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RESEARCH ARTICLE

Uncompromised NK cell activation is essential for virus-specific CTL activity during acute influenza virus infection

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Natural killer (NK) cells are indispensable components of both the innate and adaptive immune response. However, their precise roles in the cross-talk between innate and adaptive immunity during influenza virus infection remain controversial. By comparing NK cell dynamics and activity under a sub-lethal dose and high dose of influenza virus infection, we showed that influenza virus PR8 directly infected NK cells during natural infection, which was consistent with our previous findings obtained from an *in vitro* investigation of human NK cells. The impairments in cytotoxicity and IFN-γ production by spleen NK cells following high-dose infection were accompanied by decreased virus-specific killing mediated by cytotoxic T lymphocytes (CTLs). Importantly, the weakened CTL activity could be reversed by adoptive transfer of spleen NK cells harvested from low-dose-infected mice but not healthy donors. Taken together, our data provide direct evidence supporting the contribution of NK cells to antiviral T-cell responses. This study also indicates that a novel NK-targeted immune evasion strategy is used by influenza virus to shrink both innate and adaptive immune responses.
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INTRODUCTION

Influenza A virus causes seasonal epidemics and worldwide pandemics and thus represents a global threat to human health.[1] Despite the presence of both a physical barrier and innate and adaptive immune responses, influenza virus establishes infection by compromising the host defense system.[2] First, antigenic drift and shift derived from mutation or reassortment are the most best known methods used by influenza virus to escape recognition by the host adaptive immune response, which consists of both pre-existing neutralizing antibodies and cytotoxic T lymphocytes (CTLs).[3] Second, proteins encoded by influenza virus gene segments, such as non-structural protein 1 (NS-1) and PB1-F2, evade the recognition and amplification of the innate immune response during the early phase of infection.[4–8] Because the transmission of influenza virus from an infected to a susceptible host typically occurs during the first few days of infection, the evasion of innate immunity by the virus might increase viral propagation and the risk of an epidemic.

Natural killer (NK) cells are a major component of innate immunity. However, despite intensive investigation, the roles of NK cells in the battle against influenza virus remain a matter of debate. Both human and mouse NK cells have been shown to efficiently constrain viral replication by recognizing influenza virus-infected cells through NKp46 and the killing of target cells.[9] However, NK cells might also play detrimental roles in the exacerbation of local inflammation and the aggravation of clinical symptoms.[10,11] In addition to their roles as effector cells, NK cells may also function as coordinators by shaping both innate and adaptive immune responses through cytokine

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production or cell–cell interactions.[12,13] In 1995, Engleman and colleagues demonstrated that mouse NK cells contributed to the differentiation of alloantigen-stimulated CD8 T cells into effector CTLs *in vitro*.[14] In a subsequent publication, the authors reported that the generation of CTLs induced by influenza virus infection in the mouse model was impaired in the absence of NK cells.[15] During the early phase of infection, NK cells represent the main source of IFN-γ,[16] which together with perforin stored in NK cells helps prime the CTL response to influenza virus infection.[17] Activated NK cells accelerate the maturation of dendritic cells (DCs) through tumor necrosis factor alpha (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion, thereby indirectly modifying the adaptive immune response. In contrast to these positive effects, NK cells may also negatively regulate T-cell-mediated immune responses by producing the inhibitory cytokine IL-10 or directly killing activated $CD8^+$ T cells in a NKG2D/perforin-dependent manner under certain circumstances.[18,19] Previously, we and other groups reported that both human and mouse NK cells could be directly infected by influenza virus.[20–22] Our study further demonstrated that influenza virus infection in human NK cells led to their dysfunction and even apoptosis,[20] which was partially mediated by contact between the influenza hemagglutinin (HA) protein and the corresponding receptors on NK cells.[23] However, whether the direct attack of influenza virus on NK cells sequentially affects the regulation or the induction of the CTL response is unknown.

Antigen-specific adaptive immune responses induced by vaccination or previous infection can efficiently eliminate specific pathogens while avoiding excessive destruction of normal tissue. The efficiency of the adaptive immune response can be modulated by the dose of virus used for infection or vaccination. By comparing the outcome of different infectious doses of PR8 (H1N1) virus, Powell *et al.*[24] demonstrated that mice infected with even a low dose of virus could establish a strong immune response and that the recruitment of leukocytes, including effector $CD8^+$ T cells, to the lung and the generation of circulating virus-specific antibodies were relatively dose-independent. Interestingly, another study by Hatta *et al.*[25] suggested that the high replication rate and administration of a high virus dose might cause premature contraction of $CD8^+$ T cells by accelerating cell apoptosis instead of improving the activation and IFN-γ production of $CD8^+$ T cells. More recently, Marois *et al.*[26] used different doses of H3N2 influenza virus to infect mice and demonstrated that high-dose virus infection led to increased infiltration of innate immune cells and virus-specific $CD8^+$ T cells into the affected lung, whereas low-dose virus infection induced a comparable level of specific antibodies but a smaller pool of central and effector memory $CD8^+$ T cells, which resulted in a poor recall response upon secondary challenge. Nevertheless, whether and how NK cells are involved in the distinct magnitudes of virus-specific CTLs evoked by a distinct infection dose remain largely unknown.

In the current study, we compared NK cell- and CTL-mediated immune responses in mice suffering from low and high dose of influenza virus infection. Circulating NK cells were infected by the PR8 virus under both low and high dose of influenza infection conditions, whereas only high-dose-infected mice resulted in the loss and dysfunction of spleen NK cells. Correspondingly, the specific killing ability and cytokine secretion of CTLs were decreased in high-dose-infected mice despite the comparable levels of virus-specific antibodies and quantities of $CD8^+$ T cells induced in the low and high dose of infection models. Importantly, the compromised virus-specific CTL response could be reversed by the adoptive transfer of uncompromised spleen NK cells harvested from low-dose-infected mice. Taken together, our data support the contribution of NK cells to competent virus-specific CTL activity during acute infection and suggest a novel NK-targeted strategy applied by the virus to escape both innate and adaptive immunity.

MATERIALS AND METHODS

Ethics statement

All animal manipulations were performed in compliance with the Animals (Scientific Procedures) Act of 1986 (UK) (amended in 2013) and approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR), Hong Kong (approval number: CULATR 2378-11).

Animals

Female C57BL/6 and BALB/C mice at 6–8 weeks of age were purchased from and maintained in the Laboratory Animal Unit at the University of Hong Kong.

Virus and infections

The mouse-adapted influenza virus PR8 (H1N1) was cultured in Madin-Darby canine kidney (MDCK) cells as described in our previous study.[27] The virus titer was determined by plaque-forming assay with MDCK cells (purchased from the American Type Culture Collection, Manassas, VA, USA, and routinely maintained by the Department of Pediatrics and Adolescent Medicine, The University of Hong Kong). The 50% median tissue culture infective dose ($TCID_{50}$) was calculated in accordance with the Reed-Muench formula. Low and high dose of influenza virus infection models were established by treating C57BL/6 mice with a sub-lethal (1×10^2 $TCID_{50}$ in 25 μl of PBS) or LD_{50} (1×10^4 $TCID_{50}$ in 25 μl of PBS) dose of PR8 virus via intranasal inoculation under anesthesia. PBS-treated mice were used as the mock controls.

Determination of the virus titer and cytokine and chemokine production from lung homogenates

At the indicated time points post infection, lungs were harvested from two groups of mice and homogenized in 2 ml of cold PBS. Then, the supernatants of the homogenates were collected after centrifugation at 1500*g* for 15 min at 4 °C The virus titer in the supernatant was determined by the plaque-forming assay with MDCK cells, and the concentrations of

inflammatory cytokines and chemokines were determined by Bender MedSystems as previously described.[27,28]

Histological examination

The lungs were harvested from mice infected by two initial infectious dose at the indicated time points and preserved in 75% ethanol after fixation overnight with 10% formalin to prepare paraffin-embedded tissue sections. Hematoxylin–eosin (H&E) staining was performed according to the standard protocols.[29]

***In vivo* NK cytotoxicity assay**

The *in vivo* cytotoxicity assay developed by Vaknin *et al.*[30] was used here with minor modifications. Briefly, splenocytes from C57BL/6 (syngeneic) and BALB/c mice (allogeneic) were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Carlsbad, CA, USA) at final concentrations of 5 μM ($CFSE^{high}$) and 0.5 μM ($CFSE^{low}$), respectively. A total of 5×10^6 $CFSE^{high}$ and $CFSE^{low}$ cells were mixed at a 1:1 ratio and injected intravenously into recipient C57BL/6 mice. Single-cell suspensions of different tissues (peripheral blood, lung, mesenteric lymph nodes (MLNs) and spleen) were harvested 12 h post injection, and the experimental ratio of $CFSE^{high}$:$CFSE^{low}$ cells was determined by FACS. Allogeneic cell clearance was calculated according to the following formula: allogeneic cell clearance rate = [1 − original ratio of $CFSE^{high}$:$CFSE^{low}$/experimental ratio of $CFSE^{high}$:$CFSE^{low}$] × 100 in uninfected recipient mice.

***In vivo* CTL cytotoxicity assay**

The analysis of *in vivo* CTL cytotoxicity was performed according to the previously described protocol.[31] Splenocytes from C57BL/6 mice were labeled with high (5 μM) or low (0.5 μM) CFSE concentrations. After washing, the cells labeled with 5 μM CFSE were pulsed with 5 μg/ml of NP peptide for 1 h in 10% FBS RPMI at 37 °C, whereas the cells labeled with 0.5 μM CFSE were pulsed with the control peptide (MOG). Then, 5×10^6 NP-loaded cells ($CFSE^{high}$) and control peptide-loaded cells ($CFSE^{low}$) were mixed at a 1:1 ratio and injected intravenously into recipient C57BL/6 mice. Single-cell suspensions of different tissues (peripheral blood, lung, MLN and spleen) were harvested 12 h post injection, and the peptide-specific killing rate was calculated as follows: specific killing rate = [1 − original ratio of $CFSE^{low}$:$CFSE^{high}$/ experimental ratio of $CFSE^{low}$:$CFSE^{high}$] × 100 in uninfected recipient mice.

Stimulation of CTLs *in vitro*

Splenocytes were collected on day 9 post infection and incubated in 2 ml of 1× ammonium–chloride–potassium (ACK) buffer on ice for 5 min to lyse the red blood cells. Following two washes with PBS, the cells were seeded into 96-well plates at a density of 1×10^6 cells per well and stimulated with 1 μg/ml of the NP peptide (Genscript, Piscataway, NJ, USA) or MOG peptide (Chinese Peptide Company, Hangzhou, China) in 200 μl of 10% FBS-RPMI medium. The cells were incubated for 16 h with the addition of Brefeldin A (BFA) for the final 4 h. The expression of surface markers and intracellular molecules was detected as previously described.[28]

Adoptive transfer of NK cells

Splenocytes were harvested from untreated mice or low dose-infected mice on day 3 post infection. Then, the NK cells were purified following the instructions of the mouse NK isolation kit (Miltenyi Biotec, Miltenyi, San Diego, CA, USA). The NK cell purity was at least 80%. A total of 0.5×10^6 freshly isolated NK cells were re-suspended in 200 μl of PBS and intravenously injected into high-dose-infected mice. On day 9 post infection, the *in vivo* cytotoxicity of the CTLs was determined as described above.

Single-cell suspensions from different organs

At the indicated time points post infection, single-cell suspensions were prepared from the spleens, lungs and MLNs. Spleens and MLNs were ground in cold PBS. The lung tissues were pre-treated with collagenase/DNase I (Roche, Basel, Switzerland) at 37 °C for 1 h prior to grinding. All cell suspensions were filtered through a 75-μm nylon mesh to remove the connective tissue mass. Heparin was used to prepare blood samples for cytological examination. Plasma was obtained from the blood by centrifugation at 300*g* and stored at − 80 °C.

Antibodies and flow cytometry

The following monoclonal antibodies were used: Pacific Blue-labeled anti-mouse CD3, antigen-presenting cell (APC)-labeled anti-mouse CD8, APC-labeled anti-mouse CD49b (all from eBioscience, Waltham, MN, USA), FITC-labeled anti-mouse NK1.1, Pacific Blue-labeled anti-mouse NK1.1 (Biolegend, San Diego, CA, USA), and FITC-labeled anti-influenza virus NP (Thermo Fisher, Waltham, MN, USA). For the quantitative determination of cell numbers, counting beads (Invitrogen, Waltham, MA, USA) were added to the samples prior to the flow cytometry assay.

Statistical analysis

Data are shown as the means ± s.e.m. Differences in survival among different groups were determined by the Kaplan–Meier log-rank test. A multiple regression analysis was used to compare differences in weight loss among the groups. Comparisons of cell numbers and cytokine or chemokine concentrations between the low- and high-dose-infected groups were analyzed with an unpaired two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Influenza virus infected peripheral NK cells in infected mice

In our previous study, using an *in vitro* system, we found that human NK cells succumbed to influenza virus infection.[20] To investigate NK cell dynamics during influenza virus infection, mild and severe infection models were established by treating C57BL/6 mice with a low (1×10^2 $TCID_{50}$ of PR8 in 25 μl of PBS) and high dose (1×10^4 $TCID_{50}$ of PR8 in 25 μl of PBS) of

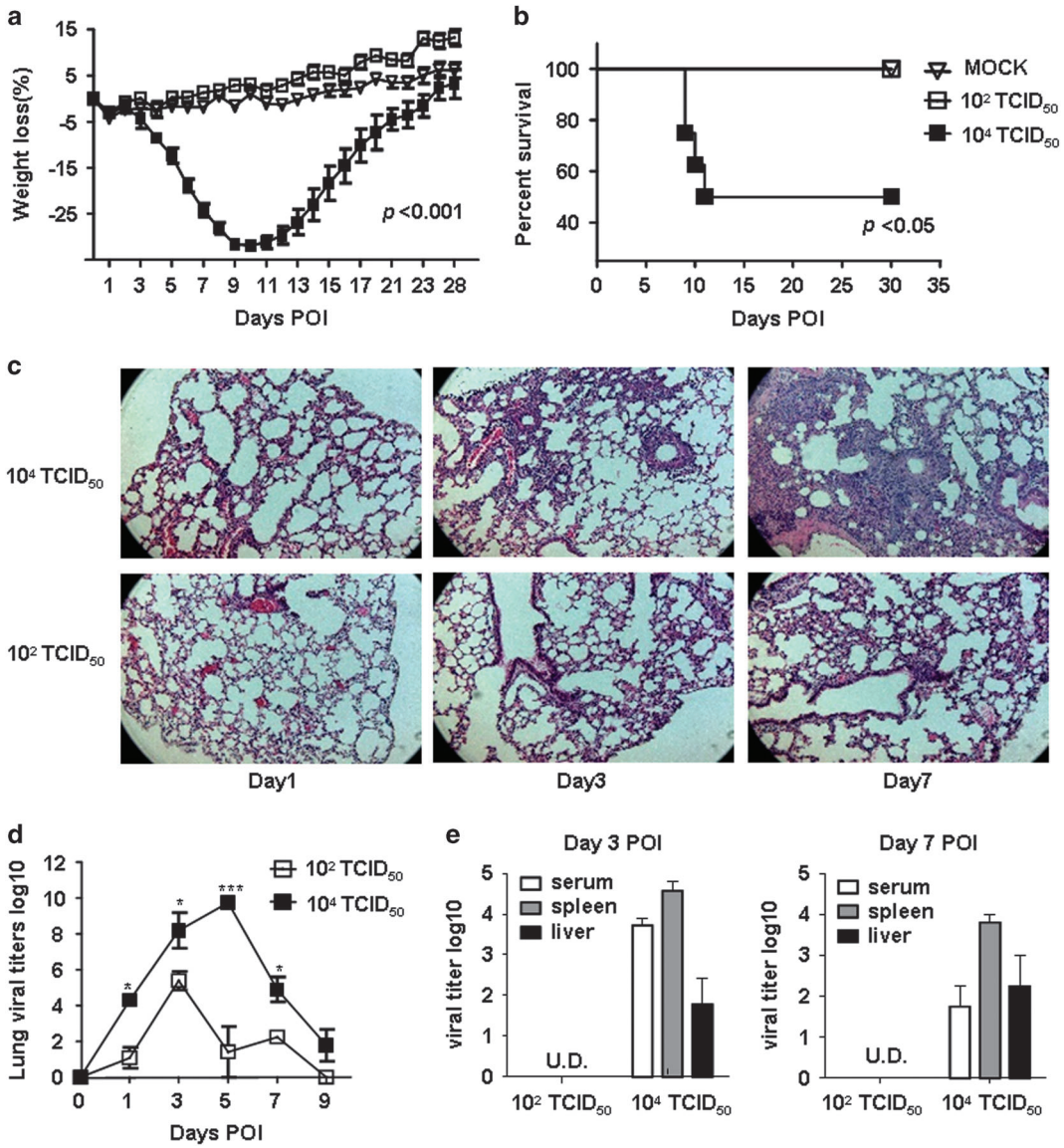

Figure 1 Establishment of high and low PR8 infection models. C57/B6 mice were infected with 1×10^2 $TCID_{50}$ (low dose) or 1×10^4 $TCID_{50}$ (high dose) of PR8 via the intranasal route. Weight loss (**a**) and survival (**b**) were monitored daily. (**c**) Lungs were collected from low and high dose PR8-infected mice on days 1, 3 and 7 post infection (POI). The histological examination was performed by H&E staining of lung sections. (**d**, **e**) At the indicated time points post infection, the virus titers in the lungs (**d**), serum, spleen and liver (**e**) were determined by the plaque-forming assay with Madin–Darby canine kidney (MDCK) cells. Data are shown as the means ± s.e.m. and represent three independent experiments. (*$P<0.05$, ***$P<0.001$). UD, undetectable.

influenza virus. As shown in Figures 1a and b, mice infected with the high dose of PR8 virus exhibited prominent weight loss and a 50% mortality rate. In contrast, the low dose of PR8 virus did not cause death, and all mice in this group recovered soon after infection. In support of these results, the histological examination demonstrated that inflammation in the lungs of mice suffering from high-dose infection was much more severe than that caused by the low-dose infection on days 3 and 7 post infection (Figure 1c). Consistent with this finding, the virus titer in the lungs of the high-dose-infected mice was higher than the lung titer in the low-dose-infected model, although the dynamics were similar between the two groups (Figure 1d). Importantly, live viruses in the spleen and liver were identified in high-dose-infected mice, whereas no active virus was detected in the sera or tissue homogenates of the low-dose-infected mice by the $TCID_{50}$ assay (Figure 1e).

To determine whether NK cells could be directly infected by influenza virus *in vivo*, the expression of the influenza virus nucleoprotein (NP) in peripheral NK cells was screened by intracellular staining. As shown in Figure 2, high-dose infection induced significantly higher NP expression in lung NK cells than low-dose infection. Taken together, our data confirmed the direct interaction between influenza virus and NK cells during natural infection.

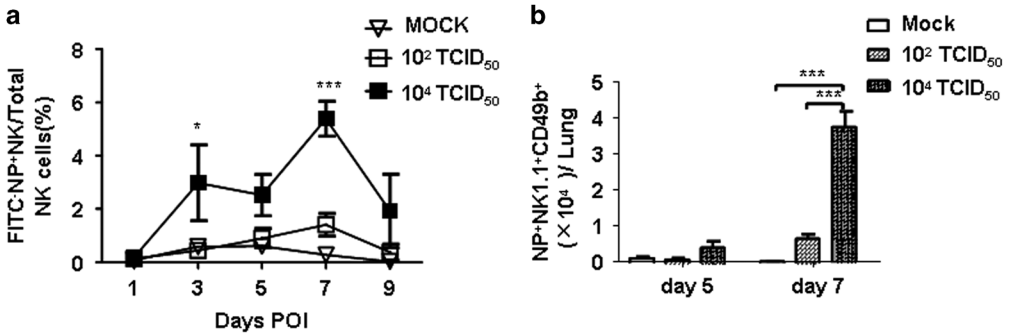

Figure 2 Infection of NK cells in high- and low-dose-infected mice. C57/B6 mice were infected as previously described. At the indicated time points post infection (POI), the proportion (**a**) and the number (**b**) of virus-infected NK cells in different organs was determined by intracellular staining for influenza virus NP. Data are shown as the means ± s.e.m. and represent three independent experiments (*$P<0.05$, ***$P<0.001$).

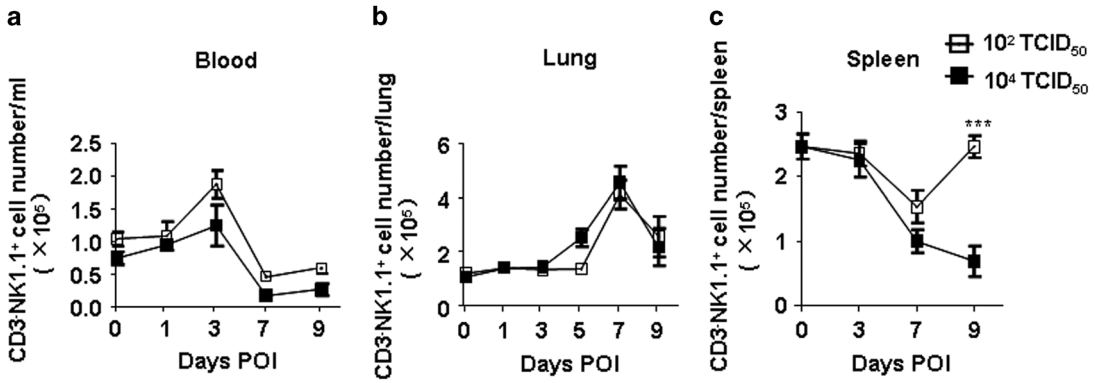

Figure 3 Altered spleen NK cell dynamics in high-dose infection. C57/B6 mice were infected as previously described. At the indicated time points post infection, the absolute number of NK cells in the blood (**a**), lung (**b**) and spleen (**c**) was determined using flow cytometry. Data are shown as the means ± s.e.m. and represent three independent experiments (***$P<0.001$).

Altered spleen NK cell dynamics in high-dose-infected mice

Consistent with the NP expression levels in NK cells, the initial dose of virus infection led to dynamic changes in peripheral NK cells. As shown in Figures 3a and b, both blood and lung NK cells exhibited similar dynamics during acute infection in the low- and high-dose infection groups. However, the number of spleen NK cells in high-dose-infected mice was significantly lower than in the low-dose-infected mice on day 9 post infection (Figure 3c). This finding was consistent with the delayed recovery of high-dose-infected mice, as shown in Figure 1.

Biological functions of spleen NK cells under low and high dose of influenza infection conditions

In addition to the changes in the NK cell quantity, we investigated the impact of influenza virus infection on the biological functions of peripheral NK cells. First, *in vivo* cytotoxicity mediated by NK cells from the spleen, lung, blood and MLN) was compared between low and high-dose infection models by adoptively transferring a mixture of allogeneic and syngeneic splenocytes at a 1:1 ratio. As shown in Figure 4, most of the allogeneic cells ($CFSE^{low}$ population) in the periphery of the low-dose infection model were eliminated by 12 h post injection. In contrast, the ratio of allogeneic to syngeneic cells ($CFSE^{low}$:$CFSE^{high}$) in the peripheral blood, lungs, spleens and MLN of high-dose-infected mice exhibited only minor changes at the same time point, which suggested that the cytotoxicity mediated by NK cells was impaired in the high-dose infection model.

To comprehensively compare NK cell characteristics between the two models, the expression of cell activation markers (CD25, CD27, CD69, NKG2A, NKG2D and NKp46) and effector-related molecules (TNF-related apoptosis inducing ligand (TRAIL), CD107a, perforin, TNF-α and IFN-γ) in spleen NK cells was determined by surface and intracellular staining (Figure 5). Consistent with our previous *in vitro* work (19), CD69 expression in spleen NK cells was increased in both groups and was accompanied by a decrease in lysis receptor NKp46-expressing NK cells, which indicated that both low- and high-dose infection activated spleen NK cells (Figures 5a and b). Notably, spleen NK cells from low-dose infection model mice displayed higher IFN-γ production but lower TNF-α production than their analogs in the high-dose-infected group, whereas a similar level of TRAIL, CD107a and perforin

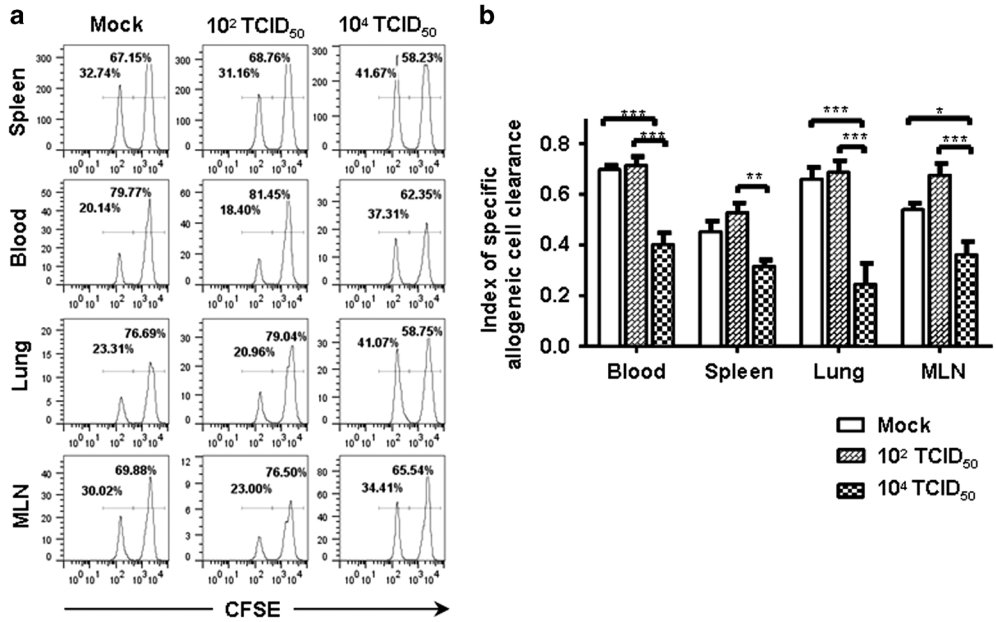

Figure 4 Impaired cytotoxicity of NK cells due to influenza infection. C57/B6 mice were infected as previously described. On day 6 post infection, 5×10^6 splenocytes from BALB/c and C57/B6 mice were stained with 0.5 or 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE), respectively, and an equal mixture was intravenously injected into recipient mice. Twelve hours later, cells were collected from the blood, spleen, lung and mesenteric lymph node (MLN), and the experimental ratio (**a**) and index of specific allogeneic cell clearance (**b**) of the $CFSE^{high}$:$CFSE^{low}$ populations was determined by FACS. The data shown here represent three independent experiments (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

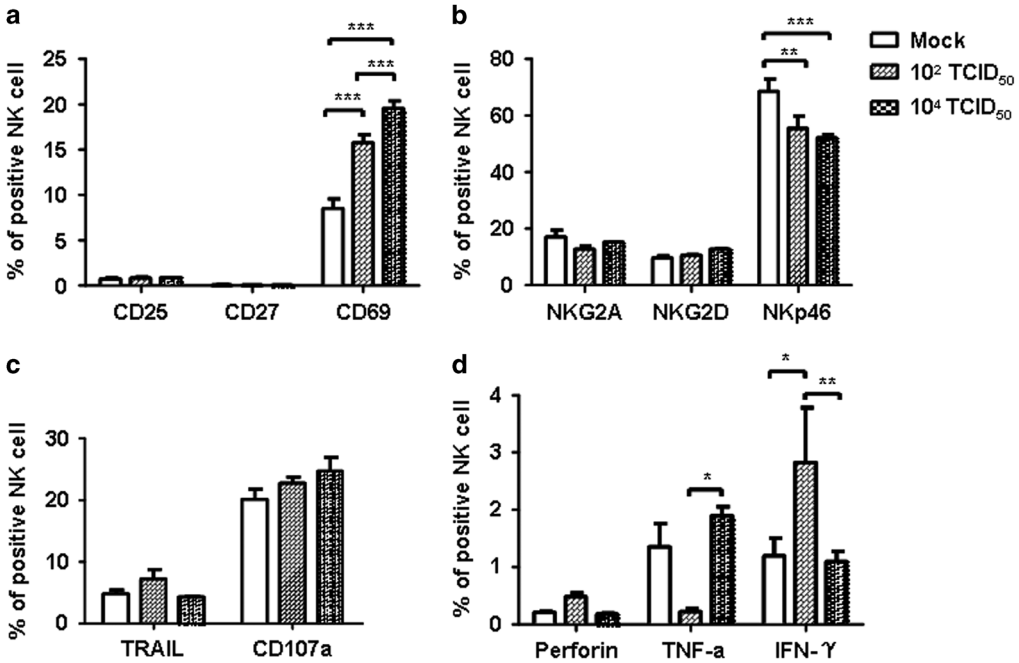

Figure 5 Phenotypes of spleen NK cells in high- and low-dose infection model mice. C57/B6 mice were treated as previously described. On day 3 post infection, spleens were isolated from the mice, and single-cell suspension were prepared for surface staining of activation markers (CD25, CD27 and CD69) (**a**), natural cytotoxicity receptors (NKG2A, NKG2D and NKp46) (**b**) and cytotoxicity-related markers (TRAIL and CD107a) (**c**) and intracellular staining for perforin, TNF-α and IFN-γ (**d**) in spleen NK cells and analyzed by flow cytometry (**b**–**d**). The proportion of positive NK cells was calculated for statistical analysis. Data are shown as the means ± s.e.m. and represent three independent experiments. (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

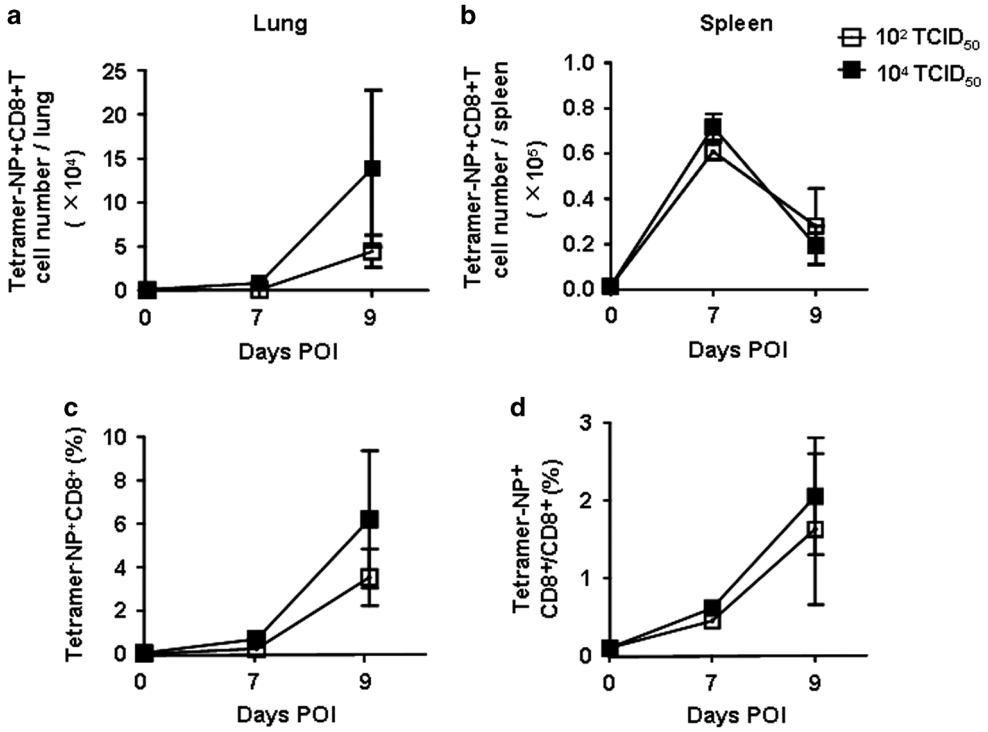

Figure 6 Dynamics of antigen-specific cytotoxic T lymphocytes during acute influenza virus infection. C57BL/6 mice were infected as previously described. The absolute number and the proportion of tetramer-NP-positive CD8 T cells in the lung (**a**, **c**) and spleen (**b**, **d**) were determined on days 0, 7 and 9 post infection using flow cytometry. Data are shown as the means ± s.e.m. and represent three independent experiments.

was detected in the two groups (Figures 5c and d). Taken together, our data showed that spleen NK cells were directed into distinct functional status under different infection doses and that their cytotoxicity and IFN-γ production were impaired by direct infection with influenza virus.

Antigen-specific CTL activity was impaired in high-dose-infected mice

To determine whether the adaptive immune response might also be impaired during acute influenza infection, we examined the quantity and quality of CTLs in the low and high dose of infection models. As shown in Figure 6, the number and proportion of antigen-specific CTLs in the lungs (Figures 6a and c) and spleens (Figures 6b and d), as determined by NP-loaded tetramer staining, showed no significant differences between the two groups on days 0, 7 and 9 post infection.

To determine the specific killing capacity of CTLs, influenza virus NP peptide- and unrelated peptide-pulsed splenocytes were stained with different concentrations of CFSE and mixed at a 1:1 ratio prior to injection into low- or high-infection model mice on day 9 post infection. Four hours after injection, single-cell suspensions were prepared from the harvested organs to determine the specific clearance of the injected cells in accordance with a previous report.[26] CTLs in the blood, lungs and MLNs of the high-dose-infected mice showed a reduced capacity to eliminate NP-loaded cells ($CFSE^{high}$ population) compared to their analogs in the low-dose-infected group (Figures 7a and b), suggesting that high dose of infection compromised the response mediated by antigen-specific CTLs as well as NK cells during acute infection.

Adoptive transfer of activated but uncompromised spleen NK cells reversed the impaired virus-specific CTL response

To investigate the direct correlation between the biological functions of spleen NK cells and antigen-specific CTL activity during acute influenza virus infection, spleen NK cells harvested from untreated mice or low-dose-infected mice were adoptively transferred into high-dose-infected recipients on day 3 post infection. On day 9 post infection, antigen-specific CTL cytotoxicity was determined *in vivo* and compared among the different groups. Surprisingly, the decreased CTL activity in the high-dose-infected recipients could be reversed only by the adoptive transfer of spleen NK cells from the low-dose-infected group but not those from naive donors (Figure 8a). Consistent with this result, the production of IFN-γ and TNF-α by CTLs in the high-dose-infected mice could also be partially rescued by the adoptive transfer of NK cells from the low-dose infection group (Figure 8b and c). These data demonstrated that activated but uncompromised spleen NK cells made an essential contribution to intact antigen-specific CTL activity during acute influenza virus infection.

DISCUSSION

In our previous publication, we demonstrated that human NK cells could be directly bound by the influenza HA protein and infected by various influenza A viruses *in vitro*.[20] In addition,

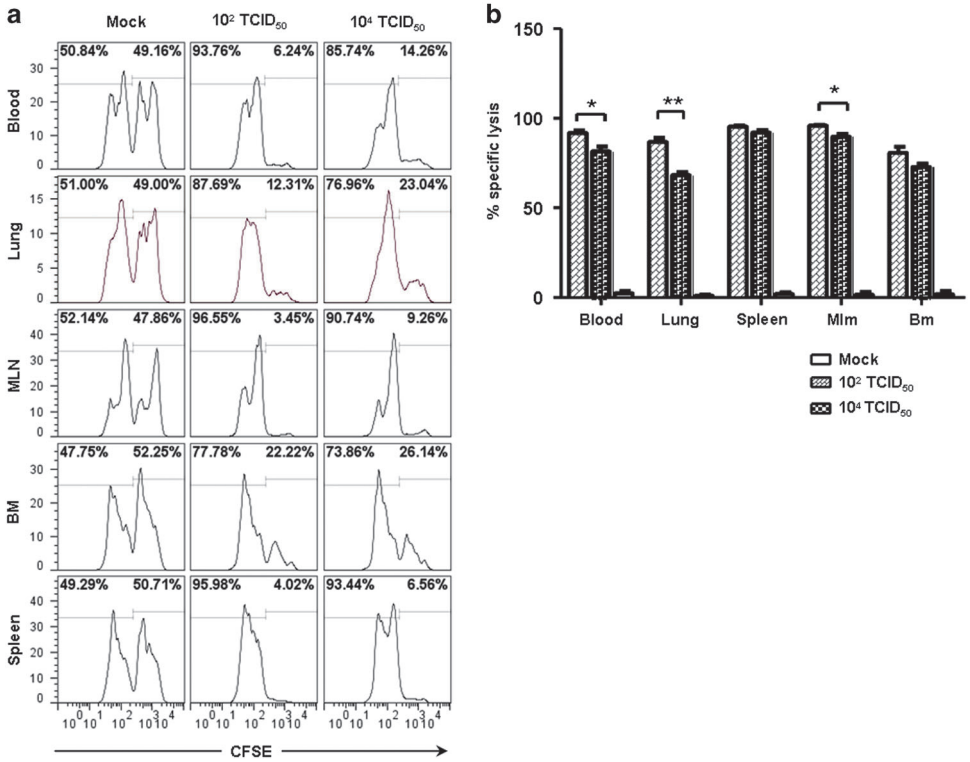

Figure 7 High dose of influenza infection impaired antigen-specific cytotoxic T lymphocyte activity. C57/B6 mice were infected as previously described. On day 9 post infection, splenocytes from C57BL/6 mice were labeled with high (5 μM) or low (0.5 μM) carboxyfluorescein diacetate succinimidyl ester (CFSE) concentrations. After washing, the 5 μM CFSE-labeled cells were pulsed with 5 μg/ml of NP peptide for 1 h in 10% FBS RPMI at 37 °C, whereas the 0.5 μM CFSE-labeled cells were pulsed with the control peptide (MOG). Then, 5×10^6 NP-loaded cells ($CFSE^{high}$) and control peptide-loaded cells ($CFSE^{low}$) were mixed at a 1:1 ratio and injected intravenously into recipient C57BL/6 mice. Single-cell suspensions of different tissues (peripheral blood, lung, mesenteric lymph node (MLN) and spleen) were harvested 12 h post injection, and the experimental ratio (**a**) and index of specific lysis (**b**) were calculated as previously described. The data shown here represent three independent experiments. ($^*P<0.05$, $^{**}P<0.01$).

influenza virions and free HA proteins suppressed the cytotoxicity of freshly isolated and interleukin-2 (IL-2)-activated human NK cells.[23] In agreement with these findings, our current study showed that mouse NK cells could be infected by the mouse-adapted influenza A virus PR8 during natural infection. Importantly, the loss and dysfunction of spleen NK cells due to severe infection was followed by impaired virus-specific CTL activity. Interestingly, the decreased virus-specific killing mediated by CTLs could be reversed by the adoptive transfer of spleen NK cells harvested from mildly infected mice but not untreated donors. Thus, our results demonstrate a close interaction between NK cells and CTLs that might be exploited by influenza virus to evade both innate and adaptive immunity.

The outcome of influenza virus infection is largely determined by the infectious dose and viral virulence. Severe influenza virus infections might progress to pneumonia or develop into acute respiratory distress syndrome. Here by comparing mice inoculated with different doses of PR8, we showed that high dose of influenza virus infection led to severe inflammation within the first few days post infection (Supplementary Figure S1) and subsequently resulted in significant weight loss, lung damage, viremia, multiple organ failure and even death. This model mimicked severe influenza virus infection in humans, which is marked by excessive pro-inflammatory cytokines/chemokines and massive infiltration of leukocytes into the lung. Unfortunately, the accumulation of immune cells might not necessarily lead to the elimination of pathogens and the recovery of the infected host. As shown in Figures 2 and 3, the virus-specific cytotoxicity of CTLs in multiple organs from high-dose-infected mice was impaired, although the absolute number of CTLs was comparable between the high- and low-dose-infected models. In contrast to most previous reports claiming that the decrease in the absolute number of CTLs was caused by high dose of influenza infection,[17,32,33] our data demonstrate a novel strategy applied by the virus to disable host adaptive immunity by impairing the quality of CTLs.

Several previous studies have shown that influenza viruses can infect diverse immune cells, including DCs, macrophages, B cells and even NK cells,[20–22,34,35] in addition to respiratory tract epithelial cells. Similarly, we also detected expression of the PR8 virus NP protein in lung NK cells under both high and low dose of infection conditions. However, only treatment with

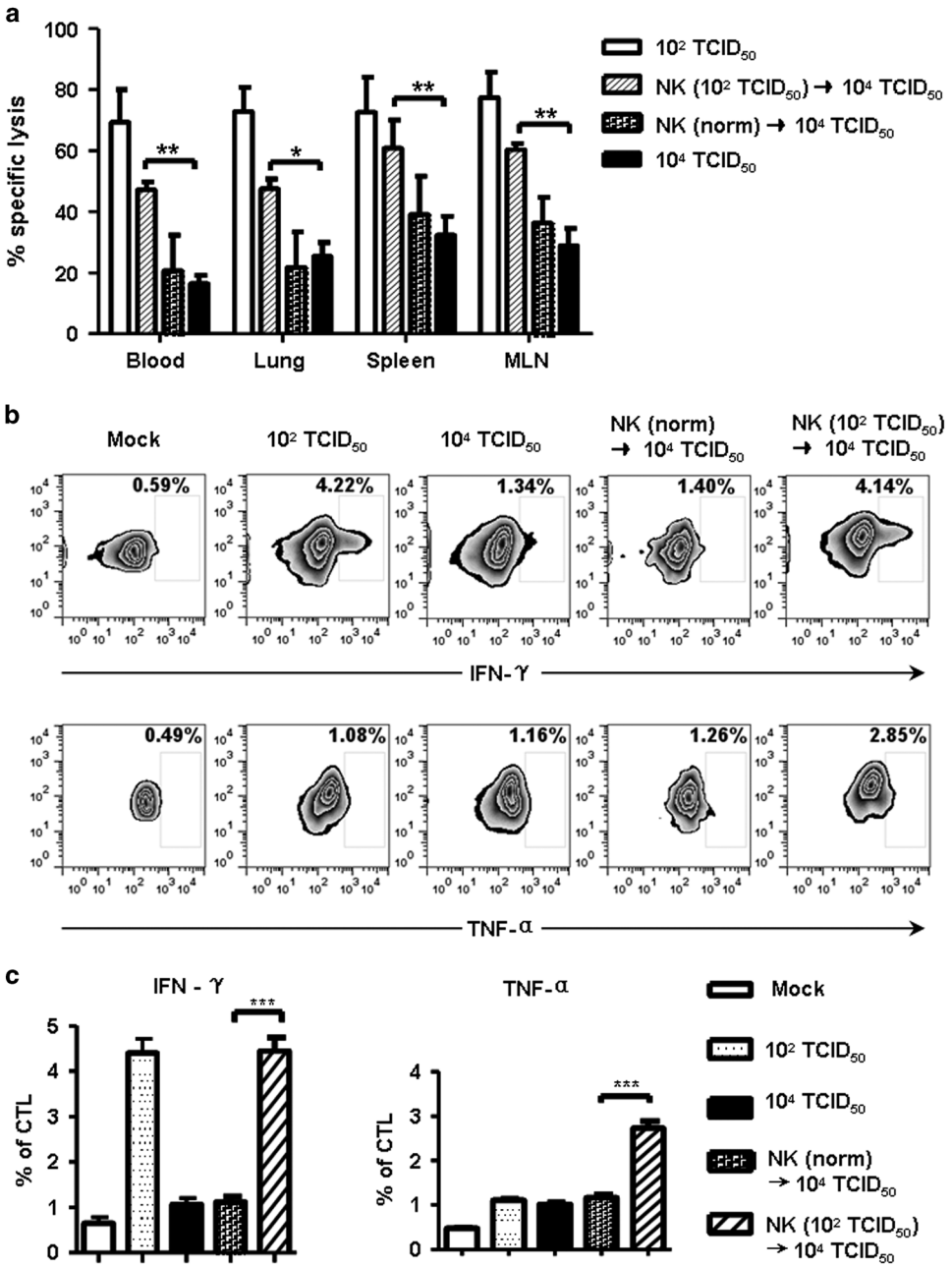

Figure 8 Decreased cytotoxic T lymphocyte (CTL) activity can be reversed by adoptive transfer of spleen NK cells from low-dose-infected mice. C57/B6 mice were infected as previously described. On day 3 post infection, spleen NK cells were isolated from normal mice or low-dose-infected mice using a NK isolation kit. A total of 0.5×10^6 NK cells in 200 μl of PBS from each group were intravenously injected into high-dose-infected mice. (**a**) On day 9 post infection, peptide-specific CTL cytotoxicity was determined as described above. (**b**, **c**) Splenocytes were harvested from the different groups on day 9 post infection and seeded into 96-well plates at a density of 1×10^6 cells/well. Then, the cells were stimulated with 1 μg/ml of the NP peptide or control peptide in 10% FBS-RPMI medium for 16 h with the addition of BFA for the final 4 h. TNF-α and IFN-γ expression in the $CD8^+$ T cells was determined by intracellular staining. The data shown here represent three independent experiments. ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

the LD_{50} virus led to direct infection of lung NK cells and impairment of their cytotoxicity and IFN-γ production, which is a strong enhancer of the CTL-mediated immune response.[36] As introduced previously, NK cells can accelerate the initiation and exemplification of the T-cell response by secreting cytokines (GM-CSF, TNF-α and IFN-γ) or promoting the functional maturation of DCs.[17,37–39] NK cells were also found to improve the ability of $CD8^+$ T cells to secrete IFN-γ and lyse *Mycobacterium tuberculosis*-infected cells.[40] Moreover, several ligands for T-cell co-stimulatory molecules, such as CD86, CD70 and OX40L, can be expressed by NK cells. The expression of these co-stimulators might favor the adoption of some APC-like characteristics by NK cells to activate T cells directly.[41] Moreover, cytotoxicity mediated by NK cells against

target cells upregulates antigen-specific $CD4^+$ T-cell-mediated immune responses, which subsequently improve the priming of $CD8^+$ T cells and the production of IgG.[42] To assess the roles of NK cells in influenza infection, Engleman *et al.*[14,15] first demonstrated that the generation of an influenza virus-specific CTL response required the participation of NK cells based on an *in vitro* model. Consistent with these previous reports,[17,42,43] we also found a decrease in the absolute number (Supplementary Figure S2a) and proportion (Supplementary Figure S2b) of virus-specific CTLs induced by influenza infection in mice depleted of NK cells. However, the precise contribution of NK cells to the activity of virus-specific CTLs under the natural *in vivo* system remains a matter of debate and may differ under distinct doses of influenza infection. By comparing NK cell- and CTL-mediated immune responses in mice suffering from low and high dose of infection, we found that both virus-specific killing and inflammatory cytokine secretion by CTLs were impaired in high-dose-infected mice following the loss and dysfunction of spleen NK cells, although no significant differences were identified in the absolute numbers and percentages of CTLs between the high- and low-dose infection models (Figure 6). Taken together, these data strongly support the hypothesis that NK cells make an important contribution to the generation of functional CTLs.

To illustrate the direct effects of NK cells on the CTL response under different doses of influenza virus infection, an adoptive transfer system was established for the high-dose-infected model in this study. Surprisingly, only NK cells isolated from low-dose-infected mice, but not from untreated mice, reversed the impaired cytotoxicity and cytokine production by CTLs in high-dose-infected mice. Consistent with a previous report showing that the higher potential of IFN-γ production by spleen NK cells could effectively contribute to the initiation of $CD8^+$ T-cell-mediated responses and the migration of DCs during influenza virus infection,[17] we also found that spleen NK cells isolated from low-dose-infected mice showed a high potential for IFN-γ production, which might help explain their benefits in reversing the impaired CTL activity compared to NK cells from healthy donors. In contrast to a report suggesting that mouse NK cells contributed to the production of virus-specific antibodies,[42] we found that the antibody levels were comparable between the two groups, which indicated that the impaired NK cells in the severe influenza infection group did not affect the humoral response in our model (Supplementary Figure S3a). We also compared the protective effects mediated by immune memory between mice infected with different doses of PR8 by challenging recovered mice with a lethal dose of PR8. The magnitude of immune memory established in the high and low dose of infection models was similar (Supplementary Figure S3b), suggesting that the effects of NK cells on CTL activity primarily occurred during the acute phase of infection.

During the early phase of virus infection, innate immunity represents the first line of host defense against the virus and may provide the foundation for the initiation of the adaptive immune response. To the best of our knowledge, this report is the first to demonstrate direct modulation of the quantity and quality of NK cells by influenza virus and the relationship between NK cell characteristics and virus-specific CTL activity *in vivo*. Furthermore, our study provides direct evidence in support of the beneficial role of mouse NK cells in the generation of functional antigen-specific CTLs and provides important information for the development of NK cell-targeted treatments to reduce the immune evasion of influenza virus during the early stage of infection.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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