Cross-protective immunity against influenza pH1N1 2009 viruses induced by seasonal influenza A (H3N2) virus is mediated by virus-specific T-cells

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Influenza A (H1N1) viruses of swine origin were introduced into the human population in 2009 and caused a pandemic. The disease burden in the elderly was relatively low, which was attributed to the presence of cross-reacting serum antibodies in this age group, which were raised against seasonal influenza A (H1N1) viruses that circulated before 1957. It has also been described how infection with heterosubtypic influenza viruses can induce some degree of protection against infection by a novel strain of influenza virus. Here, we assess the extent of protective immunity against infection with the 2009 influenza A (H1N1) pandemic influenza virus that is afforded by infection with a seasonal influenza A (H3N2) virus in mice. Mice that experienced a primary A (H3N2) influenza virus infection displayed reduced weight loss after challenge infection and cleared the 2009 influenza A (H1N1) virus infection more rapidly. To elucidate the correlates of protection of this heterosubtypic immunity to pandemic H1N1 virus infection, adoptive transfer experiments were carried out by using selected post-infection lymphocyte populations. Virus-specific CD8⁺ T-cells in concert with CD4⁺ T-cells were responsible for the observed protection. These findings may not only provide an explanation for epidemiological differences in the incidence of severe pandemic H1N1 infections, they also indicate that the induction of cross-reactive virus-specific CD8⁺ and CD4⁺ T-cell responses may be a suitable approach for the development of universal influenza vaccines.

INTRODUCTION

Influenza A viruses (IAVs) are an important cause of respiratory-tract disease. Variants of seasonal IAV caused by antigenic drift are responsible for annual epidemics that are associated with excess morbidity and mortality. In addition, zoonotic transmission of antigenically unrelated IAV may spark a pandemic outbreak. In 2009, influenza A (H1N1) viruses of swine origin caused a worldwide outbreak of IAV that started in Mexico (pH1N1) (WHO, 2009). Furthermore, highly pathogenic avian IAVs of the H5N1 subtype are sporadically transmitted from infected poultry to humans and have caused more than 500 human cases, with a 60 % fatality rate, since 2003 (WHO, 2010). However, these viruses are inefficiently transmitted from human to human.

The viral haemagglutinin (HA) is a receptor binding protein, and mediates the first step in the virus replication cycle. Antibodies that can block binding of HA to its receptors can prevent infection of cells. Therefore, the presence of serum antibodies directed against HA correlates with protection against infection IAV, provided that these antibodies match the virus strain causing the infection (de Jong et al., 2003). During the 2009 influenza pandemic, the incidence of patients developing disease displayed a disproportionate age distribution. Elderly people were less likely to develop the disease than children and young adults (Hancock et al., 2009). This correlated with the presence of antibodies that cross-reacted with the H1N1 pandemic strain and that were originally induced after infection with influenza A (H1N1) viruses that were in circulation before 1957. These findings were also confirmed in animal models, demonstrating that vaccine- or infection-induced antibodies directed against historical H1N1 strains cross-neutralized the H1N1 2009 pandemic strain and protected against infection with this virus (Guo et al., 2011; Kash et al., 2010; Laurie et al., 2010; Manicasamy et al., 2010; Medina et al., 2010; Min et al., 2010; Skountzou et al., 2010).
The absence or presence of antibodies cross-reacting with the pH1N1 2009 virus does not fully explain the high incidence of disease caused by pH1N1 in children and does not explain why some children developed severe disease while others did not.

Children may not have been infected with seasonal IAV early in life, as was demonstrated recently in a seroepidemiological study (Bodewes et al., 2011), and therefore may not have developed heterosubtypic immunity that could protect against infection with an antigenically unrelated strain of IAV. The latter has been shown to afford various degrees of protection in animal models and humans, depending on the combination of subtypes of IAV used for priming and challenge infection, respectively (Benton et al., 2001; Grebe et al., 2008; Kreijtz et al., 2007, 2009; McMichael et al., 1983; Nguyen et al., 1999; O’Neill et al., 2000; Schulman & Kilbourne, 1965; Seo & Webster, 2001).

Since understanding of the nature of infection-induced heterosubtypic immunity may aid the development of more universally applicable vaccines, we wished to investigate to what extent infection with a seasonal IAV of the H3N2 subtype could afford protection against the H1N1 2009 pandemic strain. In addition, the correlates of protection were investigated by performing adoptive transfer experiments by using post-infection serum and post-infection B- and T-lymphocyte populations.

Here, we demonstrate that both virus-specific CD8+ and CD4+ T-cells are needed to afford protection against a heterologous challenge with pH1N1 virus in the mouse model. Therefore this study highlights the importance of designing new vaccines that are able to induce virus-specific CD8+ and CD4+ T-cells and afford protection against multiple IAV subtypes.

RESULTS

Infection of mice with seasonal H3N2 influenza virus (sH3N2) protects against challenge with pH1N1

Upon infection with pH1N1 virus, naïve control mice (group 2) lost body weight up until 7 days post-infection (p.i.) when the animals were taken out of the experiment (Fig. 1a). In contrast, priming by infection with sH3N2 (group 3) prevented the development of severe signs and limited the loss of body weight after infection with pH1N1 virus. This reduction of disease severity correlated with a reduction in virus replication (Fig. 1c). Especially at day 7 post-challenge infection the lung titres were significantly reduced (10 000-fold, \( P < 0.05 \)), compared with unprimed control mice (group 4) [\( 10^{2.8} \text{TCID}_{50} \) (g of tissue)\(^{-1}\) and \( 10^{6.8} \text{TCID}_{50} \) (g of tissue)\(^{-1}\), respectively].

When the order of infection with sH3N2 and pH1N1 virus was reversed, similar results were obtained. Thus, priming by infection with pH1N1 virus (group 5) prevented loss of body weight, which would otherwise have been caused by infection with sH3N2 virus, as in the unprimed control mice (group 4). Again, the observed protection correlated with reduced clinical signs and faster viral clearance in the lungs (Fig. 1).

Primary infection does not induce cross-neutralizing antibodies

After infection with sH3N2 virus, mice of group 3 developed virus-specific antibodies that did not cross-react with pH1N1 in the haemagglutination inhibition (HI) assay and the virus neutralization (VN) assay. Twenty-eight days after priming, the geometric mean titres (GMT) to the

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**Fig. 1.** sH3N2 virus infection induces protective immunity against infection with pH1N1. Mice were primed by inoculation with sH3N2 (\( \Delta \)) or pH1N1 (\( \square \)), or were mock infected by instilling PBS (\( \bigcirc \)). Twenty-eight days later, mice were challenged with pH1N1 (\( \triangle \), \( \triangle \)) or sH3N2 (\( \square \), \( \square \)), or received PBS (\( \bigcirc \)). Subsequently their body weight was determined daily and expressed as the percentage of the original body weight at day 0, before challenge infection (a). Six mice were used per experimental group. Lung virus titres were determined on day 4 p.i. (b) and on day 7 p.i. (c). *, \( P < 0.005 \) (Mann–Whitney test). The dotted line indicates the cut-off value.
homologous virus were 220 and 413, as measured in the HI and VN assays, respectively (Fig. 2a–d). Mice of group 5 developed a homologous antibody response to influenza virus pH1N1 with HI and VN GMT of 453 and 441, respectively. Also, these antibodies did not cross-react or cross-neutralize the heterologous virus sH3N2 (Fig. 2a–d). Upon challenge infection with pH1N1, both primed mice of group 3 and unprimed mice of group 2 developed pH1N1-specific antibodies (Fig. 2a, b). Upon challenge infection of mice of groups 4 and 5 with sH3N2, modest antibody-titre responses were observed against this strain at 7 days p.i. (Fig. 2c, d).

**Induction of virus-specific CD8⁺ T-cells after challenge infection**

Seven days after challenge infection, virus-specific CD8⁺ T-cell responses in the spleens were examined. Splenocytes were stimulated with peptides representing the PA subunit of the polymerase (PA)224–233 and nucleoprotein (NP)366–374 epitope variants of IAVs sH3N2 and pH1N1, and the induction of IFN-γ production was assessed. The mice of group 3 that were primed by infection with IAV sH3N2 developed strong CD8⁺ T-cell responses to both PA epitope variants (Fig. 2e). Also a CD8⁺ T-cell response to the pH1N1 NP epitope was observed, but not to the sH3N2 epitope.

Also, in mice of group 5 that were infected with both viruses in reversed order, CD8⁺ T-cell responses to both PA epitopes were observed. These mice also developed a response to the sH3N2 variant of the NP epitope.

Seven days after primary infection with IAV pH1N1, a response to the PA epitope was observed, while virus-specific CD8⁺ T-cells were virtually undetectable after primary sH3N2 infection. In control mice, low background levels of IFN-γ⁺ CD8⁺ T-cells were observed.

**Virus-specific T-cells afford clinical protection**

To determine which lymphocyte population provided protection against pH1N1 virus infection, adoptive transfer experiments were carried out (Fig. 3).

In a first experiment, naïve recipient mice received 200 μl of serum, 2.8 × 10⁶ B-cells or 2.6 × 10⁶ T-cells from

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**Fig. 2.** Antibody and T-cell responses after challenge infection. Mice were primed by infection with sH3N2 (Δ) or pH1N1 (□), or were mock infected by instilling PBS (△, □). Twenty-eight days later, mice were challenged with pH1N1 (△, □) or sH3N2 (Δ, □). Serum antibodies against pH1N1 (a, b) and sH3N2 (c, d) viruses were determined before and 7 days after challenge infection in the VN assay (a, c) and HI assay (b, d). The dotted line indicates the cut-off value. Six mice were used per experimental group. Virus-specific T-cell responses in the spleen on day 7 p.i. were assessed by determining the frequency of CD8⁺ interferon-gamma secreting (IFN-γ⁺) T-cells after stimulation of the splenocytes with peptides representing the NP366–374 or the PA224–233 epitopes derived from pH1N1 or sH3N2, as indicated (e). *, P<0.005 (Mann–Whitney test).
Virus-specific CD8 T-cells confer protection against pH1N1

To further delineate the subpopulations of T-cells responsible for protection against pH1N1 infection, we performed additional experiments by using purified CD8+ and CD4+ T-cell populations in addition to purified non-separated T-cells obtained from sH3N2-infected donor mice. A larger number of cells was used for adoptive transfer to increase the effect that was observed in the previous experiment. Groups of six recipient mice received 6.7 × 10^6 T-cells, 4.1 × 10^6 CD8+ T-cells or 6 × 10^6 CD4+ T-cells that had been obtained from mock or sH3N2-infected donor mice.

As shown in Fig. 5(a), none of the T-cell populations obtained from mock-infected donor mice prevented the loss of body weight caused by pH1N1 virus infection. Also the transfer of post-infection CD4+ T-cells did not prevent the loss of body weight, whereas the transfer of CD8+ T-cells afforded protection to a certain extent. In particular, the transfer of un-separated post-infection T-cells afforded robust protection against infection with IAV pH1N1. The observed clinical protection correlated with a 6 log reduction of the lung virus titre measured at 7 days p.i. with IAV pH1N1, which was comparable to that of mice that had been infected with sH3N2 virus (Fig. 5b). Thus, virus-specific T-cells induced by infection with IAV sH3N2 can afford robust protection against challenge infection with pH1N1.

The reduction of body-weight loss and virus replication in the lungs correlated with the absence of virus-infected cells in lung tissue, as demonstrated by immunohistochemistry (Fig. 6). In the lungs of mice that received un-separated T-cells from sH3N2-infected mice and in the lungs of mice that were infected with sH3N2 prior to challenge, virus-infected cells were undetectable 7 days p.i. with pH1N1. In all other groups, virus-infected cells were detected in the bronchiolar epithelium. In these mice, approximately 50% of the lung surface showed pulmonary consolidation macroscopically. Microscopically, the presence of infected cells correlated with the development of histopathological changes which were diagnosed as multifocal moderate broncho-interstitial pneumonia (Supplementary Fig. S1, available in JGV Online).

This lesion was characterized by histopathological changes in alveoli, bronchioles and pulmonary blood vessels. Alveolar lumina were filled with many neutrophils and macrophages mixed with abundant T-cell debris, some red blood cells and oedema fluid. The alveolar walls were thickened owing to the presence of neutrophils and mononuclear cells. There were a moderate number of neutrophils and mononuclear cells in the bronchiolar walls, and moderate numbers of mononuclear cells around pulmonary blood vessels.

The transfer of post-infection T-cells prevented the development of these histopathological changes (Supplementary Fig. S1). Also, macroscopically, no lesion was observed in this group of mice. As expected, a prior infection with sH3N2 virus also prevented the development of severe histopathological and macroscopical changes to a great extent.

Fig. 3. Experimental design of adoptive transfer experiments. Serum, purified T-cells or B-cells from mice inoculated with IAV sH3N2 or mock-infected mice were transferred to naïve recipient mice via the intraperitoneal route. Four hours later, six recipient mice were inoculated with pH1N1 (a). In a second experiment, purified T-cells, CD8+ T-cells or CD4+ T-cells from sH3N2-infected or mock-infected mice were transferred to six naïve recipient mice that were subsequently inoculated with pH1N1 virus (b).
However, in these mice, infiltrates of lymphocytes and neutrophils were observed around bronchioles and blood vessels. These mice developed perivascular moderate proliferation of inducible bronchus associated lymphoid tissue (iBALT). These infiltrates were not observed in mice that received T-cells obtained from sH3N2-infected donor mice. Mice that received post-infection CD8$^{+}$ T-cells only had reduced macroscopical lesions compared with naïve control mice (10 versus 50%). Compared with control mice, more neutrophils relative to mononuclear cells were observed, thus indicating that the development of lesions was delayed in these mice. Lymphocytes and mononuclear cells were present perivascularly.

**Virus-specific T-cell response 7 days post-challenge infection with pH1N1**

Splenocytes and lung cells were tested for the presence of CD8$^{+}$ T-cells specific for the PA epitope variants of sH3N2 and pH1N1 viruses. As shown in Fig. 5(c, d), positive-control mice that were primed by sH3N2 virus infection developed strong PA224-233$^{-}$ T-cell responses upon challenge infection with pH1N1. However, the PA-specific CD8$^{+}$ T-cell response in lungs and spleen of the recipient mice, including those that received un-separated T-cells or purified CD8$^{+}$ T-cells of primed mice, was not significantly different from that of naïve control mice.

**DISCUSSION**

In the present study we have shown that primary infection with a seasonal IAV of the H3N2 subtype induced protective immunity against pandemic IAV pH1N1 in mice. This heterosubtypic immunity was mediated by virus-specific T-cells, as demonstrated in adoptive transfer experiments. Both CD4$^{+}$ and CD8$^{+}$ T-cells were required to induce protection against challenge infection.
Fig. 5. The effect of adoptive transfer of post-infection T-cells, or CD4⁺ or CD8⁺ T-cell subpopulations on the outcome of pH1N1 virus infection. Donor mice were inoculated with sH3N2 virus or not and 28 days later purified T-cells and purified CD8⁺ or CD4⁺ T-cells were collected for adoptive transfer experiments. Naïve recipient mice received T-cells from mock-infected animals (○) or sH3N2-infected animals (●), CD8⁺ T-cells from mock-infected animals (□) or infected animals (◼), or CD4⁺ T-cells from mock-infected animals (○) or infected animals (●) and their body weight was determined after challenge infection with pH1N1 virus (a). Mock-infected animals were included as a negative control (△) and mice that were primed by infection with sH3N2 virus were included as a positive control group (▴). Seven days post-challenge infection with pH1N1 virus, the lung virus titres were determined (b). The dotted line indicates the cut-off value. The horizontal bars represent the mean virus titre of the respective groups. Virus-specific T-cell responses on day 7 p.i. were calculated in the spleen (c) and in the lungs (d) after stimulation of the cells by NP_366–374 or PA_224–233 derived from pH1N1 or sH3N2 and determination of the frequency of CD8⁺ T-cells secreting IFN-γ. *, P<0.005 (Mann–Whitney test).
Prior infection with sH3N2 virus induced protection against pH1N1 challenge. The observed protection correlated with a CD8+ T-cell response specific for the PA224–233 epitope but not the NP366–374 epitope. This discrepancy might be explained by the antigenic similarity of the sH3N2 and pH1N1 variants of the PA224–233 epitope (one amino acid substitution), while NP366–374 displays four amino acid differences in the amino acid sequence and
therefore the two variants lack cross-reactivity. It has been shown that in the absence of a response to the dominant NP-366–374 epitope caused by mismatch of the amino acid sequence, the PA224–233 epitope gains immunodominance (Chen et al., 2004). Whether or not PA224–233-specific CD8+ T-cells are protective is still a matter of debate (Crowe et al., 1995; Song et al., 2011; Skountzou et al., 2011). To demonstrate that virus-specific T-cell responses not only correlated with protection but were also directly responsible for heterosubtypic immunity, we performed adoptive transfer experiments. Although protective antibodies have been described that are specific for conserved epitopes in the stem region of the HA molecule (Corti et al., 2010; Ekiert et al., 2009; Steel et al., 2010; Wang et al., 2010), the matrix-2 protein (Fan et al., 2004; Heinen et al., 2002; Slepushkin et al., 1995; Song et al., 2011; Tompkins et al., 2007) and the nucleoprotein (Carthaguer et al., 2008; LaMere et al., 2011a, b; Rangel-Moreno et al., 2008), the transfer of serum obtained from mice infected with sH3N2 virus failed to protect against infection with heterologous pH1N1 virus. Either antibodies to these conserved epitopes were not induced or the titres were too low to be protective. In contrast, transfer of post-infection serum afforded protection against the homologous sH3N2 strain, confirming that HA-specific antibodies can neutralize virus efficiently provided that they match the strain causing the infection. Also, the transfer of post-infection B-cells did not protect recipient mice from infection with heterologous pH1N1 virus. In contrast, transfer of post-infection T-cells protected recipient mice from severe weight loss caused by pH1N1 infection. When the number of transferred cells was large enough, the pH1N1 virus infection was cleared by day 7 p.i. with pH1N1, and weight loss was prevented completely. However, no sterile immunity was achieved, since low virus titres were observed 7 days p.i. and all mice sero-converted (data not shown). To further delineate the subpopulations of T-cells responsible for the protective effect, post-infection CD4+ and CD8+ T-cells were transferred to naïve recipient mice. The transfer of post-infection CD4+ T-cells alone was insufficient to afford protection against pH1N1 infection. However, the adoptive transfer of post-infection CD8+ T-cells prevented weight loss caused by pH1N1 infection to a certain extent. It also reduced the pathological changes in the lungs compared with mice that had received CD8+ T-cells obtained from mock-infected control mice. Based on the higher proportion of neutrophils than mononuclear cells in the pulmonary lesions, we hypothesize that the kinetic of the pH1N1 challenge infection was delayed in the mice receiving virus-specific CD8+ T-cells, resulting in histological lesions resembling those observed at more recent time points in unprimed control mice (Wareing et al., 2007). From these results we conclude that virus-specific CD8+ T-cells are indispensable for heterosubtypic immunity, but the presence of virus-specific CD4+ T-cells largely increases its efficiency in clearing IAV infection. Thus CD8+ T-cells induced after infection need memory CD4+ T-cells for their activation and to target virus-infected cells. The relationship between CD4+ and CD8+ T-cells has been studied extensively, and it seems that memory CD8+ T-cells are impaired in the absence of memory CD4+ T-cells, leading to increased cell death and a decreased secondary T-cell response (Cui & Kaech, 2010).

Our findings coincide well with those obtained by others (Guo et al., 2011) who showed that depletion of both CD4+ T-cells and CD8+ T-cells from mice primed by infection with IAV X31 abrogated the heterosubtypic immunity to pH1N1 virus strain A/California/4/09, although in this study CD4+ T-cells were as protective as CD8+ T-cells. In the late 1970s it had already been demonstrated that CD8+ T-cells contribute to protective immunity (Yap et al., 1978). Furthermore, CD8+ T-cells also afforded heterosubtypic protection in chickens (Seo & Webster, 2001).

Surprisingly, adoptive transfer of post-infection T-cells more efficiently prevented weight loss and histopathological changes in the lungs after pH1N1 challenge infection than primary infection with sH3N2. We hypothesize that mice primed by infection with sH3N2 developed stronger local and systemic immune responses upon challenge infection with pH1N1, which may have caused more weight loss. In both groups the infection was cleared by day 7 p.i.

Collectively, the present study demonstrates that the induction of T-cells specific for sH3N2 IAVs affords strong protection against infection with antigenically unrelated 2009 pandemic H1N1 viruses. Since human T-cells specific for seasonal IAVs also display strong cross-reactivity with IAVs of other subtypes (Jameson et al., 1999; Kreijtz et al., 2008; Lee et al., 2008) and pH1N1 2009 viruses (Richards et al., 2010; Tu et al., 2010), it can be hypothesized that individuals with a history of IAV infection were protected from pH1N1 virus infections to a certain extent. This would imply that young children that are supposedly immunologically naïve for IAV would be more at risk for developing severe disease. This would, at least partially, explain the disproportionate age distribution of pH1N1 IAV infections in 2009 and the high disease incidence amongst infants. Indeed, a large proportion of children <2 years of age have not experienced IAV, as demonstrated in a recent sero-epidemiological study (Bodewes et al., 2011). However, it remains unknown as to why adolescents who most probably experienced one or more
IAV infection developed disease [Health Protection Agency, 2009; Nishiura et al., 2009; Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, 2009]. Of course, the low incidence of severe pH1N1 virus infections among the elderly can be attributed to the presence of cross-protective antibodies induced by infection with IAV related to the 2009 pH1N1 virus and that circulated before 1950 (Hancock et al., 2009).

Our findings also imply that targeting the induction of the CD4+ and CD8+ T-cell response specific for conserved proteins of IAV may be a viable approach for the development of more universal influenza vaccines that could afford protection against ‘mismatching’ seasonal IAVs and newly introduced pandemic IAVs. Since children are immunologically naïve to influenza, this vulnerable age group may benefit especially from these novel vaccines.

Methods

Viruses. Influenza viruses A/HongKong/2/68 (sH3N2) and A/Netherlands/602/09 (pH1N1) were propagated in Madin–Darby canine kidney (MDCK) cells as described previously (Rimmelzwaan et al., 1998). The culture supernatants of the infected MDCK cells were clarified by low-speed centrifugation (10 min at 300 g), aliquoted and stored at –80 °C until use. Infectious-virus titres were determined as described previously (Rimmelzwaan et al., 1998).

Mice. Female, 6–8-week-old, specific-pathogen-free C57BL/6 J (H-2b) mice were purchased from Charles River Laboratories. The animals were housed in individual ventilated cages and had access to food and water ad libitum. The experimental protocol was approved by an independent animal ethics committee.

Forty-three mice were divided into five groups. Groups 1, 2 and 4 were mock infected intranasally with PBS (pH 7.4) under anaesthesia with 2% isoflurane in oxygen. The mice of group 3 were inoculated intranasally with 10⁴ TCID₅₀ of pH1N1 (for the dose, see Bodewes et al., 2010). Mice of group 5 were inoculated intranasally with 10⁵ TCID₅₀ of pH1N1. Seven days after challenge infection, the mice were euthanized and their lungs and spleen were collected. In addition, mock-infected mice were included as negative and positive controls in the pH1N1 challenge procedure. They were primed with 5 × 10⁵ TCID₅₀ of sH3N2 or received PBS and were challenged 4 weeks later with 10⁶ TCID₅₀ of pH1N1 virus. They were euthanized 7 days after challenge. Six mice were used per experimental group.

Assessment of the CD8+ T-cell response. Suspensions containing single cells of lungs and spleens were prepared 7 days post-challenge infection as previously described (Bodewes et al., 2010). CD8+ T-cell responses were measured by incubating splenocytes or lung cells for 6 h at 37 °C in the presence of 5 μM of either NP₁₆₆–₁₇₄ (NP₁₆₆–₁₇₄), ASNENMDAM or PA₂₂₄–₂₃₃ (PA₂₂₄–₂₃₃) (Eurogentec) peptides derived from influenza virus sH3N2 or NP₁₆₆–₁₇₄ (NP₁₆₆–₁₇₄), ASNENVEIM or PA₂₂₄–₂₃₃ (PA₂₂₄–₂₃₃) (SpleenFLRA) peptides (Eurogentec) derived from influenza virus pH1N1, in Iscove’s modified Dulbecco’s medium with 5% FCS and GolgiStop (BD PharMingen). These peptides represent two immunodominant epitopes of IAV in C57BL/6 J mice (H-2b) (Belz et al., 2000; Doherty et al., 1978). After incubation, cells were stored overnight at 4 °C. The cells were then stained for CD3, CD8, IFN-γ and whether they were alive or dead and were analysed by flow-cytometry as described previously (Bodewes et al., 2009). Peptide-specific responses were calculated by subtracting the percentage of CD8+ IFN-γ+ T-cells after incubation with medium and GolgiStop only from the percentage of CD8+ IFN-γ+ T-cells after peptide stimulation.

Lung virus titres. Lungs of mice collected 4 or 7 days after inoculation were snap frozen and stored at –80 °C. Lungs were homogenized and quadruplicate tenfold serial dilutions of these samples were used to inoculate MDCK cells as described previously (Bodewes et al., 2009). Seven days later, the HA activity of the culture supernatants was used to determine the virus titre according to the Spearman–Kärber method (Kärber, 1931).

Serology. Twenty-eight days after priming and 7 days after challenge infection, serum samples were collected. They were stored at –20 °C until further use. After treatment with cholera filtrate and heat inactivation, they were tested for the presence of HA-specific antibodies against IAV A/HongKong/2/68 and A/Netherlands/602/09 by using an HI assay as described previously (Palmer et al., 1975). Virus-neutralizing antibodies were also assessed after heat inactivation of the sera by using a VN assay as described previously (Frank et al., 1980).

Histopathology and immunohistochemistry. After euthanasia, one half of the lungs was inflated with 10% neutral buffered formalin.
After fixation and embedding in paraffin, lungs were cut into 3 µm sections and stained with haematoxylin and eosin, and the tissue sections were examined microscopically. Using an immunoperoxidase method, sequential slides were also stained with a mAb directed against the nucleoprotein of IAV, as described previously, to visualize IAV antigen expression (Rimmelzwaan et al., 2001).

Statistical analysis. The data were analysed using the Mann–Whitney test. Two groups were considered statistically different when P<0.05.

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