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## The frequency and function of nucleoprotein-specific CD8<sup>+</sup> T cells are critical for heterosubtypic immunity against influenza virus infection

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### ETHICS APPROVAL

Animal research was conducted using the guidelines of the CDC's Institutional Animal Care and Use Committee in the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), an accredited animal facility.

### ADDITIONAL FILES

The following material is available [online](#).

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## Abstract

Cytotoxic T lymphocytes (CTLs) mediate host defense against viral and intracellular bacterial infections and tumors. However, the magnitude of CTL response and their function needed to confer heterosubtypic immunity against influenza virus infection are unknown. We addressed the role of CD8<sup>+</sup> T cells in the absence of any cross-reactive antibody responses to influenza viral proteins using an adenoviral vector expressing a 9mer amino acid sequence recognized by CD8<sup>+</sup> T cells. Our results indicate that both CD8<sup>+</sup> T cell frequency and function are crucial for heterosubtypic immunity. Low morbidity, lower viral lung titers, low to minimal lung pathology, and better survival upon heterosubtypic virus challenge correlated with the increased frequency of NP-specific CTLs. NP-CD8<sup>+</sup> T cells induced by differential infection doses displayed distinct RNA transcriptome profiles and functional properties. CD8<sup>+</sup> T cells induced by a high dose of influenza virus secreted significantly higher levels of IFN- $\gamma$  and exhibited higher levels of cytotoxic function. The mice that received NP-CD8<sup>+</sup> T cells from the high-dose virus recipients through adoptive transfer had lower viral titers following viral challenge than those induced by the low dose of virus, suggesting differential cellular programming by antigen dose. Enhanced NP-CD8<sup>+</sup> T-cell functions induced by a higher dose of influenza virus strongly correlated with the increased expression of cellular and metabolic genes, indicating a shift to a more glycolytic metabolic phenotype. These findings have implications for developing effective T cell vaccines against infectious diseases and cancer.

## Keywords

influenza; CTL; adeno vector; RNA-seq

With an estimated 12,000–61,000 people dying annually from infection in the United States, the influenza virus continues to be a pathogen of importance despite being a vaccine-preventable infectious disease (1). Vaccine-induced antibody responses primarily target the head region of hemagglutinin (HA), which blocks virus attachment to hosts' cell surfaces and prevents infection (2, 3). However, due to the lack of proofreading mechanisms of the virally encoded RNA-dependent RNA polymerases, frequent mutations occur during influenza virus genome replication leading to antigenic drift (4). Because antibodies induced against the major virus surface protein, HA, tend to be strain-specific, vaccines must be updated annually to match the circulating strains (5). Cytotoxic T lymphocytes (CTLs) recognize highly conserved viral proteins and play a significant role in heterosubtypic immunity across influenza strains (6–9). CD8<sup>+</sup> CTLs against the polymerase-binding protein (PB1), matrix 1 (M1), and nucleoprotein (NP) play an essential role in immune responses against influenza virus infection (10–12). Recent studies during the 2009 pandemic with H1N1 virus demonstrated that individuals with higher frequencies of preexisting cross-reactive CTLs had milder symptoms and a lower risk of virus shedding (13). In addition,

a study using low- and high-avidity CTL lines induced with a recombinant vaccinia virus expressing HIV-GP16 and stimulated *in vitro* with different concentrations of peptides suggests that the quality of CTLs is as important as the quantity of CTLs (14). However, the role of CTL frequency and quality for heterosubtypic protection against influenza virus infection remains unclear.

CTL responses against influenza virus infection in C57Bl/6 mice include H-2D<sup>b</sup>-restricted NP<sub>366–374</sub>, D<sup>b</sup>PA<sub>224–233</sub>, D<sup>b</sup>PB1-F2<sub>62–70</sub>, K<sup>b</sup>PB1<sub>703–711</sub>, K<sup>b</sup>NS2<sub>114–121</sub>, and K<sup>b</sup>M1<sub>128–135</sub>. However, NP<sub>366–374</sub> is abundant and CTL responses against NP are also dominant post-infection, while responses to other epitopes are subdominant. Hence, we addressed CD8<sup>+</sup> T cell responses against the dominant epitope NP<sub>366–374</sub>. In this study, using a mouse model with an H1N1 (PR8 virus) primary infection followed by an H3N2 (HK68 virus) challenge, we demonstrated that the increased frequency of NP-CD8<sup>+</sup> T cells is essential for heterosubtypic protection without causing significant lung pathology. We further confirmed this finding using an adenoviral vector expressing the H1N1 NP CD8<sup>+</sup> T cell epitope (HAd-NPCD8) in the absence of any NP-specific antibody response. Results showed that a high frequency of NP-specific CD8<sup>+</sup> T cells correlated with increased survival after heterosubtypic challenge with the HK68 virus in the absence of antibodies against NP. In addition, NP-CD8<sup>+</sup> T cells induced by different virus doses displayed differential transcriptional reprogramming profiles and are different in cellular and metabolic profiles. Furthermore, CD8<sup>+</sup> T cells induced by the higher virus dose secreted higher levels of IFN- $\gamma$ , exhibited higher levels of cytotoxic function, and reduced viral titer following viral challenge on adoptive transfer. Therefore, the quantity and function of NP-CD8<sup>+</sup> T cells are both essential in heterosubtypic immunity against influenza virus infection.

## RESULTS

### The frequency of NP-specific CD8<sup>+</sup> T cells induced is proportional to the infectious dose

Mice were inoculated with increasing doses [2, 10, and 20 50% mouse infectious dose (MID<sub>50</sub>)] of PR8 (H1N1) influenza virus and monitored for body weight changes. Mice infected with the highest dose, 20 MID<sub>50</sub>, displayed morbidity between days 6 and 11 post-infection compared to the naive group (Fig. 1A). The duration of morbidity was shorter for mice infected with the 10 MID<sub>50</sub> PR8 virus occurring from day 7 to 10 post-infection. No differences were observed in body weight change between the 2 MID<sub>50</sub> infection dose and the naive group (Fig. 1A). We examined the frequency of NP-specific CD8<sup>+</sup> T cells 4 weeks post-infection using flow cytometry with NP<sub>366–374</sub>-H-2D<sup>b</sup> tetramers in conjunction with antibodies specific for CD8 and the activation marker CD44. The HK68-specific response allowed us to examine the cross-reactive T cell pool size before the heterosubtypic challenge. HK68 epitope has D and A at P7 and P8 positions, while PR8 has E and T at P7 and P8 positions, respectively; however, PR8 NP-reactive cells could cross-react with HK68 NP tetramers. Mice immunized with formalin-inactivated (FI) PR8 virus intramuscularly (I.M.) induced little-to-no CD8<sup>+</sup> T cell responses (Fig. 1B and C). NP-specific CD8<sup>+</sup> T cell responses showed dose-dependent increases, and mice infected with the highest dose of the virus, the 20 MID<sub>50</sub>, had the highest frequency of NP-CD8<sup>+</sup> T cells in all tissues examined (Fig. 1B). Especially in the lungs, PR8 virus-infected mice displayed a significantly higher

frequency of antigen-specific CD8<sup>+</sup> T cells than the naive or I.M. immunized group (Fig. 1B). The HK68-specific response demonstrated a similar trend as the PR8-specific response. Furthermore, in all three tissues examined (spleen, lymph node, and lung), mice infected with the 20 MID<sub>50</sub> infectious dose had a statistically significant increase of antigen-specific T cells as compared to the naive or I.M. immunized group (Fig. 1C). Thus, the frequency of reactive and cross-reactive NP-specific CD8<sup>+</sup> T cells induced is proportional to the virus dose. All three groups had various levels of detectable NP-specific CD8<sup>+</sup> T cells post-primary infection, as demonstrated in Fig. 1. NP 366–374 epitope for D<sup>b</sup> is well conserved among several influenza viruses, and their sequence homology is shown in Fig. 2. D<sup>b</sup> allele-specific anchor residues warrant peptides to have “S” at position 2, “N” at position 5 and “M,” “I,” “L,” or “V” at position 9 (15–17). In addition, P3 can serve as a secondary anchor position. P4, P6, P7, and P8 serve as TCR contact residues. Both the PR8- and HK68-specific NP CD8<sup>+</sup> T cells were detected in the lymph node, spleen, and lung tissues.

### **Morbidity and survival are proportional to NP-specific CD8<sup>+</sup> T cell frequency on the heterosubtypic challenge**

Mice immunized with different doses of PR8 (H1N1) influenza virus were challenged with 2 LD<sub>50</sub> of HK68 (H3N2) influenza virus and monitored for morbidity, survival, lung histopathology, serological responses against PR8 and HK/68, and lung viral titers. As shown in Fig. 3A and B, mice that received the high infectious doses (20 MID<sub>50</sub> and 10 MID<sub>50</sub>) of PR8 lost the least body weight, and all mice survived upon heterosubtypic challenge. Mice infected with 2 MID<sub>50</sub> of the PR8 virus had moderate weight loss, and 90% of those mice survived the challenge. However, groups of mice with no NP-specific CD8<sup>+</sup> T cell response, the naive and I.M. group, succumbed to the challenge by day 6 (Fig. 3B). We also examined the lung pathology on day 5 post-HK68 virus challenge using hematoxylin and eosin (H&E) staining of lung sections (Fig. 3C). Naive mice without viral challenge did not contain any inflammatory cells (score = 0). However, mice with HK/68 challenge had moderate peribronchiolar and alveolar inflammation (score = 2). Mice primed with 2, 10, or 20 MID<sub>50</sub> PR8 and challenged with HK68 had an average inflammation score of 1.

Before the heterosubtypic virus challenge, we measured the HI titers to the PR8 and HK68 viruses to rule out the possibility of cross-reactive antibodies from the PR8-infected or PR8-immunized mice. We observed a dose-dependent increase in HI titers against the PR8 virus (Fig. 3D). Responses were significantly different for the 10 MID<sub>50</sub> and 20 MID<sub>50</sub> groups compared to the other groups. Mice infected with 2 MID<sub>50</sub> of PR8 virus or immunized I.M. with formalin-inactivated PR8 virus had similar levels of antibody titers. Most importantly, there were no cross-reactive HI antibodies against the challenge HK68 virus (Fig. 3E). However, we cannot rule out cross-reactive antibody responses against the stem as the HI test cannot detect them. In addition, on day 5 post-heterosubtypic challenge, the frequency of PR8-specific NP-CD8<sup>+</sup> T cells was much higher than the HK68-specific NP-CD8<sup>+</sup> T cells (Fig. 3F) in the previously PR/8-infected mice, possibly due to original antigen sin of CD8<sup>+</sup> T cells (18). In addition, mice that received the higher infecting doses of the PR8 virus displayed lesser cellular infiltration and more normal lung tissue structure when compared to those that received the lower doses of the virus. There was a significant reduction in viral titers in all infection dose groups in lungs 5 days post-HK68 viral challenge ( $P = 0.00390$ )

compared to naive mice challenged with HK68 virus (Fig. 3G). Although there was a dose-dependent reduction in lung viral titers, it was not significant. These data demonstrated that increased frequencies of NP-specific CD8<sup>+</sup> T cells result in lower morbidity, enhanced survival, reduced lung pathology, and reduced viral titers following the heterosubtypic virus challenge.

### **Increased frequency of NP-specific CD8<sup>+</sup> T cells is sufficient for enhanced survival after heterosubtypic challenge**

To rule out the effect of NP-specific antibodies, we immunized mice with  $1 \times 10^6$ ,  $1 \times 10^7$ ,  $1 \times 10^8$ , or  $1 \times 10^9$  PFU/mouse of a replication-defective adenovirus with deletions in the E1 and E3 regions expressing the PR8 NP epitope (HAd-NPCD8). We examined the NP-CD8<sup>+</sup> T cell responses prior to the heterosubtypic virus challenge. As controls, mice were immunized intranasally (I.N.) with  $1 \times 10^9$  PFU/mouse with the same vector but without any influenza epitopes (HAd- E1E3) or PBS. As shown in Fig. 4A, there was a dose-dependent increase in the number of NP-CD8<sup>+</sup> T cells in the lungs. Mice receiving  $1 \times 10^8$  and  $1 \times 10^9$  PFU/mouse of the HAd-NPCD8 vector had similar numbers of NP-CD8<sup>+</sup> T cells, while mice infected with HAd- E1E3 displayed only very low background staining (Fig. 4A). We also examined the histopathological changes in the lungs following the HK68 challenge and scored the changes in lungs from 0 (no histologic change) to 16, exhibiting variably severe lesions including suppurative bronchitis (asterisks), endarteritis/vasculitis (white arrowheads), hypertrophic vascular endothelium (black arrowhead), and peribronchiolar and perivascular inflammation (arrows) (Fig. 4C and D). The mice inoculated with HAd-NPCD8 ( $10^7$  PFU/animal) and HAd-NPCD8 ( $10^9$  PFU/animal) show a statistically significant decrease in histopathology scores compared to the mice inoculated with HAd- E1E3.

Anti-NP mucosal IgA and serum IgG antibodies were detected by ELISA in mucosal washes and sera of mice inoculated with HAd-NP FL (Fig. 4E). However, no NP-specific antibodies were detected either in nasal secretions or sera of mice inoculated with HAd-NPCD8. Thus, increased frequency of NP-specific CD8<sup>+</sup> T cells results in enhanced viral clearance after heterosubtypic challenge in the absence of NP-specific antibodies.

### **Transcriptomic profiles of NP-specific CD8<sup>+</sup> T cells induced by different infection doses**

The presence of a higher frequency of CD8<sup>+</sup> T cells aids in the recovery post-heterosubtypic virus challenge; however, it is not known if there are functional differences in terms of transcriptomic profiles between CD8<sup>+</sup> T cells induced by different doses of initial priming. To address this issue, we infected mice intranasally with 2 or 20 MID<sub>50</sub> of the PR8 virus and boosted them with the same dose 2 weeks later. We harvested lung tissues 5 days post-boost, and NP-CD8<sup>+</sup> T cells were fluorescence activated cell sorter (FACS) sorted. We subjected an equal number of NP-specific CD8<sup>+</sup> T cells from mice immunized with high or low doses of PR8 virus for global RNA transcriptome analysis. There were 1,567 differentially expressed genes (DEGs) identified when we compared NP-CD8<sup>+</sup> T cells between high and low doses of PR8 infection, which accounts for 6.63% of the total genes mapped (Fig. 5A). Among these, 888 genes were upregulated and 679 genes were downregulated. Gene Ontology (GO) functional and pathway enrichment analysis was performed using the clusterProfiler (19) package in R. NP-CD8<sup>+</sup> T cells induced by 20 MID<sub>50</sub> PR8 virus,

as compared to 2 MID<sub>50</sub> infection dose, significantly enriched genes primarily associated with response to the virus, cytokine-mediated signaling, cell adhesion, and cell growth (Fig. 5B). Pathways associated with T cell function, including T cell proliferation and activation, regulation of immune effector process, actin filament organization, and migration were also substantially enriched in NP-CD8<sup>+</sup> T cells induced with the higher dose of infection. As shown in Fig. 5C, the expression of genes associated with the effector CD8<sup>+</sup> T cell response to virus infection, including CD8 T cell expansion and activation (CD86, CD63, CD81, Adam17, Pld2, Mertk, Mbp, and Irak1) and inflammatory cytokine production (IL-15), was upregulated in NP-CD8<sup>+</sup> T cells from mice infected with the higher dose of PR8.

Furthermore, increased expression of genes associated with CTL migration (Cxcl13, Ccl8, Cx3cr1, Alox5, and Rara), clonal expansion, differentiation, survival (Rara), and cytotoxicity effect (CD74, Vcam1, Cav1, Camp, and Prkcd) were observed in NP-CD8<sup>+</sup> T cells induced by the higher dose of PR8 infection. On the other hand, interferon-stimulated genes (Isg15 and Isg20), interferon regulatory factor 7 (Irf-7), interferon induced protein with tetratricopeptide repeats 3 (Ifit3b), known to be involved in type 1 IFN-mediated antiviral responses as shown in Fig. 4C, were significantly downregulated in response to the high dose of PR8 infection as compared to the low dose of PR8. Therefore, the comparative transcriptome analysis revealed a distinct gene profile of NP-CD8<sup>+</sup> T cells induced by the higher dose of HAd-NPCD8 associated with potent CTL function, which contributes to protective heterosubtypic immunity.

Metabolic pathways are essential regulators of immune differentiation, activation, and function and, as such, are central regulators of immune cell functions (20–22). Since changes in the metabolic profiles of immune cells are linked to altered functional outcomes (23), we identified that 43 genes related to metabolic pathways are altered in the NP-CD8<sup>+</sup> T cells between high- and low-dose PR8 virus infection. NP-CD8<sup>+</sup> T cells from the high dose infection are characterized by the upregulation of genes involved in the glycolytic pathway, tricarboxylic acid cycle (TCA), and fatty acid metabolic pathways, indicating cells have altered cellular metabolic profile compared to cells obtained from the low dose of the virus. As shown in Fig. 5D, the commitment of the CD8<sup>+</sup> T cells from the high dose of virus infection toward glycolysis was reflected by the upregulation of genes involved in the glycolytic pathway, which included adenosine kinase (Adk) and aldolase C (AldoC), the key enzymes of the glycolytic pathway. Additionally, genes mapping to the TCA and mitochondrial electron transport chain, electron transfer flavoprotein regulatory factor 1 (Etf1), NADH: ubiquinone oxidoreductase complex assembly factor 1 (Ndufa1), coenzyme Q10A (Coq10a), as well as the fatty acid metabolism genes [Acyl-CoA synthetase family member 2 (Acsf2), Acyl-Coenzyme A oxidase 1, and palmitoyl (Acox1)], were also upregulated in the CD8<sup>+</sup> T cells isolated from the high virus dose. The gene expression changes support the observation that the cells from high and low virus doses are qualitatively different, with the cells from the high dose exhibiting higher expression of genes involved in the glycolytic pathway.



## Functional characterization of NP-specific CD8<sup>+</sup> T cells induced by different infection doses

To further characterize the function of NP-specific CD8<sup>+</sup> T cells induced by different infectious doses, we stimulated an equal number of NP-specific CD8<sup>+</sup> T cells from lungs or spleens from mice infected with 2 or 20 MID<sub>50</sub> of PR8 virus with NP peptide *in vitro* and examined their proliferation, cytokine production, and cytotoxic activity. NP-CD8<sup>+</sup> T cells induced by different infection doses displayed similar proliferation kinetics measured by carboxyfluorescein succinimidyl ester (CFSE) dilution (data not shown). However, NP-CD8<sup>+</sup> T cells from the spleen induced by a higher infection dose secreted significantly higher levels of IFN- $\gamma$  and TNF- $\alpha$  as compared to those from mice infected with a lower dose of PR8 virus (Fig. 6A). A similar trend of cytokine production profile was detected in the NP-CD8<sup>+</sup> T cells isolated from the lungs, although the difference is not statistically significant (Fig. 6B). An equal number of NP-specific T cells from mice infected with 2 or 20 MID<sub>50</sub> PR8 virus were co-cultured with CFSE-labeled EL4 cells pulsed with NP peptide to assess their cytotoxic activity. We determined the target cell death amount by quantifying 7-aminoactinomycin D (7-AAD)-positive EL4 cells. We observed two times higher 7-AAD<sup>+</sup> EL4 cells in co-cultures containing splenocytes from 20 MID<sub>50</sub> infection dose compared to the co-culture containing splenocytes from 2 MID<sub>50</sub> infection dose ( $22.2\% \pm 5.3\%$  versus  $52.24\% \pm 4.0\%$ , Fig. 6C). Taken together, NP-CD8<sup>+</sup> T cells induced by higher PR8 infection dose displayed more cytokine production and cytotoxic activity. To further address the functional property of NP-CD8<sup>+</sup> T cells *in vivo*, an equal number of pooled NP-CD8<sup>+</sup> T cells isolated from lungs, lymph nodes, and spleens of mice infected with 2 or 20 MID<sub>50</sub> PR8 viruses were adoptively transferred to naive mice. The antibodies used to characterize and gating strategy for lung and splenic NP-specific CD8<sup>+</sup> T cells are shown in Fig. S1 and S2, respectively. The transferred populations consist of similar frequencies of naive, central, and effector memory cell populations (Fig. S3). Mice were challenged with the HK68 virus 24 h later, and the viral titers in the lung were quantitated. The results presented in Fig. 6D demonstrated that mice receiving cells from 20 MID<sub>50</sub> PR8 dose had significantly lower viral load in the lung, as compared to mice receiving cells from 2 MID<sub>50</sub> PR8, suggesting that NP-CD8<sup>+</sup> T cells induced by higher PR8 infection dose displayed more protective heterosubtypic immunity against the HK68 virus challenge. Therefore, besides the frequency, the functional quality of influenza NP-CD8<sup>+</sup> T cells is also critical for heterosubtypic immunity.

## DISCUSSION

The seminal observation that adoptively transferred influenza-specific CTLs in mice can mediate influenza virus clearance by Yap et al. (24) in 1978 has led to an interest in delineating their role in mediating protection from influenza infection (25–29). Since CTLs recognize conserved epitopes (Fig. 2), developing vaccines that induce CTLs can confer protection against continuously evolving influenza viruses (30–34). In addition to viral clearance, influenza virus-specific CD8<sup>+</sup> T cells have been implicated in immunopathology in the lungs where the virus is replicating (35, 36). For example, when F5 TCR transgenic mice, which have a very high frequency of anti-influenza NP CTL precursors, were infected with different doses of influenza virus, exacerbated viral pathology and high mortality were

observed only at high viral doses (37). In fatal cases with novel swine H1N1 influenza infection, Mauad et al. (38) showed the presence of many CTLs and granzyme B<sup>+</sup> cells within the human lung tissues. We examined whether CD8<sup>+</sup> CTL frequency and/or function contribute to the protection and/or immunopathology following the heterosubtypic virus challenge.

We first used an infection-challenge model where mice were infected first with different doses (2, 10, or 20 MID<sub>50</sub>) of an H1N1 (PR8) virus and then challenged with an H3N2 (HK68) virus. We observed that the frequency of reactive and cross-reactive NP-specific CD8<sup>+</sup> T cells is proportional to the virus infection dose before the challenge in the lymph node, spleen, and lung tissues. In addition, PR8-infected mice with increased NP-CD8<sup>+</sup> T cells displayed enhanced survival and less morbidity against the H3N2 virus challenge compared to those that could only generate antibodies with low/no detectable levels of NP-CD8<sup>+</sup> T cells (I.M. immunization using FI PR8 virus) (Fig. 3A, B, and F). Furthermore, there was a dose-dependent clearance of the H3N2 virus in the lungs (Fig. 3G) with a corresponding decrease in pathological changes in the lungs (Fig. 3C). I.M. immunization with FI-PR8 and HK/68 challenge resulted in diffuse moderate to severe peribronchiolar and alveolar inflammation (score = 2–3). The severe tissue injury observed in the FI-PR8 group challenged with HK/68 is similar to those observed with the FI-RSV vaccine administered to children in 1960s, which resulted in severe inflammation of the lungs and case fatalities post-vaccination when exposed to RSV (39). It has been shown that formalin inactivation of RSV altered pre- and post-fusion protein ratios, which could lead to pathology (40). Subtle alterations and generation of reactive oxygen species tend to tilt the system toward Th2 responses and associated pathologies despite the protection (41). A similar observation was made with the FI Japanese B encephalitis vaccine and others (42). The enhanced pathology observed in the FI-PR8-immunized group compared to the PBS group may be due to the altered influenza viral antigens by formalin treatment. No structural or immunogenicity data exist comparing HA, NA, and M2e before and after formalin inactivation. Our findings suggest that an increased frequency of cross-reactive CD8<sup>+</sup> T cells does not cause immunopathology and appears to be critical for survival following heterosubtypic challenges. We employed a system that does not artificially increase the burst size of the NP-specific CD8<sup>+</sup> T cell response that happens in the adoptive transfer or transgenic models (37). Our findings also demonstrated a correlation between increased frequency of NP-specific CD8<sup>+</sup> T cells with enhanced survival upon heterosubtypic virus challenge. However, we could not rule out the contribution of non-HI but cross-reactive antibodies to other regions of HA, such as the stem, for survival upon heterosubtypic virus challenge (29, 43). To address this, we employed a replication-defective adenovirus expressing only the CD8<sup>+</sup> T cell epitope of the PR8 virus (HAd-NPCD8). Mice immunized with HAd-NPCD8 had no detectable anti-NP antibodies in the sera or nasal washes but generated NP-CD8<sup>+</sup> T cells, which protected mice against the HK68 virus challenge by reducing lung viral titers with corresponding reduction in lung pathology, which correlated with the frequency of NP-specific CD8<sup>+</sup> T cells (Fig. 4). Thus, antigen-specific CD8<sup>+</sup> T cells alone were sufficient and critical to recover from influenza without any antibody-mediated protective mechanisms. We further demonstrated that having a higher frequency of NP-specific CD8<sup>+</sup> T cells is crucial for heterosubtypic challenge.



The intrinsic quality of CTL has been shown to be as necessary as the frequency of CTL for the control and clearance of virus infection in adoptive transfer studies with HIV GP160-specific CD8<sup>+</sup> T cell lines induced with a recombinant vaccinia virus expressing GP160 and maintained *in vitro* by stimulating with peptides. Adoptive transfer of established CD8<sup>+</sup> T cell lines that have either high or low avidity as determined by anti-CD3 antibody-redirected lysis of Fc-receptor-expressing target cells, P815, demonstrated that low avidity CD8<sup>+</sup> T cells were inefficient in clearing recombinant vaccinia virus infection (14). These findings prompted us to examine whether influenza NP-CD8<sup>+</sup> T cells induced by a high virus dose are qualitatively different from the ones induced by a lower virus dose. Our study used CD8<sup>+</sup> T cells induced *in vivo* without subsequent *in vitro* manipulation or culture. Next, we observed significant differences in transcriptomics signatures between NP-specific T cells induced by different doses of PR8 infection. Specifically, 888 genes were significantly upregulated by higher doses of PR8, while 679 genes were substantially downregulated.

Upon influenza infection, naive CD8<sup>+</sup> T cells are primed by antigen-presenting cells in secondary lymphoid organs, which leads to CD8<sup>+</sup> T cell activation and expansion. Activation of CD8<sup>+</sup> T cells depends on three signals: TCR engagement (signal 1), costimulatory (signal 2), and inflammatory stimulus (signal 3) (44). The functional annotation analysis of NP-CD8<sup>+</sup> T cells for enrichment of GO Biological Process (BP) terms identified 386 significantly enriched terms with adjusted *P*-value < 0.05. Among these, 159 (41.2%) enriched GO terms are related to T cell functions. These results demonstrated that a high dose of PR8 increased the expression of pathways necessary for CD8<sup>+</sup> T cell activation and expansion, including pathways response to the virus, cytokine-mediated signaling, regulation of cell growth, and cell-cell adhesion (Fig. 5B). Specifically, the induction of CD86 and upregulation of CD63, CD81, Mbp, and Mertk was observed on NP-CD8<sup>+</sup> T cells from mice infected with the higher dose of PR8 virus. These molecules were shown to be critical for CD8<sup>+</sup> effector function as costimulatory or activation molecules (45–49). The inflammatory cytokine IL-15 was reported to induce CD8<sup>+</sup> T cells to acquire functional NK receptors capable of modulating cytotoxicity and cytokine secretion. A higher dose of PR8 significantly induced this gene compared to a lower dose (50). After activation, T cells undergo clonal expansion and gain different effector functions. The expression of genes Adam17, Pld2, and Irak1 was substantially increased in NP-CD8<sup>+</sup> T cells from the high-dose group. Several studies have reported that these genes are involved in CD8<sup>+</sup> T cell proliferation (51–53). Upon activation, CD8<sup>+</sup> T cells exit the lymph node and home toward inflamed tissues under the guidance of adhesion and chemokine expressed by the inflamed tissue (54). Cxcl13 and Ccl8 mediate the recruitment of CD8<sup>+</sup> T cells to control viral infection (55–57). Similarly, Rara plays an important role in the homing of T cells, their differentiation, expansion, and survival as Rara-deficient CD8<sup>+</sup> T cells failed to control *Listeria monocytogenes* infection (58, 59). *In vitro* studies demonstrated the critical role of Cx3cr1 in effector lymphocyte trafficking (60). In our study, the increased expression of these genes in NP-CD8<sup>+</sup> T cells from the high dose of PR8 indicates the efficient trafficking of activated CD8<sup>+</sup> T cells toward the infection site to combat the pathogen (55, 56, 58–60). The CTLs target virus-infected host cells and kill them through three known mechanisms: perforin/granzyme-mediated cytotoxicity, apoptosis mediated by FasL/Fas, and TRAIL/TRAIL-DR signaling (54). Related genes associated with the cytotoxicity

killing, such as CD74, Vcam1, Cav1, Camp, and Prkcd, were upregulated by the higher infection dose. While CD74, Camp, and VCAM-1 were demonstrated to boost CD8<sup>+</sup> T cell immunity (61, 62), Cav1 was reported to modulate CD8<sup>+</sup> effector function through membrane organization and integrin function (63), and Prkcd directly mediates TCR signals leading to granule exocytosis-mediated cytotoxicity (64). It has been shown that innate immune response genes in adaptive immune cells, such as CD8<sup>+</sup> T cells, are modulated in acute and chronic infections to induce antiviral or anti-tumor responses (57, 65, 66). Isg15, Isg20, Irf7, and Ifit3b play a significant role in antiviral immunity mediated by type 1 IFN induced by innate immune sensors (67). However, they are downregulated in activated CD8<sup>+</sup> T cells induced by high-dose PR8 virus. These CD8<sup>+</sup> T cells are likely programmed to perform a lytic function, as demonstrated in Fig. 5C, compared to the CD8<sup>+</sup> T cells induced by the low-dose virus, which may rely on conventional lytic function and innate immune responses. Investigation of kinetics of induction and activation of CD8<sup>+</sup> T cells in response to antigen dose at transcriptome and proteome levels will delineate the role of innate genes in CD8<sup>+</sup> T cell-mediated heterosubtypic immunity. Changes in the antigen-presenting cells, priming microenvironment, and cytokine milieu may contribute to the observed changes in CD8<sup>+</sup> T cells. For example, Boon et al. (68) demonstrated that the concentration of IL-2 during *in vitro* expansion of influenza M1- and NP-specific cells had a profound effect on their functionality.

Metabolic reprogramming of innate immune cells influences the functions and differentiation of immune cells and the course of an immune response (20–22, 69–72). Recent evidence suggests that different classes of T cells have distinct metabolic profiles to meet the cells' energy demands. For example, naive T cells use glucose and fatty acid as an energy source, activated T cells are known to upregulate glycolytic pathways even in the presence of sufficient oxygen, effector T cells are more dependent on glucose, and memory T cells use fatty acid oxidation. Here, we have shown that CD8<sup>+</sup> T cells from the high antigen dose have an altered bioenergetic profile compared to CD8<sup>+</sup> T cells induced by a low-dose antigen. To gain insight into the molecular basis for observed different effects of the two different antigen doses on the functional outcome of the CD8<sup>+</sup> T cells, we examined the role of glycolysis during the induction/activation of CD8<sup>+</sup> T cells. Our results show that glycolytic enzymes are upregulated in CD8<sup>+</sup> T cells. These findings reveal that modifications of metabolic glycolytic and OXPHos pathways regulate the cellular functions of CD8<sup>+</sup> T cells. To further characterize the functional properties of NP-CD8<sup>+</sup> T cells, memory CD8<sup>+</sup> T cells were generated upon infection with 2 or 20 MID<sub>50</sub> of the PR8 virus. Cells were then re-stimulated *in vitro* using NP peptide to recall NP-specific CD8<sup>+</sup> T cell response. The recall proliferation potential of NP-CD8<sup>+</sup> T cells was monitored by CFSE dilution, and no difference was observed between NP CD8<sup>+</sup> T cells induced by two infection doses. However, spleen and lung NP-CD8<sup>+</sup> T cells from mice infected with 20 MID<sub>50</sub> PR8 produced substantially higher levels of IFN- $\gamma$ - and TNF- $\alpha$ -secreting cells, compared to NP-CD8<sup>+</sup> T cells induced by a lower infection dose. High infection dose programmed CD8<sup>+</sup> T cells differently than the low infection dose, with genes involved in glycolytic pathways being highly expressed in cells induced by the high dose signifying their functional differences.

Furthermore, NP-CD8<sup>+</sup> T cells from higher infection doses demonstrated significantly more cytotoxic activity. The *in vivo* transfer study directly demonstrated that NP-CD8<sup>+</sup> T cells from mice infected with 20 MID<sub>50</sub> PR8 provide better protective immunity than the same number of NP-CD8<sup>+</sup> T cells from mice infected with 2 MID<sub>50</sub> PR8 when an equal number of them were transferred, and the naive, central, and effector memory composition of the adoptively transferred cells is similar. NP is highly conserved among influenza A virus strains, and NP-specific CTLs can confer protective immunity against antigenically distinct influenza A virus strains (73, 74). Therefore, in our model, NP-specific CD8<sup>+</sup> CTLs induced by PR8 infection or HAd-NP vaccination cleared HK68-infected cells and conferred heterosubtypic protection.

In conclusion, influenza-virus-specific CD8<sup>+</sup> T cells are critical for survival from heterosubtypic challenges without causing immunopathology. Furthermore, the functional quality and frequency of CD8<sup>+</sup> T cells are both crucial in conferring heterosubtypic protection. Hence, developing next-generation influenza vaccines that can induce/recall humoral immune responses and enhanced frequency with the quality of CD8<sup>+</sup> T cells is crucial to control epidemics and pandemics.

## MATERIALS AND METHODS

### Generation and characterization of replication-defective HAd-NPCD8, HAd-NP FL, and HAd- E1E3 vectors

HAd-NPCD8, HAd-NP FL, and HAd- E1E3 vectors were constructed as described previously (75). Briefly, HAd-NP FL or HAd-NPCD8 represents HAd- E1E3 expressing either the full-length coding region of the NP gene of the PR8 influenza virus or one of the NP CD8 epitopes of the PR8 virus (366–374, ASNENMETM), respectively. The gene construct was under the control of cytomegalovirus promoter and bovine growth hormone polyadenylation signal (polyA). HAd- E1E3 represents the E1 and E3 deleted HAd vector. The vectors were plaque purified, and their genomes were analyzed by restriction enzyme digestion and sequencing to confirm the presence or absence of a foreign gene cassette. HAd- E1E3, HAd-NP FL, or HAd-NPCD8 were grown in HEK 293 cells (76), purified by cesium chloride density-gradient centrifugation (76), and titrated by PFU assay in BHH-2C (bovine human hybrid 2C) cells (77).

### Influenza viruses

Influenza viruses used in this study include A/Puerto Rico/8/34 (PR8, H1N1) and A/Hong Kong/1/68 (HK68, H3N2). Viruses were propagated for 2 days in the allantoic cavity of 10-day-old embryonated chicken eggs. Pooled allantoic fluid was clarified by centrifugation, sequenced, titered, aliquoted, and stored at –80°C until use.

### Immunization and virus infections

Six-week-old female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA) were anesthetized by inhalation of isoflurane and infected intranasally with 2, 10, and 20 MID<sub>50</sub> of PR8 virus in a final volume of 50 µL. Similarly, mice were immunized I.N. with various doses of ( $1 \times 10^6$ ,  $1 \times 10^7$ ,  $1 \times 10^8$ , and  $1 \times 10^9$  PFU/mouse) replication-

deficient adenoviruses, HAd-NPCD8, HAd-NP FL, or HAd- E1E3. In addition, control mice were immunized intramuscularly with 500 hemagglutinin units in 50  $\mu$ L of formalin-inactivated PR8 virus in each quadriceps muscle. Sera were obtained 3 weeks post-infection/immunization to determine antibody responses. Four weeks post-infection/immunization, mice were challenged with 2 50% lethal dose (2 LD<sub>50</sub>) of HK68 (H3N2) influenza virus and monitored for weight loss and mortality or lung virus titers. For RNA sequencing analysis, mice were infected I.N. with 2 or 20 MID<sub>50</sub> of the PR8 virus and boosted with the same dose 2 weeks later. Lung tissues were harvested 5 days post-boost, and NP-CD8<sup>+</sup> T cells were FACS sorted. Mice losing more than 25% of their pre-infection body weight were humanely euthanized.

### Flow cytometry and cell sorting

Mice received a 100  $\mu$ L intravenous (IV) injection retro-orbitally of 1  $\mu$ g fluorescently conjugated CD45 (IV-label) 5 min prior to sacrifice to delineate cells in circulation versus those that are tissue-resident. Lung tissues were harvested at sacrifice into gentleMACS c-tubes (Miltenyi) containing 2.4 mL of serum-free 1 $\times$  RPMI-1640 (Gibco) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1 $\times$  nonessential amino acids, 1 $\times$  antibiotic/antimycotic solution (all from Corning; sf-RPMI-1640), along with a final concentration of 10 mg/mL Liberase TL (Roche) and 100 mg/mL DNaseI (Sigma) at 4°C. Tissues were homogenized using a gentleMACS Octo Dissociator with two cycles of the m\_lung\_01\_02 protocol, each followed by a 10-min incubation at 37°C. Following the second 10-min incubation, c-tubes were returned to the dissociator and the m\_lung\_02\_01 protocol was run followed by a 10-min incubation at 37°C. After the last 10-min incubation at 37°C (30 min in total), the c-tubes were returned to the dissociator and the m\_lung\_01\_02 protocol and m\_lung\_02\_01 protocol were run in immediate succession. Cells were then pelleted by centrifugation for 5 min at 1,400 rpm and 4°C and then the lung digestion supernatant was aspirated off. Cells were resuspended in 10 mL of a custom deficient RPMI-1640 media containing 3% newborn calf serum (NBCS; Life Technologies) and 0.1  $\mu$ L/mL Benzonase (Sigma; hereafter FACS buffer) and passed through a 70  $\mu$ m cell strainer (Miltenyi). Single-cell suspensions were pelleted and resuspended in 1 mL of ammonium-chloride-potassium (ACK) lysis buffer to lyse red blood cells (RBCs) for ~2 min at room temperature (RT). Following RBC lysis, 10 mL FACS buffer was added, and cells were pelleted through a 2 mL 100% NBCS serum layer prior to downstream processing.

Spleen cells were passed through a 70  $\mu$ m cell strainer to obtain single-cell suspensions, and RBCs were lysed with ACK lysis buffer. Following RBC lysis, 10 mL FACS buffer was added, and cells were pelleted through a 2 mL 100% NBCS serum layer prior to downstream processing.

Cells (up to 10<sup>7</sup> total) were resuspended in 44  $\mu$ L FACS buffer and pre-incubated with 1  $\mu$ L TruStain FcX (BioLegend) and 5  $\mu$ L CellBlox (ThermoFisher Scientific; final volume 50  $\mu$ L) for 10 min at 4°C in 5 mL FACS tubes (Corning). The 20-color extracellular staining master mix (Fig. S1) was prepared 2 $\times$  in BD Horizon Brilliant Stain Buffer (BD Biosciences), added 1:1 (50  $\mu$ L) to cells, and then incubated for 30 min at 4°C. Following staining, cells were washed with ~4 mL of FACS buffer. Cells were fixed in 1.6 $\times$  BD FACS

lysing solution (10× stock diluted in dH<sub>2</sub>O) for 30 min at room temperature. Cells were washed in ~4 mL FACS buffer, then resuspended in 200–1,000 µL FACS buffer with 10 µL of Precision Count Beads (BioLegend) for acquisition using BD FACSDiva Software on a BD FACSsymphony A5. To distinguish auto-fluorescent cells from cells expressing low levels of a particular surface marker, we established upper thresholds for auto-fluorescence by staining samples with fluorescence-minus-one control stain sets in which a reagent for a channel of interest is omitted. Data were analyzed with FlowJo version 10.8.1 (BD Biosciences). Mice received a 100 µL IV injection retro-orbitally of 1 µg fluorescently conjugated CD45 (IV-label, see Fig. S2) 5 min prior to sacrifice to delineate cells in circulation versus those that are tissue-resident. Lung tissues were harvested at sacrifice into gentleMACS c-tubes (Miltenyi) containing 2.4 mL of serum-free 1× RPMI-1640 (Gibco) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1× nonessential amino acids, 1× antibiotic/antimycotic solution (all from Corning; sf-RPMI-1640), along with a final concentration of 10 mg/mL Liberase TL (Roche) and 100 µg/mL DNaseI (Sigma) at 4°C. Tissues were homogenized using a gentleMACS Octo Dissociator with two cycles of the m\_lung\_01\_02 protocol, each followed by a 10-min incubation at 37°C. Following the second 10-min incubation, c-tubes were returned to the dissociator and the m\_lung\_02\_01 protocol was run followed by a 10-min incubation at 37°C. After the last 10-min incubation at 37°C (30 min in total), the c-tubes were returned to the dissociator and the m\_lung\_01\_02 protocol and m\_lung\_02\_01 protocol were run in immediate succession. Cells were then pelleted by centrifugation for 5 min at 1,400 rpm and 4°C and then the lung digestion supernatant was aspirated off. Cells were resuspended in 10 mL of a custom deficient RPMI-1640 media [see reference (4)] containing 3% newborn calf serum (Life Technologies) and 0.1 µL/mL Benzonase (Sigma; hereafter FACS buffer) and passed through a 70 µm cell strainer (Miltenyi). Single-cell suspensions were pelleted and resuspended in 1 mL of ACK lysis buffer to lyse red RBCs for ~2 min at room temperature. Following RBC lysis, 10 mL FACS buffer was added, and cells were pelleted through a 2 mL 100% NBCS serum layer prior to downstream processing.

Spleen cells were passed through a 70 µm cell strainer to obtain single-cell suspensions, and RBCs were lysed with ACK lysis buffer. Following RBC lysis, 10 mL FACS buffer was added, and cells were pelleted through a 2 mL 100% NBCS serum layer prior to downstream processing. PR8 or HK68-specific NP CD8<sup>+</sup> T cells in the lungs, lymph nodes, and spleens were identified using H-2D<sup>b</sup>/ASNENMETM or H-2D<sup>b</sup>/ASNENMDAM tetramer, respectively (NIH tetramer core facility, Emory University, Atlanta, GA, USA).  $1 \times 10^6$  cells from the lung and spleen of mice infected with the PR8 virus for 3 weeks were stimulated *in vitro* with NP peptide (2 µM) for 12 h with GolgiPlug (BD Bioscience, San Jose, CA, USA) to detect intracellular cytokine production. Cells were surface stained with anti-CD44, anti-CD8 antibody, and NP tetramer, followed by intracellular staining with anti-IFN-γ and anti-TNF-α antibody (BD Bioscience). Splenocytes from mice infected with the PR8 virus for 3 weeks were labeled with 5 µM carboxyfluorescein succinimidyl ester and incubated with NP peptide for 5 days. Cells were then surface stained with CD8 and NP tetramer. Samples were analyzed using an LSR Fortessa Flow cytometer (BD Biosciences), and the cytometric data were analyzed using FlowJo software version 9.3.3 (Tree Star, Inc). For adoptive transfer experiments, two groups each of 15–20 mice were infected with either

2 or 20 MID<sub>50</sub> PR8 virus and boosted 2 weeks later. Lung, lymph node, and spleen were harvested 5 days post-boost pooled from 15 to 20 mice for each virus dose, and NP CD8<sup>+</sup> T cells were sorted using a FACS Aria II (BD).  $5 \times 10^5$  sorted cells were then adoptively transferred via the tail vein in 100  $\mu$ L volume to each recipient mouse.

### ***In vitro* cytotoxicity assay**

Effector cells were prepared from the spleens of mice infected with 2 or 20 MID<sub>50</sub> PR8 virus for 7 days or spleens of mice infected for 21 days and expanded *in vitro* with NP peptide (2  $\mu$ M) for another 3 days. Target cells EL4 were pulsed with NP peptide for 1 h each at 26°C and 37°C and then labeled with 5  $\mu$ M of CFSE. Effector and target cells were co-cultured at a 50:1 (E/T) ratio for 4 h and then stained with 7-aminoactinomycin D (BD Biosciences) for 30 min. Cells were acquired on an LSR Fortessa Flow cytometer to detect the death of EL4 cells as an indication of CD8<sup>+</sup> T cell-mediated killing.

### **Quantitation of viral titers in the lung**

Lung tissues were harvested 5 days post-challenge and homogenized in 2 mL of cold PBS. Confluent monolayers of MDCK cells in 6-well plates were washed with DMEM and infected with serial 10-fold dilutions of lung homogenates for 1 h at 37°C in 5% CO<sub>2</sub>. Cells were washed with DMEM and overlaid with 1.6% SeaKem LE agarose (Lonza, Basel, Switzerland) mixed 1:1 with 2 $\times$  L15 medium (Lonza, Basel, Switzerland) containing 4 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 mM L-glutamine, 5  $\mu$ g/mL gentamycin, 1.5 mg/mL sodium bicarbonate, and 1  $\mu$ g/mL TPCK-treated trypsin (Sigma-Aldrich, St. Louis, MO, USA). Virus plaques were stained with 0.3% crystal violet solution (BD, Sparks, MD, USA) after 72-h incubation at 37°C in 5% CO<sub>2</sub> and counted.

### **Antibody assays**

Sera collected from immunized mice were treated with receptor-destroying enzyme (RDE, Denka Seiken) at 37°C overnight and titered against PR8 and HK68 viruses by the standard HI assay using 1% turkey RBCs. Briefly, 25  $\mu$ L of 1 $\times$  PBS was added to wells of a 96-well V-bottom plate (Corning). This was followed by adding 50  $\mu$ L of RDE-treated sera to each column and then serially diluted. Next, 25  $\mu$ L of 4 HA units of PR8 or HK68 virus was added to each well and incubated at RT for 30 min. Finally, 50  $\mu$ L of 1% standardized turkey RBCs in PBS was added to each well and incubated at RT for exactly 30 min. The reciprocal serial dilutions of the sera that showed complete inhibition of hemagglutination were recorded as the HI titer. NP antigen-specific ELISA was performed to determine IgA and IgG antibody titers in sera and lung washes.

### **Histopathology**

On the designated days post-viral challenge, the lung samples were collected and fixed in 10% neutral buffered formalin. After 72 h, the samples were transferred to 70% ethanol, sectioned, and stained with H&E. The lung pathology scores are with the following description: 0, no inflammation; 1, mild inflammation; 2, moderate inflammation; and 3, severe inflammation.



## RNA isolation, sequencing, alignment, and quantification

RNA samples were purified from  $10^5$  FACS-sorted NP-CD8<sup>+</sup> T cells with Direct-zol Microprep kit (Zymo, CA, USA). RNA concentration was measured by Bioanalyzer 2100 with Agilent RNA 6000 Pico kit (Agilent, CA, USA). Thirteen nanograms of total RNA (RIN 6.8–7.9) was used for the construction of NGS libraries with QIAseq FX Single Cell RNA Library Kit (Qiagen, MD, USA). The libraries were quantified by Bioanalyzer 2100 with Agilent High Sensitivity DNA Kit (Agilent, CA, USA) and sequenced on a NovaSeq6000 system with NovaSeq 6000 SP reagent kit v1 (100 cycles) (Illumina, CA, USA). Sequences were mapped to the mm10 Mus musculus Ensembl Transcriptome v96 and quantified using Kallisto (78) and Taximport (79).

## Differential expression analysis

Differential expression analysis was performed in R with the package DESeq2 (80–82). Transcripts that have more than five mapped reads in at least two samples were kept for analysis. Genes with low counts were filtered for low expression by their normalized mean counts, and raw *P*-values were adjusted for multiple testing using the Benjamini-Hochberg correction. Genes considered significantly differentially expressed are those that passed the filters: (i) adjusted *P*-values controlled at false discovery rates (FDR) < 0.05 threshold; and (ii) the absolute value of log<sub>2</sub> fold change > 1. A volcano plot was produced in the R with the package ggplot2 (83) and showed the log<sub>2</sub> fold change of each gene plotted against its false discovery rate (–log<sub>10</sub>FDR), both of which were calculated in DESeq2. Enrichment of all DEGs in BP GO terms was analyzed in R with the package clusterProfiler (19), and the top enriched GO terms are visualized in a bar plot. A subset of 46 important DEGs was selected to generate the heatmap in R with the package ComplexHeatmap (84). Processed data and code are available via link: [yyw-informatics/RNA-seq-InfluenzaNP-CD8-T-cells \(github.com\)](https://yyw-informatics.github.io/RNA-seq-InfluenzaNP-CD8-T-cells/).

## Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software). A one-way ANOVA with multiple comparisons was used to determine the significance of the NP-specific CD8<sup>+</sup> T cell data and HI titer among all groups. In addition, the Student's *t*-test was used to analyze differences among treatments for intracellular cytokine production, *in vitro* cytotoxicity assay, and virus titer. Data were presented as mean ± SEM. All differences were considered significant when the *P*-value was < 0.05.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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S.S., S.A., W.C., E.E.S., and W.-C.W. designed and performed experiments. E.E.S., W.-C.W. and S.K.M. constructed, characterized, purified, and titrated HAd- E1E3, HAd-NP FL, and HAd-NPCD8. W.S. and N.W. performed lung section histopathology. S.R. and D.J.E. performed flow cytometry and in vitro cytotoxicity assays. A.K. quantitated the viral titers in the lungs and A.K. and S.D.S. performed HI assays. C.B. performed the mice adoptive transfer studies. P.R. purified RNA for sequencing. M.M., M.S., J.H., R.A., J.L., J.P., M.B., and R.J. performed RNA sequencing. Y.W., R.A., and R.J. performed data analysis. S.G. assisted and ensured that the animal experiments are carried out as per the protocols.

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## DATA AVAILABILITY

The data that support the findings of this study are available on request from the corresponding author, S.S.

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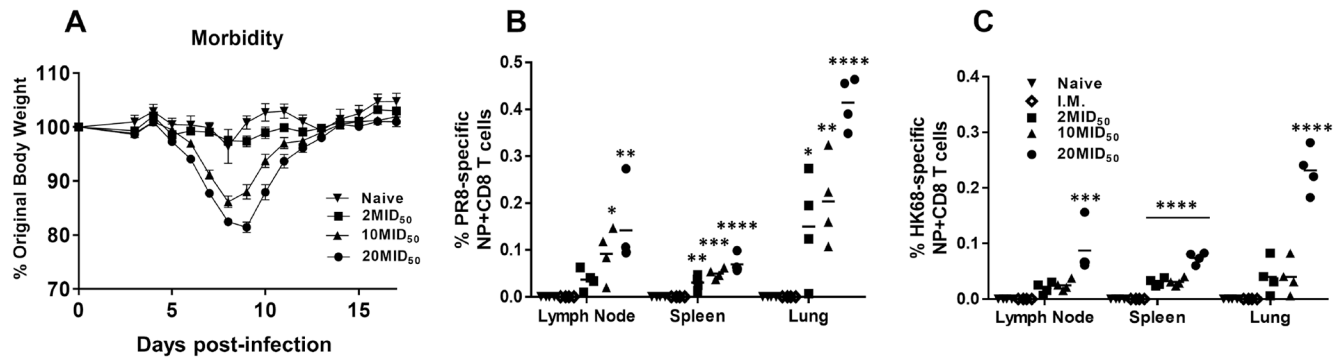
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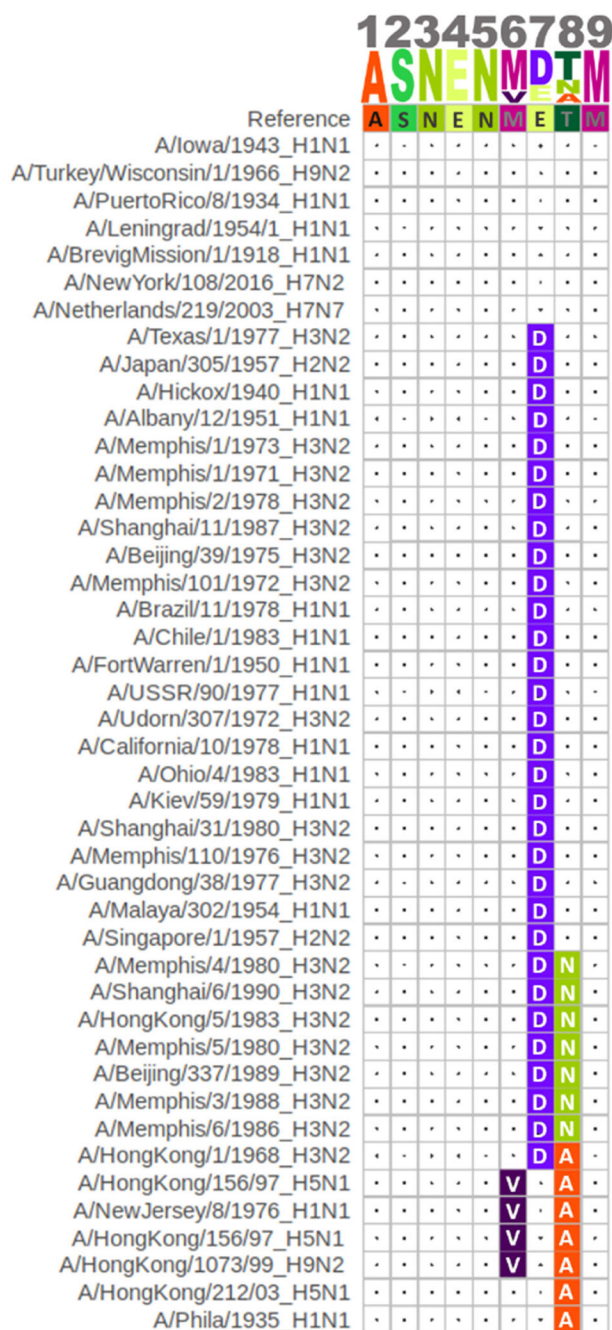
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**IMPORTANCE**

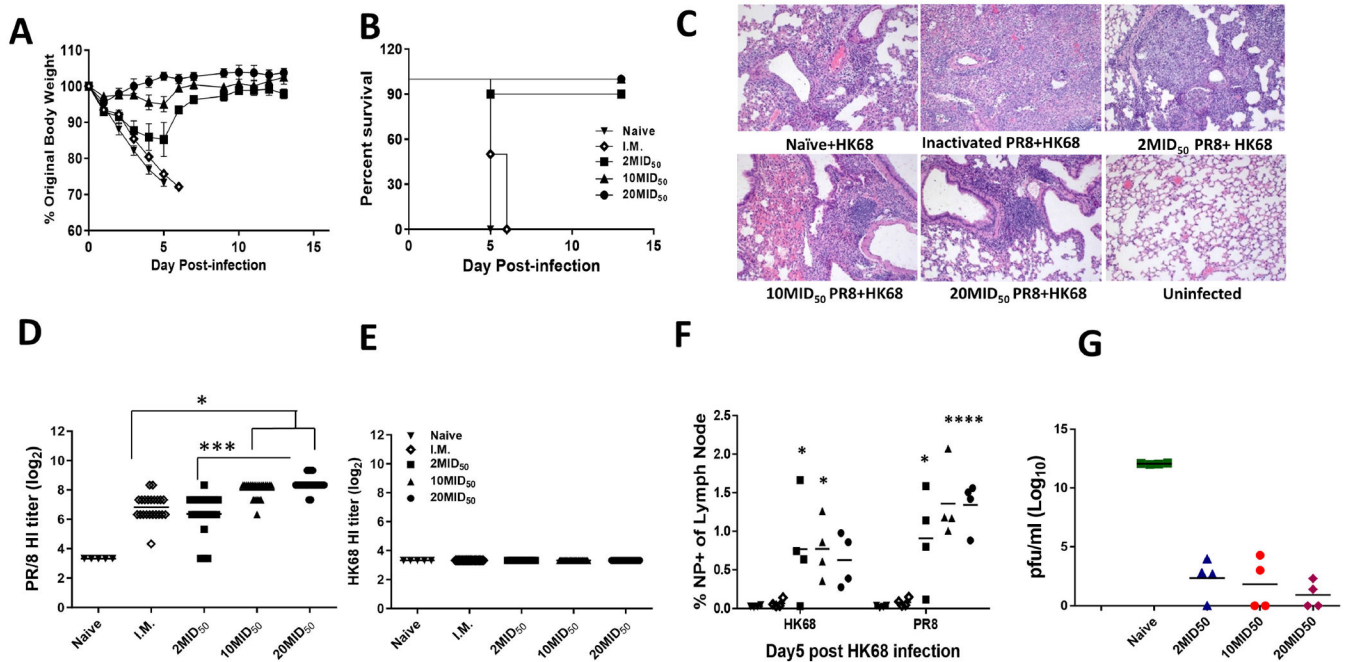
Cytotoxic T lymphocytes (CTLs) are an important component of the adaptive immune system that clears virus-infected cells or tumor cells. Hence, developing next-generation vaccines that induce or recall CTL responses against cancer and infectious diseases is crucial. However, it is not clear if the frequency, function, or both are essential in conferring protection, as in the case of influenza. In this study, we demonstrate that both CTL frequency and function are crucial for providing heterosubtypic immunity to influenza by utilizing an Ad-viral vector expressing a CD8 epitope only to rule out the role of antibodies, single-cell RNA-seq analysis, as well as adoptive transfer experiments. Our findings have implications for developing T cell vaccines against infectious diseases and cancer.

**FIG 1.**

The frequency of NP-specific CD8<sup>+</sup> T cells induced is proportional to the influenza virus dose. Six-week-old female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA) were anesthetized by inhalation of isoflurane and infected intranasally with 2, 10, and 20 MID<sub>50</sub> of PR8 virus in a final volume of 50  $\mu$ L. Control mice were either given PBS or immunized with a dose of 500 hemagglutinin units in 50  $\mu$ L of formalin-inactivated PR8 virus in each quadriceps muscle. (A) The mice were monitored for body weight changes up to 17 days post-PR8 virus infection. Data are an aggregate of 38 mice per group from three independent experiments for the infected groups and four for the naive group (two independent experiments). (B and C) Four weeks post-infection, the draining lymph nodes, spleen, and lung tissues were harvested, and single-cell suspensions were prepared. Activated CD8<sup>+</sup> T cells in the tissues were identified using anti-CD8 and anti-CD44 antibodies. PR8 (B) or HK68 (C) virus NP-specific CD8<sup>+</sup> T cells in the tissues were identified using H-2D<sup>b</sup>/ASNENMETM or H-2D<sup>b</sup>/ASNENMDAM tetramers, respectively. Data are from two independent experiments with at least four mice per group. One-way ANOVA with multiple comparisons was used to analyze the differences between treated and naive control groups. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$  compared to the naive control group.

**FIG 2.**

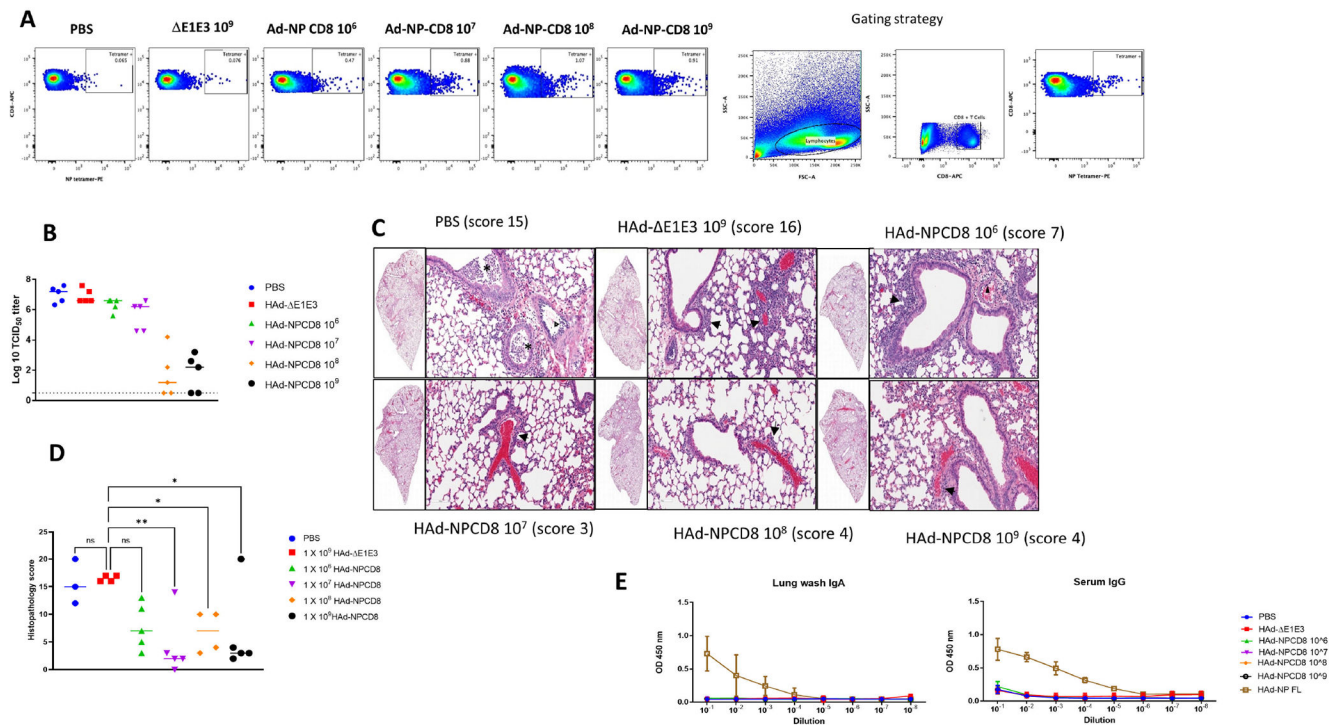
Sequence homology of NP across different influenza viruses. P2, P5, and P9 serve as primary anchors for D<sup>b</sup> allele-specific binding with a requirement for “S” at position 2, “N” at position 5, and “M,” “I,” “L,” or “V” at position 9. In addition, P3 can serve as a secondary anchor position. P4, P6, P7, and P8 serve as TCR contact residues.

**FIG 3.**

Increased NP-specific CD8<sup>+</sup> T cell frequency correlates with low morbidity, increased survival, and enhanced viral clearance on a heterosubtypic challenge. Six-week-old female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA) were anesthetized by inhalation of isoflurane and infected intranasally (I.N.) with 2, 10, and 20 MID<sub>50</sub> of PR8 virus in a final volume of 50  $\mu$ L. Control mice received PBS or immunized I.M. with a dose of 500 hemagglutinin units (HAU) in 50  $\mu$ L of formalin-inactivated PR8 virus in each quadriceps muscle. (A and B) Mice were challenged 4 weeks post-infection with 2 LD<sub>50</sub> of HK68 (H3N2) influenza virus and monitored for morbidity (A) and mortality (B). Data represented two independent experiments with at least four mice per group. (C) Five days post-heterosubtypic challenge, lungs were harvested and fixed in 10% neutral-buffered formalin. After 72 h, the samples were transferred to 70% ethanol, sectioned, and stained with hematoxylin and eosin. (D and E) Six-week-old female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA) were anesthetized by inhalation of isoflurane and infected I.N. with 2, 10, and 20 MID<sub>50</sub> of PR8 virus in a final volume of 50  $\mu$ L. Control mice received PBS or were immunized with a dose of 500 HAU in 50  $\mu$ L of formalin-inactivated PR8 virus by I.M. route in each quadriceps muscle. Four weeks post-infection, sera were collected and examined for HI titers to PR8 virus (D) or HK68 virus (E). Data are from 22 mice from two independent experiments for infected or vaccinated groups and 10 from three independent experiments for the naive group. (F) Four weeks post-infection, mice were challenged with 2 LD<sub>50</sub> of HK68 (H3N2) influenza virus. Lung tissues were harvested, and single-cell suspensions were prepared. Activated CD8<sup>+</sup> T cells in the tissues were identified using anti-CD8 and anti-CD44 antibodies. PR8- or HK68-specific NP CD8<sup>+</sup> T cells in the tissues were identified using H-2D<sup>b</sup>/ASNENMETM or H-2D<sup>b</sup>/ASNENMDAM tetramers, respectively. Data represent two independent experiments with at least four mice per group. One-way ANOVA with multiple comparisons was used to analyze the differences

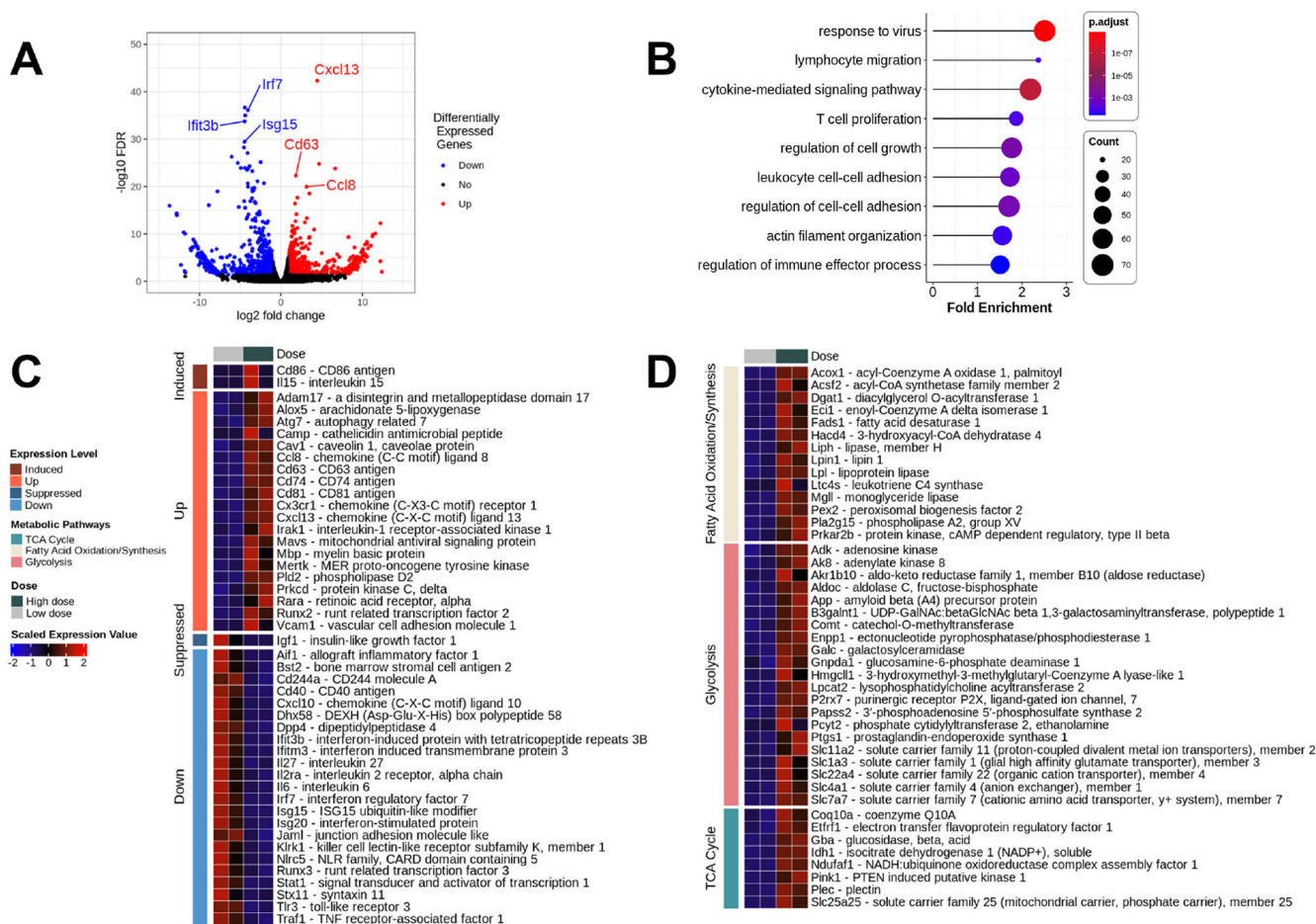
between treatment and naive control groups.  $*P < 0.05$  and  $****P < 0.0001$  compared to the naive control group. (G) Lungs were also collected to determine viral titers on day 3 post-HK68 challenge, and the study was repeated at least twice with a minimum of four mice per group.



**FIG 4.**

Increased frequency of NP-specific CD8<sup>+</sup> T cells is sufficient for improved survival after heterosubtypic challenge. Six-week-old female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA) were anesthetized and inoculated I.N. with  $1 \times 10^6$ ,  $1 \times 10^7$ ,  $1 \times 10^8$ , and  $1 \times 10^9$  PFU/mouse HAd-NPCD8 or  $1 \times 10^9$  PFU/mouse HAd-  $\Delta$ E1E3. Control mice were inoculated I.N. with PBS. (A) Four weeks post-inoculation, the lungs were harvested from mice, and single-cell suspensions were prepared. PR8 virus NP-specific CD8<sup>+</sup> T cells in the lung tissues were identified using H-2D<sup>b</sup>/ASNENMETM tetramer. (B) Four weeks post-inoculation, mice were challenged with 2 LD<sub>50</sub> of HK68 virus. Three days post-challenge, the lungs were collected to determine viral titers. Data are a representation of two independent experiments. (C) Animals were euthanized at 3-day post-challenge, and the lung lobe samples were collected and processed for histopathology. The lung tissue sections in low magnification (H&E, 10 $\times$ ) are shown as insets on the left side of each higher magnification photo (H&E, 200 $\times$ ). At low magnification, darker foci correspond to areas with higher cellularity of inflammatory cells. At higher magnification, the lung sections with histopathology score from 0 (no histologic change) to 16 exhibit variably severe lesions, including suppurative bronchitis (asterisks), endarteritis/vasculitis (white arrowheads), hypertrophic vascular endothelium (black arrowhead), and peribronchiolar and perivascular inflammation (arrows). (D) Lung histopathology scores of the mice groups after challenge with A/Hong Kong/1/68(H3N2) (HK68) compared to the HAd-  $\Delta$ E1E3-inoculated group. The mice inoculated with HAd-NPCD8 ( $10^7$  PFU/animal) and HAd-NPCD8 ( $10^9$  PFU/animal) show a statistically significant decrease in histopathology scores compared to the mice inoculated with HAd-  $\Delta$ E1E3. (E) Three weeks post-inoculation with HAd-NP FL vectors, lung washes and sera were collected to determine recombinant PR8 NP-specific IgA and IgG titers by ELISA. ns, non-significant at  $P > 0.05$ ; \*, significant at  $P < 0.05$ ; and \*\*, significant at  $P < 0.01$ .

significant at  $P < 0.01$ . Each experimental group consisted of at least five mice, and each experiment was repeated at least twice.

**FIG 5.**

Transcriptomic profile of NP-CD8<sup>+</sup> T cells induced by different infection doses. NP-CD8<sup>+</sup> T cells from 10 to 15 mice each immunized with 2 or 20 MID<sub>50</sub> PR8 viruses were sorted by FACS, and RNA content was sequenced with the Illumina platform. (A) Differentially expressed genes between the low- and high-dose groups are identified based on the adjusted *P*-value < 0.05 and |log2 fold change| > 1.0 from the DESeq2 analysis. The results are presented in a volcano plot. A total of 888 upregulated genes are shown in red and 679 downregulated genes are shown in blue. Essential genes among the top 10 based on adjusted *P*-value and fold change are labeled with gene symbols. (B) The 1,567 DEGs identified in the previous figure were analyzed for GO terms corresponding to the biological process. Among the 386 significantly enriched terms, top terms related to T cell functions were visualized in a bar plot. The length of the bars represents the count of genes associated with the GO term, and the color represents the adjusted *P*-value associated with the enrichment test. (C) The scaled expression values of key DEGs related to T cell functions between low- and high-dose groups are presented in a heatmap. Each column represents a sample in an experimental group, and each row represents a gene. Induced genes are genes with fold changes increased from zero under low-dose conditions to a positive value under high-dose conditions and vice versa for suppressed genes. (D) The scaled expression values of critical metabolic genes between the low- and high-dose groups are presented in a

heatmap. Columns represent samples from low- and high-dose groups, and rows represent genes grouped by vital metabolic pathways, such as the glycolytic pathway, tricarboxylic acid (TCA) cycle, and fatty acid oxidation and synthesis. Both heatmaps were derived from RNA-seq experiments by comparing the RNA levels between the cells from a high and low dose with two biological replicates at each stage.

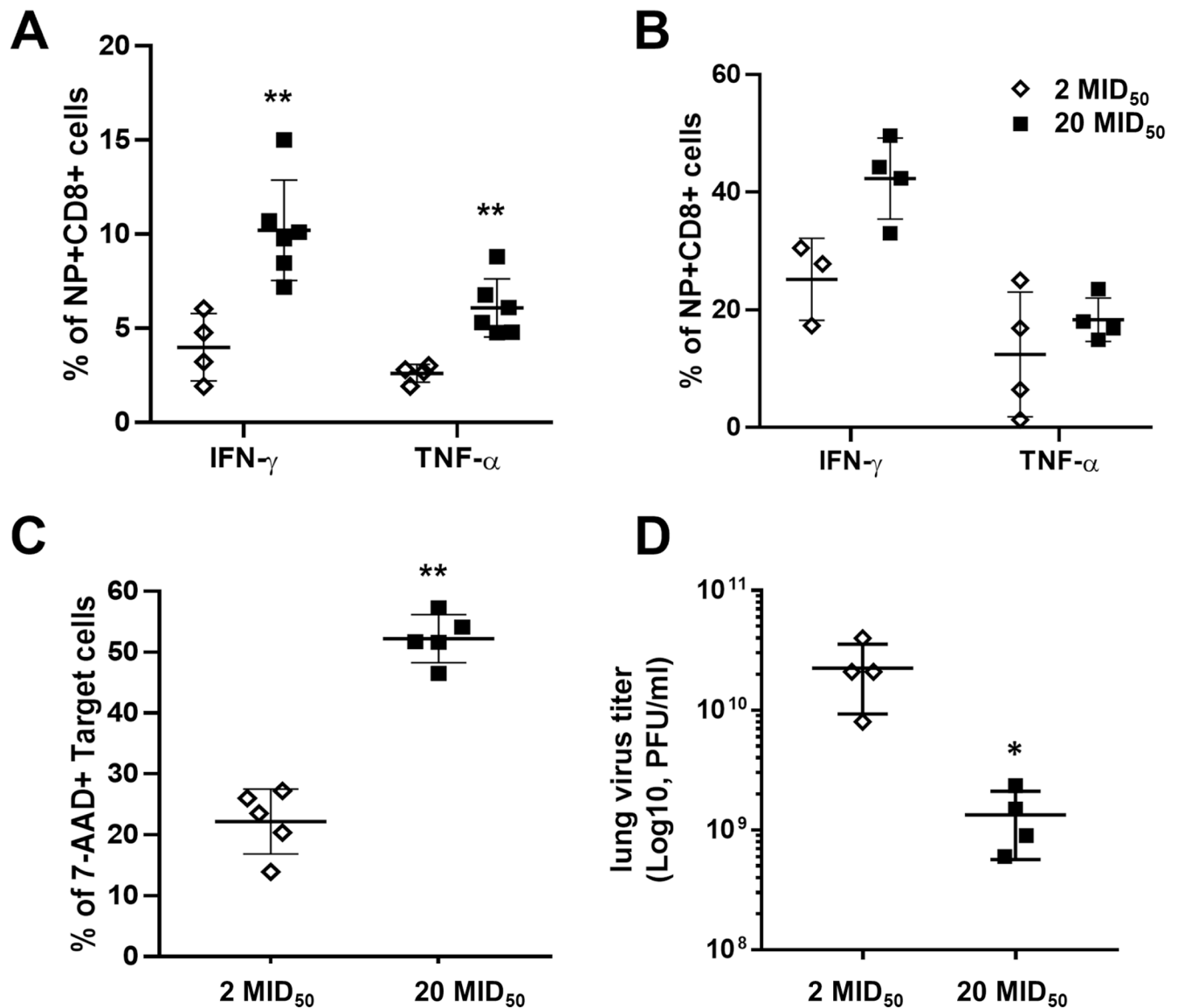


FIG 6.

Functional characterization of NP-CD8<sup>+</sup> T cells induced by different infection doses. (A and B) Single-cell suspension of the lungs (A) and spleen (B) from 10 to 15 mice each infected with 2 or 20 MID<sub>50</sub> PR8 virus for 3 weeks was *in vitro* stimulated with NP peptide (2  $\mu$ M) in the presence of GolgiPlug for 12 h. The percentages of IFN- $\gamma$ - or TNF- $\alpha$ -producing cells in the CD8<sup>+</sup> NP-tetramer<sup>+</sup> populations are presented. (C) Single-cell suspension of the spleens from mice infected with 2 or 20 MID<sub>50</sub> PR8 virus for 3 weeks was *in vitro* stimulated with NP peptide for another 3 days and used as effector cells. EL4 cells pulsed with 2  $\mu$ M of NP peptide and labeled with CFSE were used as target cells. Effector cells from the 2 MID<sub>50</sub> dose were co-cultured with target cells at a ratio of 50:1. Adjusted amount of effector cells from the 20 MID<sub>50</sub> dose was used for co-culture with target cells so that both groups have the same number of NP-CD8<sup>+</sup> T cells as effector cells. After 4 h of co-culture, 7-AAD was added to detect cell death. The percentages of 7-AAD<sup>+</sup> cells of total

EL4 cells are presented. (D) NP-CD8<sup>+</sup> T cells were sorted from 10 to 15 mice each mouse infected with 2 or 20 MID<sub>50</sub> PR8 virus for 2 weeks and boosted after 5 days.  $5 \times 10^5$  cells were adoptively transferred to live mice via tail vein injection, and recipient mice were challenged with 2 LD<sub>50</sub> HK68 virus 24 h later. Lung viral titer at day 5 post-challenge was determined by plaque assay. Data are a representation of two independent experiments. \* $P < 0.05$  and \*\* $P < 0.01$  compared to the 2 MID<sub>50</sub> group.