

# A Bovine Adenoviral Vector-Based H5N1 Influenza -Vaccine Provides Enhanced Immunogenicity and Protection at a Significantly Low Dose

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Several human and nonhuman adenovirus (AdV) vectors including bovine AdV type 3 (BAdV-3) were developed as gene delivery vectors to supplement and/or elude human AdV (HAdV)-specific neutralizing antibodies (vector immunity). Here we evaluated the vaccine immunogenicity and efficacy of BAdV-3 vector (BAd-H5HA) expressing hemagglutinin (HA) of a H5N1 influenza virus in a dose escalation study in mice with the intranasal (IN) or intramuscular (IM) route of inoculation in comparison with the HAdV type C5 (HAdV-C5) vector (HAd-H5HA) expressing HA of a H5N1 influenza virus. Dose-related increases in the immune responses were clearly noticeable. A single IM inoculation with BAd-H5HA resulted in enhanced cellular immune responses compared with that of HAd-H5HA and conferred complete protection following challenge with a heterologous H5N1 virus at the dose of  $3 \times 10^7$  plaque-forming units (PFUs), whereas a significant amount of influenza virus was detected in the lungs of mice immunized with  $1 \times 10^8$  PFUs of HAd-H5HA. Similarly, compared with that of HAd-H5HA, a single IN inoculation with BAd-H5HA produced significantly enhanced humoral (HA-specific immunoglobulin [IgG] and its subclasses, as well as HA-specific IgA) and cellular immune responses, and conferred complete protection following challenge with a heterologous H5N1 virus. Complete protection with BAd-H5HA was observed with the lowest vaccine dose ( $1 \times 10^6$  PFUs), but similar protection with HAd-H5HA was observed at the highest vaccine dose ( $1 \times 10^8$  PFUs). These results suggest that at least 30-fold dose sparing can be achieved with BAd-H5HA vector compared with HAd-H5HA vaccine vector.

## INTRODUCTION

Adenovirus (AdV) vector-based vaccines have been shown to elicit balanced humoral and cell-mediated immune (CMI) responses<sup>1,2</sup> by activating innate immunity through both Toll-like receptor (TLR)-dependent as well as TLR-independent pathways.<sup>3,4</sup> Due to the high prevalence of AdV in humans, the development of AdV-specific neutralizing antibodies, known as “pre-existing vector immunity,”<sup>5-7</sup> is one of the potential concerns for several human AdV

(HAdV) vector-based vaccine delivery systems. To address this concern, a number of less prevalent HAdVs or nonhuman AdVs have been developed as vaccine delivery vectors.<sup>8,9</sup> These nonhuman AdV vectors are based on bovine AdV (BAdV), simian AdV (SAdV), porcine AdV (PAdV), ovine AdV (OAdV), canine AdV (CAdV), avian AdV (AAAdV), and murine AdV (MAdV).<sup>8,9</sup>

It has been demonstrated that there were no reductions in humoral and CMI responses against the vaccine immunogen and the resultant protection efficacy of a BAdV type 3 (BAdV-3) vector-based H5N1 influenza vaccine even in the presence of exceptionally high levels of pre-existing HAdV vector immunity.<sup>10</sup> In addition, pre-existing HAdV-neutralizing antibodies in humans did not cross-neutralize BAdV-3,<sup>11</sup> and HAdV-specific CMI responses did not cross-react with BAdV-3.<sup>4</sup> Bio-distribution, pathogenesis, transduction, and persistence studies in animal models and cell lines have suggested that the safety aspects of BAdV vectors are similar to that of HAdV vectors.<sup>12,13</sup> It has been illustrated that the cell internalization of BAdV-3 is independent of the HAdV type C5 (HAdV-C5) receptors [Coxsackievirus-AdV receptor (CAR) and  $\alpha v\beta 3$  or  $\alpha v\beta 5$  integrin],<sup>14</sup> but it is indicated that the  $\alpha(2,3)$ -linked and  $\alpha(2,6)$ -linked sialic acid receptors serve as major receptors for BAdV-3 internalization.<sup>15</sup> It appears that BAdV-based vectors may serve as excellent vaccine vectors for humans without any concerns of pre-existing HAdV vector immunity.

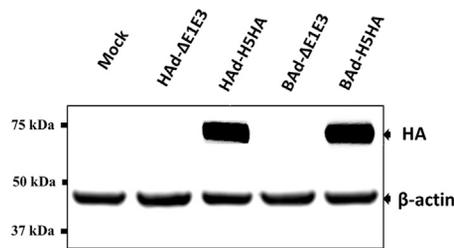
Vaccine formulation features that are important for developing effective pre-pandemic influenza vaccine strategies include the development of balanced humoral and CMI responses that could offer cross-protection, safety, and efficacy with a single dose, dose sparing

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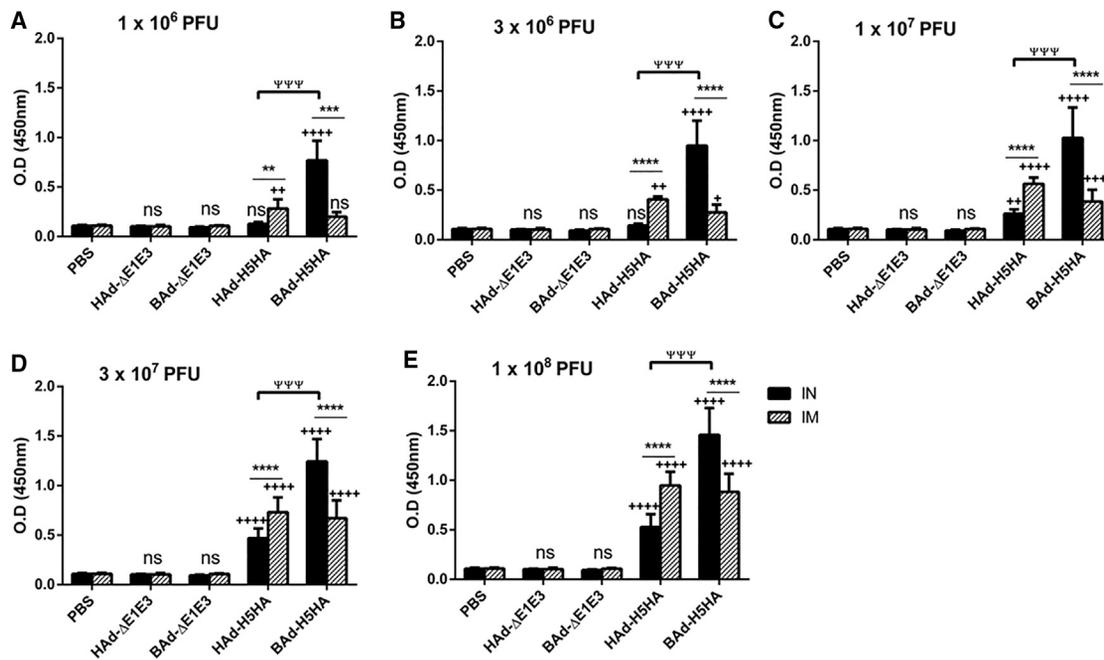
**Figure 1. HA Expression Levels by HAd-H5HA or BAd-H5HA in Infected Cells**

BHH3 cells were either mock infected or infected with HAd-ΔE1E3, HAd-H5HA, BAd-ΔE1E3, or BAd-H5HA at an MOI of 10 PFUs per cell. At 48 hr post-infection, the cells were harvested and processed for immunoblotting. HA expression was detected using an HA-specific polyclonal antibody. The expression levels of β-actin were monitored to demonstrate equal loading. The molecular weight markers in kDa are shown on the left.

for vaccine delivery to a large number of individuals, and the capacity to produce a large number of vaccines at short notice. In our previous studies, we have demonstrated that AdV vector-based vaccines could elicit potent humoral and CMI responses in mice conferring cross-

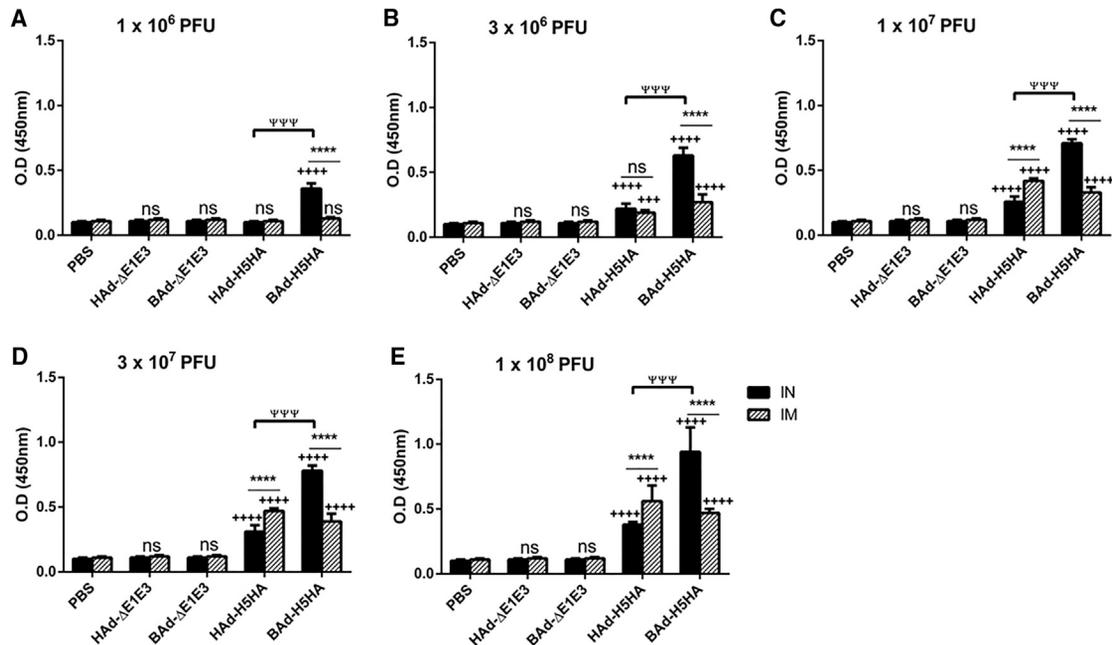
protection depending on the immunogen(s) of choice.<sup>16,17</sup> Because AdV vectors have been evaluated for their efficacy as gene delivery vehicles in many clinical trials in humans,<sup>18–21</sup> it is well understood how to produce a clinical grade of purified AdV vector lots in exceptionally large quantities under good laboratory practice (GLP) conditions in certified cell lines in a short time span.<sup>1,22–24</sup>

In this study, we have compared the immunogenicity and efficacy of BAdV-3 vector (BAd-H5HA) expressing hemagglutinin (HA) of a H5N1 influenza virus in a mouse model with that of HAdV-C5 vector (HAd-H5HA) expressing the same HA. The vaccine doses [ $1 \times 10^6$ ,  $3 \times 10^6$ ,  $1 \times 10^7$ ,  $3 \times 10^7$ , or  $1 \times 10^8$  plaque-forming units (PFUs)] and the routes of immunization [intranasal (IN) or intramuscular (IM)] were evaluated to determine whether the BAdV vector system will serve as a better vaccine vector compared with the HAdV vector. Overall, significantly higher levels of humoral and CMI responses accompanied with higher protection efficacy were observed with the BAd-H5HA vector compared with that of HAd-H5HA. The best protection efficacy with a significantly lower vaccine dose was observed in the mouse group inoculated IN with BAd-H5HA. These results suggest that the BAdV-3-based vector system is a better vaccine delivery vehicle for developing pre-pandemic influenza vaccines.



**Figure 2. HA-Specific Serum IgG Antibody Responses in Mice Immunized Once with BAd-H5HA or HAd-H5HA**

Mice were immunized intramuscularly (IM) or intranasally (IN) once with  $1 \times 10^6$  (A),  $3 \times 10^6$  (B),  $1 \times 10^7$  (C),  $3 \times 10^7$  (D), or  $1 \times 10^8$  (E) PFUs of BAd-H5HA or HAd-H5HA. For all dose groups, mice inoculated IM or IN with PBS or  $1 \times 10^6$  PFUs of BAd-ΔE1E3 or HAd-ΔE1E3 served as negative or internal controls, respectively. Four weeks after inoculation, serum samples were collected, diluted to 1:500, and the development of HA-specific IgG antibody responses were monitored by ELISA. Data are represented as the mean ± SD of the optical density (OD) readings. Statistically significant responses are shown as compared with the PBS group (+), IN versus IM route of inoculation in the same group (\*), or BAd-H5HA vector versus HAd-H5HA vector (ψ). \*\* or ++, significant at  $p < 0.01$ ; \*\*\*, +++, or ψψψ, significant at  $p < 0.001$ ; \*\*\*\* or +\*\*\*\*, significant at  $p < 0.0001$ . The statistical analysis was done by Bonferroni post-test and two-way ANOVA using GraphPad Prism 6. BAd-ΔE1E3, BAd empty vector; BAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of A/Hong Kong/156/97(H5N1) influenza virus; HAd-ΔE1E3, HAd empty vector; HAd-H5HA, human adenoviral vector expressing HA of A/Hong Kong/156/97(H5N1) influenza virus; ns, no significance at  $p > 0.05$ .



**Figure 3. HA-Specific Serum IgG1 Antibody Responses in Mice Immunized Once with BAd-H5HA or HAd-H5HA**

Mice were immunized intramuscularly (IM) or intranasally (IN) once with  $1 \times 10^6$  (A),  $3 \times 10^6$  (B),  $1 \times 10^7$  (C),  $3 \times 10^7$  (D), or  $1 \times 10^8$  (E) PFUs of BAd-H5HA or HAd-H5HA. For all dose groups, mice inoculated IM or IN with PBS or  $1 \times 10^8$  PFUs of BAd- $\Delta$ E1E3 or HAd- $\Delta$ E1E3 served as negative or internal controls, respectively. Four weeks after inoculation, serum samples were collected, diluted to 1:500, and the development of HA-specific IgG1 antibody responses was monitored by ELISA. Data are represented as the mean  $\pm$  SD of the optical density (OD) readings. Statistically significant responses are shown as compared with the PBS group (+), IN versus IM route of inoculation in the same group (\*), or BAd-H5HA vector versus HAd-H5HA vector ( $\psi$ ). +, +++, +++++, significant at  $p < 0.001$ ; and \*\*\*\* or +++++, significant at  $p < 0.0001$ . The statistical analysis was done by Bonferroni post-test and two-way ANOVA using GraphPad Prism 6. BAd- $\Delta$ E1E3, BAd empty vector; BAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of A/Hong Kong/156/97(H5N1) influenza virus; HAd- $\Delta$ E1E3, HAd empty vector; HAd-H5HA, human adenoviral vector expressing HA of A/Hong Kong/156/97(H5N1) influenza virus; ns, no significance at  $p > 0.05$ .

## RESULTS

### HA Expression Levels by HAd-H5HA or BAd-H5HA in Infected Cells

HAd- $\Delta$ E1E3-, HAd-H5HA-, BAd- $\Delta$ E1E3-, or BAd-H5HA-infected BHH3 cells were analyzed to determine the levels of H5 HA expression by immunoblotting using an HA-specific rabbit polyclonal antibody. Roughly equal expression levels of  $\beta$ -actin demonstrate equal loading in each lane (Figure 1). Similar densities of HA-specific bands at approximately 72 kDa indicated that HAd-H5HA or BAd-H5HA resulted in a similar level of HA expression (Figure 1).

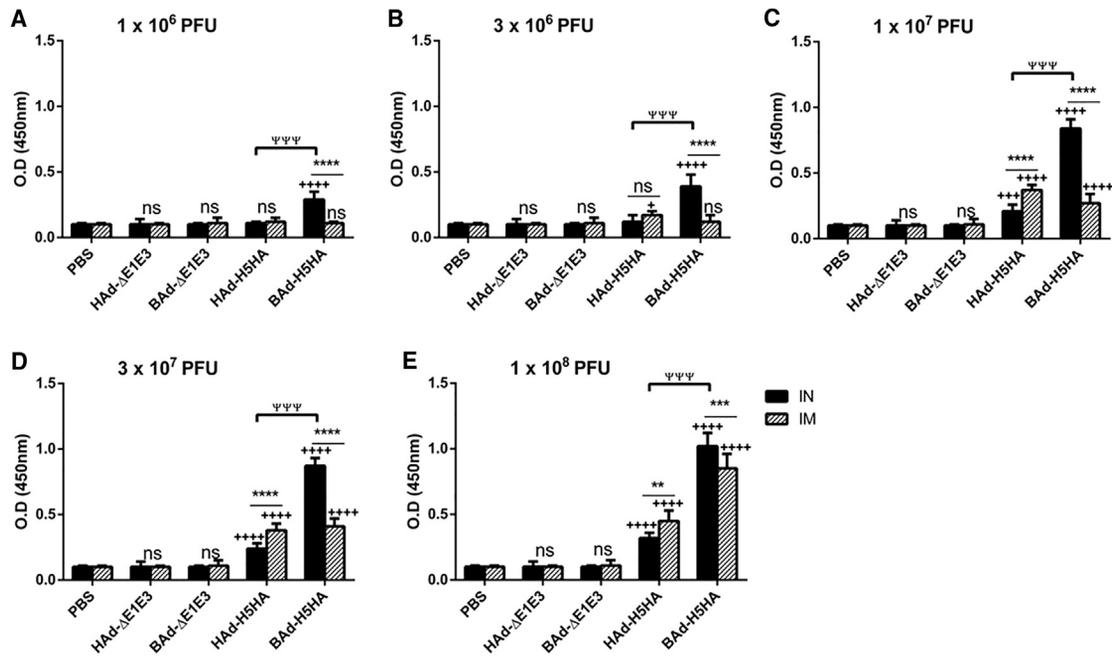
### Induction of Enhanced Humoral Immune Responses with BAd-H5HA Compared with HAd-H5HA

The mouse groups inoculated IN or IM once with  $1 \times 10^6$  (Figure 2A),  $3 \times 10^6$  (Figure 2B),  $1 \times 10^7$  (Figure 2C),  $3 \times 10^7$  (Figure 2D), or  $1 \times 10^8$  (Figure 2E) PFUs of HAd-H5HA or BAd-H5HA elicited dose-dependent increases in anti-HA immunoglobulin G (IgG) antibody levels. These levels in the IM-inoculated HAd-H5HA groups were similar to or slightly better than those observed in the similarly inoculated BAd-H5HA groups. In the IN-inoculated BAd-H5HA groups, however, anti-HA IgG antibody levels were significantly higher than those in the similarly or IM-inoculated HAd-H5HA

groups, or the IM-inoculated BAd-H5HA groups. The control groups inoculated IN or IM with PBS, HAd- $\Delta$ E1E3, or BAd- $\Delta$ E1E3 did not yield anti-HA IgG antibody levels above background (Figure 2).

We have shown previously that animals immunized with AdV vectors expressing HA produce high levels of hemagglutination inhibition (HI) and virus-neutralizing (VN) antibody titers against homologous influenza virus strains.<sup>16,17,25–27</sup> Therefore, here we only tried to determine HI and VN titers against an antigenically distinct influenza virus (VN/1203/RG) that was used for the challenge studies, and we noticed that none of the vaccinated groups developed any detectable HI and VN titers above the empty vector controls (data not shown). These findings were not unexpected because similar results were observed in our previous studies.<sup>17,28</sup>

To determine whether the route of immunization or the vector type will influence the levels of IgG subclasses, the serum samples collected from the mouse groups inoculated IN or IM once with  $1 \times 10^6$  (Figures 3A, 4A, and 5A),  $3 \times 10^6$  (Figures 3B, 4B, and 5B),  $1 \times 10^7$  (Figures 3C, 4C, and 5C),  $3 \times 10^7$  (Figures 3D, 4D, and 5D), or  $1 \times 10^8$  (Figures 3E, 4E, and 5E) PFUs of HAd-H5HA or BAd-H5HA were analyzed for anti-HA IgG1 (Figure 3), IgG2a



**Figure 4. HA-Specific Serum IgG2a Antibody Responses in Mice Immunized Once with BAAd-H5HA or HAAd-H5HA**

Mice were immunized intramuscularly (IM) or intranasally (IN) once with  $1 \times 10^6$  (A),  $3 \times 10^6$  (B),  $1 \times 10^7$  (C),  $3 \times 10^7$  (D), or  $1 \times 10^8$  (E) PFUs of BAAd-H5HA or HAAd-H5HA. For all dose groups, mice inoculated IM or IN with PBS or  $1 \times 10^8$  PFUs of BAAd- $\Delta$ E1E3 or HAAd- $\Delta$ E1E3 served as negative or internal controls, respectively. Four weeks after inoculation, serum samples were collected, diluted to 1:50, and the development of HA-specific IgG2a antibody responses was monitored by ELISA. Data are represented as the mean  $\pm$  SD of the optical density (OD) readings. Statistically significant responses are shown as compared with the PBS group (+), IN versus IM route of inoculation in the same group (\*), or BAAd-H5HA vector versus HAAd-H5HA vector ( $\psi$ ), significant at  $p < 0.05$ ; \*\*, significant at  $p < 0.01$ ; \*\*\*, +++, or  $\psi\psi\psi$ , significant at  $p < 0.001$ ; and \*\*\*\* or +++++, significant at  $p < 0.0001$ . The statistical analysis was done by Bonferroni post-test and two-way ANOVA using GraphPad Prism 6. BAAd- $\Delta$ E1E3, BAAd empty vector; BAAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of A/Hong Kong/156/97(H5N1) influenza virus; HAAd- $\Delta$ E1E3, HAAd empty vector; HAAd-H5HA, human adenoviral vector expressing HA of A/Hong Kong/156/97(H5N1) influenza virus; ns, no significance at  $p > 0.05$ .

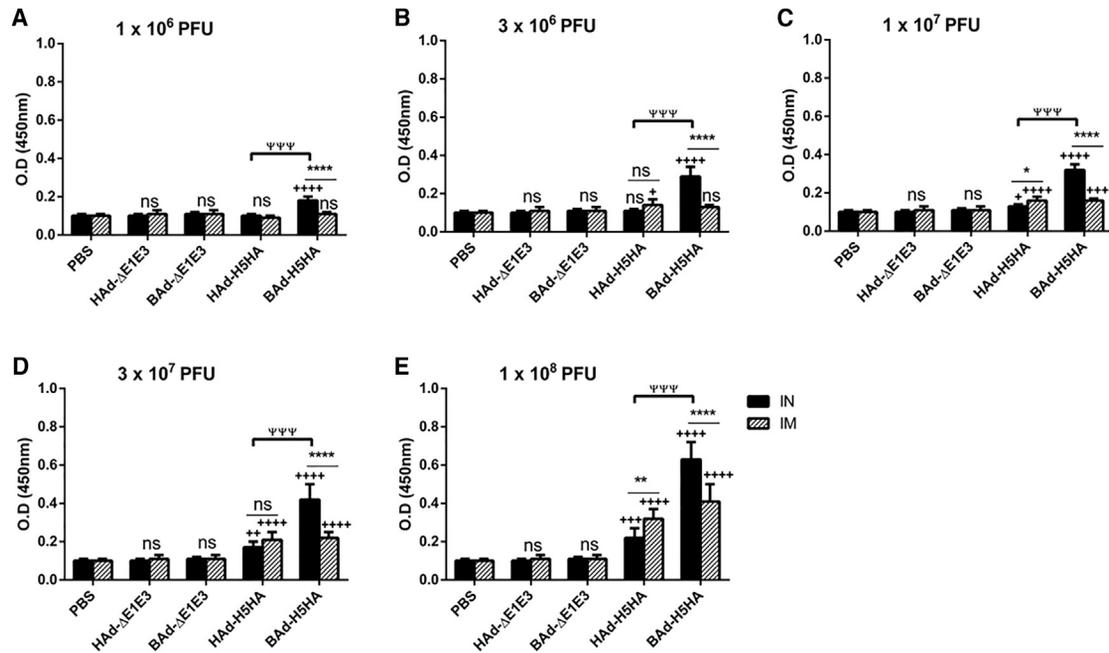
(Figure 4), and IgG2b (Figure 5) levels by ELISA, respectively. As expected, there were dose-dependent increases in anti-HA IgG1, IgG2a, and IgG2b antibody levels. These levels in the IM-inoculated HAAd-H5HA groups were similar to or slightly better or lower than those observed in the IM-inoculated BAAd-H5HA groups. However, in the IN-inoculated BAAd-H5HA groups, anti-HA IgG1, IgG2a, and IgG2b antibody levels were significantly higher than those in the IN- or IM-inoculated HAAd-H5HA groups or the IM-inoculated BAAd-H5HA groups (Figures 3, 4, and 5). Control groups inoculated IN or IM with PBS, HAAd- $\Delta$ E1E3, or BAAd- $\Delta$ E1E3 did not yield anti-HA IgG1 (Figure 3), IgG2a (Figure 4), or IgG2b (Figure 5) antibody levels above background.

Furthermore, to ascertain whether the route of immunization or the vector type will influence the development of HA-specific IgA responses at the mucosal level, the lung washes (Figure 6), as well as nasal washes (Figure 7) from the mouse groups, inoculated IN or IM once with  $1 \times 10^6$  (Figures 6A and 7A),  $3 \times 10^6$  (Figures 6B and 7B),  $1 \times 10^7$  (Figures 6C and 7C),  $3 \times 10^7$  (Figures 6D and 7D), or  $1 \times 10^8$  (Figures 6E and 7E) PFUs of HAAd-H5HA or BAAd-H5HA, were analyzed for anti-HA IgA levels by ELISA. As expected, there were dose-dependent increases in anti-HA IgA anti-

body levels in the lung washes as well as nasal washes. IgA antibody levels in the lung washes and nasal washes in the IM-inoculated BAAd-H5HA or HAAd-H5HA groups were detected only with doses of  $1 \times 10^7$  PFUs and onward (Figures 6 and 7). IgA antibody levels in the lung washes and nasal washes of the IM-inoculated BAAd-H5HA groups were similar to or slightly higher than those in the IM-inoculated HAAd-H5HA, whereas IgA antibody levels in the lung washes and nasal washes in the IN-inoculated BAAd-H5HA or HAAd-H5HA groups were detected with the lowest dose of  $1 \times 10^6$  PFU and onward. In the IN-inoculated BAAd-H5HA groups, anti-HA IgA antibody levels in the lung washes (Figure 6) and nasal washes (Figure 7) were significantly higher than those in the IN- or IM-inoculated HAAd-H5HA groups or the IM-inoculated BAAd-H5HA groups. The control groups inoculated IN or IM with PBS, HAAd- $\Delta$ E1E3, or BAAd- $\Delta$ E1E3 did not yield anti-HA IgA antibody levels above background (Figures 6 and 7).

#### Induction of Enhanced CMI Responses with BAAd-H5HA Compared with HAAd-H5HA

CMI responses against influenza viruses are important for virus clearance following infection and play an important role in heterologous as well as heterosubtypic protection against influenza viruses.<sup>17,29</sup> To



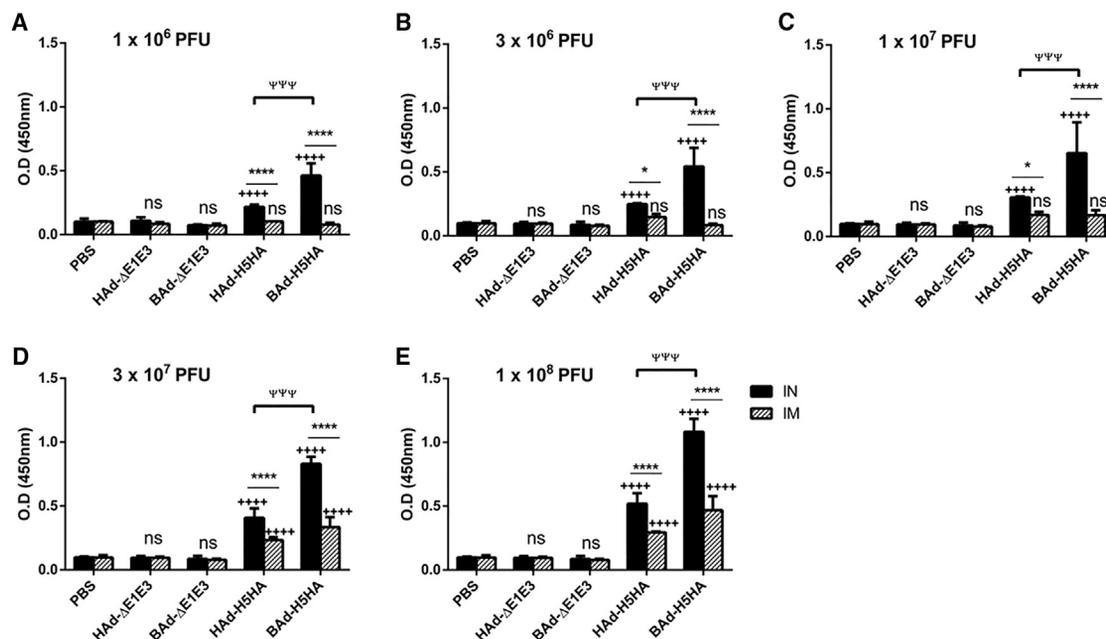
**Figure 5. HA-Specific Serum IgG2b Antibody Responses in Mice Immunized Once with BAd-H5HA or HAd-H5HA**

Mice were immunized intramuscularly (IM) or intranasally (IN) once with  $1 \times 10^6$  (A),  $3 \times 10^6$  (B),  $1 \times 10^7$  (C),  $3 \times 10^7$  (D), or  $1 \times 10^8$  (E) PFUs of BAd-H5HA or HAd-H5HA. For all dose groups, mice inoculated IM or IN with PBS or  $1 \times 10^8$  PFUs of BAd- $\Delta$ E1E3 or HAd- $\Delta$ E1E3 served as negative or internal controls, respectively. Four weeks after inoculation, serum samples were collected, diluted to 1:50, and the development of HA-specific IgG2b antibody responses was monitored by ELISA. Data are represented as the mean  $\pm$  SD of the optical density (OD) readings. Statistically significant responses are shown as compared with the PBS group (+), IN versus IM route of inoculation in the same group (\*), or BAd-H5HA vector versus HAd-H5HA vector ( $\psi$ ). \* or +, significant at  $p < 0.05$ ; \*\* or ++, significant at  $p < 0.01$ ; +++ or  $\psi\psi\psi$ , significant at  $p < 0.001$ ; and \*\*\*\* or +++, significant at  $p < 0.0001$ . The statistical analysis was done by Bonferroni post-test and two-way ANOVA using GraphPad Prism 6. BAd- $\Delta$ E1E3, BAd empty vector; BAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of A/Hong Kong/156/97(H5N1) influenza virus; HAd- $\Delta$ E1E3, HAd empty vector; HAd-H5HA, human adenoviral vector expressing HA of A/Hong Kong/156/97(H5N1) influenza virus; ns, no significance at  $p > 0.05$ .

verify whether the route of immunization or the vector type will impact the development of the CMI responses against influenza in vaccinated mice, we analyzed splenocytes (Figure 8), pooled respiratory area lymph node (RLN) cells (Figure 9), pooled inguinal lymph node (ILN) cells (Figure 10), and pooled lung lymphocytes (Figure 11) from AdV vector-inoculated groups for HA-specific CMI responses following *in vitro* stimulation with HA518 peptide using an interferon-gamma (IFN $\gamma$ )-specific enzyme-linked immunospot (ELISpot) assay. The numbers of IFN $\gamma$ -secreting HA518-specific CD8 T cells from the mouse groups inoculated IN or IM once with  $1 \times 10^6$  (Figures 8A, 9A, 10A, and 11A),  $3 \times 10^6$  (Figures 8B, 9B, 10B, and 11B),  $1 \times 10^7$  (Figures 8C, 9C, 10C, and 11C),  $3 \times 10^7$  (Figures 8D, 9D, 10D, and 11D), or  $1 \times 10^8$  (Figures 8E, 9E, 10E, and 11E) PFUs of HAd-H5HA or BAd-H5HA are shown. There were dose-dependent increases in the numbers of IFN $\gamma$ -secreting HA518-specific CD8 T cells in splenocytes (Figure 8), pooled RLN cells (Figure 9), pooled ILN cells (Figure 10), and pooled lung lymphocytes (Figure 11) of the vaccinated groups compared with the empty vector (Ad- $\Delta$ E1E3) or PBS control groups. There were significantly higher numbers of IFN $\gamma$ -secreting HA518-specific CD8 T cells in splenocytes of the IM- or IN-inoculated BAd-H5HA groups compared with the IM- or IN-inoculated HAd-H5HA groups (Figure 8), and the numbers

were consistently higher in the IM-inoculated vaccine groups compared with that of the IN-inoculated vaccine groups.

There were substantially higher numbers of IFN $\gamma$ -secreting HA518-specific CD8 T cells in the RLNs of the IM- or IN-inoculated BAd-H5HA groups compared with those of the IM- or IN-inoculated HAd-H5HA groups (Figure 9), and the numbers were consistently higher in the IN-inoculated vaccine groups compared with those of the IM-inoculated vaccine groups. Whereas in ILNs of the IM-inoculated vaccine groups, higher numbers of IFN $\gamma$ -secreting HA518-specific CD8 T cells were detected compared with those of the empty vector (Ad- $\Delta$ E1E3) or PBS control groups, or the IN-inoculated vaccine groups (Figure 10), and the numbers were consistently higher in the IM-inoculated vaccine groups compared with those of the IN-inoculated vaccine groups. Furthermore, considerably elevated numbers of IFN $\gamma$ -secreting HA518-specific CD8 T cells in the lung lymphocytes of the IM- or IN-inoculated BAd-H5HA groups compared with those of the IM- or IN-inoculated HAd-H5HA groups, respectively, were visualized (Figure 11), and the numbers were consistently higher in the IN-inoculated vaccine groups compared with those of the IM-inoculated vaccine groups.



**Figure 6. HA-Specific IgA Antibody Responses in Lung Washes of Mice Immunized Once with BAd-H5HA or HAd-H5HA**

Mice were immunized intramuscularly (IM) or intranasally (IN) once with  $1 \times 10^6$  (A),  $3 \times 10^6$  (B),  $1 \times 10^7$  (C),  $3 \times 10^7$  (D), or  $1 \times 10^8$  (E) PFUs of BAd-H5HA or HAd-H5HA. For all dose groups, mice inoculated IM or IN with PBS or  $1 \times 10^8$  PFUs of BAd-ΔE1E3 or HAd-ΔE1E3 served as negative or internal controls, respectively. Four weeks after inoculation, nasal wash samples were collected, diluted to 1:5, and the development of HA-specific IgA antibody responses was monitored by ELISA. Data are represented as the mean  $\pm$  SD of the optical density (OD) readings. Statistically significant responses are shown as compared with the PBS group (+), IN versus IM route of inoculation in the same group (\*), or BAd-H5HA vector versus HAd-H5HA vector ( $\psi$ ). \*, significant at  $p < 0.05$ ;  $\psi\psi\psi$ , significant at  $p < 0.001$ ; and \*\*\*\* or +++++, significant at  $p < 0.0001$ . The statistical analysis was done by Bonferroni post-test and two-way ANOVA using GraphPad Prism 6. BAd-ΔE1E3, BAd empty vector; BAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of A/Hong Kong/156/97(H5N1) influenza virus; HAd-ΔE1E3, HAd empty vector; HAd-H5HA, human adenoviral vector expressing HA of A/Hong Kong/156/97(H5N1) influenza virus; ns, no significance at  $p > 0.05$ .

#### Development of Enhanced Protection in Mice Immunized with BAd-H5HA Compared with HAd-H5HA

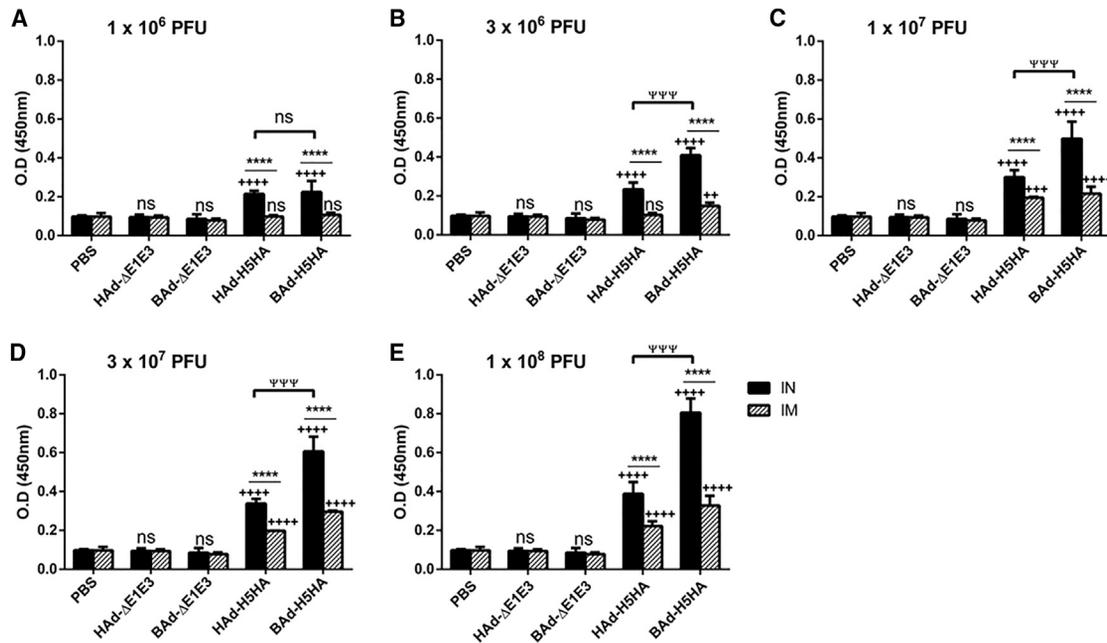
Because the influenza virus VN/1203/RG, which was used as a challenge virus to evaluate the efficacy of protection, does not cause consistent morbidity or mortality in mice,<sup>16,17</sup> significant reductions in lung viral titers in vaccinated animals following challenge are a useful measure of the virus clearance and vaccine protective efficacy. The mouse groups were immunized IN or IM once with  $1 \times 10^6$  (Figure 12A),  $3 \times 10^6$  (Figure 12B),  $1 \times 10^7$  (Figure 12C),  $3 \times 10^7$  (Figure 12D), or  $1 \times 10^8$  (Figure 12E) PFUs of HAd-H5HA or BAd-H5HA, and subsequently challenged with 100 mouse infectious dose 50 (MID<sub>50</sub>) of VN/1203/RG. For the IN route of inoculation, the lowest vaccine dose of  $1 \times 10^6$  PFUs of BAd-H5HA conferred complete protection following challenge (Figure 12A). The lowest vaccine dose for IN-inoculated HAd-H5HA that yielded complete protection following challenge was  $3 \times 10^7$  PFUs (Figure 12E). For the IM route of inoculation, the lowest vaccine dose for BAd-H5HA that conferred complete protection following challenge was  $3 \times 10^7$  PFUs (Figure 12D), but for the IM-inoculated HAd-H5HA animal group, the log lung virus titer with a dose of  $1 \times 10^8$  PFUs was  $1.84 \pm 1.14$  compared with  $4.90 \pm 0.42$  and  $4.6 \pm 0.46$  log lung virus titers in the empty vector (HAd-ΔE1E3) or PBS control groups, respectively (Figure 12E). Even at a vaccine dose of  $3 \times 10^8$  PFUs per

animal for IM-inoculated HAd-H5HA animal group, the log lung virus titer of  $0.84 \pm 0.5$  was observed following challenge (data not shown).

#### DISCUSSION

Several studies using AdV vector-based influenza vaccines have been conducted both in animal models<sup>25,26,30</sup> and as clinical trials in humans<sup>19,31</sup> to explore the potential of this vector system. Previously we have shown that AdV vector-based vaccines can provide complete protection against challenge with homologous as well as heterologous (antigenically distinct) influenza virus strains.<sup>25</sup> Moreover, AdV vector vaccines containing multiple HAs from different HA subtypes or expressing nucleoprotein (NP) of H5N1 influenza conferred either complete protection or significant reduction in lung virus titers, respectively, following challenge with pre-pandemic H5, H7, or H9 influenza viruses.<sup>16,17</sup>

The purpose of this study was to determine some of the parameters that are important for a pre-pandemic influenza vaccine including route of immunization, dose sparing, protection from a single dose, and protection against a heterologous influenza virus strain. This study was based on the hypothesis that a combination of



**Figure 7. HA-Specific IgA Antibody Responses in Nasal Washes of Mice Immunized Once with BAD-H5HA or HAd-H5HA**

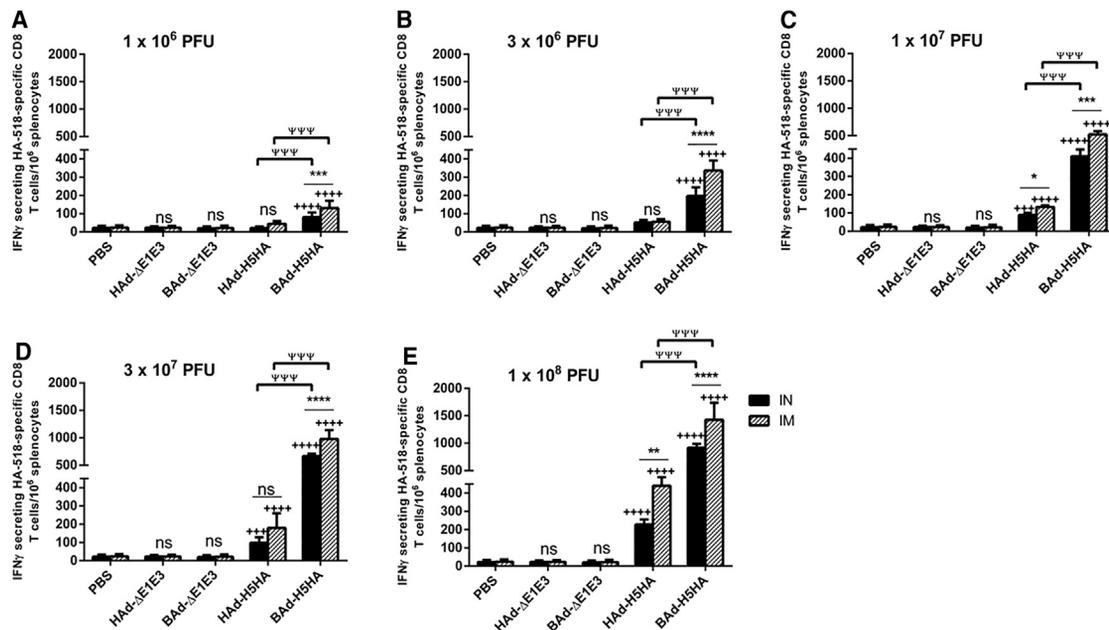
Mice were immunized intramuscularly (IM) or intranasally (IN) once with  $1 \times 10^6$  (A),  $3 \times 10^6$  (B),  $1 \times 10^7$  (C),  $3 \times 10^7$  (D), or  $1 \times 10^8$  (E) PFUs of BAD-H5HA or HAd-H5HA. For all dose groups, mice inoculated IM or IN with PBS or  $1 \times 10^6$  PFUs of BAD-ΔE1E3 or HAd-ΔE1E3 served as negative or internal controls, respectively. Four weeks after inoculation, lung wash samples were collected, diluted to 1:10, and the development of HA-specific IgA antibody responses was monitored by ELISA. Data are represented as the mean  $\pm$  SD of the optical density (OD) readings. Statistically significant responses are shown as compared with the PBS group (+), IN versus IM route of inoculation in the same group (\*), or BAD-H5HA vector versus HAd-H5HA vector ( $\psi$ ). ++, significant at  $p < 0.01$ ; +++, significant at  $p < 0.001$ ; and \*\*\*\* or +\*\*\*\*, significant at  $p < 0.0001$ . The statistical analysis was done by Bonferroni post-test and two-way ANOVA using GraphPad Prism 6. BAD-ΔE1E3, BAD empty vector; BAD-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of A/Hong Kong/156/97(H5N1) influenza virus; HAd-ΔE1E3, HAd empty vector; HAd-H5HA, human adenoviral vector expressing HA of A/Hong Kong/156/97(H5N1) influenza virus; ns, no significance at  $p > 0.05$ .

HA-specific CMI responses and cross-reactive (although not necessarily cross-neutralizing) humoral immune responses will provide heterologous protection against an antigenically distinct H5N1 influenza virus. In addition, this study also focused on determining the vector type and route of immunization for enhanced immunogenicity conferring efficient protection.

Overall, there were no significant differences in the serum anti-HA IgG, IgG1, IgG2a, and IgG2b or mucosal IgA responses in mouse groups inoculated IM with either BAD-H5HA or HAd-H5HA. However, mice inoculated with BAD-H5HA had higher numbers of IFN $\gamma$ -secreting HA518-specific CD8 T cells in the spleen, lung, and RLNs than the groups inoculated with HAd-H5HA. In contrast, there were no significant differences in the numbers of IFN $\gamma$ -secreting HA518-specific CD8 T cells in the ILNs of the mouse groups inoculated IM with either BAD-H5HA or HAd-H5HA. An intravenous bio-distribution study in mice has demonstrated that the BADV vector genome persists longer and with higher copy numbers in the spleen and lungs than that of the HADV vector.<sup>12</sup> Similar bio-distribution following the IM route of immunization may be responsible for the higher level of CMI responses with the BAD-H5HA vector. Additional studies will be required to determine the mechanism(s) of these observations.

The HK/156 HA expressed in the AdV vectors, and the HA in the challenged virus VN/1203/RG are antigenically different, so it was not surprising that the serum samples from either BAD-H5HA- or HAd-H5HA-inoculated animal groups did not have detectable levels of HI or VN titers against the challenge virus. We purposely opted for antigenically distinct HAs as an immunogen and a challenge virus to test our hypothesis that a combination of CMI responses and non-neutralizing antibodies will provide heterologous protection. The mouse group inoculated IM with  $3 \times 10^7$  PFUs of BAD-H5HA were completely protected from the challenge with VN/1203/RG, whereas detectable levels ( $0.84 \pm 0.5$  log) of lung virus titers were observed in the mouse group IM-inoculated with  $3 \times 10^8$  PFUs of HAd-H5HA. These observations suggest that significantly higher levels of CMI responses and non-neutralizing antibodies elicited with BAD-H5HA may be responsible for enhanced protection at a significantly lower dose.

In the mouse groups inoculated IN with BAD-H5HA, levels of serum anti-HA IgG, IgG1, IgG2a, and IgG2b, and levels of mucosal anti-HA IgA were significantly higher than the groups inoculated IM with BAD-H5HA or inoculated IM or IN with HAd-H5HA. Also, the numbers of IFN $\gamma$ -secreting HA518-specific CD8 T cells in the spleen, lung, and RLNs were much higher in BAD-H5HA groups than the



**Figure 8. HA518 Epitope-Specific IFN $\gamma$ -Secreting CD8<sup>+</sup> T Cells in the Spleens of Mice Immunized Once with BAd-H5HA or HAd-H5HA**

Mice were immunized intramuscularly (IM) or intranasally (IN) once with  $1 \times 10^6$  (A),  $3 \times 10^6$  (B),  $1 \times 10^7$  (C),  $3 \times 10^7$  (D), or  $1 \times 10^8$  (E) PFUs of BAd-H5HA or HAd-H5HA. For all dose groups, mice inoculated IM or IN with PBS or  $1 \times 10^6$  PFUs of BAd- $\Delta$ E1E3 or HAd- $\Delta$ E1E3 served as negative or internal controls, respectively. Four weeks after inoculation, the spleens were collected, and the splenocytes were evaluated for HA-specific cell-mediated immune responses using IFN $\gamma$ -ELISpot assay. The data represent mean  $\pm$  SD of the number of spot-forming units (SFUs). Statistically significant responses are shown as compared with the PBS group (+), IN versus IM route of inoculation in the same group (\*), or BAd-H5HA vector versus HAd-H5HA vector ( $\psi$ ). \*, significant at  $p < 0.05$ ; \*\*, significant at  $p < 0.01$ ; \*\*\*, +, or  $\psi\psi\psi$ , significant at  $p < 0.001$ ; and \*\*\*\* or +++++, significant at  $p < 0.0001$ . The statistical analysis was done by Bonferroni post-test and two-way ANOVA using GraphPad Prism 6. BAd- $\Delta$ E1E3, BAd empty vector; BAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of A/Hong Kong/156/97(H5N1) influenza virus; HAd- $\Delta$ E1E3, HAd empty vector; HAd-H5HA, human adenoviral vector expressing HA of A/Hong Kong/156/97(H5N1) influenza virus; ns, no significance at  $p > 0.05$ .

HAd-H5HA groups. Additional experiments are required to determine whether the high levels of humoral (systemic and mucosal) and CMI responses are due to better transduction and/or the levels and duration of persistence of the BAdV vector genomes following immunization with BAd-H5HA. Because BAdV-3 utilizes the  $\alpha(2,3)$ -linked and  $\alpha(2,6)$ -linked sialic acid receptors as the major receptors for virus internalization,<sup>15</sup> whereas HAdV-C5 uses CAR<sup>32–34</sup> for virus entry in the susceptible cells, we expected that BAd-H5HA will better transduce the respiratory tract following IN inoculation compared with HAd-H5HA. Moreover, the levels and duration of persistence of the BAdV vector genome copy numbers in the lungs were found to be significantly higher than that of the HAdV vector in an intravenous bio-distribution study.<sup>12</sup> There is a possibility that a similar situation may occur following the IN inoculation.

Complete protection from challenge with an antigenically distinct H5N1 influenza virus VN/1203/RG was observed even in the mouse group inoculated IN with  $1 \times 10^6$  PFUs of BAd-H5HA, whereas a similar level of protection with HAd-H5HA was obtained only with a much higher vector dose of  $3 \times 10^7$  PFUs. These observations suggest that BAdV vector-based IN vaccine delivery system has considerable promise for dose sparing because an approximately 30-fold lower vaccine dose of BAd-H5HA compared with HAd-H5HA conferred

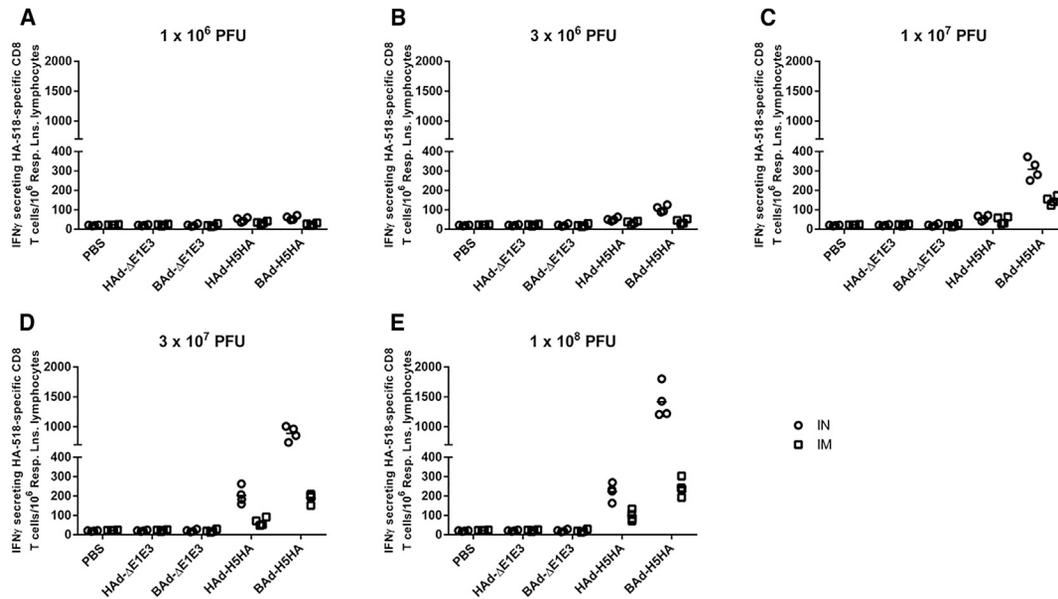
complete protection. Dose sparing not only lowers vaccine costs, but also increases the capacity to produce a large number of doses especially in an event similar to the influenza pandemic. Of course, additional studies will be needed to determine the long-term efficacy of cross-protective humoral and CMI responses induced by BAdV-3-based vectors.

The results described in this manuscript suggest that the BAdV-3-based vector system has many advantages over HAdV systems as a vaccine delivery vehicle for developing pre-pandemic influenza vaccines. Further studies in another animal model of influenza, such as ferrets, will be essential to fully explore the potential of this vaccine delivery system. Additional studies will be required to determine the best combinational antigens or immunogenic domains that could elicit broadly cross-protective immune responses when delivered through the BAdV vector system for developing a universal influenza vaccine for pandemic preparedness and for offering a better vaccine option against seasonal influenza viruses.

## MATERIALS AND METHODS

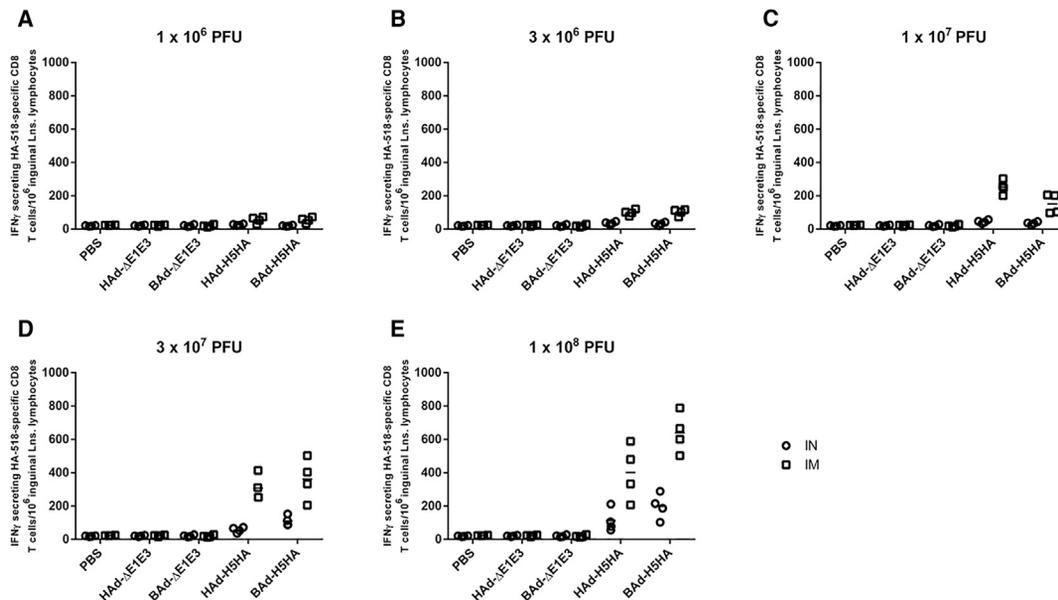
### Cell Lines, AdV Vectors, and Influenza Viruses

BHH3 (bovine-human hybrid clone 3),<sup>35</sup> BHH2C (bovine-human hybrid clone 2C),<sup>35</sup> 293 (human embryonic kidney cells expressing



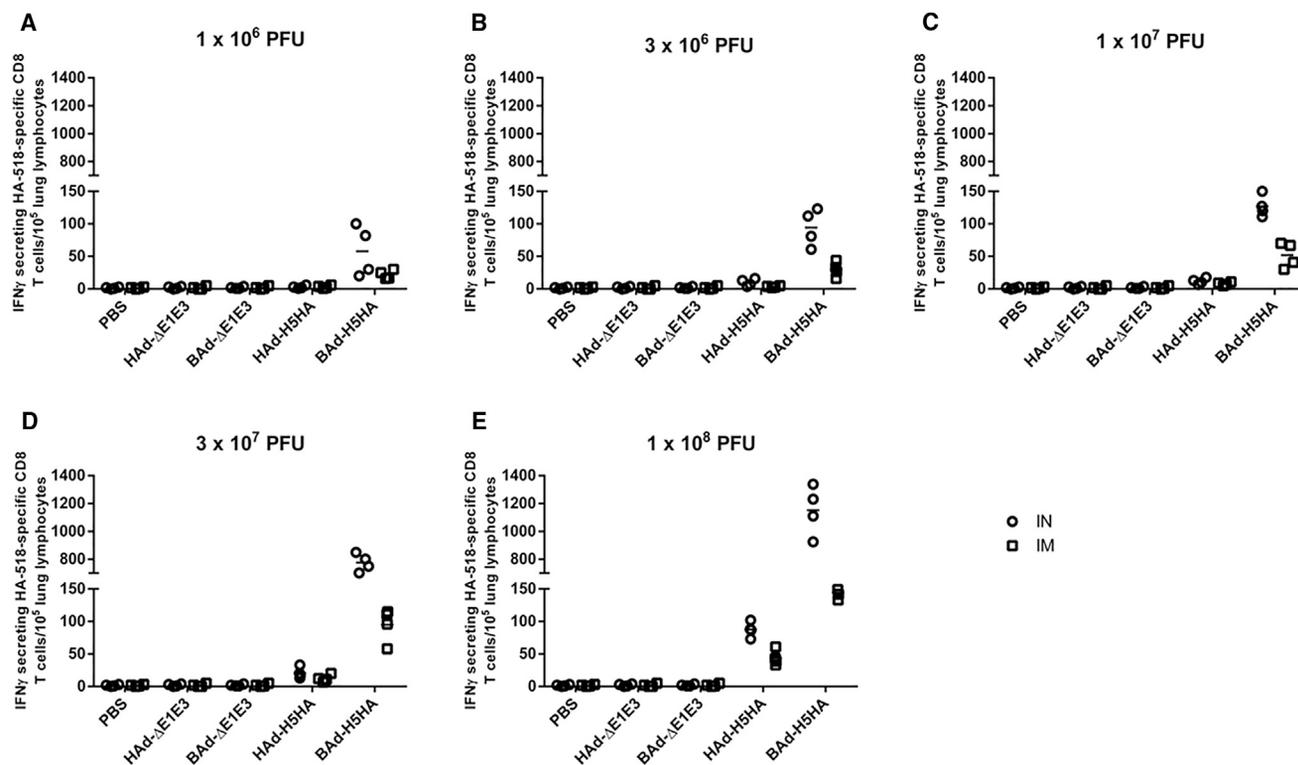
**Figure 9. HA518 Epitope-Specific IFN $\gamma$ -Secreting CD8<sup>+</sup> T Cells in the Respiratory Lymph Nodes (RLNs) of Mice Immunized Once with BAD-H5HA or HAd-H5HA**

Mice were immunized intramuscularly (IM) or intranasally (IN) once with 1 x 10<sup>6</sup> (A), 3 x 10<sup>6</sup> (B), 1 x 10<sup>7</sup> (C), 3 x 10<sup>7</sup> (D), or 1 x 10<sup>8</sup> (E) PFUs of BAD-H5HA or HAd-H5HA. For all dose groups, mice inoculated IM or IN with PBS or 1 x 10<sup>8</sup> PFUs of BAd- $\Delta$ E1E3 or HAd- $\Delta$ E1E3 served as negative or internal controls, respectively. Four weeks after inoculation, the RLNs were collected, and the pooled RLN cells were evaluated for HA-specific cell-mediated immune responses using the IFN $\gamma$ -ELISpot assay. The data represent the individual replicate numbers of spot-forming units (SFUs) from pooled samples. BAd- $\Delta$ E1E3, BAd empty vector; BAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of A/Hong Kong/156/97(H5N1) influenza virus; HAd- $\Delta$ E1E3, HAd empty vector; HAd-H5HA, human adenoviral vector expressing HA of A/Hong Kong/156/97(H5N1) influenza virus.



**Figure 10. HA518 Epitope-Specific IFN $\gamma$ -Secreting CD8<sup>+</sup> T Cells in the Inguinal Lymph Nodes (ILNs) of Mice Immunized Once with BAD-H5HA or HAd-H5HA**

Mice were immunized intramuscularly (IM) or intranasally (IN) once with 1 x 10<sup>6</sup> (A), 3 x 10<sup>6</sup> (B), 1 x 10<sup>7</sup> (C), 3 x 10<sup>7</sup> (D), or 1 x 10<sup>8</sup> (E) PFUs of BAD-H5HA or HAd-H5HA. For all dose groups, mice inoculated IM or IN with PBS or 1 x 10<sup>8</sup> PFUs of BAd- $\Delta$ E1E3 or HAd- $\Delta$ E1E3 served as negative or internal controls, respectively. Four weeks after inoculation, the ILNs were collected, and the pooled ILN cells were evaluated for HA-specific cell-mediated immune responses using IFN $\gamma$ -ELISpot assay. The data represent the individual replicate numbers of spot-forming units (SFUs) from pooled samples. BAd- $\Delta$ E1E3, BAd empty vector; BAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of A/Hong Kong/156/97(H5N1) influenza virus; HAd- $\Delta$ E1E3, HAd empty vector; HAd-H5HA, human adenoviral vector expressing HA of A/Hong Kong/156/97(H5N1) influenza virus.



**Figure 11. HA518 Epitope-Specific IFN $\gamma$ -Secreting CD8 $^+$  T Cells in the Lungs of Mice Immunized Once with BAd-H5HA or HAAd-H5HA**

Mice were immunized intramuscularly (IM) or intranasally (IN) once with  $1 \times 10^6$  (A),  $3 \times 10^6$  (B),  $1 \times 10^7$  (C),  $3 \times 10^7$  (D), or  $1 \times 10^8$  (E) PFUs of BAd-H5HA or HAAd-H5HA. For all dose groups, mice inoculated IM or IN with PBS or  $1 \times 10^6$  PFUs of BAd- $\Delta$ E1E3 or HAAd- $\Delta$ E1E3 served as negative or internal controls, respectively. Four weeks after inoculation, the lungs were collected, and the pooled lung lymphocytes were evaluated for HA-specific cell-mediated immune responses using IFN $\gamma$ -ELISpot assay. The data represent the individual replicate numbers of spot-forming units (SFUs) from pooled samples. BAd- $\Delta$ E1E3, BAd empty vector; BAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of A/Hong Kong/156/97(H5N1) influenza virus; HAAd- $\Delta$ E1E3, HAAd empty vector; HAAd-H5HA, human adenoviral vector expressing HA of A/Hong Kong/156/97(H5N1) influenza virus.

HAAdV-C5 E1 proteins),<sup>36</sup> and MDCK (Madin-Darby canine kidney) cell lines were propagated as monolayer cultures in minimum essential medium (MEM) (Life Technologies from Thermo Fisher Scientific, Waltham, MA, USA) containing either 10% reconstituted fetal bovine serum or fetal calf serum (Hyclone from Thermo Fisher Scientific) and gentamycin (50  $\mu$ g/mL).

The construction and characterization of BAd-H5HA [BAdV-3 E1 and E3 deleted vector expressing HA of A/Hong Kong/156/97(H5N1) (HK/156)],<sup>10</sup> BAd- $\Delta$ E1E3 (BAdV-3 E1 and E3 deleted empty vector),<sup>11</sup> HAAd-H5HA [HAAdV-C5 E1 and E3 deleted vector expressing HA of HK/156],<sup>25</sup> and HAAd- $\Delta$ E1E3 (HAAdV-C5 E1 and E3 deleted empty vector)<sup>37</sup> have been described previously. BAd-H5HA and BAd- $\Delta$ E1E3 were grown and titrated in BHH3 cells as described previously,<sup>10</sup> and HAAd-H5HA and HAAd- $\Delta$ E1E3 were grown in 293 cells and titrated in BHH2C cells as described previously.<sup>17</sup> These vectors were purified by cesium chloride density gradient ultracentrifugation as described previously.<sup>38</sup>

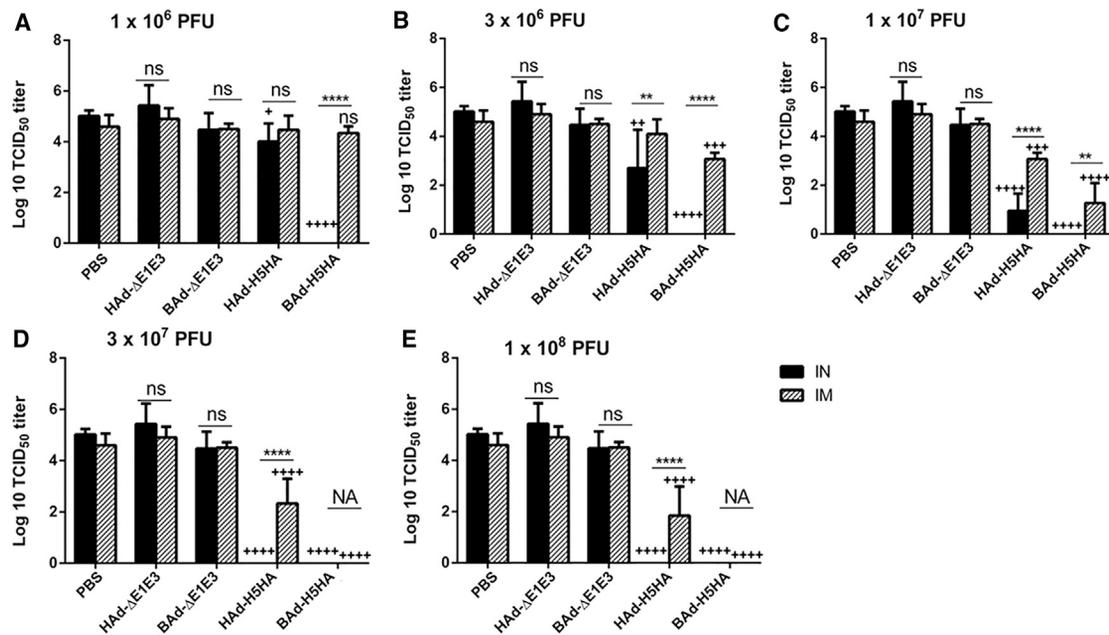
A/Vietnam/1203/2004(H5N1)-PR8/CDC-RG [VN/1203/RG] that was created by reverse genetics (RG) in the A/PR/8/1934(H1N1)

[PR8] background with a deletion in the polybasic cleavage site of the HA gene segment was grown in embryonated hen eggs and titrated in the eggs and/or MDCK. The HA gene in the vaccine vectors was from HK/156, which is antigenically distinct from the HA gene in the challenge virus VN/1203/RG.

#### Immunogenicity and Protection Studies in Mice

All animal experiments were conducted following the approvals and guidelines of the Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee (IBC). All immunization and protection studies in mice were performed in a US Department of Agriculture (USDA)-approved BSL-2 $^+$  facility with the approvals of the IACUC and the IBC. Six-to-eight-week-old BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN, USA) served as the subjects for immunization and protection studies following approved guidelines.

The mouse groups (10 animals/group) were mock inoculated with PBS (pH 7.2) or inoculated IN or IM with  $1 \times 10^6$ ,  $3 \times 10^6$ ,  $1 \times 10^7$ ,  $3 \times 10^7$ , or  $1 \times 10^8$  PFUs of BAd-H5HA or HAAd-H5HA. The mouse groups inoculated IN or IM with



**Figure 12. Lung Influenza Virus Titers in Mice Immunized Once with BAΔ-H5HA or HAΔ-H5HA**

Mice were immunized intramuscularly (IM) or intranasally (IN) once with  $1 \times 10^6$  (A),  $3 \times 10^6$  (B),  $1 \times 10^7$  (C),  $3 \times 10^7$  (D), or  $1 \times 10^8$  (E) PFUs of BAΔ-H5HA or HAΔ-H5HA. For all dose groups, mice inoculated IM or IN with PBS or  $1 \times 10^6$  PFUs of BAΔ-E1E3 or HAΔ-E1E3 served as negative or internal controls, respectively. Four weeks after immunization, mice were challenged with 100 MID<sub>50</sub> of A/Vietnam/1203/2004(H5N1)-PR8/CDC-RG influenza virus. Three days after the challenge, mice were euthanized and the lungs were collected to determine lung virus titers. The data are shown as mean Log<sub>10</sub> TCID<sub>50</sub> ± SD, and the detection limit was 0.5 Log<sub>10</sub> TCID<sub>50</sub>/mL. Statistically significant responses are shown as compared with the PBS group (+) or IN versus IM route of inoculation in the same group (\*). +, significant at  $p < 0.05$ ; \*\*, significant at  $p < 0.01$ ; +++, significant at  $p < 0.001$ ; and \*\*\*\* or +\*\*\*\*, significant at  $p < 0.0001$ . The statistical analyses were done by Bonferroni post-test and two-way ANOVA using GraphPad Prism 6. BAΔ-E1E3, BAΔ empty vector; BAΔ-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of A/Hong Kong/156/97(H5N1) influenza virus; HAΔ-E1E3, HAΔ empty vector; HAΔ-H5HA, human adenoviral vector expressing HA of A/Hong Kong/156/97(H5N1) influenza virus; NA, not applicable; ns, no significance at  $p > 0.05$ .

$1 \times 10^8$  PFUs of BAΔ-E1E3 or HAΔ-E1E3 served as vector controls. Four weeks post-inoculation, five animals per group were anesthetized with 50 μL ketamine-xylazine solution (90 mg/kg ketamine and 10 mg/kg xylazine in PBS) by intraperitoneal injections, the blood samples were collected via retro-orbital puncture, nasal washes were collected by washing the nasal passage with 0.5 mL of PBS, and the lung washes were prepared after homogenizing one lung from each animal in 1 mL of PBS as described previously.<sup>39</sup> The serum samples, nasal washes, and lung washes were used to evaluate the humoral immune responses. The second lung lobe was processed to collect CD3<sup>+</sup> T cells from the lung cells using MagniSort Mouse CD3 Positive Selection Kit following the manufacturer's instructions (Affymetrix eBioscience, San Diego, CA, USA) and used to monitor CMI responses. The spleens, RLNs, and ILNs were also collected to evaluate CMI responses.

The remaining five animals per group were challenged IN with 100 MID<sub>50</sub> of VN/1203/RG. Three days post-challenge, the animals were euthanized under ketamine-xylazine anesthesia as described earlier, and the lungs were collected for determination of the lung virus titers as described previously.<sup>17</sup>

### Immunoblotting

HA expression levels in BHH3 cells infected with either HAΔ-H5HA or BAΔ-H5HA were determined by immunoblotting following a previously described protocol.<sup>10,40</sup> An H5 HA-specific polyclonal rabbit antibody was used in immunoblotting.

### ELISA

ELISA was performed as described earlier.<sup>41,42</sup> 96-well ELISA plates (eBioscience) were coated with purified HA protein (0.5 μg/mL) of HK/156 (MyBioSource, San Diego, CA, USA) and incubated overnight at 4°C. Following blocking with 1% BSA in PBS, diluted serum samples (1:500 dilution for IgG and IgG1 and 1:50 for IgG2a and IgG2b), 1:5 diluted nasal washes, or 1:10 diluted lung washes were added and incubated at room temperature for 2 hr. During the standardization process, various dilutions of each type of sample were tested to establish the best dilution. The horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b, or IgA antibodies (Invitrogen from Thermo Fisher Scientific) at a recommended dilution for each antibody was added and incubated at room temperature for 2 hr. The color development was obtained with a BD OptEIA ELISA TMB substrate (Thermo Fisher Scientific). The reaction was stopped with a 2N sulfuric acid solution, and the

optical density readings were obtained at 450 nm using a SpectraMax i3x microplate reader (Molecular Devices, Sunnyvale, CA, USA).

### ELISpot Assays

The ELISpot assays were performed as described previously.<sup>25</sup> The splenocytes, lung lymphocytes, RLNs, and ILNs were used for IFN $\gamma$  ELISpot assays after stimulating the cells with HA518 (IYSTVASSL) peptide (H-2K<sup>d</sup>-restricted CTL epitope for HA). The number of spot-forming units (SFUs) was counted using AID ELISpot reader 8.0 (Autoimmun Diagnostika, Germany).

### Statistical Analyses

One- and two-way ANOVAs with Bonferroni post-test were performed to determine statistical significance. The statistical significance was set at  $p < 0.05$ .

### AUTHOR CONTRIBUTIONS

Conceptualization, S.K.M., S.S.; Data Curation, E.E.S., A.O.H., R.K., S.V.V., W.C.; Formal Analysis, E.E.S., A.O.H., S.S., S.K.M.; Funding Acquisition, S.K.M., S.S., I.Y.; Investigation, E.E.S., A.O.H., R.K., W.C., I.Y., S.G., S.S., S.K.M.; Methodology, E.E.S., A.O.H., R.K., W.C.; Project Administration, S.K.M., S.S.; Resources, S.K.M., S.S., I.Y., S.G.; Supervision, S.K.M., S.S.; Validation, E.E.S., A.O.H., R.K., W.C., I.Y., S.G., S.S., S.K.M.; Writing – Original Draft: E.E.S., S.K.M.; Writing – Review and Editing: E.E.S., R.K., A.O.H., W.C., I.Y., S.G., S.S., S.K.M.

### CONFLICTS OF INTEREST

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention, U.S. Department of Health and Human Services. None of the authors have any conflicts of interest.

### ACKNOWLEDGMENTS

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