Evaluation of heterosubtypic cross-protection against highly pathogenic H5N1 by active infection with human seasonal influenza A virus or trivalent inactivated vaccine immunization in ferret models

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Since 1997, highly pathogenic avian influenza (HPAI) H5N1 viruses have circulates among domestic poultry and wild birds in Asia, Africa and Europe and are believed to have undergone genetic evolution (Bragstad et al., 2007; Cattoli et al., 2009; Claas et al., 1998). The World Health Organization (WHO) reported 641 cases of HPAI H5N1 virus isolation from humans since 2003, producing a high mortality rate of 59%, that were mainly due to direct contact with infected birds (Beigel et al., 2005; WHO, 2013). Therefore, there is a growing concern that HPAI H5N1 viruses might cause the next pandemic (Pappaioanou, 2009).

Vaccination is the most effective measure to prevent the spread of novel and potentially pandemic influenza viruses. Although several pre-pandemic H5N1 vaccines are commercially available, two types of seasonal vaccines are commonly used to prevent seasonal influenza virus infection: trivalent inactivated influenza vaccine (TIV) and live attenuated influenza vaccine (LAIV) (Lambert & Fauci, 2010; Nabel & Fauci, 2010). TIVs induce relatively strain-specific neutralizing antibodies. In contrast, LAIVs elicit similar immunity with natural infection providing protection against homologous virus and strong immunity against different virus subtypes (Schulman & Kilbourne, 1965; Yewdell et al., 1985). Although a number of studies have shown that different influenza vaccine preparations could provide some degree of cross-protective immunity against HPAI H5N1 strains (Brewoo et al., 2013; Kreijtz et al., 2008, 2009; Lee et al., 2013; Wang et al., 2010), there is only limited information on whether recent human seasonal TIVs can actually provide heterosubtypic immunity. Therefore, we investigated heterosubtypic protection and induction of T-cell responses in ferret models by currently circulating seasonal influenza A virus [A/California/07/2009 (H1N1) or A/Perth/16/2009 (H3N2)] infection or immunization with a 2011–2012 human seasonal TIV (Novartis) formulation containing A/California/07/2009 (H1N1), A/Victoria/210/2009 (H3N2) and B/Brisbane/60/2008 as antigens. Individual treatments were followed by HPAI A/Vietnam/1203/2004 (H5N1) virus challenge. All experiments with H5N1 influenza virus (serology, virus challenge and titration) were conducted in a BSL3+ containment facility approved by the Korea Centers for
Disease Control and Prevention (permit number: BLS-ABSL-12-001) and followed general animal care guidelines required by the Institutional Animal Care and Use Committee of Chungbuk National University, Cheongju, Republic of Korea.

Groups of 12 seronegative ferrets (Mustela putorius furo; Marshall, New York, USA) were inoculated intranasally with $10^{5.5}$ TCID$_{50}$ ml$^{-1}$ of human seasonal H1N1 (group B) or H3N2 (group C) virus to imitate natural infection. An additional group ($n=12$) was vaccinated with seasonal TIV (group D) (3 times, with a 2-week interval). A reference group that only received sterile PBS served as a control treatment (group A). Four weeks after PBS treatment, active infection or TIV immunization, antibody titres were measured against influenza A H1N1, H3N2, H5N1 and B viruses by haemagglutinin inhibition (HI) and virus neutralization (VN) assays (Fig. 1a, b). Ferrets that were actively infected with H1N1 (group B) and H3N2 (group C) viruses displayed high geometric mean titres (GMT) only against A/California/07/2009 (H1N1) (359 HI units) and A/Perth/16/2009 (H3N2) (226 HI units) (Fig. 1a). Despite three vaccine regimens, TIV immunized ferrets (group D) showed moderate immunogenic titres against the H1N1 and H3N2 vaccine strains (80 and 44 HI units, respectively). Only modest antibody titres (15 HI units) were observed against the type B (B/Brisbane/60/2008) virus in group D (Fig. 1a). A similar pattern of neutralization was observed in VN assays correlating with results obtained in HI assays (Fig. 1b).

At 12 weeks post-treatment (infection or immunization), serological assays showed that the antibody titres appeared to have significantly waned (Fig. 1c, d). Ferrets from groups B and C displayed at least fourfold reductions in antibody titres, and no detectable antibody responses against any vaccine strain was observed in group D (Fig. 1c, d). Notably, none of the treatment groups presented any detectable cross-reactivity against the HPAI A/Vietnam/1203/2004 (H5N1) virus (Fig. 1). These results demonstrated that active infection of influenza virus induces stronger and persistent immune responses compared with repeated TIV immunizations.

To investigate cross-protective efficacy, ferrets in each of the treatment groups were intranasally and intratracheally challenged with $10^3$ 50% egg infective dose per millilitre (EID$_{50}$ ml$^{-1}$) HPAI A/Vietnam/1203/2004 (H5N1) 12 weeks after the last treatment. In group A, body temperatures increased by up to 2.6 °C from normal ranges, with

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**Fig. 1.** Antibody responses in groups of twelve ferrets after treatment with PBS (group A), active infection with human seasonal influenza viruses [H1N1 (group B) or H3N2 (group C)], or immunization with TIV (group D). HI titres (a, c) and VN titres (b, d) against H1N1, H3N2, H5N1 and B viruses were determined in sera collected 4 weeks (a, b) and 12 weeks (c, d) after the last treatments. Dotted lines indicate the lower limit of detection (<10 units). Error bars indicate SEM.
substantial mean weight loss (10.0 to 13.5%) at 5 days post-infection (p.i.). Group D also displayed high fever that increased by up to 2.3 °C at 7 days p.i., with a substantial mean weight loss of 11–16.8%. In contrast, group B exhibited modest clinical disease signs (only ~5% weight loss at 5 days p.i.) and did not show signs of fever. Group C demonstrated moderate mean weight loss of approximately 8% until 5 days p.i., with mild fever (Fig. 2a, b). All ferrets that were actively infected in groups B and C eventually recovered and survived until 14 days p.i.; notably, group B showed the mildest clinical disease following H5N1 infection and the most rapid recovery rate (Fig. 2a, b). Meanwhile, all the ferrets in groups A and D succumbed to death within 11 days p.i.; the mortality of challenged ferrets in group D was delayed compared with those in group A (Fig. 2c).

To further demonstrate the correlation between mortality and the cross-protection induced by active infection or TIV immunization of ferrets against HPAI H5N1 virus infection, viral titres in nasal wash samples were determined following A/Vietnam/1203/2004 (H5N1) virus infection, viral titres in nasal wash samples were determined following A/Vietnam/1203/2004 (H5N1) virus challenge. Ferrets in the control group A shed the highest nasal wash titres (peak titres at 7 days p.i.) relative to the other groups that lasted until 7 days p.i., whereas group D ferrets persistently shed the challenge virus at high titres up to 9 days p.i. (Fig. 2d). In contrast, the actively infected groups (B and C) demonstrated a decreasing trend of nasal wash titres that eventually became undetectable at 7 days p.i.

On day 5 post-H5N1 challenge, we sacrificed two animals from each group for tissue viral titration in various organs (Fig. 3a). Ferrets in groups A and D exhibited significantly (P<0.05) higher tissue titres in the lungs compared with those in groups B and C (6 and 5.0 versus 2.0 and 2.5 log<sub>10</sub> EID<sub>50</sub> g<sup>-1</sup>, respectively). Brain and spleen tissue titres in the latter two groups were also significantly lower compared with those in groups A and D (P<0.05); A/Vietnam/1203/2004 (H5N1) could not produce titres beyond the limit of virus detection (0.5 log<sub>10</sub> EID<sub>50</sub> g<sup>-1</sup>) in spleen. Interestingly although the virus was detected in the liver of animals of all the groups, those in group A demonstrated the highest viral titres (4.0 log<sub>10</sub> EID<sub>50</sub> g<sup>-1</sup>) (P<0.05), followed by group D (3.5 log<sub>10</sub> EID<sub>50</sub> g<sup>-1</sup>). Thus, none of the groups could completely inhibit replication of the H5N1 virus in the ferret tissues. However, actively H1N1- or

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**Fig. 2.** Measurement of (a) body weight, (b) temperature, (c) survival and (d) titration of nasal wash titres. At 12 weeks post-treatment, ferrets were challenged with 10<sup>5</sup> EID<sub>50</sub> ml<sup>-1</sup> A/Vietnam/1203/2004 (H5N1) and monitored daily for 14 days p.i. (a) Body weight losses are presented as the mean percentage of the animals’ weight differences relative to their weight on the day prior to inoculation. (b) Rectal temperature is shown as changes in temperature. (c) Survival is recorded for 14 days p.i. (d) Nasal wash samples were collected at 1, 3, 5, 7 and 9 days p.i. Data shown are mean ± SD virus titres of six ferrets per group. Dotted lines indicate the lower limit of detection (0.5 log<sub>10</sub> EID<sub>50</sub> ml<sup>-1</sup>). Asterisks indicate significant differences (*P<0.05, **P<0.001 and ***P<0.0001) as determined by (c) log rank (Mantel–Cox) test on Kaplan–Meier versus group A for survival test or (d) two tailed unpaired t-test for nasal wash titres (GraphPad Prism version 5). #, No samples collected because the ferrets in this group died.

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H3N2-infected ferrets showed less virus replication in the organs tested than TIV-immunized ferrets. Despite no serological evidence of antibody titres, group B and C ferrets were able to survive lethal HPAI H5N1 virus infection suggesting the role of heterosubtypic T-cell immune response in cross-protection. We therefore analysed T-cell responses in the lungs. Total RNA was isolated from lung tissues (n=2) collected at 1 and 3 days p.i. and real-time reverse-transcription (RT) PCR was performed in triplicate using primer sets aimed at determining the mRNA levels of CD4^+^, CD8^+^ and IFN-γ. The amount of specific mRNA was normalized to endogenous β-actin and presented as an increase over values obtained from PBS-treated groups. RT-PCR were performed with each primer set of: CD4^+^ sense: 5'-ctgctcggagcttcctc-3', CD4^+^ antisense: 5'-tcacctctgtgcatgtc-3'; CD8^+^ sense: 5'-cctcagaacgaacctg-3', CD8^+^ antisense: 5'-tagcaccgattgctg-3'; IFN-γ sense: 5'-agagttgctttcctt-3', IFN-γ antisense: 5'-tacctgacgattctgtc-3'; β-actin sense: 5'-ctctccagcttactgctgct-3', β-actin antisense: 5'-gcctccagcttactgctgct-3'. Intrapulmonary CD4^+^, CD8^+^ and IFN-γ were increased more than twofold in groups B and C but not in group D relative to the control group A at 1 day p.i. (Fig. 3b–d). CD4^+^ and IFN-γ were further robustly elevated at 3 days p.i. Although CD8^+^ expression levels were relatively unchanged at 3 days p.i. in groups B and C, values remained significantly higher (P<0.001) than those in PBS-treated and TIV-immunized ferrets. These findings suggest that both CD4^+^ and CD8^+^ T-cells contributed to IFN-γ production in ferrets that were previously infected with human seasonal H1N1 and H3N2 viruses, potentially affording protection against the HPAI H5N1 virus challenge.

The majority of the human population is serologically naïve to HPAI H5N1 viruses, even after annual vaccination with seasonal TIV (Beigel et al., 2005). Thus, it remains a question whether pre-existing immunity due to vaccination or exposure to human seasonal influenza viruses could provide some degree of cross-protection against HPAI H5N1 virus infection. We showed here that despite three immunization regimens, a commercial 2011–2012 TIV could not sustain high titres against the human seasonal vaccine strains at 3 months post-vaccination. We also found that latent immune responses induced by TIV immunization were not sufficient to protect ferrets from fatal H5N1 virus infection. Thus, our data substantiate previous findings that seasonal inactivated vaccination may not induce but rather hamper the mounting of potent heterosubtypic immunity against influenza A/H5N1 virus (Bodewes et al., 2009, 2011). In contrast to the TIV group (group D), the groups actively infected with human seasonal viruses (group B and C) demonstrated better homologous virus immunogenicity even at 12 weeks p.i.
indicating induction of more persistent immune responses compared to the repeated killed vaccinations.

Serological assays performed prior to animal challenge did not reveal any detectable cross-reactive titres against the A/Vietnam/1203/2004 (H5N1) virus. Despite that, all ferrets in group B and C, but not group D, were protected from the lethal HPAl H5N1 virus challenge similar to findings obtained by Kreijtz et al. (2009) in mice. Active infection may have a similar effect with LAIVs in terms of inducing more heterosubtypic immunity compared to TIV through the induction of cross-reactive T-cell immune response (Hoft et al., 2011). The influenza A virus protein sequences of avian and human strains share highly conserved sequences, as in matrix 2 (M2) and nucleocapsid proteins, most of which are predicted to have immune relevance as T-cell epitopes (Heiny et al., 2007; Lee et al., 2008; Tompkins et al., 2007). Indeed, concomitant with the cross-protection was the robust elevation of lung CD4+ and CD8+ T-cell mRNA expression levels along with IFN-γ in groups B and C but not in group D. With respect to LAIV immunizations, the percentage of CD4+ and CD8+ T-cells was also increased in 5- to 8-year-old children (He et al., 2006). Therefore, our data support the critical role of cross-reactive T-cell immune responses elicited by the live-virus infections that promoted heterosubtypic immunity against the HPAI A/Vietnam/1203/2004 (H5N1) virus.

T-cell responses induced by active infections have also been proposed to enhance recovery by promoting virus clearance and reducing clinical symptoms in infected hosts (Moskophidis & Kioussis, 1998). The H1N1-infected ferrets (group B) displayed more attenuated clinical disease signs and virus titres than H3N2-infected ferrets (group C). This may be due to the closer genetic relatedness between the HPAI H5N1 and seasonal H1N1 viruses. The haemagglutinin (HA) genes of A/Vietnam/1203/2004 (H5N1) and A/California/07/2009 (H1N1) collectively belong to the H1 clade (e.g. H1, H2, H5 and H6) of group 1 influenza viruses which contain common HA neutralizing epitopes (Air, 1981; Sakabe et al., 2010). Moreover, they possess the same although not identical neuraminidase (NA) subtype, which can be responsible for the similar antibodies that conferred additional cross-protective immunity against H5N1 (Chen et al., 2012). Lastly, the surface M2 viral proteins, also known to elicit virus-specific antibodies (Lamb et al., 1985; Tompkins et al., 2007), of A/Vietnam/1203/2004 (H5N1) and A/California/07/2009 (H1N1) are genetically more homologous (90.8%) than A/Perth/16/2009 (H3N2) (83.7%). It is also noteworthy that live-virus infection groups had lower tissue titres in various organs tested. Thus, the hampered HPAI H5N1 viral dissemination in general may have further contributed to the attenuated infection and to the overall cross-protection observed in ferrets.

Altogether, this study suggests that immune response induced by immunization with recent human seasonal TIVs is insufficient to protect against lethal infection with the HPAI A/Vietnam/1203/2004 (H5N1) virus. Rather, our data imply that exposure to or natural infection with seasonal human influenza A viruses could elicit more potent cross-protective immunity in our ferret model even after a few months of infection. Such degree of cross-protection could be beneficial in the event of a looming outbreak until such a time that a well-matched vaccine becomes available. Therefore, although the pandemicity of HPAI H5N1 viruses remains of great concern, the predicted high mortality caused by newly emerging viruses might be overestimated. Finally, this work highlights the need for development of improved universal influenza A virus vaccine that could promote heterosubtypic immunity.

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