Vaccination with whole inactivated virus vaccine affects the induction of heterosubtypic immunity against influenza virus A/H5N1 and immunodominance of virus-specific CD8\(^+\) T-cell responses in mice


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It was recently shown that the use of an experimental subunit vaccine protected mice against infection with a human A/H3N2 influenza virus, but consequently affected the induction of heterosubtypic immunity to a highly pathogenic A/H5N1 influenza virus, which was otherwise induced by the A/H3N2 infection. As whole inactivated virus (WIV) vaccines are widely used to protect against seasonal influenza and also contain inner viral proteins such as the nucleoprotein (NP), the potential of a WIV vaccine to induce protective immunity against infection was tested with a homologous A/H3N2 (A/Hong Kong/2/68) and a heterosubtypic A/H5N1 influenza virus (A/Indonesia/5/05). As expected, the vaccine afforded protection against infection with the A/H3N2 virus only. In addition, it was demonstrated that the use of WIV vaccine for protection against A/H3N2 infection affected the induction of heterosubtypic immunity that was otherwise afforded by A/H3N2 influenza virus infection. The reduction in protective immunity correlated with changes in the immunodominance patterns of the CD8\(^+\) T-cell responses directed to the epitopes located in the acid polymerase subunit of the viral RNA polymerase (PA 224–233) and the NP (NP 366–374). In unvaccinated mice that experienced infection with the A/H3N2 influenza virus, the magnitude of the CD8\(^+\) T-cell response to both peptides was similar on secondary infection with A/H5N1 influenza virus. In contrast, prior vaccination with WIV affected the immunodominance pattern and skewed the response after infection with influenza virus A/Indonesia/5/05 towards a dominant NP 366–374-specific response. These findings may have implications for vaccination strategies aimed at the induction of protective immunity to seasonal and/or pandemic influenza.

INTRODUCTION

Influenza viruses are a major cause of respiratory tract infections and are responsible for annual excess morbidity and mortality mainly in the elderly, in infants and in patients with underlying disease (World Health Organization, 2009). In addition to annual epidemics, influenza A viruses occasionally cause pandemics. New influenza A/H1N1 viruses of swine origin caused the recent pandemic that started in Mexico in the spring of 2009 (Centers for Disease Control and Prevention, 2009; Chan, 2009). Because influenza A viruses of various subtypes can be transmitted to humans from animal reservoirs and potentially could cause novel pandemic outbreaks, there is interest in immunity that could protect against multiple subtypes of influenza A viruses, so-called heterosubtypic immunity, as a basis for the development of universal vaccines (Cassetti et al., 2005; Kaiser, 2006).

This type of immunity induced by infection with influenza A virus was recognized in the 1960s and has been demonstrated in various animal models (Epstein et al., 1997; Liang et al., 1994; O’Neill et al., 2000; Schulman & Kilbourne, 1965; Yetter et al., 1980). Heterosubtypic immunity induced by infection has been shown to be long lasting (Yetter et al., 1980). It does not prevent infection, as virus-neutralizing antibodies against the viral glycoproteins – the haemagglutinin (HA) and neuraminidase (NA) – by definition will not cross-react with those of other subtypes. However, heterosubtypic immunity does afford a certain degree of protection and reduces morbidity and mortality otherwise caused by infection with an influenza virus of an
alternative subtype (reviewed by Grebe et al., 2008). Evidence for the existence of heterosubtypic immunity in humans is circumstantial, but probably contributes to protection against infection with (pandemic) influenza viruses of novel subtypes (Epstein, 2006; McMichael et al., 1983; Sonoguchi et al., 1985). Heterosubtypic immunity may be relevant in particular for protection against infection with highly pathogenic avian influenza viruses such as those of the H5N1 subtype, which have infected almost 500 people since 1997 with a case fatality rate of 59% (World Health Organization, 2010).

Elucidation of the mechanism of heterosubtypic immunity has been the subject of numerous studies. It has been demonstrated in mice that multiple arms of the immune system contribute to heterosubtypic immunity, including virus-specific CD4 and CD8 T cells, local virus-specific antibodies and B cells (Benton et al., 2001; Grebe et al., 2008; Neirynck et al., 1999; Nguyen et al., 1999, 2007; Slepushkin et al., 1995; Tumpey et al., 2001).

CD8 T lymphocytes (CTLs) in particular are thought to contribute to heterosubtypic immunity, because they predominantly recognize cross-reactive epitopes located in conserved proteins such as the nucleoprotein (NP) and matrix (M) protein. The cross-reactive nature of influenza virus-specific CTLs has been confirmed with T-cell populations obtained from mice (Taylor & Askonas, 1986; Wells et al., 1981; Yap et al., 1978) and humans (Greenbaum et al., 2009; Jameson et al., 1999; Kreijtz et al., 2008; Lee et al., 2008; Powell et al., 2007). Furthermore, the presence of CTL immunity correlates with protection against infection and inversely correlates with the extent and duration of virus replication in mice and humans (Flynn et al., 1998; Kreijtz et al., 2007; McMichael et al., 1983).

As infection with seasonal influenza A viruses induces strong virus-specific CTL responses, it can be hypothesized that the use of vaccines that induce protective antibody responses to seasonal influenza viruses subsequently prevents the induction of virus-specific CTL responses. This could affect the induction of heterosubtypic immunity against infection with viruses of novel subtypes. We recently tested this hypothesis in a mouse model using an alum-adjuvanted subunit preparation to protect against infection with an A/H5N1 influenza virus. We found that H3N2-vaccinated mice did not develop heterosubtypic immunity and were no longer protected against a lethal challenge with an A/H5N1 influenza virus (Bodewes et al., 2009b).

In the present study, we used a whole inactivated influenza A virus (WIV) vaccine based on an A/H3N2 virus comparable to vaccine preparations used for the vaccination of human subjects to mimic the situation in humans more closely. This vaccine preparation also contained internal viral proteins such as NP and M1 protein in addition to HA and NA. Upon vaccination, this WIV vaccine preparation induced protective immunity to a human seasonal influenza A/H3N2 virus and virus-specific CD8+ T-cell responses against the conserved proteins, but failed to induce protection against infection with influenza A/H5N1 virus. Furthermore, vaccination with the WIV preparation affected the induction of heterosubtypic immunity induced by experimental infection with a human influenza A/H3N2 virus, similar to the use of adjuvanted subunit vaccine. In addition, use of the WIV vaccine affected the immunodominance pattern of the CD8+ T-cell response to epitopes of the NP (NP366-374) and the acid polymerase (PA) subunit of the viral RNA polymerase (PA224-233).

## RESULTS

### Antibody and CD8+ T-cell responses induced by vaccination with WIV

At 28 days after administration of WIV X-31 vaccine, mice of groups 2 and 5 (vaccinated with WIV and infected with A/HK/2/68 and/or A/IND/5/05, respectively; see Table 1) developed HA-specific antibodies against influenza virus A/Hong Kong/2/68 (A/HK/2/68; H3N2) as detected in a haemagglutination inhibition assay [geometric mean titre (GMT) of 234] and virus neutralization assay (GMT of 19) (Fig. 1a, b). At 28 days after the second vaccination, the GMTs increased to 915 and 144, respectively (Fig. 1a, b). Influenza virus-specific antibody responses were detected in none of the mock-vaccinated mice. The induction of virus-specific CTL responses by WIV vaccination was assessed by determining the frequency of CD8+ T lymphocytes in the spleens specific for the immunodominant NP366-374 epitope from influenza A virus X-31 (ASNENMETM) by tetramer staining using TmX-31, a tetramer specific for this epitope. Eight days after the first vaccination, the mean percentage of CD8+ TmX-31+ T lymphocytes was 4.7 ± 2.2 (mean ± SD), which was significantly higher than in mock-vaccinated mice (P = 0.02) (Fig. 1c). Subsequently, the frequency of CD8+ TmX-31+ T lymphocytes declined but remained higher than in mock-vaccinated mice (0.25 and 0.05%, respectively; P = 0.04).

### Table 1. Overview of experimental groups of mice used in this experiment

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<th>Experimental group</th>
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The booster vaccination did not increase the frequency of NP_{366–374}-specific CD8^{+} T cells. At 28 and 56 days after the second vaccination, the percentages of CD8^{+} T_{mX-31} cells were no longer different from those determined in mock-vaccinated mice (P=0.13 and P=1.00, respectively). The frequencies of CD8^{+} T_{mX,31}^{+} T lymphocytes in the spleens of control mice infected with influenza virus X-31 were 8.9±3.0 and 1.0±0.7 at 8 and 28 days post-infection (p.i.), respectively. The proportion of cells that expressed CD62L or CD127 did not differ significantly between vaccinated and infected mice (data not shown).

**Outcome of infection with influenza virus A/HK/2/68 (H3N2)**

Upon infection with influenza virus A/HK/2/68, mock-vaccinated mice developed mild to moderate clinical signs and lost body weight from days 3 to 7 p.i. (Fig. 2a). From day 7 onwards, these mice recovered from infection. In contrast, mice vaccinated with the WIV X-31 vaccine (group 2) did not develop any clinical signs following infection and did not display loss of body weight, comparable to mock-infected mice (groups 1, 4 and 5). The development of clinical signs correlated with virus titres in the lungs at 4 days p.i. In the lungs of mock-vaccinated mice, the virus replicated to mean titres of 10^{8.6} 50% tissue culture infective doses (TCID_{50}) per gram of tissue (Fig. 2b). In contrast, infectious virus could not be detected in the lungs of mice that were vaccinated with the WIV X-31 vaccine preparation and in lungs of mock-infected control mice.

**Fig. 1.** Antibody and CD8^{+} T-cell responses after vaccination of mice with WIV X-31 (H3N2). (a, b) Virus-specific serum antibody levels were measured by a haemagglutination inhibition (HI) assay (a) and a virus neutralization (VN) assay (b) at 28 days after the first (dp 1st) and second (dp 2nd) vaccinations. (c) Splenic CD8^{+} T-cell responses directed against the NP_{366–374} epitope of influenza A X-31 (ASNENMETM). Open bars represent mean results ± SD for mock-vaccinated mice (groups 1, 3 and 4) and shaded bars represent mean results ± SD for WIV X-31-vaccinated mice (groups 2 and 5). *, P<0.05 compared with mice of group 1. ND, Not detectable.

**Fig. 2.** Outcome of infection with influenza virus A/HK/2/68 (H3N2). (a) At 28 days after the second vaccination, mice were mock infected (groups 1, 4 and 5; open circles) or infected with influenza A/H3N2 virus (group 2, shaded circles; group 3, filled circles). The mice were weighed daily and the mean weight loss ± SEM was calculated. (b) At 4 days p.i., lung virus titres were determined for mice of groups 1, 2 and 3. Bars represent mean virus titres ± SD of four mice per group. The dashed line indicates the cut-off value for obtaining a positive result.
**CD8⁺ T-cell responses after infection with influenza A/H3N2**

**Tetramer staining.** At 12 and 28 days after infection with influenza virus A/HK/2/68 (H3N2), the frequency of CD8⁺ T cells specific for the NP366–374 epitope present in influenza virus A/HK/2/68 (H3N2) (ASNENMDAM) was assessed in the spleen and lungs by staining using a tetramer specific for this epitope (TmH3N2). At 12 days p.i., the mean percentage of CD8⁺ TmH3N2⁺ T cells in the spleens and lungs of mock-vaccinated, H3N2-infected mice of group 3 were 0.83±0.36 (mean±SD) and 4.00±1.00, respectively. In contrast, the mean frequency of CD8⁺ TmH3N2⁺ T lymphocytes was significantly lower in the WIV-vaccinated mice [0.22±0.10 and 0.93±0.54% in spleens (P=0.004) and lungs (P=0.02), respectively] and comparable to the frequencies found in naïve control mice (Fig. 3a, b).

At 28 days p.i., the percentage of CD8⁺ TmH3N2⁺ T cells in the spleens and lungs had declined somewhat (Fig. 3c, d). The percentage in the spleens and lungs was still lower in vaccinated, H3N2-infected mice compared with unvaccinated, H3N2-infected mice. This difference was statistically significant for the spleens (P=0.01) and approached statistical significance for the lungs (P=0.05) at this time point.

**Cross-reactivity and differentiation of CD8⁺ TmH3N2⁺ T cells.** The cross-reactivity of CD8⁺ TmH3N2⁺ T cells with the epitope variant of the influenza A/H5N1 virus (ASNENMEVM) was analysed using a tetramer derived from influenza virus A/Indonesia/5/05 (A/IND/5/05) NP366–374 (TmH5N1). Only a few CD8⁺ TmH3N2⁺ T cells induced after infection with influenza virus A/HK/2/68 (H3N2) cross-reacted with the H5N1-derived epitope. Furthermore, the percentages of CD8⁺ TmH5N1⁺ T cells did not differ between the respective groups after influenza A/H3N2 infection (data not shown).

The CD8⁺ TmH3N2⁺ T cells were also phenotyped according to their expression of CD62L and CD127. CD62Lhigh/CD127low cells were regarded as central memory (CM), CD62Llow/CD127high cells as effector memory (EM) cells and CD62Llow/CD127low as effector (E) cells (Bachmann et al., 2005). In the spleens and lungs of mock-vaccinated, H3N2-infected mice (group 3) at 12 and 28 days p.i., most CD8⁺ TmH3N2⁺ T cells were of the EM phenotype (Fig. 4). The percentage of EM CD8⁺ TmH3N2⁺ T cells of these mice was significantly higher than that of the WIV X-31-vaccinated group of mice 2 (P<0.05) in both spleens (12 and 28 days p.i.) and lungs (only at 12 days p.i.). At 12 days p.i., the percentage of E CD8⁺ TmH3N2⁺ T cells was higher in mice of group 3 compared with mice of group 2 in both lungs and spleens. On day 28 p.i., this population was virtually absent in mice of all groups. At 12 days p.i., an increase in the percentage of CM CD8⁺ TmH3N2⁺ T cells was observed in the spleens, but not in the lungs, of H3N2-infected mice compared with mock-infected animals or those that were vaccinated and infected (P=0.002). At 28 days p.i., these differences between groups were no longer detectable.

**Specificity of the CD8⁺ T-cell response (intracellular gamma interferon (IFN-γ) staining).** To assess the cross-reactivity of the CD8⁺ T-cell response with epitope variants present in the influenza virus strain A/IND/5/05 (H5N1), splenocytes and lung cells obtained from mice at 12 days after infection with influenza virus A/HK/2/68 (H3N2) were stimulated with peptides NP366–374 and PA224–233 derived from both viruses. Following infection with the H3N2 strain, peptide-specific CD8⁺ T-cell responses were observed in spleens and lungs, which also cross-reacted with the peptide from the heterosubtypic strain (Fig. 5). The response to NP366–374 (H5N1) was significantly higher in mice that were vaccinated with WIV X-31 prior to infection with A/HK/2/68 (H3N2) than in mock-vaccinated mice. Otherwise, vaccination reduced the magnitude of the CD8⁺ T-cell response following infection with the H3N2 strain.

After infection of mock-vaccinated mice, virus-specific CD8⁺ T cells to the homologous NP366–374 peptide and the H3N2 and H5N1 PA224–233 peptide variants were also observed in the lungs. These responses were significantly higher than those of mice that were vaccinated against the H3N2 strain. At 28 days p.i., peptide-specific CD8⁺ T cells were no longer detectable by intracellular IFN-γ staining in
either the lungs or spleens of any of the mice (data not shown).

**Vaccination prevents heterosubtypic immunity against influenza A/H5N1**

At 28 days after infection with influenza A/H3N2, mice of groups 2–5 were infected with a 90% lethal dose of influenza A/IND/5/05 (H5N1). All H5N1-infected mice developed clinical signs including weight loss from 2 days p.i. onward. Eleven out of 12 unvaccinated, H3N2-infected mice (group 3) lost weight until day 6 or 7 and then started to recover and gained weight. In contrast, all mice of groups 4 and 5 lost weight until they reached humane end points for euthanasia according to animal welfare regulations (weight loss of more than 20% and severe clinical signs). Of the WIV X-31-vaccinated, H3N2-infected mice, eight out of 12 (67%) mice had to be euthanized. Mice of groups 2 and 3 that survived the challenge and were not taken out of the experiment to assess lung virus titres or CD8+ T-cell responses continued to gain weight and reached their original body weight by 14 days p.i. The difference in weight loss and survival between mock-vaccinated and WIV X-31-vaccinated H3N2-infected mice was statistically significant (both P<0.01) (Fig. 6a, b). No significant differences were observed between virus titres in the lungs of mice of groups 2 and 3 at 4 (data not shown) and 7 days p.i., but the mean virus titre at 7 days p.i. was the lowest in mice of group 3, which survived the lethal challenge (P=0.32) (Fig. 6c). No virus was detected in the lungs of mice that survived the lethal challenge until 14 days p.i.

**Virus-specific CD8+ T-cell responses after H5N1 infection**

At 7 days after infection with influenza virus A/IND/5/05 (H5N1), the presence of CD8+ T cells specific for the NP<sub>H5N1</sub>, NP<sub>H3N2</sub>, NP<sub>X-31</sub> and PA<sub>H5N1</sub> epitopes in the spleen was assessed by tetramer staining and/or intracellular IFN-γ staining after stimulation with peptides. In naive mice, virus-specific CD8+ T-cell responses were virtually undetectable at 7 days p.i. with influenza virus A/
IND/5/05 (H5N1) (Fig. 6d). In contrast, a prior infection with influenza virus A/HK/2/68 (H3N2) (group 3 mice) primed mice for a CD8$^+$ T-cell response for both the NP366–374 and PA 224–233 epitope derived from the H5N1 strain (PA$_{H5N1}$). The magnitude of the response to both epitopes was comparable, indicating that the responses were co-dominant. In contrast, the response in group 5 mice, which were vaccinated with WIV X-31 before infection with the A/H5N1 virus, was dominated by an NP366–374-specific response. A similar result was observed in mice of group 2, which were vaccinated with WIV X-31 and subsequently infected with influenza virus A/HK/2/68 before infection with influenza virus A/IND/5/05 (H5N1). In this group, the response to PA$_{224-233}$ was virtually absent and significantly lower than that observed in mice of group 3 ($P<0.001$).

The results obtained with intracellular IFN-γ staining were in agreement with those obtained with NP$_{366-374}$-based tetramer staining (Table 2). In addition, this method allowed the assessment of the cross-reactivity of the CD8$^+$ T cells that were induced against this epitope. Prior infection with influenza virus A/HK/2/68 (H3N2) (containing NP$_{366-374}$ epitope ASNENMDAM) did not predispose to the induction of CD8$^+$ T cells cross-reactive with PA$_{H5N1}$ (ASNENMEVM). In contrast, vaccination with WIV X-31 (containing ASNENMETM), primed mice for the induction of CD8$^+$ T-cell responses not only cross-reactive with the H5N1 variant of the epitope ASNENMEVM but also with the H3N2 variant ASNENMDAM (Table 2).

**DISCUSSION**

In the present study, it was demonstrated that vaccination of mice with an inactivated influenza A/H3N2 vaccine induced protective immunity to infection with a corresponding A/H3N2 virus. By preventing productive infection with influenza virus A/HK/2/68 (H3N2), the protective potential of heterosubtypic immunity afforded by infection with the A/H3N2 virus was severely reduced. As a result, WIV X-31-vaccinated, H3N2-infected mice suffered more from clinical signs such as weight loss and displayed higher mortality rates after infection with the highly pathogenic influenza virus A/IND/5/05 (H5N1) than unvaccinated, H3N2-infected mice. In addition, WIV X-31 vaccination altered the immunodominance patterns of CD8$^+$ T-cell responses induced after infection with the A/H5N1 virus and the extent of virus shedding.

In a previous study, we showed that the use of an experimental subunit vaccine exclusively containing the HA and NA proteins of influenza virus X-31 prevented the induction of heterosubtypic immunity against an influenza A/H5N1 virus infection, which is otherwise induced by infection with influenza virus A/HK/2/68 (H3N2) (Bodewes et al., 2009b). As the viral inner proteins were absent, this vaccine preparation failed to induce CD8$^+$ T-cell responses to, for example, NP.

In this study, we evaluated the use of WIV vaccines for several reasons: firstly, WIV vaccines are more immunogenic than subunit vaccines and are widely used in humans as
unadjuvanted influenza vaccines. Secondly, as WIV vaccines contain inner viral proteins, it was anticipated that they would also induce CD8+ T-cell responses to these conserved proteins (Webster & Askonas, 1980), albeit inefficiently, and therefore may afford some level of heterosubtypic immunity. Indeed, vaccination with WIV X-31 induced detectable virus-specific CD8+ T-cell responses, probably through cross-priming (Bevan, 2006), in addition to virus-specific antibody responses, and protected mice from infection with the corresponding H3N2 influenza virus A/HK/2/68. However, the T-cell responses to the NP366–374 epitope were transient and were only detected shortly after the first vaccination. In addition, they were modest compared with the responses observed after infection with influenza virus X-31. Furthermore, vaccination severely impaired the CD8+ T-cell response to NP3H2N2, PAH3N2 and PAH5N1, but not the response to NP5H1N1, after infection with influenza virus A/HK/2/68 (H3N2). Apparently, there is cross-reactivity between the X-31 (ASNENMETM), H3N2 (ASNENMDAM) and H5N1 (ASNENMEVM) variants of the NP366–374 epitope, which has also been reported by others (Haanen et al., 1999).

Vaccination with WIV largely prevented the formation of CM and especially EM cells specific for NP3H2N2, which are otherwise induced after infection with influenza virus A/HK/2/68 (H3N2). However, vaccination did prime for a secondary CD8+ T-cell response specific for the heterologous NP366–374 epitope derived from influenza virus A/IND/5/05 (H5N1) (ASNENMETM vs ASNENMEVM). Apparently, these two variants are antigenically similar and are highly cross-reactive, and CD8+ T-cell responses were induced of which the relative responses to the NP366–374 and PAH5N1 epitopes resembled that of a typical secondary influenza virus-specific CTL response in C57BL/6 mice (Belz et al., 2000). Nevertheless, WIV X-31 vaccination did not afford protection against the development of clinical signs and mortality caused by A/IND/5/05 infection and did not reduce virus replication in the lungs.

In contrast, a primary infection with influenza virus A/HK/2/68 (H3N2) afforded partial protection against infection with influenza virus A/IND/5/05 (H5N1). These mice started to regain body weight from 7 days p.i. onward and fully recovered. This protection correlated with the induction of co-dominant CD8+ T-cell responses against the NP5H1N1 and PAH5N1 epitopes. Although the difference with naïve mice of group 4 and WIV-vaccinated mice of group 5 was striking, it is uncertain whether the response, in particular to the PA224–233 epitope, was responsible for the protective effect of a prior A/H3N2 infection. Firstly, it has been demonstrated that the PA224–233 epitope is presented by professional antigen-presenting cells such as dendritic cells, but not by virus-infected epithelial cells (Crowe et al., 2003), indicating that PA224–233-specific CD8+ T cells are unable to eliminate virus-infected cells from the lung efficiently. In addition, it has been shown that vaccination with dendritic cells loaded with the PA224–233 peptide induces an epitope-specific CD8+ T-cell response but fails to control subsequent infection with influenza virus, and even delays virus clearance (Crowe et al., 2003). Secondly, only a minority of NP366–374-specific CD8+ T cells induced after infection with influenza virus A/HK/2/68 (H3N2) was cross-reactive with the peptide variant derived from the H5N1 virus, which could explain the co-dominant PA224–233-specific response. Although the infection with influenza virus A/IND/5/05 (H5N1) resulted in the selective expansion of cross-reactive NP366–374-specific cells, this response was much lower than observed after a secondary infection with an influenza virus carrying the identical epitope sequence (Chen et al., 2004). In the absence of a dominant response to NP366–374, the response to PA224–233 can become co-dominant (Chen et al., 2004).

Vaccination with WIV X-31 (group 2) completely eliminated the response to the PA224–233 epitope observed after consecutive infections with influenza viruses A/HK/2/68 and A/IND/5/05, but not the response to the NP366–374 epitope. With regard to the response to the latter epitope, two contradictory mechanisms could be at work simultaneously: firstly, vaccination could reduce the induction of CD8+ T cells specific for NP3H2N2 (ASNENMDAM) cross-reactive with NP5H1N1 (ASNENMEVM), as was also observed after vaccination with a subunit vaccine (Bodewes et al., 2009b). Secondly, WIV vaccination could prime for a secondary response to NP366–374 as was seen for the mice of group 5.

In addition, vaccination may prevent the induction of other arms of the adaptive immune response, which are otherwise

Table 2. Specificity and cross-reactivity of NP366–374-specific CD8+ T cells in spleens of mice at 7 days after infection with influenza A/IND/5/05 (H5N1) as detected by tetramer (Tm) staining.

| Group | NP3H2N2+ | NP5H1N1+ |
|-------+---------+---------|
| 2     | 1.1 (1.0)| 0.8 (0.7)|
| 3     | ND      | 2.0 (2.7)|
| 4     | 0.1 (0.1)| 0.0 (0.1)|
| 5     | 2.9 (3.3)| 0.9 (0.9)|

| Group | NP5H1N1+ |
|-------+---------|
| 2     | 1.2 (1.8)|
| 3     | ND      |
| 4     | ND      |
| 5     | 3.6 (6.9)|

Data are represented as the percentage of Tm+ cells within the CD8+ T-cell population; SD is shown in parentheses. ND, No data collected.
induced by infection, such as virus-specific CD4\(^+\) T cells, antibodies to the extracellular domain of the matrix protein (Benton et al., 2001; Jegerlehner et al., 2004; Liang et al., 1994; Neirynck et al., 1999; Nguyen et al., 1999, 2000, 2001, 2007; Rangel-Moreno et al., 2008; Slepushkin et al., 1995; Straight et al., 2008) and the induction of tertiary lymphoid structures (Moyron-Quiroz et al., 2004). Although it has been suggested recently that antibodies to the NP also contribute to heterosubtypic immunity (Carragher et al., 2008), it is unlikely that they played a role in the present study, as all vaccinated mice developed NP-specific antibodies, which did not correlate with protection against H5N1 infection (data not shown).

Collectively, this study demonstrated that vaccination with WIV X-31 induced virus-specific CD8\(^+\) T-cell responses, induced protective immunity against homologous infection with influenza A/H3N2 and primed for secondary CD8\(^+\) T-cell responses after infection with A/H5N1 influenza virus. However, despite its capacity to induce memory CD8\(^+\) T-cell immunity, vaccination with WIV, like the use of subunit vaccines, did not afford protection against influenza A/H5N1 infection.

In addition, the use of WIV vaccines affected the induction of heterosubtypic immunity by infection, as with the use of a subunit vaccine (Bodewes et al., 2009b). Thus, regardless of the nature of the inactivated vaccine that is used for protection against seasonal influenza, the induction of heterosubtypic immunity is affected. This may be an unwanted effect of seasonal influenza vaccination using inactivated vaccines and is at present a matter of debate, as it was recently recommended in a number of countries including the USA that all healthy children aged 6–59 months of age should be vaccinated against seasonal influenza (Bodewes et al., 2009a; Fiore et al., 2008; Heikkinen & Peltola, 2009; Heikkinen et al., 2006).

The interference with the induction of heterosubtypic immunity correlated with changes in the immunodominance patterns of the virus-specific CD8\(^+\) T-cell response. However, it is unlikely that these changes were responsible for the lack of protection per se and they probably reflect differences in imprinting of CD8\(^+\) T-cell responses by the absence or presence of priming of NP\(_{366-374}\)-specific T-cell memory responses. Detailed analysis of the outcome of immune responses upon consecutive infections with influenza viruses of different subtypes may provide a better insight into the advantages and disadvantages of the use of inactivated vaccines in immunologically naïve subjects.

**METHODS**

**Viruses.** Virus stocks of influenza viruses A/HK/2/68 (H3N2) and A/IND/5/05 (H5N1) were prepared by infecting confluent Madin–Darby canine kidney (MDCK) cells, and infectious virus titres were determined in MDCK cells as described previously (Bodewes et al., 2009b).

**Vaccine preparation.** Egg-grown concentrated and purified influenza A X-31 virus (a reassortant vaccine strain of A/Aichi/2/68 and A/PR/8/34 of which the HA and NA proteins resemble those of influenza A/HK/2/68 closely) was inactivated by treatment with 0.05% formaldehyde for 7 days under continuous stirring at 4 °C. After inactivation, antigen was dialysed against PBS. The purity of the vaccine preparation was tested by SDS-PAGE and inactivation was confirmed by failure to passage on MDCK cells. The protein concentration was determined using a BCA Protein Assay kit (Pierce).

**Immunization and infection of mice.** Female, 6–8-week-old, specific-pathogen-free C57BL/6 J (H-2b) mice were purchased from Charles River Laboratories. Mice were divided into five groups, and animals in groups 1 (n=42), 3 (n=35) and 4 (n=17) were mock immunized intramuscularly twice with an interval of 4 weeks with 100 μl PBS in two hind legs, whilst mice of groups 2 (n=47) and 5 (n=23) were immunized twice with 15 μg WIV X-31 in PBS (total volume 100 μl) (Table 1). Eight and 28 days after the first and 28 and 56 days after the second vaccination, four mice of groups 1 and 2 were bled and their spleens were resected. As a positive-control group for the induction of virus-specific CD8\(^+\) T-cell responses after vaccination, eight mice were also infected with 2 \(\times\) 10⁴ TCI⁰₉₀ influenza A X-31 intranasally in a total volume of 50 μl, and euthanized at 8 and 28 days p.i.

Four weeks after the second vaccination, mice of groups 2 and 3 were infected intranasally with 5 \(\times\) 10⁴ TCI⁰₉₀ influenza virus A/HK/2/68 (H3N2) in a volume of 50 μl. Mice of groups 1, 4 and 5 were mock infected with PBS. Following infection, mice were weighed daily to monitor their weight loss as a clinical indicator of infection. At 4, 12 and 28 days p.i., six mice of groups 1, 2 and 3 were bled and their lungs and spleens were resected. Four weeks after infection with influenza virus A/HK/2/68 (H3N2), all mice except those of group 1 were infected intranasally with 2 \(\times\) 10⁴ TCI⁰₉₀ influenza virus A/IND/5/05 (H5N1). This dose was chosen as it is the minimal dose reproducing a lethal infection in >90% mice (Bodewes et al., 2009b; Kreijtz et al., 2009). Mice of group 1 were mock-infected with PBS. The day before infection with influenza virus A/IND/5/05 (H5N1), six mice of each group except group 4 were euthanized and their lungs and spleens were resected. After infection with influenza virus A/IND/5/05 (H5N1), mice were weighed daily and monitored for clinical signs. Mice were euthanized at 4 (n=5 per group), 7 (n=8 or more per group) and 14 (n=4 or fewer per group) days after challenge and their spleens and lungs were resected. Vaccinations, intranasal infections, blood withdrawal and euthanasia were performed under anaesthesia with isoflurane in O₂. All experiments with influenza A/H5N1 virus were performed under Biosafety Level 3 (BSL-III) conditions. An independent animal ethics committee approved the experimental protocol before the start of the experiments.

**Serology.** Before vaccination, 4 weeks after the first vaccination, 4 weeks after the second vaccination and 4 weeks after infection with influenza A/H3N2 virus, serum samples of mice were collected and tested for the presence of HA-specific antibodies against influenza viruses A/H3N2 and A/H5N1 using a haemagglutination inhibition assay and for virus-neutralizing antibodies using a virus neutralization assay as described previously (Bodewes et al., 2009b; Frank et al., 1980; Palmer et al., 1975).

**Lung virus titres.** Lungs of mice collected 4 and 12 days after infection with influenza A/H3N2 and 4, 7 and 14 days after challenge with influenza A/H5N1 virus were snap frozen and stored at −70 °C until further processing. Lungs were homogenized and quintuplicate tenfold serial dilutions of these samples were used to inoculate MDCK cells as described previously (Bodewes et al., 2009b). The HA activity of the culture supernatants collected 5 days p.i. was used as an
indicator of infection. Titres were calculated according to the Spearman–Karber method (Karber, 1931).

**Flow cytometry of virus-specific CD8+ T cells**

**Preparation of single-cell suspensions of lung cells and splenocytes.** Twelve and 28 days after influenza A/H3N2 virus infection, mice were bled and the lungs of three to four mice per group were subsequently collected in gentleMACS tubes (Miltenyi Biotec) with Iscove’s modified Dulbecco’s medium (IMDM; Lonza) supplemented with 5% fetal calf serum (FCS), 100 μg streptomycin ml−1 and 100 IU penicillin ml−1 and a single-cell suspension was prepared following the manufacturer’s protocol. The lung suspension was then filtered with a 100 μm cell strainer (BD Falcon) and subsequently treated with red blood cell lysis buffer (Roche). Single-splenocyte suspensions were prepared as described previously (Kreijtz et al., 2007).

**Tetramer staining.** After vaccination or infection with influenza A/H3N2, single-cell suspensions of splenocytes and lung cells were washed and stained with the fluorescently labelled monoclonal antibodies (mAbs) phycoerythrin (PE)—Cy7-conjugated CD3ε, fluorescein isothiocyanate-conjugated CD8b.2 (both from BD), allophycocyanin (APC)—Cy7-conjugated CD62L (BioLegend), Pacific Blue-conjugated CD127 (eBioscience) and either a PE-labelled H-2Db tetramer with the immunodominant NP366–374 epitope derived from influenza A virus X-31 (ASNENMETM; TmH3N2) or influenza A/HK/2/68 (ASNENMDM; TmH5N1) and an APC-labelled tetramer derived from influenza A/IND/5/05 NP366–374 (ASNENMEVM; TmH5N1) (Altman et al., 1996). Tetramers were purchased from Sanquin Research. To exclude dead cells in the analysis, cells of vaccinated or H3N2-infected mice were also stained with LIVE/DEAD Aqua Fixable Dead Cell Stain (Invitrogen). Following incubation with the tetramers and mAbs for 20 min, cells were washed twice and analysed with a FACSCalibur and analysed with CellQuest Pro software (both BD).

After H5N1 infection (under BSL-III conditions), single-cell suspensions of spleens were washed and stained with mAbs against CD3ε, CD8b.2 and with the PE-labelled H-2Db tetramer TmH3N2 or TmH5N1 or the APC-labelled tetramer TmH5N1. After incubation, cells were washed, fixed with Cytofix (BD), acquired on a FACSCalibur and analysed with CellQuest Pro software (both BD).

**Peptides and intracellular IFN-γ staining.** CD8+ T-cell responses were measured by incubation of splenocytes or lung cells with peptides representing two immunodominant epitopes of influenza A viruses in C57BL/6 J mice (H2–b), PA224–233 and NP366–374 (Belz et al., 2000; Doherty et al., 1978). Peptides were manufactured at Eurogentec. Splenocytes or lung cells were cultured for 6 h at 37°C in the presence of 5 μM of either NP366–374 ASNENMDM (NP1H5N1) or PA224–233 SCLLENFRAY (PA1H5N1) peptide derived from influenza A/HK/2/68 (H3N2) virus or NP366–374 ASNENMEVM (NPH5N1) or SSSLNFRAY (PAH5N1) peptide (differences underlined) derived from influenza A/IND/5/05 virus in IMDM with 5% FCS and GolgiStop (BD). The PAH5N1 peptide is also a known epitope in the influenza A X-31 vaccine strain that was used. After incubation, cells were stored overnight at 4°C and the presence of intracellular IFN-γ in CD8+ T cells was analysed as described previously (Bodewes et al., 2009b). 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.

**Statistical analysis.** Data for weight loss after infection, lung virus loads, tetramer staining and peptide pulsing were analysed statistically using a Mann–Whitney test. Survival was analysed using a logrank test. Differences were considered significant at P<0.05.

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