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 μl of a virus suspension (1 μl 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).

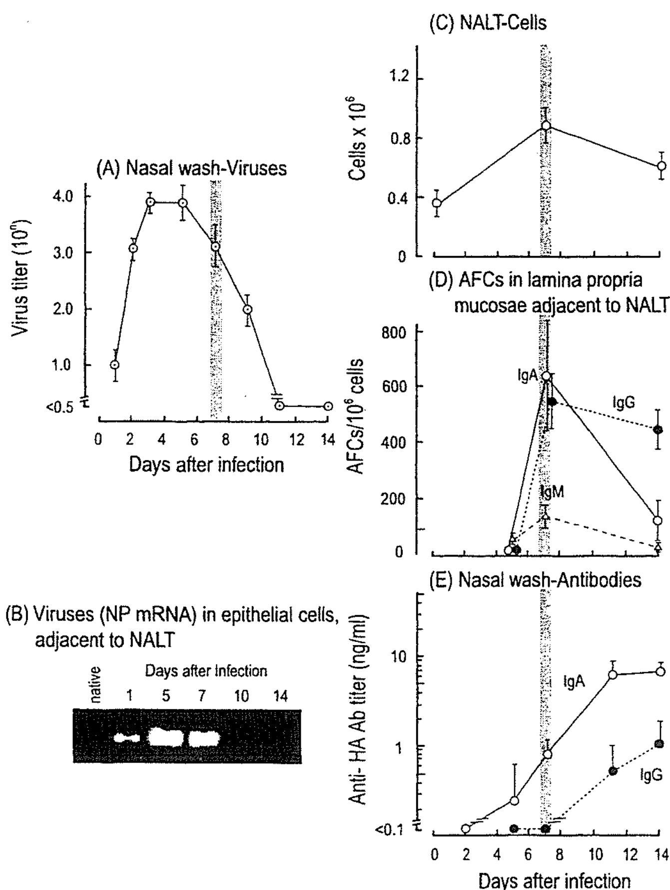


Fig. 4. Kinetics of virus titers in the nasal wash (A), expression of NP mRNA in the epithelial cells (B), number of lymphoid cells in the isolated NALT (C), AFC responses in the isolated NALT, including the mucosa (D), and Ab responses in the nasal wash in mice infected with a small volume of A/PR8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

5-1-3. 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 $CD8^+$ 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 $CD8^+$ 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-γ (42,73).

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-γ mRNA expressions are induced in the NALT of infected mice during the early days of infection (74). IFN-γ mRNA is expressed by both $CD4^+$ and $CD8^+$ 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-γ, 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-β 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-γ 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, $CD4^+$ T-cell-dependent anti-viral Ab responses, as well as $CD8^+$ 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 $CD4^+$ T cells and MHC class II+/+ bone marrow cells in the short term radiation chimeras made with MHC class II–/– recipients are able to clear influenza virus from MHC class II–/– lung cells (86). This implies that immune $CD4^+$ 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 $CD8^+$ 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 (µg/ml)			Local IgA/serum IgA
				IgA	IgG	IgA	IgG	(IgA/IgG)	(Local IgG/serum IgG)
Upper RT	Mucosal epithelia	Nasal cavity Pharynx	Mucous	73.6	5.8	22.0	5.7	(3.9)	137 (0.46)
Lower RT	Mucosal epithelia	Trachea	Mucous	5.1	0.3	21.4	3.6	(5.9)	134 (0.29)
Lower RT	Mucosal epithelia	Bronchi ~ Bronchioli	Mucous	17.4	3.2	5.9	3.6	(1.6)	37 (0.29)
Lower RT	Alveolar epithelia	Respiratory bronchioli ~ Pulmonary alveoli	Serous fluid	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 $CD8^+$ 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–/– mice (B cell-deficient mice) immunized with live virus and challenged with a lethal dose of influenza virus (101). $CD4^+$ and $CD8^+$ 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, β2-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 $CD4^+$ 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-

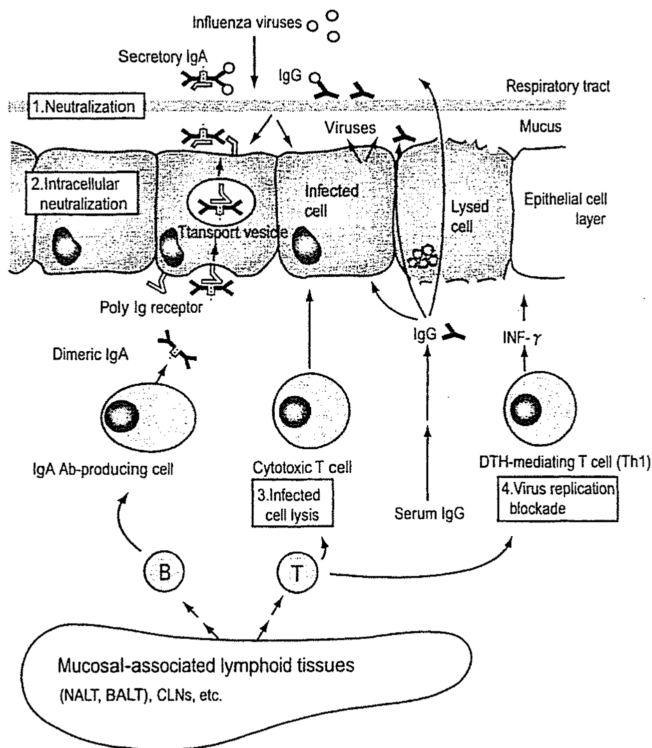


Fig. 6. Major adaptive immune responses involved in defense against influenza in the respiratory tract mucosa.

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 IFN-γ 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.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Professor Hiroshi Kiyono, Division of Mucosal Immunology, the Institute of Medical Science, University of Tokyo, for his interest and invaluable advice, to Miss Atsuko Noguchi for preparing the Figures of the manuscript, to all participants in their research works cited in this review for their cooperation and to the Research Foundation for Microbial Diseases of Osaka University for supporting the works.

REFERENCES

1. Lamb, R. A. and Krug, R. M. (1996): Orthomyxoviridae: The viruses and their replication. p. 1353-1395. *In* Field, B. N., Knipe, D. N., et al. (eds), Virology. Lippincott-Raven Publishers, Philadelphia.
2. Murphy, B. R. and Webster, R. G. (1996): Orthomyxoviruses. p. 1397-1445. *In* Field, B. N., Knipe, D. N., et al. (eds), Virology. Lippincott-Raven Publishers, Philadelphia.
3. Ada, G. L. and Jones, P. D. (1986): The immune response to influenza infection. Curr. Top. Microbial. Immunol., 128, 1-54.
4. Yoshikawa, T., Matsuo, Ke., Matsuo, Ka., Suzuki, Y., Nomoto, A., Tamura, S.-I., Kurata, T. and Sata, T. (2004): Total viral genome copies and virus-Ig complexes after infection with influenza virus in the nasal secretions of immunized mice. J. Gen. Virol. (in press).
5. Burnet, F. M. (1948): Mucins and mucoids in relation to influenza virus action. IV. Inhibition by purified mucoid of infection and haemagglutinin with the virus strain WSB. Aust. J. Exp. Biol. Med. Sci., 26, 227-233.
6. Shugars, D. C. (1999): Endogenous mucosal antiviral factors of the oral cavity. J. Infect. Dis., 179, Suppl. 3, S431-S435.
7. Husseini, R. H., Sweet, C., Collie, M. H. and Smith, H. (1982): The relation of interferon and nonspecific inhibitors to virus levels in nasal washes of ferrets infected with influenza viruses of differing virulence. Br. J. Exp. Pathol., 62, 87-93.
8. Wyde, P. R., Wilson, M. R. and Cate, T. R. (1982): Interferon production by leucocytes infiltrating the lungs of mice during primary influenza virus infection. Infect. Immun., 38, 1249-1255.
9. Monteiro, J. M., Harvey, C. and Trinchieri, G. (1998): Role of interleukin-12 in primary influenza virus infection. J. Immunol., 72, 4825-4831.
10. Mak, N. K., Leung, K. N. and Ada, G. L. (1982): The generation of "cytotoxic" macrophages in mice during infection with influenza A or Sendai virus. Scand. J. Immunol., 15, 553-561.
11. Fujimoto, I., Pan, J., Takizawa, T. and Nakanishi, Y. (2000): Virus clearance through apoptosis-dependent phagocytosis of influenza A virus-infected cells by macrophages. J. Virol., 74, 3399-3403.
12. Husseini, R. H., Sweet, C., Collie, M. H. and Smith, H. (1981): Elevation of nasal viral levels by suppression of fever in ferrets infected with influenza viruses of different virulence. J. Infect. Dis., 145, 520-524.
13. Welsh, R. M. (1981): Natural cell-mediated immunity during viral infections. Curr. Top. Microbiol. Immunol., 92, 83-103.
14. Stein-Streinlein, J., Bennett, M., Mann, M. D. and Kumor, V. (1983): Natural killer cells in mouse lung: surface phenotype, target preference and response to local influenza virus infection. J. Immunol., 131, 3699-2704.
15. Biron, C. A., Nguyen, K. B., Pien, G. C., Cousens, L. P. and Salazar-Mather, T. P. (1999): Natural killer cells in antiviral defence: function and regulation by innate cytokines. Annu. Rev. Immunol., 17, 189-220.
16. Kägi, D., Ledermann, B., Bürki, K. et al. (1994): Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature, 369, 31-37.
17. Hicks, J. T., Ennis, F. A., Kim, E. and Verbonitz, M. (1978): The importance of an intact complement pathway in recovery from a primary viral infection: influenza in decomplemented and in C5-deficient mice. J. Immunol., 121, 1437-1445.
18. Kopf, M., Abel, B., Gallimore, A., Carroll, M. and Bachmann, M. F. (2002): Comlement component C3 promotes T-cell priming and lung migration to control acute influenza virus infection. Nat. Med., 8, 373-378.
19. Matsuo, K., Yoshikawa, T., Asanuma, H., Iwasaki, T., Hagiwara, Y., Chen, Z., Kadowaki, S., Tsujimoto, H., Kurata, T. and Tamura, S.-I. (2000): Induction of innate immunity by nasal influenza vaccine administered in combination with an adjuvant (cholera toxin). Vaccine, 18, 2713-2722.
20. Gangemi, J. D., Hightower, J. A., Jackson, R. A., Marker, M. H., Welsh, M. G. and Sigel, M. M. (1983): Enhancement of natural resistance to influenza virus in lipopolysaccharide -responsive and non-responsive mice by *Propionibacterium acnes*. Infect. Immun., 39, 726-735.
21. Mak, N. K., Schiltknecht, E. and Ada, G. L. (1983): Protection of mice against influenza virus infection: enhancement of nonspecific cellular responses by *Corynebacterium parvum*. Cell. Immunol., 78, 314-325.
22. Abe, T., Takahashi, H., Hamazaki, H., Miyano-Kurosaki, N., Matsuura, Y. and Takaku, H. (2003): Baculovirus induces an innate response and confers protection from lethal influenza virus infection in mice. J. Virol., 171, 1133-1139.
23. Hemmi, H., Takeuchi, O., Kawai, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S. (2000): A Toll-like receptor recognizes bacterial DNA. Nature, 408, 656-660.
24. Alexopoulou, L., Holt, A. C., Medzhitov, R. and Flavell, R. A. (2001): Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature, 423, 732-738.
25. Akira, S., Takeda, K. and Kaisho, T. (2001): Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immunol., 2, 675-680.
26. Krieg, A. M., Yi, A. K., Manson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A. and Klinman, D. M. (1995): CpG motifs in bacterial DNA trigger direct B-cell activation. Nature, 374, 546-549.
27. Tokunaga, T., Yamamoto, T. and Yamamoto, S. (1999): How BCG led to the discovery of immunostimulatory DNA. Jpn. J. Infect. Dis., 52, 1-11.
28. Moldoveanu, Z., Love-Homan, L., Huang, W. Q. and Krieg, A. M. (1998): CpG DNA, a novel immune enhancer for systemic and mucosa immunization with influenza virus. Vaccine, 16, 1216-1224.
29. Kaisho, T. and Akira, S. (2002): Toll-like receptors as adjuvant receptors. Biochem. Biophys. Acta, 1589, 1-13.
30. Zinkernagel, R. M. and Doherty, P. C. (1979): MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T cell restriction specificity, function and responsiveness. Adv. Immunol., 27, 51-77.
31. Nguyen, H. N., Boyaka, P. N., Moldoveanu, Z., Novak, M. J., Kiyono, H., Mcghee, J. R. and Mestecky, J. (1998): Influenza virus-infected epithelial cells present

viral antigens to antigen-specific CD8+ cytotoxic T lymphocytes. J. Virol., 72, 4534-4536.
32. Tulp, A., Verwoerd, D., Dobberstein, B. et al. (1994): Isolation and characterization of the intracellular MHC class II compartment. Nature, 369, 660-662.
33. Schulta-Cherry, S., Dybdahl-Sissoko, N., Neumann, G., Kawaoka, Y. and Hinshaw, V. S. (2001): Influenza virus NS1 protein induces apoptosis in cultured cells. J. Virol., 75, 7875-7881.
34. Albert, M. L., Sauter, B. and Bhardwaj, N. (1998): Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature, 392, 86-89.
35. Lyons, C. R. and Lipscomp, M. F. (1983): Alveolar macrophages in pulmonary immune responses. I. Role in the initiation of primary immune responses and in the selective recruitment of T lymphocytes to the lung. J. Immunol., 130, 1113-1119.
36. Mosmann, T. R. and Coffman, R. L. (1989): Heterogeneity of cytokine secretion patterns and functions of helper T cells. Adv. Immunol., 46, 111-147.
37. Ridge, J. P., Rosa, F. D., and Matzinger, P. (1998): A conditional dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature, 393, 474-478.
38. Riberdly, J. M., Christensen, J. P., Branum, K. and Doherty, P. C. (2000): Diminished primary and secondary influenza virus-specific CD8+ T-cell responses in CD4-depleted Ig−/− mice. J. Virol., 74, 9762-9765.
39. Murphy, B. R. (1994): Mucosal immunity to viruses. p. 333-343. *In* Ogra, P. L., Lamm, M. E., McGhee, J. R., Mestecky, J., Strober, W. and Bienenstock, J. (eds), Handbook of Mucosal Immunology. Academic Press, San Diego, CA.
40. Cher, D. J. and Mosmann, T. R. (1987): Two types of murine helper T cell clone. II. Delayed-type hypersensitivity is mediated by Th1 clones. J. Immunol., 138, 3688-3694.
41. Liew, F. Y., Russell, S. M. and Brand, C. M. (1979): Induction and characterization of delayed-type hypersensitivity to influenza virus in mice. Eur. J. Immunol., 9, 783-790.
42. Tamura, S.-I., Miyata, K., Matsuo, K., Asanuma, H., Takahashi, H., Nakajima, K., Suzuki, Y., Aizawa, C. and Kurata, T. (1996): Acceleration of influenza virus clearance by Th1 cells in the nasal site of mice immunized intranasally with adjuvant-combined recombinant nucleoprotein. J. Immunol., 156, 3892-3900.
43. Nonacs, R., Humborg, C., Tam, J. P. and Steinman, R. M. (1992): Mechanisms of mouse dendritic cell function in the generation of influenza-specific, cytolytic T lymphocytes. J. Exp. Med., 176, 519-529.
44. Topham, D. J., Tripp, R. A. and Doherty, P. C. (1997): CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. J. Immunol., 159, 5197-5200.
45. Johnson, B. J., Costelloe, E. O., Fitzpatrick, D. R., Haanen, J. B., Schumacher, T. M. N., Brown, L. E. and Kelso, A. (2003): Single-cell perforin and granzyme expression reveals the anatomical localization of effector CD8+ Tcells in influenza virus-infected mice. Proc. Natl. Acad. Sci. USA, 100, 2657-2662.
46. Price, G. E., Gaszewska-Mastariarz, A. and Moskophidis, D. (2000): The role of alpha/beta and gamma interferons in development of immunity to influenza A virus in mice. J. Virol., 74, 3996-4003.
47. Nguyen, H. H., van Ginkel, F. W., Vu, H. L., Novak, M. J., McGhee, J. R. and Mestecky, J. (2000): Gamma interferon is not required for mucosal cytotoxic T-lymphocyte responses or heterosubtypic immunity to influenza A virus infection in mice. J. Virol., 74, 5495-5501.
48. Brandtzaeg, P. (1989): Overview of the mucosal immune system. Curr. Top. Microbial. Immunol., 146, 13-25.
49. McGhee, J. R., Mestecky, J., Dertzbaugh, M. T., Eldridge, J. H., Hirasawa, M. and Kiyono, H. (1992): The mucosal immune system: from fundamental concepts to vaccine development. Vaccine, 10, 75-88.
50. Brandtzaeg, P., Krajci, P., Lamm, M. E. and Kaetzl, C. S. (1994): Epithelial and hepatobiliary transport of polymeric immunoglobulins. p.113. *In* P. L. Ogra, M. E. Lamm, J. R. McGhee, J. Mestecky, W. Strober and J. Bienenstock (eds), Handbook of Mucosal Immunology. Academic Press, San Diego, CA.
51. Brandtzaeg, P., Baekkevold, E. S., Farstad, I. N., Jahnsen, F. L., Johansen, F.-E., Nilsen, E. M. and Yamanaka, T. (1999): Regional specialization in the mucosal immune system: what happens in the microcompartments? Immunol. Today, 20, 141-151.
52. Kuper, C. F. et al. (1992): The role of naso-pharyngeal lymphoid tissue. Immunol. Today, 13, 219-224.
53. Asanuma, H., Thompson, A. H., Iwasaki, T., Sato, Y., Inaba, Y., Aizawa, C., Kurata, T. and Tamura, S.-I. (1997): Isolation and characterization of mouse nasal-associated lymphoid tissue. J. Immunol. Meth., 302, 123-131.
54. Bienenstock, J. and Clancy, R. (1994): Bronchial mucosal lymphoid tissue. p. 529-538. *In* P. L. Ogra, M. E. Lamm, J. R. McGhee, J. Mestecky, W. Strober and J. Bienenstock (eds), Handbook of Mucosal Immunology. Academic Press, San Diego, CA.
55. Perry, M. and Whyte, A. (1998): Immunology of the tonsils. Immunol. Today, 19, 414-426.
56. Tamura, S.-I., Iwasaki, T., Thompson, A. M., Asanuma, H., Chen, Z., Suzuki, Y., Aizawa, C. and Kurata, T. (1998): Antibody-forming cells in the nasal-associated lymphoid tissue during primary influenza virus infection. J. Gen. Virol., 79, 291-299.
57. Zuercher, A. W., Coffin, S. E., Thurnheer, M. C., Fundova, P. and Cebra, J. J. (2002): Nasal-associated lymphoid tissue is a mucosal inductive site for virus-specific humoral and cellular immune responses. J. Immunol., 168, 1796-1803.
58. Hirabayahi, Y., Kurata, H., Funato, H., Nagamine, T., Aizawa, C., Tamura, S.-I., Shimada, K. and Kurata, T. (1990): Comparison of intranasal inoculation of influenza HA vaccine combined with cholera toxin B subunit with oral or parenteral vaccination. Vaccine, 8, 243-248.
59. Walker, R. I. (1994): New strategies for using mucosal vaccination to achieve more effective immunization. Vaccine, 12, 387-400.
60. Jones, P. D. and Ada, G. L. (1986): Influenza virus-specific antibody-secreting cells in the murine lung during primary influenza virus infection. J. Immunol., 60, 614-619.
61. Reneger, K. B. and Small, P. A. Jr. (1994): Passive immunization: systemic and mucosal. p. 347-356. *In* P. L. Ogra, M. E. Lamm, J. R. McGhee, J. Mestecky, W. Strober and J. Bienenstock (eds), Handbook of Mucosal

Immunology. Academic Press, San Diego, CA.
62. Asahi, Y., Yoshikawa, T., Watanabe, I., Iwasaki, T., Asanuma, H., Sato, Y., Shimada, S., Nanno, M., Matsuoka, Y., Ohwaki, M., Iwakura, Y., Suzuki, Y., Aizawa, C., Sata, T., Kurata, T. and Tamura, S.-I. (2002): Protection against influenza virus infection in poly Ig receptor-knockout mice immunized intranasally with adjuvant-combined vaccines. J. Immunol., 168, 2930-2938.
63. Murphy, B. R. and Clements, M. L. (1989): The systemic and mucosal immune response of humans to influenza A virus. Curr. Top. Microb. Immunol., 146, 107-116.
64. Peppard, J. V. and Russell, M. W. (1999): Phylogeneic development and comparative physiolody of IgA. p.163. *In* P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. Bienenstock and J. R. McGhee (eds), Mucosal Immunology. Academic Press, San Diego, CA.
65. Yetter, R. A., Lehrer, S., Ramphal, R. and Small P. A. Jr. (1980): Outcome of influenza infection: effect of site of initial infection and heterotypic immunity. Infect. Immun., 29, 654-662.
66. Tamura, S.-I. and Kurata, T. (1996): Intranasal immunization with influenza vaccine. p 425-436. *In* Kiyono, H., Ogra, P. L. and McGhee, J. R. (eds), Mucosal Vaccines. Chapter 32. Academic Press, San Diego, CA.
67. Liang, B., Hyland, L. and Hou, S. (2001): Nasal-associated lymphoid tissue is a site of long-term virus-specific antibody production following respiratory virus infection of mice. J. Virol., 75, 5416-5420.
68. Asanuma, H., Inaba, Y., Aizawa, C., Kurata, T. and Tamura, S.-I. (1995): Characterization of mouse nasal lymphocytes isolated by enzymatic extraction with collagenase. J. Immunol. Method., 187, 41-51.
69. Mazanec, M. B., Coulder, C. L. and Fletcher, D. R. (1995): Intracellular neutralization of influenza virus by immunoglobulin A anti-hemagglutinin monoclonal antibodies. J. Virol., 69, 1339-1343.
70. Wiley, J. A., Hogan, R. J., Woodland, D. L. and Harmsen, A. G. (2001): Antigen-specific CD8 (+) T cells persist in the upper respiratory tract following influenza virus infection. J. Immunol., 167, 3293-3299.
71. Flynn, K. J., Belz, G. T., Altman, J. D., Ahmed, R., Woodland, D. L. and Doherty, P. C. (1998): Virus-specific $CD8^+$ T cells in primary and secondary influenza pneumonia. Immunity, 8, 683-691.
72. Flynn, K. J., Riberdy, J. M., Christensen, J. M., Altman, J. D. and Doherty, P. C. (1999): In vivo proliferation of naive and memory influenza-specific $CD8^+$ T cells. Proc. Natle. Acad. Sci., USA, 96, 8597-8602.
73. Scherle, P. A., Palladino, G. and Gerhard, W. (1992): Mice can recover from pulmonary influenza virus infection in the absence of class-I-restricted cytotoxic T cells. J. Immunol., 148, 212-217.
74. Matsuo, K., Iwasaki, T., Asanuma, H., Yoshikawa, T., Chen, Z., Tsujimoto, H., Kurata, T. and Tamura, S.-I. (2000): Cytokine mRNA in the nasal-associated lymphoid tissue during influenza virus infection and nasal vaccination. Vaccine, 18, 1344-1350.
75. Coffman, R. L., Lebman, D. A. and Shrader, B. (1989): Transforming growth factor β specifically enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes. J. Exp. Med., 170, 1039-1044.
76. Sonoda, E., Matsumoto, R., Hitoshi, Y. et al. (1989): Transforming growth factor β induced IgA production and acts additively with interleukin 5 for IgA production. J. Exp. Med., 170, 1415-1420.
77. Beagley, K. W., Eldridge, J. H., Kiyono, H. et al. (1988): Recombinant murine IL-5 induced high rate IgA synthesis in cycling IgA-positive Peyer's patch B cells. J. Immunol., 141, 2035-2042.
78. Beagley, K. W., Eldridge, J. H., Lee, F. et al. (1989): Interleukins and IgA synthesis: human and murine IL-6 induce high rate IgA serection in IgA-committed B cells. J. Exp. Med. 169, 2133-2148.
79. Snapper, C. M. and Paul, W. E. (1987): Interferon-γ and B-cell stimulatory factor-I reciprocally regulate Ig isotype production. Science, 236, 944-947.
80. Yap, K. L., Ada, G. L. and McKenzie, I. F. C. (1978): Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus. Nature, 173, 238-239.
81. Mackenzie, C. D., Taylor, P. M. and Askonas, B. A. (1989): Rapid recovery of lung histology correlates with clearance of influenza virus by specific CD8 cytotoxic T cells. Immunology, 67, 375-381.
82. Eichelberger, M., Allan, W., Zijlstra, M., Jaenisch, R. and Doherty, P. C. (1991): Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8+ T cells. J. Exp. Med., 174, 857-880.
83. Bender, B. S., Crogham, T., Zhang, L. and Small, P. A. (1992): Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. J. Exp. Med., 175, 1143-1145.
84. Gerhard, W., Mozdzanowska, K., Furchner, M., Washko, G. and Maiese, K. (1997): Role of the B-cell response in recovery of mice from primary influenza virus infection. Immunol. Rev., 159, 95-103.
85. Palladino, G., Mozdzanowska, K., Washko, G. and Gerhard, W. (1995): Virus-neutrlizing antibodies of immunoglobulin G (IgG) but not of IgM or IgA isotypes can cure influenza virus pneumonia in SCID mice. J. Virol., 69, 2075-2081.
86. Topham, D. J., Tripp, R. A., Sarawar, S. R. et al. (1996): Immune $CD4^+$ T cells promote the clearance of influenza virus from major histocompatibility complex class II -/- respiratory epithelium. J. Virol., 70, 1288-1291.
87. Iwasaki, T. and Nojima, T. (1977): Defence mechanisms against primary influenza virus infection in mice. I. The role of interferon and neutralizing antibodies and thymus dependence of interferon and antibody production. J. Immunol., 118, 256-263.
88. Kris, R. M., Asofsky, R., Evans, C. B. and Small, P. A. (1985): Protection and recovery in influenza virus-infected mice immunosuppressed with anti-IgM. J. Immunol., 134, 1230-1235.
89. Tamura, S.-I., Funato, H., Hirabayashi, Y., Kikuta, K., Suzuki, Y., Nagamine, T., Aizawa, C., Nakagawa, M. and Kurata, T. (1990): Functional role of RT haemagglutinin-specific IgA antibodies in protection against influenza. Vaccine, 8, 479-485.
90. Tamura, S.-I., Funato, H., Hirabayashi, Y., Suzuki, Y., Nagamine, T., Aizawa, C. and Kurata, T. (1991): Cross-protection against influenza A virus infection by passively transferred RT IgA antibodies to different hemagglutinin molecules. Eur. J. Immunol., 21, 1337-

1344.
91. Renegar, K. B. and Small, P. A., Jr. (1991): Passive transfer of local immunity to influenza virus infection by IgA antibody. J. Immunol., 146, 1972-1978.
92. Renegar, K. B. and Small, P. A., Jr. (1991): Immunoglobulin A mediation of murine nasal anti-influenza virus immunity. J. Virol., 65, 2146-2148.
93. Renegar, K. B., Jackson, G. D. and Mestecky, J. (1998): In vitro comparison of the biologic activities of monoclonal monomeric IgA, polymeric IgA, and secretory IgA. J. Immunol., 160, 1219-1223.
94. Liew, F. Y., Russell, S. M., Appleyard, G., Brand, C. M. and Beale, J. (1984): Cross-protection in mice infected with influenza A virus by the respiratory route is correlated with local IgA antibody rather than serum antibody or cytotoxic T cell reactivity. Eur. J. Immunol., 14, 350-356.
95. Ramphal, R., Cogliano, R. C., Shands, J. W., Jr. and Small, P. A., Jr. (1979): Serum antibody prevents lethal murine influenza pneumonitis but not tracheitis. Infect. Immun., 25, 992-997.
96. Ito, R., Yoshikawa, T., Ozaki, Y., Hasegawa, H., Suzuki, Y., Aizawa, C., Sata, T., Kurata, T. and Tamura, S.-I. (2003): Roles of anti-hemagglutinin IgA and IgG antibodies in different sites of the respiratory tract of vaccinated mice in preventing lethal influenza pneumonia. Vaccine, 21, 2362-2371.
97. McMichael, A. (1994): Cytotoxic T lymphocytes specific for influenza virus. Curr. Top. Microb. Immunol., 189, 75-91.
98. Couch, R. B. and Kasel, J. A. (1983): Immunity to influenza in man. Ann. Rev. Microbiol., 37, 529-549.
99. McDermott, M. R., Lukacher, A. E., Braciale, V. L., Braciale, T. J. and Bienenstock, J. (1987): Characterization and in vivo distribution of influenza-virus-specific T-lymphocytes in the murine respiratory tract. Am. Rev. Respir. Dis., 135, 245-249.
100. Taylor, P. M., Esquivel, F. and Askonas, B. A. (1990): Murine $CD4^+$ T cell clones vary in function in vitro and in influenza virus infection in vivo. Int. Immunol., 2, 323-328.
101. Bot, A., Reichlin, A., Isobe, H., Schulman, J., Yokoyama, Y. M. and Bona, C. A. (1996): Cellular mechanisms involved in protection and recovery from influenza virus infection in immunodeficient mice. J. Immunol., 70, 5668-5672.
102. Benton, K. A., Misplon, J. A., Lo, C.-Y., Brutkiewicz, R. R., Prasad, S. A. and Epstein, S. L. (2001): Heterosubtypic immunity to influenza A virus in mice lacking IgA, all Ig, NKT cells, or $\gamma\delta$ T cells. J. Immunol., 166, 7437-7445.
103. Bender, B. S., Bell, W. E., Taylor, S. and Small, P. A., Jr. (1994): Class I major histocompatibility complex-restricted cytotoxic T lymphocytes are not necessary for heterotypic immunity to influenza. J. Infect. Dis., 170, 1195-1200.
104. Waldman, R. H. and Ganguly, R. (1974): Immunity to infection on secretory surfaces. J. Infect. Dis., 130, 419-440.
105. Shvartsman, Y. S. and Zykov, M. P. (1976): Secretory anti-influenza immunity. Adv. Immunol., 22, 291-330.
106. Maassab, H. F. (1967): Adaptation and growth characteristics of influenza virus at 25°C. Nature (London), 213, 612-614.
107. Ben-Yehuda, A., Joseph, A., Barenholz, Y., Zeira, E., Even-Chen, S., Louria-Hayon, I. et al. (2003): Immunogenicity and safety of a novel IL-2-supplemented liposomal influenza vaccine (INFLUSOME-VAC) in nursing home residents. Vaccine, 21, 3169-3178.
108. Muszkat, M., Greenbaum, E., Ben-Yehuda, A., Oster, M., Yau'l, E., Heimann, S., Levy, R., Friedman, G. and Zakay-Rones, Z. (2003): Local and systemic immune response in nursing-home elderly following intranasal or intramuscular immunization with inactivated influenza vaccine. Vaccine, 21, 1180-1186.
109. Tamura, S.-I., Samegai, Y., Kurata, H., Nagamine, T., Aizawa, C. and Kurata, T. (1988): Protection against influenza virus infection by vaccine inoculated intranasally with cholera toxin B subunit. Vaccine, 6, 409-413.
110. Tamura, S.-I., Yamanaka, A., Shimohara, M., Tomita, T., Komase, K., Tsuda, Y., Suzuki, Y., Nagamine, T., Kawahara, K., Danbara, H., Aizawa, C., Oya, A. and Kurata, T. (1994): Synergistic action of cholera toxin B subunit (and *Escherichia coli* heat-labile toxin B subunit) and a trace amount of cholera whole toxin as an adjuvant for nasal influenza vaccine. Vaccine, 12, 419-426.
111. Tamura, S.-I., Asanuma, H., Ito, Y., Hirabayashi, Y., Suzuki, Y., Nagamine, T., Aizawa, C., Kurata, T. and Oya, A. (1992): Superior cross-protective effect of nasal vaccination to subcutaneous inoculation with influenza hemagglutinin vaccine. Eur. J. Immunol., 22, 477-481.
112. Tamura, S-I., Ito, Y., Asanuma, H., Hirabayashi, Y., Suzuki, Y., Nagamine, T., Aizawa, C. and Kurata, T. (1992): Cross-protection against influenza virus infection afforded by trivalent inactivated vaccines inoculated intranasally with cholera toxin B subunit. J. Immunol., 149, 981-988.
113. Durrer, P., Gluck, U., Spyr, C., Lang, A. B., Zurbriggen, R., Herzog, C. and Gluck, R. (2003): Mucosal antibody responses induced with a nasal virosome-based influenza vaccine. Vaccine, 21, 4328-4334.
114. Mutsch, M., Zhou, W., Rhodes, P., Bopp, M., Chen, R. T., Linder, T., Spyr, C. and Steffen, R. (2004): Use of the inactivated intranasal influenza vaccines and the risk of Bell's palsy in Switzerland. New Eng. J. Med., 350, 896-903.
115. Kweon, Mi-Na., Yamamoto, M., Watanabe, F., Tamura, S-I., van Ginkel, F. W., Miyauchi, A., Takagi, H., Takeda, Y., Hamabata, T., Tujihashi, K., McGhee, J. R. and Kiyono, H. (2002): A nontoxic chimeric enterotoxin adjuvant induce protective immunity in both mucosal and systemic compartments with reduced IgE antibodies. J. Infect. Dis., 186, 1261-1269.

The Roles of Two IκB Kinase-related Kinases in Lipopolysaccharide and Double Stranded RNA Signaling and Viral Infection

Hiroaki Hemmi,[1] Osamu Takeuchi,[1] Shintaro Sato,[1,2] Masahiro Yamamoto,[1] Tsuneyasu Kaisho,[1,3] Hideki Sanjo,[1] Taro Kawai,[1,2] Katsuaki Hoshino,[1,3] Kiyoshi Takeda,[1,2] and Shizuo Akira[1,2]

[1]*Department of Host Defense, Research Institute for Microbial Diseases, Osaka University and* [2]*Exploratory Research for Advanced Technology Program, Japan Science and Technology Corporation, Osaka 565-0871, Japan*
[3]*Institute of Physical and Chemical Research, Research Center for Allergy and Immunology, Kanagawa 230-0045, Japan*

The Journal of Experimental Medicine

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-β gene induction. Recently, two IκB kinase (IKK)–related kinases, inducible IκB kinase (IKK-*i*) and TANK-binding kinase 1 (TBK1), were suggested to act as IRF-3 kinases and be involved in IFN-β 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-β 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-*i*–deficient cells, but completely abolished in IKK-*i*/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-*i* play a crucial role in the induction of IFN-β and IFN-inducible genes in both TLR-stimulated and virus-infected EFs.

Key words: Toll-like receptor • interferon regulatory factor 3 • NF-κB • embryonic fibroblasts • IFN-β

Downloaded from www.jem.org on April 27, 2005

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-

Address correspondence to Shizuo Akira, Dept. of Host Defense, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-8303; Fax: 81-6-6879-8305; email: sakira@biken.osaka-u.ac.jp

Abbreviations used in this paper: dsRNA, double stranded RNA; EF, embryonic fibroblast; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; IKK, IκB kinase; IKK-*i*, inducible IKK; IRF, IFN regulatory factor; ISRE, IFN-stimulated response element; JNK, c-Jun NH_2-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-β; VSV, vesicular stomatitis virus.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/2004/06/1641/10 $8.00
Volume 199, Number 12, June 21, 2004 1641–1650
http://www.jem.org/cgi/doi/10.1084/jem.20040520

ciated kinases 1 and 4 (14). This pathway leads to NF-κB 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-β gene, which is distinctly regulated from the proinflammatory cytokine genes. Molecular mechanisms for type I IFN (IFN-α/β) induction have been well studied in viral infection (19–21). Induction of IFN-β is primarily regulated at transcriptional levels. Previous papers have shown that the IFN-β gene expression is regulated by several transcription factors, such as NF-κB, 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-β gene (24), whereas IRF-7 expression depends on IFN-β, 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-β gene. These events are independent of MyD88, but dependent on another TIR domain–containing adaptor, TIR domain-containing adaptor inducing IFN-β (TRIF)/TIR-containing adaptor molecule 1 (26–30). The TRIF-related adaptor molecule is involved in TLR4- but not TLR3-mediated IFN-β 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 IκB kinase (IKK)-related kinases, inducible IKK (IKK-*i*) and TANK-binding kinase 1 (TBK1; references 33, 34). IKK-*i*, also called IKK-ε, was originally identified as an LPS-inducible kinase (35, 36). TBK1 is also known as NF-κB activating kinase or TRAF2 associated kinase (37–39). These two kinases share homology with IKK-α and IKK-β. Both kinases have been reported to interact with TRAF2 and the TRAF-binding protein TANK/I-TRAF, and function upstream of IKK-α and IKK-β (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-κB (39). Thus, these two IKK-related kinases have been implicated in NF-κB 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-β, 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-β, 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-β gene induction, analysis of IKK-*i*/TBK1 doubly deficient (IKK-$i^{-/-}$$TBK1^{-/-}$) cells clearly showed that IKK-*i* is also critically involved in poly(I:C)-induced up-regulation of IFN-β and IFN-inducible genes. Poly(I:C)-induced activation of IRF-3, but not NF-κB, was also impaired in IKK-$i^{-/-}$$TBK1^{-/-}$ cells, indicating that IKK-*i* and TBK1 act through IRF-3 to activate IFN-β. Thus, IKK-*i* and TBK1 play important roles in IFN-β 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_3CSK_4 (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-α and IL-1β 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-β 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

Downloaded from www.jem.org on April 27, 2005

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 μg/ml or 10 μg/ml LPS or 10 μg/ml poly(I:C) for the indicated periods. Nuclear extracts were prepared and incubated with a radio-labeled oligonucleotide probe containing NF-κB 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_2-terminal kinase (JNK) Ab, anti-extracellular signal-regulated kinase (ERK) Ab, and anti–NF-κB 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 μg/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-β promoter reporter plasmid using FuGENE 6 transfection reagent (Roche Diagnostics). 24 h after transfection, the cells were stimulated with 10 μg/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-

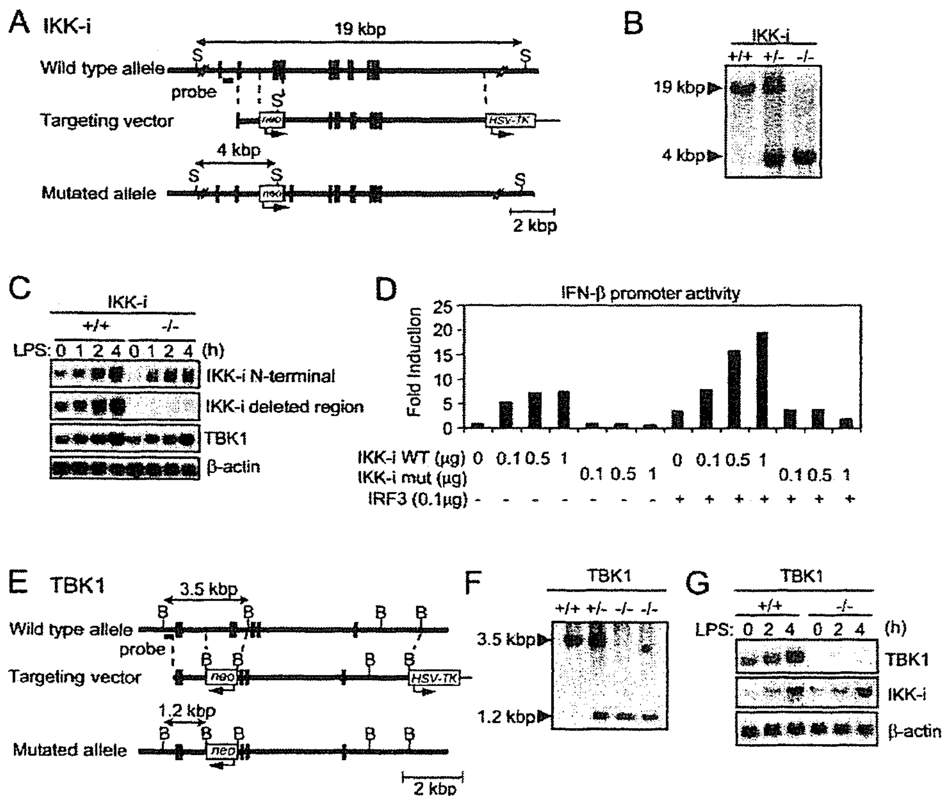


Figure 1. Generation of IKK-$i^{-/-}$ and $TBK1^{-/-}$ mice. (A) The structure of the *IKK-i* gene, the targeting vector, and the predicted mutated allele are shown. Closed boxes denote the coding exon. S, SphI. (B) Southern blot analysis of offspring from the heterozygote intercrosses. Genomic DNA was 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-$i^{-/-}$ mice were stimulated with 100 ng/ml LPS for the indicated periods. Total RNA (10 μg) was electrophoresed, transferred to a nylon membrane, and hybridized using the IKK-*i* NH_2-terminal fragment, the deleted region of *IKK-i* gene in targeting construct, TBK1, or β-actin cDNA fragment as a probe. (D) IFN-β promoter reporter assay. HEK293 cells were transiently cotransfected with the indicated expression vectors and the IFN-β luciferase reporter vector (0.1 μg). 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 heterozygous 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 $TBK1^{-/-}$ EFs after stimulation with 1.0 μg/ml LPS for the indicated periods, electrophoresed, transferred to a nylon membrane, and hybridized using the IKK-*i*, TBK1, or β-actin cDNA fragment as a probe.

Downloaded from www.jem.org on April 27, 2005

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-$i^{-/-}$ and TBK1$^{-/-}$ Mice. To investigate the physiological roles of IKK-*i* and TBK1, we generated IKK-$i^{-/-}$ and TBK1$^{-/-}$ mice by gene targeting. The targeting vector used to generate IKK-$i^{-/-}$ 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-$i^{-/-}$ mice were born at the expected Mendelian frequency, were fertile, and appeared to be healthy (Fig. 1 B). Using an NH_2-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-β 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-β induction.

The targeting vector used to generate TBK1$^{-/-}$ 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$^{-/-}$ 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$^{-/-}$ EFs, TBK1 mRNA was not detected, whereas IKK-*i* gene induction was normal (Fig. 1 G).

IL-6 Production Was Normal in IKK-$i^{-/-}$ and TBK1$^{-/-}$ Cells. IKK-*i* and TBK1 have been implicated in NF-κB activation (35–40). Because IL-6 production is dependent

Downloaded from www.jem.org on April 27, 2005

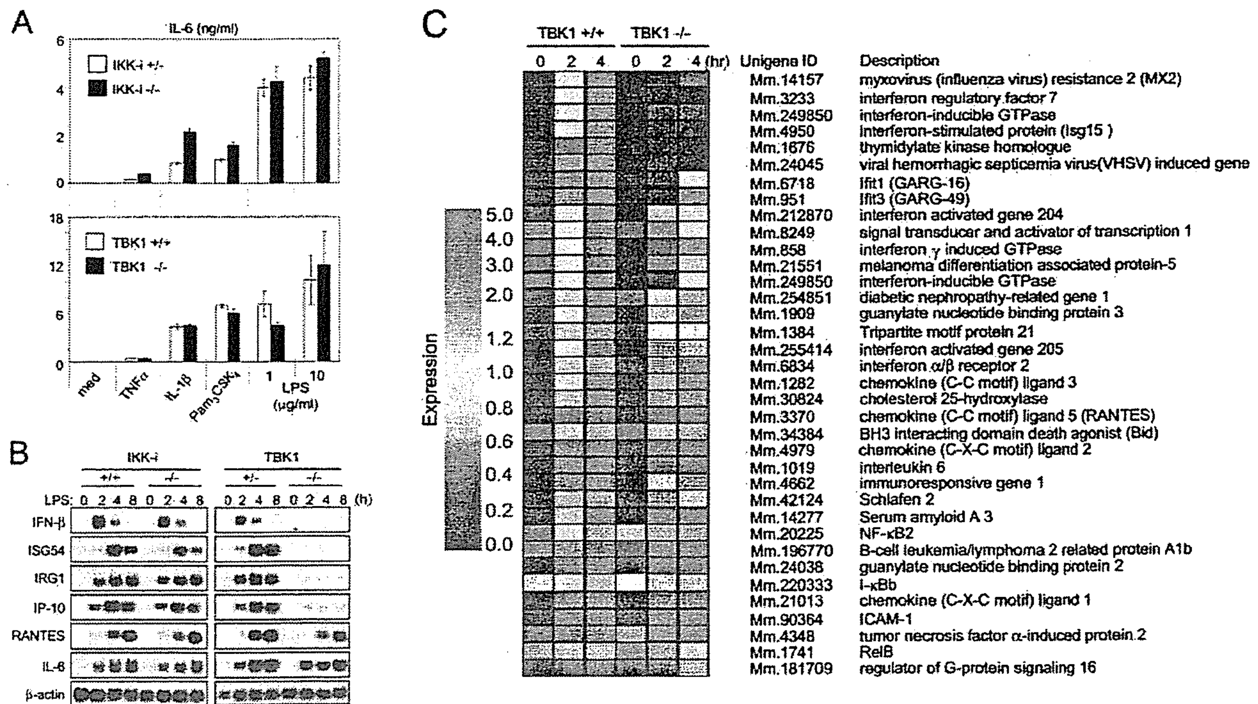


Figure 2. Impaired induction of IFN-β and IFN-inducible genes in LPS-stimulated TBK1$^{-/-}$, but not IKK-$i^{-/-}$ EFs. (A) IL-6 production by EFs. Control (IKK-$i^{+/-}$ or TBK1$^{+/+}$), IKK-$i^{-/-}$ (top), or TBK1$^{-/-}$ (bottom) EFs were stimulated with 10 ng/ml TNF-α, 10 ng/ml IL-1β, 100 ng/ml Pam_3CSK_4, or 1.0 or 10 μg/ml LPS for 24 h. The concentration of IL-6 in the culture supernatants was measured by ELISA. Data are shown as mean ± SD of triplicate samples of one representative experiment from three independent experiments. (B) Gene induction in LPS-stimulated EFs. Control (IKK-$i^{+/+}$ or TBK1$^{+/-}$), IKK-$i^{-/-}$ (left), or TBK1$^{-/-}$ (right) EFs were stimulated with 1.0 μg/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$^{-/-}$ EFs were stimulated with 1.0 μg/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-κB, we first measured IL-6 production by EFs in response to various stimuli known to activate NF-κB. EFs were stimulated with TNFα, IL-1β, the TLR2 ligand Pam_3CSK_4 (triacylated lipopeptides), and the TLR4 ligand LPS. As shown in Fig. 2 A, IKK-$i^{-/-}$ and TBK1$^{-/-}$ EFs produced similar amounts of IL-6 compared with wild-type cells in response to all stimulants tested. Moreover, EMSA analysis showed that NF-κB DNA binding activity was not affected in response to TNF-α, IL-1β, or LPS in both mutant EFs (unpublished data).

Induction of a Set of IFN-inducible Genes in Response to LPS Was Impaired in TBK1$^{-/-}$ EFs. LPS stimulates IRF-3 activation, which leads to expression of IFN-β 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-β 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-β, ISG54, IP-10, and IRG1 was severely impaired in TBK1$^{-/-}$ EFs.

Next, we performed DNA microarray analysis of LPS-stimulated EFs. Wild-type and TBK1$^{-/-}$ 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$^{-/-}$ EFs. In contrast, TBK1$^{-/-}$ EFs showed normal induction of MyD88-dependent genes such as IL-6, ICAM-1, and IκB-β (Fig. 2 C).

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

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

The Response to Poly(I:C) Is Impaired in TBK1$^{-/-}$ EFs. Next, we examined the response to the TLR3 ligand, poly(I:C). Because simple addition of poly(I:C) alone to 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-β and IFN-inducible genes was monitored by Northern blot analysis (Fig. 4 A). In wild-type EFs, gene induction of IFN-β, IFN-α, 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$^{-/-}$ cells, induction of the IFN-β, IFN-α, ISGF54, and IRG1 genes was severely reduced. Alternately, mRNA induction of RANTES, IP-10, and IL-6 was not impaired in these cells.

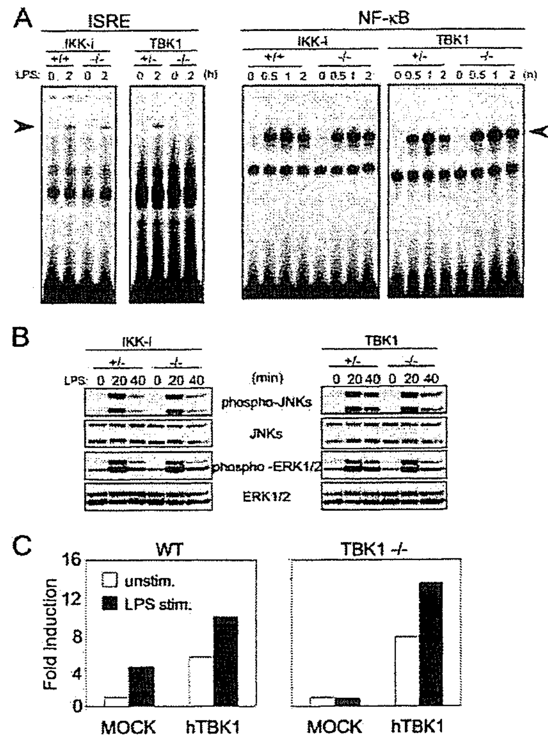


Figure 3. Requirement of TBK1 in LPS-induced ISRE-binding and activation of the IFN-β promoter. (A) Impaired ISRE-binding in TBK1$^{-/-}$ EFs. Control (IKK-$i^{+/+}$ or TBK1$^{+/-}$), IKK-$i^{-/-}$, or TBK1$^{-/-}$ EFs were stimulated with 1.0 μg/ml LPS for the indicated periods, and ISRE binding (left) and NF-κB binding activity (right) were determined by EMSA. (B) Activation of MAP kinases in LPS-stimulated EFs. Control (IKK-$i^{+/-}$ or TBK1$^{+/-}$), IKK-$i^{-/-}$, or TBK1$^{-/-}$ EFs were stimulated with 10 μg/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-β promoter in TBK1$^{-/-}$ EFs. TBK1$^{+/+}$ (WT) or TBK1$^{-/-}$ EFs were cotransfected with human TBK1 (hTBK1) and the IFN-β promoter luciferase reporter. 24 h after transfection, the cells were stimulated with or without 10 μg/ml LPS for an additional 12 h before luciferase activity was measured. Similar results were obtained from two independent experiments.

Downloaded from www.jem.org on April 27, 2005

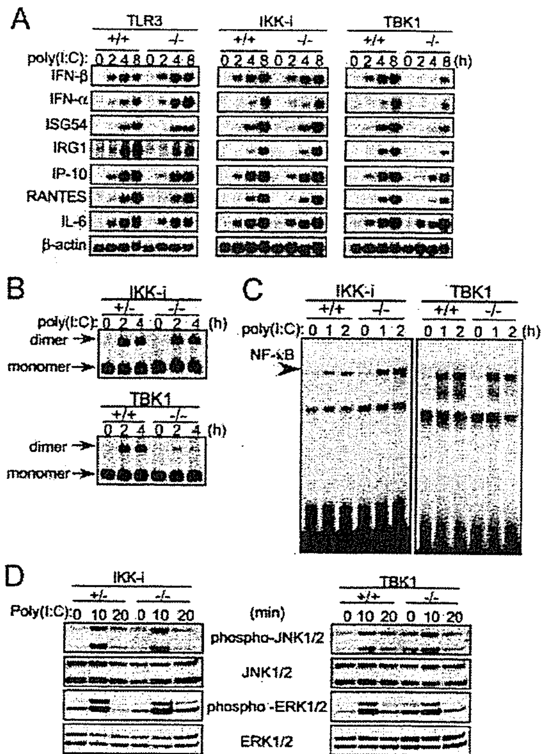


Figure 4. Impaired induction of IFN-β and IFN-inducible genes in poly(I:C)-stimulated TBK1$^{-/-}$, but not IKK-$i^{-/-}$ EFs. (A) mRNA induction in poly(I:C)-stimulated EFs. Wild-type, TLR3$^{-/-}$, IKK-$i^{-/-}$, or TBK1$^{-/-}$ EFs were transfected with 10 μg/ml poly(I:C) for the indicated period. 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-$i^{+/-}$ or TBK1$^{+/+}$), IKK-$i^{-/-}$, or TBK1$^{-/-}$ EFs were transfected with 10 μg/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-κB DNA binding activity. Wild-type, IKK-$i^{-/-}$, or TBK1$^{-/-}$ EFs were stimulated with 10 μg/ml poly(I:C) for the times indicated, and NF-κB binding was determined by EMSA. (D) MAP kinase activation in poly(I:C)-stimulated EFs. Control (IKK-$i^{+/-}$ or TBK1$^{+/+}$), IKK-$i^{-/-}$, or TBK1$^{-/-}$ EFs were stimulated with 10 μg/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-κB. IRF-3 dimerization was normally induced in IKK-$i^{-/-}$ EFs, but dramatically decreased in TBK1$^{-/-}$ EFs (Fig. 4 B). However, both IKK-$i^{-/-}$ and TBK1$^{-/-}$ EFs showed similar NF-κB DNA binding activity to that observed in wild-type cells. (Fig. 4 C). Moreover, in both IKK-$i^{-/-}$ and TBK1$^{-/-}$ 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-β 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-$i^{-/-}$ EFs, induction of mRNA for IFN-β, RANTES, and IP-10 was similar to that of wild-type cells in VSV or SeV infection. Alternately, in TBK1$^{-/-}$ cells, the expression of IFN-β 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$^{-/-}$ EFs were immunoblotted to detect IRF-3. As shown in Fig. 5 B, accumulation of IRF-3 and NF-κB p65 in the nucleus was observed in wild-type cells. The accumulation of IRF-3 was impaired in TBK1$^{-/-}$ cells, whereas NF-κB 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-β 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

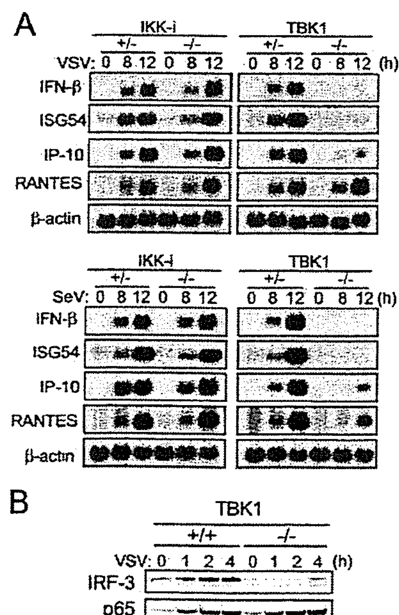


Figure 5. Requirement of TBK1 for IFN-inducible gene expression in virus-infected EFs. (A) Control (IKK-$i^{+/-}$ or TBK1$^{+/-}$), IKK-$i^{-/-}$, or TBK1$^{-/-}$ EFs were infected with recombinant VSV or SeV for the indicated periods. Total RNA was extracted and subjected to Northern blot analysis with probes for the indicated genes. Similar results were obtained from three independent experiments. (B) Nuclear translocation of IRF-3 and NF-κB 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-κB p65 Abs.

Downloaded from www.jem.org on April 27, 2005

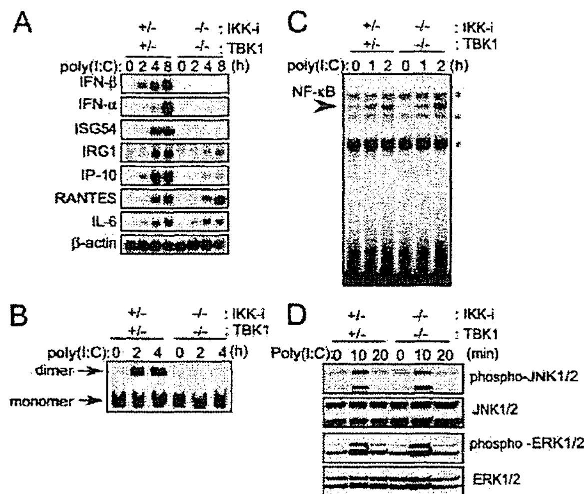


Figure 6. Involvement of IKK-*i* in IFN-β gene expression in poly(I:C) signaling. (A) Impaired mRNA induction in poly(I:C)-stimulated IKK-$i^{-/-}$TBK1$^{-/-}$ cells. Immortalized control and IKK-$i^{-/-}$TBK1$^{-/-}$ EFs were transfected with 10 μg/ml poly(I:C) and incubated for the indicated period. 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-$i^{+/-}$TBK1$^{+/-}$) and IKK-$i^{-/-}$TBK1$^{-/-}$ EFs were transfected with 10 μg/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-κB DNA binding activity. Immortalized IKK-$i^{+/-}$TBK1$^{+/-}$ and IKK-$i^{-/-}$TBK1$^{-/-}$ EFs were transfected with 10 μg/ml poly(I:C) for the indicated periods and NF-κB binding was determined by EMSA. Similar results were obtained from two lines of immortalized EFs. *, nonspecific bands. (D) MAP kinase activation. Immortalized IKK-$i^{+/-}$TBK1$^{+/-}$ and IKK-$i^{-/-}$TBK1$^{-/-}$ EFs were stimulated with 10 μg/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-β mRNA, although severely reduced, still remained in poly(I:C)-stimulated TBK1$^{-/-}$ EFs. These results led us to investigate a possibility that this residual induction of IFN-β mRNA is mediated by IKK-*i*. For this purpose, we established immortalized IKK-$i^{-/-}$TBK1$^{-/-}$ cells and analyzed gene inductions upon poly(I:C) stimulation. In control cells, the induction of the IFN-β, IFN-α, ISGF54, 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-$i^{-/-}$TBK1$^{-/-}$ cells, mRNA induction of IFN-β, IFN-α, 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-κB 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-β 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-β. 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$^{-/-}$, but not IKK-$i^{-/-}$, EFs showed a marked decrease in the induction of the IFN-β and IFN-inducible genes in response to LPS. In addition, TBK1$^{-/-}$ fibroblasts showed impaired response to poly(I:C) as demonstrated by delayed induction of IFN-β and IFN-inducible genes, and diminished IRF-3 dimerization. These responses were completely abolished in IKK-*i*/TBK1 doubly deficient fibroblasts, demonstrating that IKK-*i* is also involved in poly(I:C)-induced IRF-3 activation. However, mRNA induction of IFN-β and ISG54 was almost completely abolished in LPS-stimulated or virus-infected TBK1$^{-/-}$ EFs. Alternately, IKK-$i^{-/-}$ 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-$i^{-/-}$TBK1$^{-/-}$ mice under TNFα$^{-/-}$ 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-α and IKK-β. Overexpression of IKK-α, IKK-β, IKK-*i*, or TBK1 leads to NF-κB activation. However, IKK-*i* and TBK1 differ from IKK-α and IKK-β in the way they activate NF-κB. IKK-α and IKK-β phosphorylate both serines 32 and 36 of I-κBα, 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-γ and IKK–α/β complexes is dramatically increased in the presence of IKK-*i* or TBK1 (45), suggesting that IKK-*i* and TBK1 are involved in NF-κB activation upstream of IKK–α/β. However, a previous gene paper revealed that NF-κB DNA binding activity is up-regulated upon either TNF-α or IL-1 stimulation in TBK1$^{-/-}$ EFs (39). We have also found that up-regulation of NF-κB DNA binding activity in TBK1$^{-/-}$ EFs was comparable to that observed in wild-type cells in response to LPS as well as TNF-α and IL-1β (unpublished data). A previous work suggested that TLR4-induced signaling events leading to activation of NF-κB and IRF-3 are diverged at TRIF, and

Downloaded from www.jem.org on April 27, 2005

depend on TRAF6 and TBK1, respectively (30). Thus, IKK-*i* and TBK1 are dispensable for NF-κB activation, at least, in response to these stimuli.

TLR3 or TLR4 stimulation of macrophages leads to the immediate up-regulation of IFN-β, IP-10, and RANTES genes in an IRF-3–dependent manner (46). The present work shows that induction of IFN-β 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-β. 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-β, depending on the cell type or the stimulus applied.

When poly(I:C) is directly introduced to the cytoplasm of EFs via lipofectamin, IFN-β 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-*i*$^{-/-}$TBK1$^{-/-}$ EFs revealed that IKK-*i* 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-*i* are essential for the activation of IFN-β and IFN-inducible genes in EFs. Control of these kinases will modulate expression of IFN-β 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.

The authors would like to thank Drs. Y. Matsuura and T. Abe for recombinant VSV; Dr. T. Shioda for SeV virus; Drs. H. Kuwata and S. Uematsu for helpful discussions; M. Hashimoto for secretarial assistance; N. Okita, N. Iwami, and Y. Fukuda for technical assistance; and Mr. P. Lee for critical reading of the paper.

This work was supported by grants from Special Coordination Funds; the Ministry of Education, Culture, Sports, Science and Technology; the Naito Foundation; the Virtual Research Institute of Aging of Nippon Boehringer Ingelheim; and by research fellowships from the Japan Society for the Promotion of Science for Young Scientists. H. Hemmi is a research fellow of the Japan Society for the promotion of Science. M. Yamamoto is supported by the Junior Research Associate from the Institute of Physical and Chemical Research.

Submitted: 18 March 2004
Accepted: 26 April 2004

References

1. Janeway, C.A., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annu. Rev. Immunol.* 20:197–216. First published on October 4, 2001; 10.1146/annurev.immunol.20.083001.084359.
2. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu. Rev. Immunol.* 21:335–376. First published on December 19, 2002; 10.1146/annurev.immunol.21.120601.141126.
3. Takeuchi, O., S. Sato, T. Horiuchi, K. Hoshino, K. Takeda, Z. Dong, R.L. Modlin, and S. Akira. 2002. Role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J. Immunol.* 169:10–14.
4. Takeuchi, O., T. Kawai, P.F. Muhlradt, M. Morr, J.D. Radolf, A. Zychlinsky, K. Takeda, and S. Akira. 2001. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int. Immunol.* 13:933–940.
5. Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity.* 11:443–451.
6. Alexopoulou, L., A.C. Holt, R. Medzhitov, and R.A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature.* 413:732–738.
7. Poltorak, A., X. He, I. Smirnova, M.Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science.* 282:2085–2088.
8. Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol.* 162:3749–3752.
9. Hayashi, F., K.D. Smith, A. Ozinsky, T.R. Hawn, E.C. Yi, D.R. Goodlett, J.K. Eng, S. Akira, D.M. Underhill, and A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature.* 410:1099–1103.
10. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature.* 408:740–745.
11. Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, and S. Bauer. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science.* 303:1526–1529. First published on February 19, 2004; 10.1126/science.1093620.
12. Diebold, S.S., T. Kaisho, H. Hemmi, S. Akira, C. Reis, and E. Sousa. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science.* 303:1529–15231. First published on February 19, 2004; 10.1126/science.1093616.
13. Zhang, D., G. Zhang, M.S. Hayden, M.B. Greenblatt, C. Bussey, R.A. Flavell, and S. Ghosh. 2004. A toll-like receptor that prevents infection by uropathogenic bacteria. *Science.* 303:1522–1526.
14. Janssens, S., and R. Beyaert. 2003. Functional diversity and regulation of different interleukin-1 receptor-associated kinase (IRAK) family members. *Mol. Cell.* 11:293–302.
15. Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity.* 11:115–122.
16. Kawai, T., O. Takeuchi, T. Fujita, J. Inoue, P. F. Muhlradt, S. Sato, K. Hoshino, and S. Akira. 2001. Lipopolysaccharide stimulates the MyD88-independent pathway and results in

Downloaded from www.jem.org on April 27, 2005

activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J. Immunol.* 167: 5887-5894.

17. Hoshino, K., T. Kaisho, T. Iwabe, O. Takeuchi, and S. Akira. 2002. Differential involvement of IFN-β in Toll-like receptor-stimulated dendritic cell activation. *Int. Immunol.* 14:1225–1231.
18. Toshchakov, V., B.W. Jones, P.Y. Perera, K. Thomas, M.J. Cody, S. Zhang, B.R. Williams, J. Major, T.A. Hamilton, M.J. Fenton, and S.N. Vogel. 2002. TLR4, but not TLR2, mediates IFN-β-induced STAT1α/β-dependent gene expression in macrophages. *Nat. Immunol.* 3:392–398. First published on March 18, 2002; 10.1038/ni774.
19. Sen, G.C. 2001. Viruses and interferons. *Annu. Rev. Microbiol.* 55:255–281.
20. Grandvaux, N., B.R. tenOever, M.J. Servant, and J. Hiscott. 2002. The interferon antiviral response: from viral invasion to evasion. *Curr. Opin. Infect. Dis.* 15:259–267.
21. Taniguchi, T., and A. Takaoka. 2002. The interferon-α/β system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. *Curr. Opin. Immunol.* 14:111–116.
22. Sato, M., H. Suemori, N. Hata, M. Asagiri, K. Ogasawara, K. Nakao, T. Nakaya, M. Katsuki, S. Noguchi, N. Tanaka, and T. Taniguchi. 2000. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-α/β gene induction. *Immunity.* 13:539–548.
23. Sakaguchi, S., H. Negishi, M. Asagiri, C. Nakajima, T. Mizutani, A. Takaoka, K. Honda, and T. Taniguchi. 2003. Essential role of IRF-3 in lipopolysaccharide-induced interferon-β gene expression and endotoxin shock. *Biochem. Biophys. Res. Commun.* 306:860–866.
24. Yoneyama, M., W. Suhara, Y. Fukuhara, M. Fukuda, E. Nishida, and T. Fujita. 1998. Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *EMBO J.* 17:1087–1095.
25. Levy, D.E., I. Marie, and A. Prakash. 2003. Ringing the interferon alarm: differential regulation of gene expression at the interface between innate and adaptive immunity. *Curr. Opin. Immunol.* 15:52–58.
26. Yamamoto, M., S. Sato, K. Mori, K. Hoshino, O. Takeuchi, K. Takeda, and S. Akira. 2002. A novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-β promoter in the Toll-like receptor signaling. *J. Immunol.* 169:6668–6672.
27. Oshiumi, H., M. Matsumoto, K. Funami, T. Akazawa, and T. Seya. 2003. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-β induction. *Nat. Immunol.* 4:161–167. First published on January 21, 2003; 10.1038/ni886.
28. Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, and S. Akira. 2003. Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. *Science.* 301:640–643. First published on July 10, 2003; 10.1126/science.1087262.
29. Hoebe, K., X. Du, P. Georgel, E. Janssen, K. Tabeta, S.O. Kim, J. Goode, P. Lin, N. Mann, S. Mudd, et al. 2003. Identification of *Lps2* as a key transducer of MyD88-independent TIR signalling. *Nature.* 424:743–748. First published on July 20, 2003; 10.1038/nature01889.
30. Sato, S., M. Sugiyama, M. Yamamoto, Y. Watanabe, T. Kawai, K. Takeda, and S. Akira. 2003. Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-κB and IFN-regulatory factor-3, in the Toll-like receptor signaling. *J. Immunol.* 171:4304–4310.
31. Yamamoto, M., S. Sato, H. Hemmi, S. Uematsu, K. Hoshino, T. Kaisho, O. Takeuchi, K. Takeda, and S. Akira. 2003. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat. Immunol.* 4:1144–1150. First published on October 12, 2003; 10.1038/ni986.
32. Fitzgerald, K.A., D.C. Rowe, B.J. Barnes, D.R. Caffrey, A. Visintin, E. Latz, B. Monks, P.M. Pitha, and D.T. Golenbock. 2003. LPS-TLR4 signaling to IRF-3/7 and NF-κB involves the toll adapters TRAM and TRIF. *J. Exp. Med.* 198:1043–1055. First published on September 29, 2003; 10.1084/jem.20031023.
33. Sharma, S., B.R. tenOever, N. Grandvaux, G.P. Zhou, R. Lin, and J. Hiscott. 2003. Triggering the interferon antiviral response through an IKK-related pathway. *Science.* 300:1148–1151. First published on April 17, 2003; 10.1126/science.1081315.
34. Fitzgerald, K.A., S.M. McWhirter, K.L. Faia, D.C. Rowe, E. Latz, D.T. Golenbock, A.J. Coyle, S.M. Liao, and T. Maniatis. 2003. IKKε and TBK1 are essential components of the IRF3 signaling pathway. *Nat. Immunol.* 4:491–496. First published on April 14, 2003; 10.1038/ni921.
35. Shimada, T., T. Kawai, K. Takeda, M. Matsumoto, J. Inoue, Y. Tatsumi, A. Kanamaru, and S. Akira. 1999. IKK-*i*, a novel lipopolysaccharide-inducible kinase that is related to IκB kinases. *Int. Immunol.* 11:1357–1362.
36. Peters, R.T., S.M. Liao, and T. Maniatis. 2000. IKKε is part of a novel PMA-inducible IκB kinase complex. *Mol. Cell.* 5:513–522.
37. Pomerantz, J.L., and D. Baltimore. 1999. NF-κB activation by a signaling complex containing TRAF2, TANK and TBK1, a novel IKK-related kinase. *EMBO J.* 18:6694–6704.
38. Tojima, Y., A. Fujimoto, M. Delhase, Y. Chen, S. Hatakeyama, K. Nakayama, Y. Kaneko, Y. Nimura, N. Motoyama, K. Ikeda, et al. 2000. NAK is an IκB kinase-activating kinase. *Nature.* 404:778–782.
39. Bonnard, M., C. Mirtsos, S. Suzuki, K. Graham, J. Huang, M. Ng, A. Itie, A. Wakeham, A. Shahinian, W.J. Henzel, et al. 2000. Deficiency of T2K leads to apoptotic liver degeneration and impaired NF-κB-dependent gene transcription. *EMBO J.* 19:4976–4985.
40. Nomura, F., T. Kawai, K. Nakanishi, and S. Akira. 2000. NF-κB activation through IKK-*i*-dependent I-TRAF/TANK phosphorylation. *Genes Cells.* 5:191–202.
41. Iwamura, T., M. Yoneyama, K. Yamaguchi, W. Suhara, W. Mori, K. Shiota, Y. Okabe, H. Namiki, and T. Fujita. 2001. Induction of IRF-3/-7 kinase and NF-κB in response to double-stranded RNA and virus infection: common and unique pathways. *Genes Cells.* 6:375–388.
42. Nakaya, T., M. Sato, N. Hata, M. Asagiri, H. Suemori, S. Noguchi, N. Tanaka, and T. Taniguchi. Gene induction pathways mediated by distinct IRFs during viral infection. 2001. *Biochem. Biophys. Res. Commun.* 283:1150–1156.
43. Lin, R., C. Heylbroeck, P. Genin, P.M. Pitha, and J. Hiscott. 1999. Essential role of interferon regulatory factor 3 in direct activation of RANTES chemokine transcription. *Mol. Cell. Biol.* 19:959–966.

Downloaded from www.jem.org on April 27, 2005

44. Genin, P., M. Algarte, P. Roof, R. Lin, and J. Hiscott. 2000. Regulation of RANTES chemokine gene expression requires cooperativity between NF-κB and IFN-regulatory factor transcription factors. *J. Immunol.* 164:5352–5361.
45. Chariot, A., A. Leonardi, J. Muller, M. Bonif, K. Brown, and U. Siebenlist. 2002. Association of the adaptor TANK with the IκB kinase (IKK) regulator NEMO connects IKK complexes with IKKε and TBK1 kinases. *J. Biol. Chem.* 277: 37029–37036. First published on July 19, 2002; 10.1074/jbc.M205069200.
46. Doyle, S., S. Vaidya, R. O'Connell, H. Dadgostar, P. Dempsey, T. Wu, G. Rao, R. Sun, M. Haberland, R. Modlin, and G. Cheng. 2002. IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity.* 17:251–263.
47. Diebold, S.S., M. Montoya, H. Unger, L. Alexopoulou, P. Roy, L.E. Haswell, A. Al-Shamkhani, R. Flavell, P. Borrow, and C. Reis e Sousa. 2003. Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. *Nature.* 424:324–328. First published on June 22, 2003; 10.1038/nature01783.

Downloaded from www.jem.org on April 27, 2005

TOLL-LIKE RECEPTOR SIGNALLING

Shizuo Akira and Kiyoshi Takeda‡*

One of the mechanisms by which the innate immune system senses the invasion of pathogenic microorganisms is through the Toll-like receptors (TLRs), which recognize specific molecular patterns that are present in microbial components. Stimulation of different TLRs induces distinct patterns of gene expression, which not only leads to the activation of innate immunity but also instructs the development of antigen-specific acquired immunity. Here, we review the rapid progress that has recently improved our understanding of the molecular mechanisms that mediate TLR signalling.

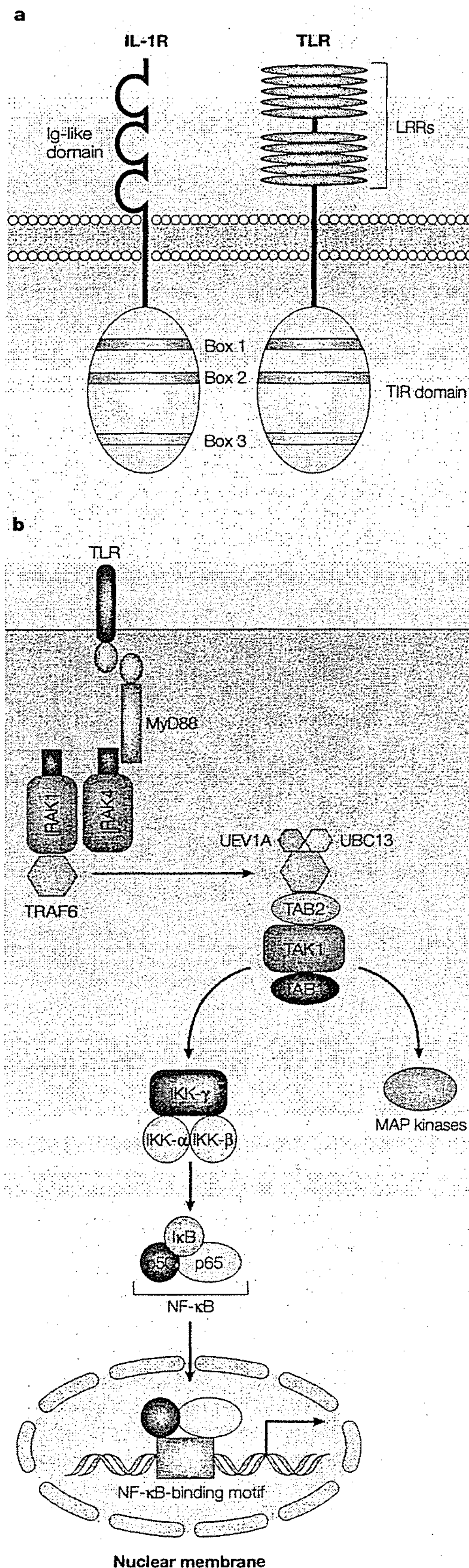
All living organisms are exposed constantly to microorganisms that are present in the environment and need to cope with invasion of these organisms into the body. The vertebrate immune response can be divided into innate and acquired immunity, with innate immunity being the first line of defence against pathogens. By contrast, acquired immune responses are slower processes, which are mediated by T and B cells, both of which express highly diverse antigen receptors that are generated through DNA rearrangement and are thereby able to respond to a wide range of potential antigens. This highly sophisticated system of antigen detection is found only in vertebrates and has been the subject of considerable research. Far less attention has been directed towards innate immunity, as it has been regarded as a relatively nonspecific system, with its main roles being to destroy pathogens and to present antigen to the cells involved in acquired immunity. However, recent studies have shown that the innate immune system has a greater degree of specificity than was previously thought and that it is highly developed in its ability to discriminate between self and foreign pathogens[1]. This discrimination relies, to a great extent, on a family of evolutionarily conserved receptors, known as the Toll-like receptors (TLRs), which have a crucial role in early host defence against invading pathogens[1,2]. Furthermore, accumulating evidence indicates that activation of the innate immune system is a prerequisite for the induction of acquired immunity, particularly for the induction of a T helper 1 (T_H1)-cell response[3,4]. This marked shift in our thinking has changed our ideas about the pathogenesis and treatment of cancers, and infectious, immune and allergic diseases. In the past few years, our knowledge of TLR signalling and the responses these receptors control has greatly increased. In this review, we discuss the TLRs, focusing on their signalling pathways.

**Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, and ERATO, Japan Science and Technology Agency, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan.
‡Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
Correspondence to S.A.
e-mail: sakira@biken.osaka-u.ac.jp*
doi:10.1038/nri1391

TLR/IL-1R superfamily: structure and function

The discovery of the TLR family began with the identification of Toll, a receptor that is expressed by insects and was found to be essential for establishing dorsoventral polarity during embryogenesis[5]. Subsequent studies revealed that Toll also has an essential role in the insect innate immune response against fungal infection[6]. Homologues of Toll were identified through database searches, and so far, 11 members of the TLR family have been identified in mammals. The TLRs are type I integral membrane glycoproteins, and on the basis of considerable homology in the cytoplasmic region, they are members of a larger superfamily that includes the interleukin-1 receptors (IL-1Rs). By contrast, the extracellular region of the TLRs and IL-1Rs differs markedly: the extracellular region of TLRs contains leucine-rich repeat (LRR) motifs, whereas the extracellular region of IL-1Rs contains three immunoglobulin-like domains (FIG. 1a).

Toll/IL-1R domain. TLRs and IL-1Rs have a conserved region of ~200 amino acids in their cytoplasmic tails, which is known as the Toll/IL-1R (TIR) domain[7]. Within the TIR domain, the regions of homology comprise three conserved boxes, which are crucial for



signalling (FIG. 1a). Amino-acid sequence conservation among the TIR domains is generally 20–30%, and these domains vary in size. The crystal structures of the TIR domains of human TLR1 and TLR2 have been obtained and analysed; they contain a central five-stranded parallel β-sheet, which is surrounded by five α-helices on each side[8]. These two secondary structural elements are connected by loops: for example, the BB loop connects the strand β-B and the helix α-B. The conserved boxes 1 and 2 and the BB loop are adjacent and display most of their side chains for interaction with adaptor molecules.

C3H/HeJ mice have a defect in their ability to respond to lipopolysaccharide (LPS) because of a missense mutation in the *Tlr4* gene[9], which alters the sequence located at the tip of the BB loop, farthest from the rest of the TIR domain. This indicates that the mutation abrogates LPS signalling not because it disrupts the TIR domain structure itself, but rather because it disrupts a direct point of contact with another molecule or molecules, specifically with other TIR-domain-containing molecules.

Leucine-rich repeats. The extracellular domain of TLRs contains 19–25 tandem copies of the LRR motif. Each repeat consists of 24–29 amino acids and contains the leucine-rich sequence XLXXLXLXX, and another conserved sequence XØXXØX$_4$FXXLX (REF. 10), where X denotes any amino acid and Ø a

Figure 1 | **TLR structure and signalling. a** | Toll-like receptors (TLRs) and interleukin-1 receptors (IL-1Rs) have a conserved cytoplasmic domain, that is known as the Toll/IL-1R (TIR) domain. The TIR domain is characterized by the presence of three highly homologous regions (known as boxes 1, 2 and 3). Despite the similarity of the cytoplasmic domains of these molecules, their extracellular regions differ markedly: TLRs have tandem repeats of leucine-rich regions (known as leucine rich repeats, LRR), whereas IL-1Rs have three immunoglobulin (Ig)-like domains. **b** | Stimulation of TLRs triggers the association of MyD88 (myeloid differentiation primary-response protein 88), which in turn recruits IRAK4 (IL-1R-associated kinase 4), thereby allowing the association of IRAK1. IRAK4 then induces the phosphorylation of IRAK1. TRAF6 (tumour-necrosis-factor-receptor-associated factor 6) is also recruited to the receptor complex, by associating with phosphorylated IRAK1. Phosphorylated IRAK1 and TRAF6 then dissociate from the receptor and form a complex with TAK1 (transforming-growth-factor-β-activated kinase), TAB1 (TAK1-binding protein 1) and TAB2 at the plasma membrane (not shown), which induces the phosphorylation of TAB2 and TAK1. IRAK1 is degraded at the plasma membrane, and the remaining complex (consisting of TRAF6, TAK1, TAB1 and TAB2) translocates to the cytosol, where it associates with the ubiquitin ligases UBC13 (ubiquitin-conjugating enzyme 13) and UEV1A (ubiquitin-conjugating enzyme E2 variant 1). This leads to the ubiquitylation of TRAF6, which induces the activation of TAK1. TAK1, in turn, phosphorylates both mitogen-activated protein (MAP) kinases and the IKK complex (inhibitor of nuclear factor-κB (IκB)-kinase complex), which consists of IKK-α, IKK-β and IKK-γ (also known as IKK1, IKK2 and nuclear factor-κB (NF-κB) essential modulator, NEMO, respectively). The IKK complex then phosphorylates IκB, which leads to its ubiquitylation and subsequent degradation. This allows NF-κB to translocate to the nucleus and induce the expression of its target genes.