Role of Itk signalling in the interaction between influenza A virus and T-cells

Kewei Fan,1,2† Yinping Jia,1,3† Song Wang,1 Hua Li,1 Defeng Wu,2 Guoshun Wang4 and Ji-Long Chen1

Correspondence
Ji-Long Chen
chenjl@im.ac.cn

1CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences (CAS), Beijing, PR China
2College of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou, PR China
3College of Animal Science and Technology, Southwest University, Chongqing, PR China
4Gene Therapy Program, Department of Medicine and Genetics, Louisiana State University Health Sciences Center, New Orleans, LA, USA

Although the T-cell-mediated immune response to influenza virus has been studied extensively, little information is available on the direct interaction between influenza virus and T-cells that pertains to severe diseases in humans and animals. To address these issues, we utilized the BALB/c mouse model combined with primary T-cells infected with A/WSN/33 influenza virus to investigate whether influenza virus has an affinity for T-cells in vivo. We observed that small proportions of CD4+ T-cells and CD8+ T-cells in spleen and thymus expressed viral proteins in infected mice. A significant proportion of mouse primary T-cells displayed expression of α2,6 sialic acid-linked influenza virus receptor and were infected directly by influenza A virus. These experiments reveal that there exists a population of T-cells that is susceptible to influenza A virus infection. Furthermore, we employed human Jurkat T-cells to investigate the virus–T-cell interaction, with particular emphasis on understanding whether Itk (interleukin-2-inducible T-cell kinase), a Tec family tyrosine kinase that regulates T-cell activation, is involved in virus infection of T-cells. Interestingly, influenza virus infection resulted in an increased recruitment of Itk to the plasma membrane and an increased level of phospholipase C-γ1 (PLC-γ1) phosphorylation, suggesting that Itk/PLC-γ1 signalling is activated by the virus infection. We demonstrated that depletion of Itk inhibited the replication of influenza A virus, whereas overexpression of Itk increased virus replication. These results indicate that Itk is required for efficient replication of influenza virus in infected T-cells.

INTRODUCTION

Influenza A virus can cause acute respiratory infection in humans and animals throughout the world. Therefore, influenza A virus has continued to be a significant public-health threat (Tscherne & García-Sastre, 2011). Much attention has consequently been given to the better understanding of mechanisms underlying host antiviral immunity. It has been shown that, in addition to humoral immunity, the immune responses for virus infection are conducted by several types of cells and cytokines involved both in innate and in adaptive immunity. In particular, T-cell-mediated adaptive immunity plays a critical role in virus clearance. For example, influenza virus infection activates the responses of virus-specific CD8+ cytolytic T lymphocytes (CTLs); CTLs in turn contribute to virus clearance by releasing perforin and granzymes from cytoplasmic granules, which leads to lysis of virus-infected cells (Hatta et al., 2010; Johnson et al., 2003; Wu et al., 2011; Zammit et al., 2006). CD4+ T-cells certainly also contribute to the control of influenza virus infection. Previous findings have suggested that, without protective antibody responses, individuals vaccinated against seasonal influenza A virus may still benefit from pre-existing cross-reactive memory CD4+ T-cells, reducing their susceptibility to the infection of pandemic strains (Ge et al., 2010; Miao et al., 2010). Together, these results have provided novel insights into the immune control of virus infection.

However, despite progress in understanding the immune responses for host defence and antiviral immunological mechanisms, there is little information available on the direct interaction between influenza virus and the host immune system that pertains to severe diseases in humans and animals (Hatta et al., 2010). Early work demonstrated...
that the pathogenesis of influenza infections was associated with alterations in the lymphohaemopoietic system and experimental infection of animals with highly virulent influenza A virus resulted in the destruction of lymphocytes and histopathological necrosis of lymphoid tissues (Hinshaw et al., 1994; Mori et al., 1995; Tumpey et al., 2000; Van Campen et al., 1989). However, what is less well understood is whether and how influenza infection could damage the immune system directly and alter its ability to destroy the infectious pathogens that ultimately are detrimental to the host. In addition, little information exists on the impacts of influenza virus infection on the anatomical organization of the lymphocytes and tissues of the immune system, which is of critical importance for the generation of effective and protective responses against influenza viruses. Moreover, it is unclear whether influenza virus has an affinity for lymphocytes of the immune system and whether virus–lymphocyte interaction depends on events that are associated with cellular activation induced by signalling pathways such as T-cell receptor (TCR) signalling.

Signalling through the TCR regulates key events in the life of T-cells. The Tec family, an intracytoplasmic tyrosine kinase family, has been recognized as the important mediator of the antigen receptor signalling in lymphocytes and myeloid cells (Andreotti et al., 2010; Felices et al., 2007; Readinger et al., 2009). Itk (interleukin-2-inducible T-cell kinase) is an important member of the Tec family expressed in T-lymphocytes, NK cells and mast cells (Smith et al., 2001). As a crucial component of the TCR signalling pathway in lymphocytes, Itk plays a key role in T-cell activation (Schwartzberg et al., 2005). Following the activation of PI3K (phosphatidylinositol 3-kinase) and accumulation of PIP3 (phosphatidylinositol trisphosphate) in the plasma membrane of the T-cell (Andreotti et al., 2010; Siliciano et al., 1992; Smith et al., 2001), Itk is recruited to the plasma membrane via its pleckstrin homology (PH) domain and phosphorylated by the Src kinase, Lck (August et al., 1997; Ching et al., 1999). Once activated, Itk can directly phosphorylate its downstream target, phospholipase C-γ1 (PLC-γ1), resulting in activation of this phospholipase. Itk signalling has also been suggested to be a critical factor in regulating human immunodeficiency virus (HIV) infection and replication in T-cells (Readinger et al., 2008). However, the relationship between Itk signalling and influenza virus infection and replication is unknown.

In this study, we utilized the BALB/c mouse model and primary T-cells infected with A/WSN/33 influenza virus (H1N1) to examine whether influenza virus has an affinity for T-cells in lymphoid organs. Furthermore, we employed the human Jurkat T-cell line to investigate the virus–Jurkat T-cell interaction, with particular emphasis on determining whether Itk signalling is involved in virus–T-cell interaction. Our results suggest that influenza A virus may directly target a special population of T-cells in vivo. We further demonstrated that influenza A virus infects human Jurkat T-cells directly and that Itk signalling plays an important role in the replication of influenza A virus in these cells.

RESULTS

Influenza A virus infection causes destruction of spleen and thymus in mice

Previous studies have suggested that destructive effects of highly virulent influenza A virus on the immune system may be one crucial factor that contributes to the fatal diseases in mammalian hosts (Tumpey et al., 2000; Yuen et al., 1998). However, the mechanisms by which infection by influenza A virus causes severe pathology and death of lymphocytes and tissues of the immune system are poorly defined. Therefore, here we used the BALB/c mouse model inoculated with A/WSN/33 influenza virus (H1N1) to determine the influence of virus infection on the maintenance of two lymphoid organs: the thymus, the site of T-cell maturation, and the spleen, the major site of immune responses to antigens.

BALB/c mice were infected intranasally with a lethal dose of the A/WSN/33 influenza virus (a mouse-adapted influenza A virus commonly used to create lethal infections in the mouse model) and their thymuses and spleens were examined pathologically to determine the ability of influenza A virus to disrupt these mammalian lymphoid organs. We observed that all infected mice died within 7 days post-infection (p.i.). A decrease in body weight of infected mice was observed during 3 days p.i. (Fig. 1a). Surprisingly, the sizes of their thymuses and spleens were markedly reduced by 3 days p.i. (Fig. 1b, c). Then single-cell suspensions were prepared and lymphocytes were isolated from these lymphoid organs following virus infection. We found that the total number of lymphocytes decreased around 10-fold in spleen and around 30-fold in thymus after infection with A/WSN/33 influenza virus (Table S1, available in JGV Online). To further investigate the pathology of these lymphoid organs, we performed haematoxylin and eosin (HE) staining of the spleens and thymuses in virus-infected mice. On day 3 p.i., the spleens showed a marked loss of lymphocytes in the red and white pulps, which led to a dramatic decrease in lymphoid nodule size of white pulps compared with the control (Fig. 1d). In the thymus, the most striking change caused by influenza virus infection was the clear cortical atrophy, due to a marked loss of lymphocytes. An increased number of dark-staining lymphocytes were observed in the medullary region of the thymus (Fig. 1d). These results suggest that infection of influenza virus may cause lymphocyte destruction in primary and secondary lymphoid organs.

A population of T-cells is susceptible to influenza A virus infection in vivo

Although it has long been reported that influenza virus causes lymphopenia, the interaction between the virus and lymphocytes remains to be clarified. Therefore, next we determined whether influenza virus has an affinity for lymphocytes in lymphoid organs. To this end, single-lymphocyte suspensions were prepared from spleens and thymuses of infected mice and Western blot analysis was performed to detect the
expression of viral protein in these cells. As shown in Fig. 2(a), nucleoprotein (NP) of influenza A virus was clearly detected in lysates of splenocytes, thymocytes and control cells from lung. This implies that influenza A virus may target lymphocytes directly in infected mice.

To confirm the virus–lymphocyte interaction in lymphoid organs, we employed immunofluorescence analysis to examine the expression of viral proteins in lymphocytes. Lymphocytes were isolated from spleens and thymuses of infected mice and stained with antiviral NP and haemagglutinin (HA) antibodies. Studies using confocal microscopy showed that a minimal number of lymphocytes in spleens and thymuses were positive for viral NP and HA expression (data not shown). The results indicate that influenza A virus may have an affinity for a special population of lymphocytes. To determine whether influenza A virus targets T-cells, we used flow cytometry to sort the CD3+ T-cells from lymphocyte suspensions harvested from the thymuses and spleens of infected mice on day 3 p.i. Immunofluorescence analysis demonstrated that a small population of CD3+ T-cells was viral NP- and HA-positive (Fig. 2b). Because of this finding, it was interesting to determine whether T-cells expressed the α-2,6 sialic acid-linked influenza virus receptor. Indeed, we detected the expression of this receptor in a
significant proportion of mouse primary CD3\(^+\) T-cells (Fig. 2c). Because our experiments presented above revealed a marked loss of lymphocytes in lymphoid organs of infected mice, apoptosis and lymphocyte survival were analysed by propidium iodide/annexin V binding. We found that infection of influenza virus promoted lymphocyte apoptosis (Fig. 2d).

Next, multicolour flow cytometry was used to examine the expression of viral proteins HA and NP in CD4\(^+\) or CD8\(^+\) T-cells in mouse thymus and spleen on day 3 p.i. with A/WSN/33 influenza virus. We observed that a small proportion (approx. 1.0–1.5\%) of CD4\(^+\) or CD8\(^+\) T-cells in spleen and thymus expressed viral proteins in infected mice, but not in control mice (Fig. 3 and data not shown). Together, these observations suggest that influenza A virus may have an ability to target a special population of T-cells in vivo.

**Primary T-cells and the Jurkat T-cell line can be infected by influenza A virus in vitro**

To confirm that a proportion of T-cells is susceptible to influenza A virus infection, mouse primary T-cells were isolated from spleen or thymus and infected with A/WSN/33 influenza virus at an m.o.i. of 2.0. Interestingly, viral NP and HA proteins were detected in T-cells by Western blot analysis (Fig. 4a). Experiments using multicolour flow cytometry demonstrated that approximately 10% of CD3\(^+\) T-cells expressed the viral proteins. Importantly, a population of CD4\(^+\) or CD8\(^+\) T-cells can be infected by influenza virus in vitro (Fig. 4b and data not shown).

As our data indicate that influenza virus can target T-cells in vitro, we next asked whether the establishment of this virus infection depends on events that are associated with T-cell activation, like HIV infection of T-cells. To this end, we wished to employ an immortal T-cell line to investigate the virus–T-cell interaction. Hence, the CD3\(^+\) human Jurkat T-cell line was used and infected with A/WSN/33 influenza virus. Surprisingly, we observed that a large percentage of Jurkat T-cells could be infected by A/WSN/33 virus at an m.o.i. of 2.0, as indicated by HA expression (Fig. 5a). The \(\alpha\)-2,6 sialic acid-linked receptor was also detected in Jurkat cells (Fig. 2c). Additionally, the expression of influenza virus NP protein was detectable in Jurkat cells at 4 h p.i. and displayed a time-dependent increase in response to virus infection (Fig. 5b). Together, the results indicate that a small portion of T-cells are susceptible to influenza A virus infection in vivo, and both primary T-cells and the Jurkat T-cell line can be infected by influenza A virus in vitro.

**Influenza A virus infection affects Itk expression and cellular localization**

Itk functions in T-cells to regulate the activity of the phospholipase PLC-\(\gamma\)1, which is important for the activation
of T-cells. It has been shown that Itk plays a critical role in HIV infection and replication in T-cells (Berg et al., 2005; Readinger et al., 2008). To determine whether Itk is involved in the infection of T-cells by influenza virus, we examined the expression of Itk in virus-infected Jurkat cells. Interestingly, the expression of Itk appeared to be increased significantly after 8 h p.i. (Fig. 6a).

Previous results showed that Itk activation requires recruitment of Itk to the plasma membrane via its PH domain (Ching et al., 1999). To determine whether the infection of influenza virus alters the localization of Itk in Jurkat cells, we performed immunofluorescence staining to investigate the cellular distribution of the Itk protein. As shown in Fig. 6(b), an increased amount of Itk was seen in the plasma membrane after virus infection. To confirm such enhanced membrane localization of Itk in Jurkat cells, protein extracts enriched in cytosol and membranes were analysed by Western blotting. Itk and PLC-γ1 were mainly found in cytosol fractions in control cells (Fig. 6c, lane 4). In contrast, the level of Itk in the membrane fraction was increased markedly in the cells infected with influenza viruses (Fig. 6c, lane 5). Collectively, these data indicate that Itk is recruited to the plasma membrane during virus infection.

**Upregulation of phosphorylated PLC-γ1 is Itk-dependent in influenza virus-infected Jurkat cells**

It has been recognized that PLC-γ1 functions downstream of Itk signalling. The PLC-γ1 phosphorylation sites required for its activation are Tyr775 and Tyr783 (Serrano et al., 2005). To identify the influence of influenza virus infection on the Itk/PLC-γ1 pathway, the phosphorylation status of PLC-γ1 was examined in Jurkat cells infected with A/WSN/33 virus at different time points p.i. As shown in Fig. 7(a), the phosphorylation of PLC-γ1 at Tyr775 and Tyr783 was detected in infected cells. Notably, the levels of phosphorylated PLC-γ1 were markedly upregulated around 24–32 h p.i., although the total amount of PLC-γ1 was not significantly changed during infection. This experiment demonstrated that influenza virus infection resulted in the activation of PLC-γ1 signalling at the late stages of infection.

It has been shown that Itk interacts with PLC-γ1 and mediates phosphorylation of PLC-γ1 on Tyr775 and Tyr783 (Bogin et al., 2007; Serrano et al., 2005). Additionally, our results revealed that infection of influenza virus caused the recruitment of Itk to the plasma membrane. In an attempt to gain a better understanding of Itk function in influenza virus-infected cells, we generated a lentiviral vector expressing Itk-specific short hairpin RNA (shRNA) to disrupt Itk expression. GFP was used as a reporter to determine positive
Using this vector, we generated stable Jurkat cell lines expressing Itk-specific shRNA. Western blot analysis demonstrated that Itk expression in these cell lines was reduced greatly (Fig. 7b).

Next, we investigated the effect of Itk deficiency on phosphorylation of PLC-γ1 in Jurkat cells infected with A/WSN/33 influenza virus at an m.o.i. of 2.0. Western blot analysis showed that the phosphorylation levels of PLC-γ1 were downregulated in Itk-deficient cells after virus infection (Fig. 7c). Unlike normal Jurkat cells, Itk-deficient cells exhibited lower levels of phosphorylated PLC-γ1 at the late stages of infection, indicating that phosphorylation of PLC-γ1 is Itk-dependent in the infected cells. These data provide strong evidence that Itk mediates the activation of PLC-γ1 induced by the influenza virus infection.

**Targeting Itk inhibits the replication of influenza A virus in Jurkat T-cells**

Because of the observed alteration of Itk/PLC-γ1 signalling in influenza virus-infected Jurkat T-cells, it was of interest to test the functional involvement of Itk signalling in the replication of influenza A virus. To this end, we infected the Jurkat cell lines stably expressing the shRNAs targeting Itk or control luciferase with A/WSN/33 influenza virus. The virus replication was examined by HA assay at different time points p.i. As shown in Fig. 8(a), the replication of influenza A virus was clearly inhibited when expression of endogenous Itk in Jurkat T-cells was disrupted by the shRNA. However, no significant difference in viral NP expression was observed at the early stages (Fig. 8b), suggesting that depletion of Itk in Jurkat T-cell may not affect virus entry and transcription, but affect other step(s) of virus replication. To confirm that the phenotype of the Itk knockdown cells indeed represented the effect of Itk depletion, mouse Itk wild-type (WT) and an inactive mutant Itk (W208K), which avoid the shRNA targeting, were expressed in the Itk-deficient Jurkat cell line (Fig. 8c). We found that overexpression of Itk WT

---

**Fig. 4.** A proportion of primary T-cells can be infected by influenza virus in vitro. (a) Primary mouse T-cells from thymus and spleen were infected with A/WSN/33 influenza virus at an m.o.i. of 2.0. Then 16 h p.i. cell lysates were analysed by Western blotting using indicated antibodies. (b) Primary mouse lymphocytes were prepared and infected with influenza virus as described in (a). Flow cytometry using antibodies targeting viral HA, mouse CD3, CD4 and CD8 was performed as described in the legend to Fig. 3. Blue and orange indicate the populations of HA^+^CD3^+^CD4^+^ and HA^+^CD3^+^CD8^+^ cells, respectively. These results are representative of three identical experiments.

**Fig. 5.** A large percentage of Jurkat T-cells can be infected by A/WSN/33 influenza A virus. (a) Immunofluorescence staining using an anti-HA antibody shows the viral HA expression in infected Jurkat T-cells. The nuclei were stained with DAPI. Bar, 10 μm. (b) Jurkat T-cells infected with A/WSN/33 influenza virus were analysed by immunoblotting to detect the expression of viral NP at different time points. These results are representative of three identical experiments.
protein clearly increased the replication of influenza A virus in Jurkat T-cells compared with that of Itk W208K mutant or the empty vector control (Fig. 8d). Together, these results establish a role for Itk in the replication of influenza A virus in Jurkat T-cells.

**DISCUSSION**

Seasonal and pandemic influenza A virus continues to pose a severe threat throughout the world (Li et al., 2010). Current studies of influenza virus immunity focus on understanding of the immune responses for host defences...
and antiviral immunological mechanisms. For example, it has been well established that T-cell-mediated adaptive immunity plays an important role in virus clearance. However, what is less well understood is whether influenza A virus could target T-cells directly and damage host lymphoid organs, leading to alterations of the ability of the immune system to destroy the infectious pathogens.

In this study, we used a mouse model, primary T-cells and cell culture to investigate the interaction between influenza A virus and T-cells. We observed that intranasal infection of mice with A/WSN/33 influenza virus resulted in a significant reduction in spleen size and thymus size, and a marked decrease in the total number of lymphocytes in spleen and thymus. These results are consistent with previous studies showing that the infection of mice with highly pathogenic H5N1 viruses was associated with progressive lymphopenia until the time of death (Tumpey et al., 2000). Furthermore, we observed that approximately 1.0–1.5 % of CD4+ T-cells and CD8+ T-cells in the spleen and thymus expressed viral proteins in infected mice. These data suggest that there exists a special population of T-cells that are susceptible to influenza A virus infection in vivo. However, the results should be confirmed with seasonal viruses in primary human lymphocytes in future studies, as some of the effect may be due to the mouse adaptation of A/WSN/33.

Overall, the cell types infected by influenza A virus in vivo were poorly understood. Our results presented here suggest that influenza A virus has an affinity for at least a minor proportion of T-cells. A previous report has revealed that a significant proportion of NK and B-cells expressed viral protein, suggesting active infection of these cells by influenza virus (Manicassamy et al., 2010). In this study, the authors also showed that a minor proportion of CD4+ and CD8+ cells were GFP-positive in mice infected with a GFP reporter influenza virus, although it is possible that some of these cells are GFP-positive due to uptake of virus-infected apoptotic cells (Manicassamy et al., 2010). In addition, we found that infection of influenza virus promoted lymphocyte apoptosis. However, how influenza virus infection could result in apoptosis and depletion of lymphocytes in lymphoid tissue remains to be elucidated.

In the 1997 outbreak of H5N1 avian influenza virus in humans, infection of viruses with high pathogenicity for...
Role of Itk in influenza virus–T-cell interaction

humans was associated with lymphopenia (Fornek et al., 2009; Yuen et al., 1998). A similar phenomenon was observed in mice infected with two representative 1997 H5N1 viruses, A/Hong Kong/483/97 (HK483) and A/Hong Kong/486/97 (HK486) (Tumpey et al., 2000). Therefore, it is possible that highly pathogenic influenza viruses may infect high levels of immune cells and/or may have preferential cellular tropism for immune cells and thereby damage host immune tissues and lead to alterations of the ability of the immune system to destroy the infectious pathogens. The mechanism by which infection of influenza virus alters the organization and function of the immune system await further investigation.

Tec family tyrosine kinase is crucial for antigen-receptor-induced lymphocyte activation (Qi & August, 2007). Previous experiments have shown that Itk plays a critical role in T-cell-mediated immune responses (Andreotti et al., 2010; Bunnell et al., 2000). These studies provide us with abundant information about Itk interacting with other signalling partners such as PLC-γ1 in T-cells. It has been documented that Itk interacts with PLC-γ1 and phosphorylates it directly, leading to activation of PLC-γ1 and generation of IP3 (inositol 1,4,5-triphosphate) and DAG (1,2-diacylglycerol), release of intracellular calcium stores, and finally activation of T-cells (Berg et al., 2005; Hogan et al., 2003). Itk-deficient mouse display decreases in interleukin-2 production and defects in TH2 cytokine production in T-cells (Berg et al., 2005; Grasis et al., 2010). In addition, Itk has been shown to be involved in the regulation of actin cytoskeleton reorganization downstream of both TCR and chemokine receptors (Berg et al., 2005). Because productive infection of T-cells with HIV requires T-cell activation, chemokine receptors and actin reorganization, inhibition of Itk blocks HIV infection by affecting multiple steps of HIV replication (Readinger et al., 2008).

In the present study, we have analysed the role of Itk signalling in the infection of Jurkat T-cells with influenza virus. Our experiments revealed that Jurkat cells could be infected directly by influenza A virus. We observed the changes in Itk expression and localization in influenza virus-infected Jurkat cells. We further found that the level of PLC-γ1 phosphorylation was increased by influenza virus infection at the late stages of infection, and the phosphorylation of PLC-γ1 was Itk-dependent in these cells. These results suggest that influenza virus infection induces the activation of the Itk/PLC-γ1 signal pathway in Jurkat cells. Moreover, our data demonstrate that depletion of Itk inhibits the replication of influenza virus, whereas overexpression of Itk promotes this process, suggesting that Itk is required for efficient replication of influenza virus in Jurkat T-cells. Taken together, our experiments have provided some insights into the role of Itk in infection of T-cells by influenza A virus. However, the details of influenza virus–T-cell interaction and the mechanisms underlying functional involvement of Itk signalling in this process remain to be further determined.

METHODS

**Virus and antibodies.** Influenza virus strain A/WSN/33 (H1N1) was kindly provided by Dr George F. Gao (Institute of Microbiology, CAS) and propagated in eggs (Wang et al., 2011). The following antibodies were used in this study: anti-Itk (Cell Signaling Technology); anti-phospho-PLC-γ1 (Tyr783) (Cell Signaling Technology); anti-PLC-γ1 (Santa Cruz Biotechnology); anti-phospho-PLC-γ1 (Tyr775) (ECM Biosciences); anti-actin (Abcam); phycoerythrin (PE)-conjugated anti-mouse CD3 (eBioscience); allophycocyanin (APC)-conjugated anti-mouse CD3, CD4 and CD8 (eBioscience); anti-influenza A virus HA kindly provided by Dr George F. Gao; and anti-influenza A virus NP was obtained from Dr Wenjun Liu (Institute of Microbiology, CAS) (Zhang et al., 2009).

**Mouse experiments and preparation of lymphocyte suspension.** Mouse experiments were performed according to the guidelines of Chinese Animal Protection Law. All animal protocols were approved by the relevant Chinese authorities and by the Institutional Authority for Laboratory Animal Care. BALB/c mice (5 weeks old) were provided by Vital River Laboratory Animal Center (Beijing, China). For each mouse experiment, three mice in each group were used, and in each case three independent experiments were performed throughout this study. Mice were inoculated intranasally with a lethal dosage of the A/WSN/33 influenza virus (1 × 10⁶ p.f.u.). To determine the 50 % mouse lethal dose (MLD₅₀) of the virus, six groups of five mice were inoculated intranasally with 10⁻⁶ dilution series of virus (Chen et al., 2004). MLD₅₀ titres were calculated by the method of Reed & Muench (1938). On day 3 p.i., the mice were euthanized and their organs (thymus, lungs, spleen) were removed aseptically. Lymphocyte suspensions were prepared from spleens or thymuses using an EZ-Sep Mouse Lymphocyte Separation kit (Dakewe Biotech Company) according to the manufacturer’s instructions. Briefly, spleen and thymus were homogenized through a cell strainer into lymphocyte separation medium. Then the suspended cells were centrifuged at 800 g for 30 min and lymphocytes were isolated on a gradient.

**Flow cytometric analysis and cell sorting.** Single-cell suspensions (5 × 10⁶ cells) of lymphocytes from spleens or thymuses were stained with anti-α/β or anti-NP following by combinations of DyLight 405-conjugated anti-rabbit secondary antibody, PE-conjugated anti-mouse CD3 and APC-conjugated anti-mouse CD4 or CD8 antibodies according to the manufacturer’s instructions. Samples were analysed or sorted by a FACSArail flow cytometer (BD Biosciences).

**DNA construction, shRNA-based knockdown of Itk and generation of cell lines.** The murine Itk gene was subcloned into the pMIG-link retrovirus vector. The W208K mutation of the Itk gene was generated by using a site-directed mutagenesis kit (Stratagene). The sequences used in the shRNAs targeting Itk were described previously (Dombroski et al., 2005). A control shRNA targeting the luciferase gene, 5’-CTTACGCTGAAGCTCAG-3’, was used. The oligonucleotide duplex for each target was cloned into the pSIH-H1-GFP shRNA expression vector. Stable cell lines expressing the shRNAs targeting Itk or luciferase were generated using viral spin infection as described previously (Hehaly et al., 2009). Western blot analysis and real-time PCR were performed to determine the interference efficiency.

**Real-time PCR.** Total RNA was prepared using TRIzol reagent (Invitrogen), following the manufacturer’s instructions. Total RNA (2 μg) was reverse-transcribed using M-MuLV Reverse Transcriptase (Promega). Real-time quantitative RT-PCR was performed with SYBR premix Ex Taq (Takara) on an ABI 7300 instrument (Applied Biosystems). β-Actin (GenBank accession no. BC013835) was chosen as a reference gene for internal standardization.
Cellular fractionation, Western blotting and immunofluorescence study. Cytoplasmic and membrane proteins were isolated as described previously (Guo et al., 2010; Nishiumi & Ashida, 2007). Western blot analysis and the immunofluorescence study were performed as described previously (Chen et al., 2005; Guo et al., 2010). For the immunofluorescence study, cells were stained with either anti-Itk, anti-HA or anti-NP and incubated with fluorescence-conjugated secondary antibodies. Fluorescein-labelled *Sambucus nigra* lectin (SNA) (Vector Laboratories), specific for the α2,6 linked sialic acid to detect HuIV-Rs (human influenza virus receptors), was used to examine influenza virus receptor. Nuclei were visualized by DAPI staining. Cells were examined by confocal microscopy (model LSCM/FV500).

**Haemagglutinin (HA) assay.** Jurkat cells (5 × 10⁶) were infected with A/WSN/33 influenza virus at an m.o.i. of 0.05. Following adsorption for 1 h at 37°C, the cells were washed with PBS and maintained in RPMI 1640 (Gibco) containing 2 μg trypsin ml⁻¹. The supernatants of cell cultures were harvested at the indicated time and virus titres were assessed by HA assay. The assay was carried out in V-bottom microtitre plates. The supernatants were diluted with PBS and mixed with an equal volume of 0.5% chicken erythrocytes. After incubation for 30–60 min, negative reactions appear as dots at the bottom of the plates, whereas positive reactions form a homogenous reddish colour across the well. Virus titres were counted from the highest dilution factors that produced a positive reading.

**Histological analysis.** Mice were euthanized on day 3 p.i. and their spleens and thymuses were fixed in an excess of neutral formalin at 4°C, processed routinely and embedded in paraffin. Thereafter, 4–5 μm thick sections were cut from each block, affixed firmly to clean microscope slides, deparaffinized and stained with HE. The sections were viewed with an Olympus BH-2 microscope.

**Statistical analysis.** Comparison among groups was made using Student’s *t*-test. Data represent means ± s.e. Differences were considered statistically significant at *P* < 0.05.

**ACKNOWLEDGEMENTS**

We thank members of the laboratory of J.-L. C. for helpful discussion. This work was supported by the Ministry of Science and Technology of China (973 project grant 2010CB534004), National Key Technology R&D Program (2009BA183B01-8), Hundreds of Talents Program of the Chinese Academy of Sciences 2009–2014 and an intramural grant from the Chinese Academy of Sciences (2010-Biols-CAS-0204 and KSCX2-EW-J-6).

**REFERENCES**


