INTRODUCTION

NK-cells are large granular lymphocytes that can kill infected cells without prior sensitization and play an important role in innate immunity to microbial pathogens. NK-cells mediate protection against viruses, bacteria and parasites through the destruction of infected cells and by the secretion of cytokines that shape the adaptive immune response (Vivier et al., 2008). Increased NK-cell cytolytic activity has been observed soon after primary viral infections, and there is a striking correlation between poor NK-cell function and susceptibility to viral and other microbial infections (Adib-Conquy et al., 2014; Biron et al., 1989; Lee et al., 2007; Yarovinsky, 2014). NK-cells are an important early source of IFN-γ, which is critical for the activation of macrophages (Eckmann & Kagnoff, 2001; Martin-Fontecha et al., 2004; Schoenborn & Wilson, 2007). A more complex view of NK-cell function has emerged from both mouse and human studies, demonstrating that NK-cells can acquire diverse phenotypes and functions depending on the immune challenge (Vivier et al., 2008). These findings include the discoveries that NK-cells can be ‘educated’ and selected during development, undergo clonal expansion during infection, and become long-lived antigen-specific memory cells (Sun & Lanier, 2009; Vivier et al., 2011). Furthermore, distinct NK-cell subsets have been identified in both mice and humans, suggesting that NK-cells exhibit a variety of functions. Although the ability of NK-cells to interfere with adaptive immunity has been broadly characterized (Paust et al., 2010; Vivier et al., 2011), several recent studies have highlighted the importance of NK-cells in immunosuppressive functions. NK-cells can inhibit antiviral T-cell responses during murine cytomegalovirus or lymphocytic choriomeningitis virus infection (Lang et al., 2012; Su et al., 2001; Waggoner et al., 2012). NK-cells are also important for controlling harmful T-cell activity in autoimmunity and transplantation settings (Lünemann et al., 2009; Smeltz et al., 1999; Soderquest et al., 2011; Zhang et al., 1997). As a result, the existence of a population of regulatory NK-cells or ‘NKregs’ has been proposed (Lünemann et al., 2009; Su et al., 2001; Zhang et al., 1997). Thus, NK-cell activity can lead to both beneficial and detrimental outcomes due to their direct and indirect immunomodulatory responses.

Influenza viruses are an important cause of respiratory tract infections, and are responsible for 3–5 million severe clinical infections and 250 000–500 000 fatal cases annually (Stöhr, 2002; van de Sandt et al., 2012). Severe influenza is a clinical syndrome characterized by pathological pneumonia and hypercytokinemia in the lungs and serum (Chowell et al., 2009; Itoh et al., 2009). Additionally, NK-cells are involved in thymic atrophy induced by influenza A virus infection

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NK-cells have traditionally been viewed as innate effector lymphocytes that serve as a first line of defence against a range of viruses and tumours. More recently, the importance of NK-cell immunoregulatory functions has been highlighted. NK-cells can inhibit antiviral T-cell responses, and also play an important role in controlling harmful T-cell activity in autoimmunity and transplantation settings. Moreover, immunopathological effects of NK-cells during infection have been reported. Nevertheless, the phenotype and function of NK-cells in the thymus during influenza virus infection is not understood. In the present study, we demonstrated that influenza A virus (IAV) infection in mice led to severe thymic atrophy caused by increased thymic T-cell apoptosis and suppressed proliferation. We found that NK-cells played a critical role in this phenotype. IFN-γ production by NK-cells was a contributing factor for thymic atrophy during IAV infection. Taken together, our data indicate that NK-cells are involved in the thymic atrophy associated with IAV infection.
infection by several subtypes of influenza viruses (i.e. H5N1 and H1N1) results in a strong reduction in T-lymphocytes, also known as lymphopenia (Cao et al., 2009; Cunha et al., 2009; Maines et al., 2008; Tran et al., 2004). Peripheral lymphopenia occurs in parallel with thymic atrophy. Thymic atrophy is a common feature in a variety of acute infections and largely reflects intense lymphocyte depletion, particularly of cortical thymocytes bearing the CD4+CD8+ phenotype. This depletion corresponds to massive thymocyte apoptosis, as has been demonstrated for a variety of infections, including viral diseases (i.e. AIDS and rabies), experimental bacterial infections (i.e. tularemia and listeriosis), diseases caused by parasites (i.e. Trypanosoma cruzi, Plasmodium chaubi) and fungal infections (exemplified by experimental infections with Paracoccidioides brasiliensis and Histoplasma capsulatum) (Savino, 2006). To date, several mechanisms have been implicated in influenza virus infection-induced thymic atrophy, such as interference with T-lymphocyte development, increased levels of glucocorticoids and elevated IFN-γ production by innate CD8+ T-cells (Liu et al., 2014; Tian et al., 2012; Vogel et al., 2010).

Several studies have highlighted the pivotal role of NK-cells in the control of influenza A virus (IAV) infection, because defects in NK-cell activity or depletion resulted in delayed viral clearance and increased morbidity and mortality (Gazit et al., 2006; Nogusa et al., 2008; Stein-Streilein & Guffee, 1986). However, there also have been examples of NK-cells exacerbating the pathological process in the lungs following exposure to lethal-dose influenza virus infection in mice (Abdul-Careem et al., 2012; Zhou et al., 2013). Moreover, NK-cells also play important roles in bridging the innate and adaptive responses to IAV. For example, it was demonstrated that NK-cells regulated CD8+ T-cell priming and dendritic cell (DC) migration during IAV infection via IFN-γ- and perforin-dependent mechanisms (Ge et al., 2012; Paust et al., 2010). However, uncovering the major and complex role of NK-cells in IAV infection requires further investigation. In the present study, we demonstrated that NK-cells play a role in thymic atrophy during influenza virus infection; this function has not previously been investigated for NK-cells. We demonstrated that IAV infection induces severe thymic atrophy and that NK-cells play an important role in this pathological process. Our results reveal a new regulatory function for NK-cells and demonstrate the complexity of NK-cell responses during IAV infection.

RESULTS

Severe thymic atrophy was caused by increased apoptosis and reduced proliferation of thymic T-cells during influenza virus infection

To study the pathological reactions and extent of thymic atrophy during influenza virus infection, time-course experiments were performed with C57BL/6 mice intranasally (i.n.) infected with 3 × 10^7 p.f.u. PR8 virus. As shown in Fig. 1(a), the mice lost weight rapidly after PR8 infection; weight loss reached ~30 % 7 days post-infection (p.i.) and gradually recovered to the original level after 2 weeks. Infection also led to a progressive shrinking of the thymus accompanied by a drastic reduction in its cellularity (Fig. 1b). The total number of thymocytes decreased to ~30 % (28.1 ± 6.8 %) compared with mock-infected mice at 7 days p.i. Haematoxylin and eosin (HE) staining confirmed these results by showing a destroyed basic structure of the thymus and a diminished number of lymphocytes (Fig. 1c). Flow cytometry analysis of the major thymocyte populations revealed significant changes in the relative proportions of double-negative (DN; CD4−CD8−), double-positive (DP; CD4+CD8+) and single-positive (CD4 SP; CD4+CD8− or CD8 SP; CD4−CD8+) populations (Fig. 1d). DP populations showed a dramatic reduction at 7 days p.i. to ~10 % of the total thymic T-cells from nearly 80 % in the mock-infected mice. The total number of DP T-cells at 7 days p.i. was reduced to <5 % of the level detected in the mock-infected mice (0.52 ± 0.22 × 10^7 at 7 days p.i. versus 10.59 ± 0.14 × 10^7 in the mock-infected mice). DP T-cells were restored to normal levels by ~14 days p.i. Accompanying the dramatic reduction in DP T-cells, the proportions of DN, CD4 SP and CD8 SP T-cells increased accordingly at 7 days p.i., but the total cell numbers of DN and CD4 SP populations were reduced compared with their respective populations in the mock-infected mice. In contrast, the total cell number of the CD8 SP population was slightly increased compared with the corresponding population in the mock-infected mice (data not shown).

To investigate the reasons for the DP T-cell depletion, we first examined the apoptosis of thymic T-cells. As shown in Fig. 1(e), apoptosis of DP and CD4 SP T-cells was significantly increased after infection, especially at 7 days p.i. (P = 0.0069 for DP and P = 0.0056 for CD4 SP, 7 days p.i. versus mock-infected group). In contrast, the DN and CD8 SP populations showed a relatively stable level of basal apoptosis. Next, we investigated whether PR8 infection affected T-cell proliferation using the bromo-deoxyuridine (BrdU) incorporation assay. Surprisingly, proliferation was significantly suppressed in all populations (P_{DN} = 0.0000017, P_{DP} = 0.0015, P_{CD4SP} = 0.00042, P_{CD8SP} = 0.000019, 7 days p.i. versus mock-infected group) after infection. After infection, proliferating DN T-cells were reduced by >75 % (23.7 ± 5.8 %) compared with the mock-infected mice. Notably, the DN T-cells showed the most vigorous basal proliferation rate amongst the thymic T-cells. Thus, these results demonstrated that influenza virus infection caused severe thymic atrophy in mice. Increased apoptosis and decreased proliferation of thymic T-cells were the main reasons for this phenomenon.

NK-cells were activated by influenza virus infection

Several cytokines (i.e. IFN-α, IFN-γ, TNF-α, IL-6 and IL-10) may be involved in the process of thymic atrophy (Chen
Fig. 1. Influenza virus infection caused pathological reactions in mice with body weight loss and thymic atrophy. (a) Body weights of mice were monitored daily after influenza virus infection. The figure showed the per cent body weight of infected
mice compared with the day of infection. (b) Cell numbers (upper) and photographs (lower; bar, 1 cm) of thymuses from mock-infected mice and mice infected with influenza virus at the indicated time points. (c) Thymic sections from naive mice and infected mice at 3 and 7 days p.i. were examined using HE staining. Original magnification × 5; bar, 0.5 mm. (d) Changes in the thymocyte populations after influenza virus infection. Upper panel: representative flow cytometry plots showing the different T-cell populations at the indicated times post-infection. The numbers below the FACS cytometry plots indicate the percentage of cells in each region. Lower panel: summary graph showing the changes in the different T-cell populations at the indicated times post-infection. (e) Summary graph of apoptosis (left panel) or proliferation (right panel) of each population after influenza virus infection analysed by flow cytometry. Results shown are representative of at least three independent experiments with at least five mice in each group of (a, b) and at least three mice in each group of (c–e) for each experiment.

et al., 2005; Fayad et al., 2005; Papadopoulou et al., 2012; Savino, 2006). Therefore, we measured the kinetics of these cytokines in the thymus after infection. As shown in Fig. 2(a), IFN-β1 and IL-6 showed a peak increase at 3 days p.i. and quickly decreased to their basal levels. The levels of IFN-α4, IL-10 and IL-22 were elevated during the early state of infection, and remained relatively high until 7 days p.i. IFN-γ and TNF-α increased steadily over time. IFN-γ production exhibited a significant increase at 7 days p.i. (P = 0.032, 7 days p.i. versus mock-infected). IFN-γ is a key cytokine involved in the protective immune response against various pathogen infections (Flynn et al., 1993; Julkunen et al., 2001), but has also been shown to participate in the induction of pathological courses, especially thymic atrophy and T-cell depletion during viral or bacterial infections (Borges et al., 2012; Deobagkar-Lele et al., 2013; Liu et al., 2014).

We validated the proportions of innate immune cells in the thymus during infection. As shown in Fig. 2(b), the percentage of several innate immune cells, including NK-cells, DCs, macrophages and neutrophils, showed varying degrees of increase amongst the total thymocytes during infection. Surprisingly, we found that the proportion of NK-cells dramatically increased during infection. The percentage of NK-cells in the total thymocytes increased >10-fold compared with the mock-infected controls (NK-cells, 11.14 ± 3.49-fold; DCs, 4.22 ± 2.19-fold; macrophages, 6.80 ± 4.93-fold; neutrophils, 6.99 ± 4.26-fold increase compared with the respective populations in the mock-infected mouse) at 7 days p.i. Additionally, the absolute number of NK-cells at 7 days p.i. was expanded by approximately threefold (18.38 ± 5.76 × 10⁵ at 7 days p.i. versus 5.87 ± 0.52 × 10⁵ in the mock-infected group) compared with the mock-infected mice. As NK-cells are one of the main sources of IFN-γ, we investigated whether NK-cells produced IFN-γ in the thymus during infection. As shown in Fig. 2(c), NK-cells produced IFN-γ after infection and the proportions of IFN-γ-producing NK-cells increased from 1 to 7 days p.i. These results demonstrated that thymic NK-cells were activated by PR8 virus infection and secreted IFN-γ.

**NK-cells played a critical role in thymic atrophy**

To determine whether NK-cells played a role in thymic atrophy during infection, we depleted NK-cells in mice 1 day prior to PR8 infection using the anti-NK1.1 mAb PK136. As shown in Fig. 3(a, b), NK-cell depletion resulted in a significant reduction in body weight loss and suppressed thymic atrophy, but did not obviously affect virus infection and replication (data not shown). The thymus size of the NK-cell-depleted group was obviously larger compared with the NK-cell-intact group at 7 days p.i. The number of thymocytes in the NK-cell-depleted group was ∼1.8 times the number in the NK-cell-intact group. As PK136 depleted NK-cells and NKT-cells, we used anti-asialo GM1 antisera to deplete NK-cells in vivo to better distinguish the role of NK-cells from other lymphocytes populations. Anti-asialo GM1 antiserum depletes NK-cells and some activated T-cells, but not NKT-cells. As shown in Fig. 3(c, d), NK-cell depletion using anti-asialo GM1 also resulted in a significant reduction in body weight loss and suppressed thymic atrophy without obviously affecting virus infection and replication (data not shown). Thus, the use of both antibodies clearly showed that NK-cells affected thymic atrophy. Unless specifically stated, all of the following experiments were performed using both PK136 and anti-asialo GM1 antiserum, and were repeated at least two times.

Flow cytometry analysis showed that NK-cell depletion dramatically rescued the depletion of DP T-cells (Fig. 3e). At 7 days p.i., DP T-cells amongst total thymocytes in the NK-cell-depleted group remained at ∼50 %, but the proportion was decreased to <20 % in the NK-cell-intact group. HE staining demonstrated the relative preservation of thymic structure and the presence of more lymphocytes in the NK-cell-depleted group at 7 days p.i. (Fig. 3f).

To investigate the mechanisms underlying NK-cell involvement in thymic atrophy, we evaluated the differences between the NK-cell-intact and -depleted groups in terms of T-cell apoptosis and proliferation. NK depletion significantly diminished the apoptosis of DP and CD4 SP T-cells at 7 days p.i. (Fig. 4a). Furthermore, NK-cell depletion also significantly restored the proliferation of DN, DP and CD8 SP cells at 7 days p.i. (Fig. 4b). These results demonstrated that NK-cells played an important role in thymic atrophy during influenza virus infection by affecting the apoptosis and proliferation of thymic T-cells, especially DP T-cells.

**NK-cells affected thymic atrophy via IFN-γ production**

To investigate the mechanism by which NK-cells affected thymic atrophy during influenza virus infection, we compared cytokine production at the mRNA level between
the NK-cell-intact and -depleted groups. As shown in Fig. 5(a), there were no significant differences between the NK-cell-intact and -depleted groups in terms of IFN-α4, IFN-β1, IL-6, IL-10, TNF-α and IL-22 production at 3, 5 and 7 days p.i. However, the IFN-γ levels in the thymuses of the NK-cell-depleted group were significantly
Fig. 3. NK-cell depletion attenuated the pathological phenotypes in the thymus after influenza virus infection. (a) Body weights of NK-cell-intact or -depleted mice using PK136 after influenza virus infection. (b) Cell numbers (upper) and photographs (lower; bar, 1 cm) of thymuses from NK-cell-intact or -depleted mice using PK136 at the indicated time points after influenza virus infection. (c, d) Body weights (c) and thymocyte numbers at 7 days p.i. (d) from NK-cell-intact or -depleted mice using anti-asialo GM1 antiserum after influenza virus infection. (e) Changes in the thymocyte populations in NK-cell-intact and
-depleted mice after influenza virus infection were analysed by flow cytometry. The numbers below the FACS graphs indicate the percentage of cells in each region. The bar graph shows the percentage of DP T-cells in two groups of mice at the indicated time points. (f) Thymic sections from NK-cell-intact (upper) and -depleted (lower) mice 7 days p.i. were examined using HE staining. Original magnification ×10 (left) and ×40 (right). Results shown are representative of at least two independent experiments with at least three mice in each group. A two-way ANOVA test was performed to compare the body weight changes between the two groups (*P < 0.05, **P < 0.001) and an unpaired two-tailed t-test was used in the other analysis.

reduced compared with the NK-cell-intact group at 5 and 7 days p.i. Moreover, the sera IFN-γ concentration in the NK-cell-depleted group was significantly lower compared with NK-cell-intact mice at 7 days p.i. (Fig. 5b). As IFN-γ was reported to cause increased apoptosis of thymocytes (Borges et al., 2012; Deobagkar-Lele et al., 2013; Liu et al., 2014), we performed in vitro culture of thymocytes in the presence of different concentrations of IFN-γ. As shown in Fig. 5(c), IFN-γ co-culture clearly resulted in increased apoptosis of thymocytes in a dose-dependent manner. In the presence of 2000 U IFN-γ ml⁻¹, there was a significant increase in the thymocyte death rate.

To investigate the role of IFN-γ in thymic atrophy during influenza virus infection, we used an anti-IFN-γ antibody to neutralize IFN-γ in vivo. IFN-γ neutralization significantly alleviated thymic atrophy, as shown by the increased total thymocytes (2.98 ± 0.42 × 10⁶ cells in the mock-treated mice versus 4.90 ± 0.72 × 10⁶ cells in the neutralized mice) at 7 days p.i. in the IFN-γ-neutralized group (Fig. 5d). However, because IFN-γ is an important antiviral cytokine, the in vivo neutralization of IFN-γ also resulted in significantly increased viral loads in the lungs of the infected mice (Fig. 5e). Overall, the results suggested that the decreased IFN-γ production due to NK-cell depletion was one of the reasons for the ameliorated thymic atrophy during influenza virus infection.

**DISCUSSION**

In the present study, we demonstrated that NK-cells were involved in the severe thymic atrophy in mice infected with influenza virus. Influenza virus infection resulted in increased apoptosis and suppressed proliferation of thymic T-cells in mice. We found that the frequency of NK-cells in the mouse thymus increased during the course of influenza infection. Depletion of NK-cells by administration of anti-asialo GM1 antiserum or anti-NK1.1 antibodies prior to infection resulted in decreased pathological processes, including less body weight loss and alleviation of thymic atrophy compared with the control influenza-infected mice. Furthermore, we demonstrated that IFN-γ production by NK-cells was one mechanism by which NK-cells modulated thymic atrophy during influenza virus infection.

Our results are partly in agreement with recent findings by Abdul-Careem et al. (2012) and Zhou et al. (2013) that NK-cells exacerbated the pathology of influenza virus infection in mice. However, these studies did not show any link between thymic atrophy and NK-cell function. The beneficial role of NK-cells in enhancing the survival and decreasing the viral load in the context of influenza viral infection was observed previously (Gazit et al., 2006; Nogusa et al., 2008; Stein-Streilein & Guffee, 1986). However, in our mouse model of influenza infection, the absence of NK-cells led to reduced weight loss and attenuated thymic atrophy without an obvious effect on viral clearance. This discrepancy might be caused by differences in the infecting virus dose, virulence and mouse strains.

Vogel et al. (2010) showed that highly pathogenic IAV could infect the thymus and interfere with T-cell development. Liu et al. (2014) showed that elevated IFN-γ produced by innate CD8⁺ T-cells might contribute to thymic atrophy induced by influenza virus infection. In contrast to their studies that showed that influenza virus-infected DCs transported virus into the thymus, we did not detect influenza virus in the thymus by either plaque assay or quantitative real-time (qRT)-PCR for the influenza virus M1 gene (data not shown). This discrepancy in the results might be due to differences in virus types and infection doses because we used a lower infection dose. Our result likely excluded the possibility that NK-cells affected thymic atrophy by directly killing the infected cells. In our infection model, NK-cells might influence this procedure by directly or indirectly affecting the production of cytokines, such as IFN-γ.

In the current study, the elevated expression of pro-inflammatory cytokines such as IL-6, IFN-α, IFN-γ and TNF-α was detected in influenza virus-infected thymus tissue (Fig. 2a). However, only IFN-γ production showed a significant difference between the NK-cell-intact and -depleted groups (Fig. 5a). IFN-γ was shown to participate in the induction of thymic atrophy and T-cell depletion during viral and bacterial infections, such as severe influenza A(H1N1)pdm09, Salmonella enteric serovar Typhimurium, and Mycobacterium avium (Borges et al., 2012; Deobagkar-Lele et al., 2013; Liu et al., 2014). We showed directly that IFN-γ increased the apoptosis of thymocytes using an in vitro culture experiment (Fig. 5c). We also found that neutralization of IFN-γ in mice significantly alleviated thymic atrophy (Fig. 5d). However, because IFN-γ is an important antiviral cytokine, the in vivo neutralization of IFN-γ also resulted in increased virus titres in the lungs of infected mice (Fig. 5e).

NK-cells are an important early source of IFN-γ. Thus, NK-cell depletion may cause the loss of early IFN-γ production and affect the activation of other cells sequentially.
Although NK depletion severely inhibited IFN-γ production in the thymus, we also detected a low level of sera IFN-γ in the NK-cell-depleted group at 7 days p.i. compared with the mock-infected group. However, the level of IFN-γ in the NK-cell-depleted group was significantly reduced compared with the infected NK-cell-intact group. This low level of IFN-γ might be produced by other activated IFN-γ-secreting cells outside of the thymus, such as CD4+ and CD8+ T-cells (Kreijtz et al., 2011; Sridhar et al., 2013; our unpublished results). This might also explain the reason why IFN-γ neutralization but not NK depletion elevated the virus titre in the lungs (Fig. 5e). Severe infection could activate the hypothalamic-pituitary-adrenal axis, resulting in the copious release of glucocorticoids (Thompson, 2003); this might be another contributing factor affecting thymic atrophy, particularly after IFN-γ neutralization or reduction.

Apoptosis of thymic T-cells (especially DP T-cells) has been reported to be the main cause of thymic atrophy (Liu et al., 2014; Savino, 2006; Vogel et al., 2010). In our study, in addition to DP T-cell apoptosis we found that influenza virus infection significantly suppressed the proliferation of thymic T-cells (especially DN T-cells), as shown by the obvious decrease in BrdU incorporation (Fig. 1e). NK depletion clearly restored thymic T-cell proliferation (Fig. 4b). However, the mechanisms by which influenza virus affects thymic T-cell proliferation and how NK-cells are involved in this process are not clear and this requires further investigation.

Taken together, our results showed that influenza virus infection might cause severe thymic atrophy by increasing apoptosis and suppressing the proliferation of thymic T-cells. NK-cells were involved in this phenomenon partially through IFN-γ production. Our results revealed a new regulatory function for NK-cells and demonstrated the complexity of NK-cell responses during IAV infection. Additionally, our findings have important implications for the development of more effective preventative and therapeutic approaches against lethal influenza virus infections.

**METHODS**

**Ethics statement.** The mouse experimental design and protocols used in this study were approved by the ‘regulation’ of the Institute of Microbiology, Chinese Academy of Sciences of Research Ethics Committee (permit PZIMCAS2012008). All mouse experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People’s Republic of China.

**Virus, mice and infections.** The mouse-adapted influenza A/Puerto Rico/8/34 (H1N1; PR8) strain was propagated in the chorio-allantoic cavities of 10-day-old specific pathogen-free (SPF) embryonated chicken eggs (Beijing Merial Vital Laboratory Animal Technology) for 48–72 h at 37 °C. Allantoic fluids were then harvested and stored in aliquots at −80 °C. Virus titres were determined by plaque assay on Madin–Darby canine kidney (MDCK) cells (ATCC CCL-34). Briefly, 10-fold serial dilutions of the virus were used to infect confluent MDCK cells in 12-well plates for 1 h at 37 °C. The virus inocula were removed by washing with PBS. Cell monolayers were overlayed with agar medium [DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 1 % low-melting-point agarose and TPCK (tosyl phenylalanil chloromethyl ketone)-treated trypsin at 1 μg ml−1] and incubated at 37 °C for 48–72 h. The plates were fixed with 4 % paraformaldehyde for 1 h and then the agarose overlays were carefully removed. Staining buffer (0.1 % crystal violet and 20 % ethanol in water) was added to the wells and incubated for at least 10 min. The staining buffer was subsequently aspirated, the plaques counted and the virus titres calculated accordingly.

Female C57BL/6 mice (B6) were purchased from Vital River. All mice were housed in an animal facility under SPF conditions. For infection experiments, mice were transferred to a Biosafety Level 2 room. Mice (7–9 weeks of age) were intraperitoneally (i.p.) anaesthetized with tri-chloroacetaldehyde hydrate [375 mg (kg body weight) −1] and inoculated another 10 μg 3 days p.i. The efficiency of depletion (normally >90 %) was always verified by flow cytometry using an anti-NKp46 antibody. To neutralize IFN-γ, an anti-mouse IFN-γ antibody (R4-6A2; eBioscience) was inoculated i.p. at a dose of 250 μg per mouse 6 h after infection. Naïve sera for each antibody were used as a control in each experiment.

**In vivo NK-cell depletion and IFN-γ neutralization.** To deplete NK-cells in vivo, 200 μg anti-NK1.1 antibody (PK136) was i.p. injected into mice 1 day prior to influenza virus infection. Alternatively, mice were inoculated i.p. with 20 μg anti-asialo GM1 antiserum (Wako Chemicals) 1 day prior to infection and administered another 10 μg 3 days p.i. The efficiency of depletion (normally >90 %) was always verified by flow cytometry using an anti-Nkpn46 antibody. To neutralize IFN-γ, an anti-mouse IFN-γ antibody (R4-6A2; ebioscience) was inoculated i.p. at a dose of 250 μg per mouse 6 h after infection. Naïve sera for each antibody were used as a control in each experiment.

**Cell preparation.** Thymuses were collected and processed individually. Cell suspensions were obtained by gentle mechanical dissociation in PBS containing 2 % FBS. Cells were washed and resuspended in RPMI 1640 medium supplemented with 10 % FBS, 10 mM HEPES, 1 × MEM NEAA (non-essential amino acids), 2 mM l-glutamine, 50 μg streptomycin ml−1, 50 U penicillin ml−1 (all from Gibco) and 50 μM 2-mercaptoethanol (Amresco) (complete RPMI...
Fig. 5. IFN-γ played an important role in thymic atrophy after influenza virus infection. (a) mRNA levels of cytokines in thymocytes from NK-cell-intact or -depleted mice before or after infection were analysed using qRT-PCR. Each symbol in the figure indicates a single mouse. (b) IFN-γ concentrations in sera from NK-cell-intact or -depleted mice before or after infection were analysed using ELISA. Each symbol in the figure indicates an individual mouse. (c) Thymocyte numbers after culture with the indicated concentration of recombinant murine IFN-γ for 48 h. Results shown are representative of two independent experiments. (d) Thymocyte numbers at 7 days p.i. of mice with/without IFN-γ neutralization. (e) Lung virus titres at 7 days p.i. of mice with/without IFN-γ neutralization. Data are from at least two independent experiments with at least three mice per group. Data were analysed with an unpaired two-tailed t-test.

Table 1. Primers and probes used for qRT-PCR

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Flow cytometric analysis. For flow cytometry analysis of thymocytes, 2×10^6 cells were stained with surface molecules at 4°C for 30 min. For intracellular staining, cells were incubated in a 96-well plate at 37°C in complete RPMI medium containing 50 IU IL-2 ml⁻¹ and 10 μg Brefeldin A ml⁻¹ (Sigma) for 2 h. The cells were stained for cell surface molecules, fixed, permeabilized and stained for intracellular molecules using a Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer’s instructions. The following antibodies were used: anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-NK1.1 (PK136), anti-CD11c (N418, all from Sungene Biotech); anti-CD8 (53-6.7), anti-Gr1 (RB6-8C5) and anti-F4/80 (BM8, all from Sungene Biotech); anti-CD11b (M1/70, BD Pharmingen); anti-IFN-γ (XMGL1.2; BD Biosciences). Stained cells were analysed with a FACSCalibur flow cytometer (BD Biosciences). Data were analysed with FlowJo software (Tree Star). For staining of apoptotic cells, an Annexin V Cell Apoptosis Analysis kit (Sungene Biotech) was used according to the manufacturer’s instructions.

BrdU incorporation assay. The BrdU incorporation assay was performed as described previously (Fang et al., 2010). Briefly, mice were i.p. injected with 2 mg Brdu at the indicated time points post-infection. After 3 h the thymuses were then removed and dissociated into single-cell suspensions. In total, 2×10^6 thymocytes were added to 96-well plates. After surface staining, the cells were fixed with 100 μl fixation solution (BD) at 4°C for 30 min. After washing twice using Perm/Wash buffer (BD), the cells were resuspended with 100 μl DNase (300 μg ml⁻¹ in PBS) and incubated at 37°C for 1 h. The cells were then washed and stained with FITC-conjugated anti-BrdU antibody (Bd20a; eBioscience) at room temperature for 20 min. The cells were washed three times with Perm/Wash buffer prior to analysis by flow cytometry.

qRT-PCR. Universal Probe Library probes were purchased from Roche. Primers for cytokines were synthesized at Sangon Biotech. The primers used for cytokines are listed in Table 1. Total RNA was extracted from thymocytes of infected mice with TRIzol (Invitrogen). First-strand cDNA was synthesized using oligo-dT primers. qRT-PCR was performed using a LightCycler 480 (Roche). The cycling conditions for RT-PCR were: 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 1 s. The fold increase in mRNA expression was determined using the ΔΔCt method relative to the values for the mock-treated samples after normalization to glyceraldehyde 3-phosphate dehydrogenase gene expression.

Measurement of sera IFN-γ. Sera of infected mice were collected at the indicated time points. IFN-γ concentrations were measured using an ELISA kit (eBioscience) according to the manufacturer’s instructions.

In vitro culture of thymocytes. Thymocytes were incubated in a 96-well plate at 37°C in complete RPMI 1640 medium containing 10 IU IL-2 ml⁻¹ and the indicated concentration of recombinant murine IFN-γ (PeproTech). After 48 h, the cells were collected and the number of viable cells was counted by Trypan blue exclusion using a haemocytometer.

Histopathology. For histological analysis, thymic tissues were removed and fixed with 4% paraformaldehyde for at least 12 h, dehydrated in a series of graded alcohols and embedded in paraffin. Tissue sections (5 μm) were cut and stained with HE. Histological sections were examined using a Zeiss Axio Imager M1 microscope equipped with an AxioCam HRc camera under control of AxioVision 4 software.

Statistical analysis. Statistical analysis was performed using Prism software (GraphPad). All statistical analyses were performed using an unpaired two-tailed Student’s t-test or two-way ANOVA test as applicable. When applicable, data are displayed as mean±SEM.

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