK- $i^{+/+}$  or TBK1- $i^{+/+}$ ), IKK- $i^{-/-}$ , or TBK1- $i^{-/-}$  EFs were stimulated with 10  $\mu$ g/ml LPS for the times indicated. Whole cell lysates were prepared and blotted with antiphospho-JNK1/2 Ab (phospho-JNK1/2) or antiphospho-ERK1/2 Ab (phospho-ERK1/2). The total amounts of JNK1/2 and ERK1/2 were also determined. One representative experiment is shown. (C) The expression of TBK1 restored activation of IFN- $\beta$  promoter in TBK1- $i^{-/-}$  EFs. TBK1- $i^{+/+}$  (WT) or TBK1- $i^{-/-}$  EFs were cotransfected with human TBK1 (hTBK1) and the IFN- $\beta$  promoter luciferase reporter. 24 h after transfection, the cells were stimulated with or without 10  $\mu$ g/ml LPS for an additional 12 h before luciferase activity was measured. Similar results were obtained from two independent experiments.

the culture did not activate EFs, a poly(I:C) complex with cationic lipid was first formed and added to the culture to stimulate the cells, and the expression of IFN- $\beta$  and IFN-inducible genes was monitored by Northern blot analysis (Fig. 4 A). In wild-type EFs, gene induction of IFN- $\beta$ , IFN- $\alpha$ , ISG54, RANTES, IP-10, and IL-6 was observed after 2 or 4 h of stimulation. Induction of these genes was also observed in TLR3-deficient EFs, suggesting that transfection of poly(I:C) to EFs activates these genes in a TLR3-independent manner. Induction of these genes in IKK- $i^{-/-}$  EFs was comparable to that observed in wild-type EFs. However, in TBK1- $i^{-/-}$  cells, induction of the IFN- $\beta$ , IFN- $\alpha$ , ISG54, and IRG1 genes was severely reduced. Alternately, mRNA induction of RANTES, IP-10, and IL-6 was not impaired in these cells.



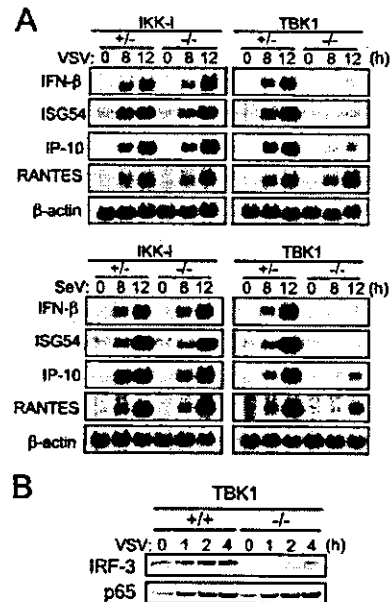
**Figure 4.** Impaired induction of IFN- $\beta$  and IFN-inducible genes in poly(I:C)-stimulated TBK1<sup>-/-</sup>, but not IKK-i<sup>-/-</sup> EFs. (A) mRNA induction in poly(I:C)-stimulated EFs. Wild-type, TLR3<sup>-/-</sup>, IKK-i<sup>-/-</sup>, or TBK1<sup>-/-</sup> EFs were transfected with 10  $\mu$ g/ml poly(I:C) for the indicated period. Total RNA was isolated and subjected to Northern blot analysis for the indicated genes. (B) Formation of IRF-3 dimer upon poly(I:C) stimulation. Control (IKK-i<sup>+/+</sup> or TBK1<sup>+/+</sup>), IKK-i<sup>-/-</sup>, or TBK1<sup>-/-</sup> EFs were transfected with 10  $\mu$ g/ml poly(I:C) and incubated for the indicated periods. Whole cell extracts were prepared and subjected to native PAGE. Monomeric and dimeric IRF-3 were detected by Western blotting. (C) NF- $\kappa$ B DNA binding activity. Wild-type, IKK-i<sup>-/-</sup>, or TBK1<sup>-/-</sup> EFs were stimulated with 10  $\mu$ g/ml poly(I:C) for the times indicated, and NF- $\kappa$ B binding was determined by EMSA. (D) MAP kinase activation in poly(I:C)-stimulated EFs. Control (IKK-i<sup>+/+</sup> or TBK1<sup>+/+</sup>), IKK-i<sup>-/-</sup>, or TBK1<sup>-/-</sup> EFs were stimulated with 10  $\mu$ g/ml poly(I:C) for the indicated periods. Whole cell lysates were prepared and blotted with antiphospho-JNK1/2 (phospho-JNK1/2) or antiphospho-ERK1/2 Ab (phospho-ERK1/2). The total amounts of JNK1/2 and ERK1/2 were also determined. One representative experiment from two independent experiments is shown.

Next, we evaluated the activation status of the transcription factors IRF-3 and NF- $\kappa$ B. IRF-3 dimerization was normally induced in IKK-i<sup>-/-</sup> EFs, but dramatically decreased in TBK1<sup>-/-</sup> EFs (Fig. 4 B). However, both IKK-i<sup>-/-</sup> and TBK1<sup>-/-</sup> EFs showed similar NF- $\kappa$ B DNA binding activity to that observed in wild-type cells (Fig. 4 C). Moreover, in both IKK-i<sup>-/-</sup> and TBK1<sup>-/-</sup> EFs, the activation of MAP kinases including JNK and ERK were normally induced as compared with wild-type cells (Fig. 4 D). Thus, the deficiency of TBK1 leads to the impairment of poly(I:C)-induced IRF-3 activation.

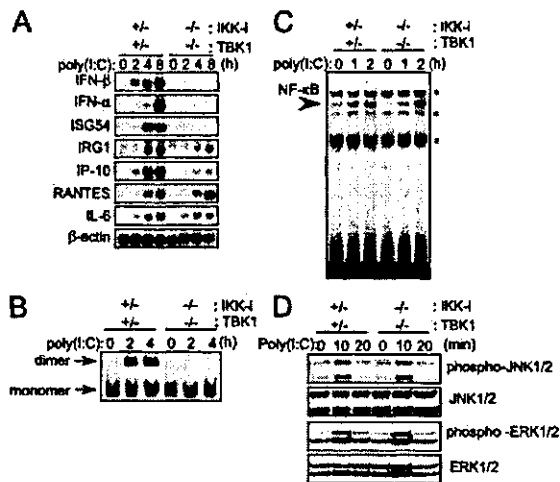
**TBK1 Is Essential for Induction of Type I IFNs and IFN-inducible Genes in Virus-infected EFs.** Viral infection also induces phosphorylation and dimerization of IRF-3, leading

to the induction of IFN- $\beta$  and other antiviral molecules (42–44). Therefore, next we examined whether virus-induced gene expression is mediated by IKK-i or TBK1 (Fig. 5 A). EFs were infected with VSV or SeV, and mRNA expression was examined by Northern blot analysis. In IKK-i<sup>-/-</sup> EFs, induction of mRNA for IFN- $\beta$ , RANTES, and IP-10 was similar to that of wild-type cells in VSV or SeV infection. Alternately, in TBK1<sup>-/-</sup> cells, the expression of IFN- $\beta$  and ISG54 mRNA was not observed, and induction of IP-10 was markedly diminished compared with wild-type cells. Although RANTES mRNA induction was almost normal in VSV infection, the induction was diminished in SeV infection. To evaluate IRF-3 activation, the nuclear proteins prepared from VSV-infected wild-type and TBK1<sup>-/-</sup> EFs were immunoblotted to detect IRF-3. As shown in Fig. 5 B, accumulation of IRF-3 and NF- $\kappa$ B p65 in the nucleus was observed in wild-type cells. The accumulation of IRF-3 was impaired in TBK1<sup>-/-</sup> cells, whereas NF- $\kappa$ B p65 translocation was not affected. These results indicate that TBK1 is an essential molecule for nuclear translocation of IRF-3 that leads to mRNA induction of IFN- $\beta$  and other antiviral molecules in viral infection.

**IKK-i/TBK1 Doubly Deficient EFs Show That IKK-i Is Also Involved in Induction of IRF3-regulated Genes.** It has been reported that both IKK-i and TBK1 phosphorylate



**Figure 5.** Requirement of TBK1 for IFN-inducible gene expression in virus-infected EFs. (A) Control (IKK-i<sup>+/+</sup> or TBK1<sup>+/+</sup>), IKK-i<sup>-/-</sup>, or TBK1<sup>-/-</sup> EFs were infected with recombinant VSV or SeV for the indicated periods. Total RNA was extracted and subjected to Northern blot analysis with probes for the indicated genes. Similar results were obtained from three independent experiments. (B) Nuclear translocation of IRF-3 and NF- $\kappa$ B p65 in response to VSV infection. EFs were infected with recombinant VSV for the indicated periods, and nuclear proteins were extracted, separated by SDS-PAGE, and blotted with anti-IRF-3 and NF- $\kappa$ B p65 Abs.



**Figure 6.** Involvement of IKK-*i* in IFN- $\beta$  gene expression in poly(I:C) signaling. (A) Impaired mRNA induction in poly(I:C)-stimulated IKK-*i*<sup>-/-</sup>TBK1<sup>-/-</sup> cells. Immortalized control and IKK-*i*<sup>-/-</sup>TBK1<sup>-/-</sup> EFs were transfected with 10  $\mu$ g/ml poly(I:C) and incubated for the indicated period. Total RNA was isolated and subjected to Northern blot analysis for the indicated genes. The similar results were obtained from two immortalized EFs. (B) Formation of IRF-3 dimer. Immortalized control (IKK-*i*<sup>-/-</sup>TBK1<sup>+/+</sup>) and IKK-*i*<sup>-/-</sup>TBK1<sup>-/-</sup> EFs were transfected with 10  $\mu$ g/ml poly(I:C) and incubated for the indicated periods. Whole cell extracts were prepared and subjected to native PAGE. Monomeric and dimeric IRF-3 proteins were detected by Western blotting. The similar results were obtained from two independently established immortalized EFs. (C) NF- $\kappa$ B DNA binding activity. Immortalized IKK-*i*<sup>-/-</sup>TBK1<sup>+/+</sup> and IKK-*i*<sup>-/-</sup>TBK1<sup>-/-</sup> EFs were transfected with 10  $\mu$ g/ml poly(I:C) for the indicated periods and NF- $\kappa$ B binding was determined by EMSA. Similar results were obtained from two lines of immortalized EFs. \*, non-specific bands. (D) MAP kinase activation. Immortalized IKK-*i*<sup>-/-</sup>TBK1<sup>+/+</sup> and IKK-*i*<sup>-/-</sup>TBK1<sup>-/-</sup> EFs were stimulated with 10  $\mu$ g/ml poly(I:C) for the times indicated. Whole cell lysates were prepared and blotted with antiphospho-JNK1/2 Ab (phospho-JNK1/2) or antiphospho-ERK1/2 Ab (phospho-ERK1/2). The total amounts of JNK1/2 and ERK1/2 were also determined. One representative experiment from two independent experiments is shown.

IRF-3 and IRF-7 in viral infection. Furthermore, as shown in Fig. 4 A, the induction of IFN- $\beta$  mRNA, although severely reduced, still remained in poly(I:C)-stimulated TBK1<sup>-/-</sup> EFs. These results led us to investigate a possibility that this residual induction of IFN- $\beta$  mRNA is mediated by IKK-*i*. For this purpose, we established immortalized IKK-*i*<sup>-/-</sup>TBK1<sup>-/-</sup> cells and analyzed gene inductions upon poly(I:C) stimulation. In control cells, the induction of the IFN- $\beta$ , IFN- $\alpha$ , ISG54, IRG1, IP-10, RANTES, and IL-6 genes was observed with the same kinetics as that observed in primary cells (Figs. 4 A and 6 A). In IKK-*i*<sup>-/-</sup>TBK1<sup>-/-</sup> cells, mRNA induction of IFN- $\beta$ , IFN- $\alpha$ , and ISG54 was completely abolished, and the induction of IRG1 and IP-10 genes was severely impaired (Fig. 6 A). Moreover, IRF-3 dimerization was abolished (Fig. 6 B). Meanwhile, RANTES and IL-6 gene expression was augmented, and activation of NF- $\kappa$ B and induction of JNK and ERK phosphorylation were normally induced in doubly deficient cells (Fig. 6 D). Thus, IKK-*i* is

also involved in poly(I:C)-induced IFN- $\beta$  induction and IRF-3 activation.

## Discussion

Activation of TLR3 by dsRNA and of TLR4 by LPS results in the induction of a set of IFN-induced genes including IFN- $\beta$ . These gene inductions are regulated in a MyD88-independent but TRIF-dependent manner and are activated by the transcription factor IRF-3 (16, 17, 23, 28, 29). Recent in vitro findings have shown that IKK-*i* and TBK1 interact with TRIF and phosphorylate IRF-3 (30, 33, 34). Here, we have examined the physiological role of these kinases through the generation of knockout mice. TBK1<sup>-/-</sup>, but not IKK-*i*<sup>-/-</sup>, EFs showed a marked decrease in the induction of the IFN- $\beta$  and IFN-inducible genes in response to LPS. In addition, TBK1<sup>-/-</sup> fibroblasts showed impaired response to poly(I:C) as demonstrated by delayed induction of IFN- $\beta$  and IFN-inducible genes, and diminished IRF-3 dimerization. These responses were completely abolished in IKK-*i*<sup>-/-</sup>TBK1<sup>-/-</sup> doubly deficient fibroblasts, demonstrating that IKK-*i* is also involved in poly(I:C)-induced IRF-3 activation. However, mRNA induction of IFN- $\beta$  and ISG54 was almost completely abolished in LPS-stimulated or virus-infected TBK1<sup>-/-</sup> EFs. Alternately, IKK-*i*<sup>-/-</sup> fibroblasts showed normal levels of gene induction after viral infection. These results indicate that the involvement of IKK-*i* in IRF-3 activation is varied, depending on stimulant. Further studies should be conducted to clarify the mechanisms of how IKK-*i* can compensate poly(I:C) stimulation, but neither LPS stimulation nor virus infection. In addition, studies using cells other than fibroblasts (e.g., by generating IKK-*i*<sup>-/-</sup>TBK1<sup>-/-</sup> mice under TNF $\alpha$ <sup>-/-</sup> background) are required to precisely determine the relative contribution of IKK-*i* and TBK1 in the responses in vivo.

IKK-*i* and TBK1 were originally identified as molecules that are structurally related to IKK- $\alpha$  and IKK- $\beta$ . Overexpression of IKK- $\alpha$ , IKK- $\beta$ , IKK-*i*, or TBK1 leads to NF- $\kappa$ B activation. However, IKK-*i* and TBK1 differ from IKK- $\alpha$  and IKK- $\beta$  in the way they activate NF- $\kappa$ B. IKK- $\alpha$  and IKK- $\beta$  phosphorylate both serines 32 and 36 of I- $\kappa$ B $\alpha$ , whereas IKK-*i* and TBK1 phosphorylate only serine 36 (35–38). Furthermore, previous papers have shown that IKK-*i* and TBK1 interact with and phosphorylate TANK/I-TRAF (37, 40), and the association of TANK with NEMO-IKK- $\gamma$  and IKK- $\alpha$ / $\beta$  complexes is dramatically increased in the presence of IKK-*i* or TBK1 (45), suggesting that IKK-*i* and TBK1 are involved in NF- $\kappa$ B activation upstream of IKK- $\alpha$ / $\beta$ . However, a previous gene paper revealed that NF- $\kappa$ B DNA binding activity is up-regulated upon either TNF- $\alpha$  or IL-1 stimulation in TBK1<sup>-/-</sup> EFs (39). We have also found that up-regulation of NF- $\kappa$ B DNA binding activity in TBK1<sup>-/-</sup> EFs was comparable to that observed in wild-type cells in response to LPS as well as TNF- $\alpha$  and IL-1 $\beta$  (unpublished data). A previous work suggested that TLR4-induced signaling events leading to activation of NF- $\kappa$ B and IRF-3 are diverged at TRIF, and

depend on TRAF6 and TBK1, respectively (30). Thus, IKK- $\beta$  and TBK1 are dispensable for NF- $\kappa$ B activation, at least, in response to these stimuli.

TLR3 or TLR4 stimulation of macrophages leads to the immediate up-regulation of IFN- $\beta$ , IP-10, and RANTES genes in an IRF-3-dependent manner (46). The present work shows that induction of IFN- $\beta$  and IP-10 transcription was dramatically decreased in TBK1 $^{-/-}$  EFs infected with VSV or SeV. However, RANTES mRNA was induced in TBK1 $^{-/-}$  EFs in response to LPS and VSV infection. Although RANTES mRNA induction appeared to be slightly diminished in TBK1 $^{-/-}$  EFs compared with wild-type EFs, the reduction may be due to the lack of secondary up-regulation by autocrine production of IFN- $\beta$ . The RANTES gene might be activated by a MyD88-dependent and IRF3-independent manner as well. These results indicate that RANTES mRNA expression is regulated differently from that of IFN- $\beta$ , depending on the cell type or the stimulus applied.

When poly(I:C) is directly introduced to the cytoplasm of EFs via lipofectamin, IFN- $\beta$  and IFN-inducible genes are induced in a TLR3-independent fashion (Fig. 4 A). This poly(I:C)-induced TLR3-independent cellular activation is also reported in dendritic cells (47). Interestingly, poly(I:C)-induced gene expression and activation of IRF-3 were diminished in TBK1 $^{-/-}$  EFs, suggesting that TBK1 also plays an important role in IRF-3 activation in the TLR3-TRIF-independent signaling pathway. Furthermore, the analysis of IKK- $\beta$  $^{-/-}$ TBK1 $^{-/-}$  EFs revealed that IKK- $\beta$  can in part compensate the deficiency of TBK1.

In conclusion, our present studies demonstrate that TBK1 as well as, albeit to a lesser extent, IKK- $\beta$  are essential for the activation of IFN- $\beta$  and IFN-inducible genes in EFs. Control of these kinases will modulate expression of IFN- $\beta$  and IFN-inducible genes without affecting induction of proinflammatory cytokines, which should enable us to obtain new therapeutic treatment for septic shock and viral infection.

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