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Research paper

Human antigen presenting cells stimulated with *Salmonella* delivered influenza antigens induce cytokine production and proliferation of human $CD4^+$ T cells *in vitro*

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ABSTRACT

This study aimed to investigate whether the human antigen presenting cells (APCs) can process and present *Salmonella* expressing H7N9 hemagglutinin (Sal-HA), neuraminidase (Sal-NA) or M2 ectodomain (Sal-M2e) to T cells and subsequently activate $CD4^+$ T cell responses *in vitro*. In this study, APCs generated from human peripheral blood mononuclear cells (PBMCs) were first treated with mitomycin-C, followed by stimulation with Sal-HA, Sal-M2e, Sal-NA or *Salmonella* alone for 24 h. Subsequently, stimulated APCs were coincubated with untreated PBMCs (1:10) of the same individual for 24 or 72 h and then analysed for cytokine induction and T cell proliferations by qRT-PCR assay and flow cytometry, respectively. Our results demonstrated that APCs stimulated with Sal-HA, Sal-M2e or Sal-NA induced significantly ($p < .05$) higher $CD3^+CD4^+$ T cell proliferations compared to the APCs treated with *Salmonella* alone. Our data further revealed that APCs treated with Sal-HA induced significantly ($p < .05$) higher $CD3^+CD4^+$ T cell responses compared to the APCs treated with either Sal-M2e or Sal-NA, which both induced almost comparable levels. The T cell proliferation responses were further measured by lymphocyte proliferation assay and the results showed that Sal-HA and Sal-M2e stimulated APCs induced significantly ($p < .05$) higher proliferations in T cells compared to the APCs stimulated with either Sal-NA or *Salmonella* alone. With respect to cytokine inductions, APCs treated with either Sal-HA or Sal-M2e induced significantly ($p < .05$) higher mRNA transcription levels of proinflammatory (IL-1β, IL-6, IL-12 and IL-23), Th1 (IFN-γ), Th17 (IL-17 and IL-21) and Th2 (IL-10 and TGF-β) cytokines in T cells compared to Sal-NA or *Salmonella* alone treated APCs. In conclusion, we show that *Salmonella* system can efficiently deliver vaccine antigens to APCs and is, thus, capable to elicit heterologous antigen-specific adaptive immunity.

1. Introduction

Influenza is a highly contagious, respiratory tract infection that causes substantial morbidity and mortality in both animal and human population. The disease is caused by Influenza A viruses which generally cause yearly epidemics and, potentially, pandemics when an influenza virus with a novel antigenically shifted hemagglutinin (HA) emerges in a population resulting in high morbidity and mortality worldwide (Martin Petric, Lorraine Comanor, 2006). The induction of neutralizing antibodies against the viral surface glycoproteins, HA and neuraminidase (NA), are primarily responsible for mediating host protection against the influenza infections. The protective role conferred by neutralizing antibodies has traditionally been limited to homosubtypic strains rather than the heterologous strains (Sridhar, 2016). However, broadly reactive neutralizing antibodies either against the HA stalk region or the extracellular domain of M2 (M2e) are capable of providing heterosubtypic protection in animal models (Deng et al., 2015; Kim et al., 2014; Steel et al., 2010; Wang et al., 2012), but the protective role played by these antibodies remains to be elucidated in humans during epidemic and/or pandemic situations. The heterosubtypic influenza immunity in animals and humans is mainly mediated by T cells, which reduce viral shedding and disease severity during influenza infections (Schotsaert et al., 2010; Ulmer et al., 1998). The T cell responses involved in conferring antiviral immunity can include both $CD4^+$ and $CD8^+$ cells, often through the action of secreted cytokines and cytolytic proteins, respectively (Ulmer et al., 1998). The role of $CD8^+$ T cells in providing antiviral immunity is well documented and clear; however, the role of $CD4^+$ T cells in mediating heterosubtypic immunity is less clear (Sridhar, 2016). Nevertheless, several studies demonstrate that $CD4^+$ T cells facilitate heterosubtypic immunity

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either through the direct cytolytic activity or indirectly by providing the help to B cells or $CD8^+$ T-cells (Brown et al., 2006, 2012). Certain types of vaccines, including subunit proteins and whole inactivated organisms, generally induce $CD4^+$ T-cell responses, but not $CD8^+$ cytotoxic T cell immunity, while live attenuated vaccines can induce both types of responses (Ulmer et al., 1998). Previously, we have shown that live attenuated *Salmonella* based H7N9 HA vaccine can induce heterologous protection and is capable of inducing both humoral and cell mediated immune (CMI) responses in chickens (Hyoung et al., 2017). In the present study, we investigated the potential of this *Salmonella*-based influenza vaccine to stimulate human T cells *in vitro*. H7N9 virus is a low pathogenic avian influenza virus which has been identified as an unusually dangerous virus for humans (Shadbolt, 2017). The H7N9 virus possess characteristic features related to the human adaptation, for instance mutations in the HA and PB2 proteins, which facilitate the virus binding to and replicating in the human respiratory tract (Gao et al., 2013; Yiu Lai et al., 2013), indicating that H7N9 virus has pandemic potential. Although whole virus inactivated H7N9 vaccine provides complete protection against the lethal H7N9 challenge in mice (Tang et al., 2014); however, these inactivated influenza vaccines require a large supply of specific-pathogen free (SPF) embryonated eggs and a long timeline that would not be feasible to use during an influenza pandemic situation affecting both animal and human population. Therefore, novel approaches are urgently needed that should allow easier manipulation and faster production of efficient vaccines. *Salmonella*-based influenza vaccines are highly economical and allow for a quick response to novel influenza viruses, as it circumvents the need for SPF embryonated eggs required for the production of conventional influenza vaccines.

In the present study, as a step forward toward the development of a *Salmonella*-based human influenza vaccine, we investigated if human antigen presenting cells (APCs), dendritic cells (DCs), from healthy adults exposed to the *Salmonella* delivered H7N9 HA, NA or M2e can process and present to human T cells and subsequent activation of $CD4^+$ T cells *in vitro*. As far as we know, there is no previous report in the literature of studies investigating the ability of human DCs to process and present *Salmonella* delivered influenza antigens to T cells *in vitro*. We show that human DCs efficiently process and present *Salmonella* delivered H7N9 HA, M2e or NA antigens to T cells and induce cytokine production and $CD4^+$ T cell responses *in vitro*.

2. Material and methods

2.1. Virus, bacteria and cell lines

H7N9 virus maintained at the Chonbuk National University, South Korea was propagated in the allantoic cavities of 9–10 days old SPF embryonated eggs at 37 °C. The virus was titered in Madin Darby Canine Kidney (MDCK) cell line as described previously (Hyoung et al., 2017). The attenuated *Salmonella* Typhimurium expressing H7N9 HA (Sal-HA), four tandem copies of M2e (Sal-M2e) or NA (Sal-NA) was cultured in Luria Bertani (LB) broth as described elsewhere (Hajam and Lee, 2017; Kim et al., 2017; Kim et al., 2018). The A549 human lung cell line used for the virus neutralization assay was cultured in DMEM media with 10% fetal bovine serum (FBS) and antibiotics, penicillin and gentamicin.

2.2. Visualization of virus neutralizing ability of vaccinated sera

Virus neutralizing ability of the vaccinated chicken sera against H7N9 virus was visualized by an IncuCyte® ZOOM System (Essen Bioscience, IncuCyte Zoom, USA). We tested vaccinated sera obtained from our previous chicken experiment wherein birds were vaccinated with *Salmonella*-HA based vaccine (Hyoung et al., 2017). The complement inactivated sera (50 µl, $n = 5$) were incubated with 100$TCID_{50}$ of H7N9 virus for 30 min. Thereafter, the incubated mixture was added to the cell monolayers of A549 human lung cells and the cells were further cultured at 37 °C for 48 h in a humidified chamber containing 5% CO_2. Virus, serum and cell controls were included in each test. After 48 h of incubation, the cells were examined under microscope for any cytopathic effects.

2.3. Preparation of human APCs and stimulation with Salmonella delivered influenza antigens

The human PBMCs (Redcross, Korea; $n = 3$) were used to prepared APCs for presentation of *Salmonella* delivered H7N9 influenza antigens to T cells *in vitro*. The PBMCs (10×10^6/ml) were stored in liquid nitrogen in complete RPMI media, 40% fetal calf serum and 10% DMSO. *Salmonella* expressing *and* delivering HA, M2e or NA was constructed as previously described and reported elsewhere (Hajam and Lee, 2017; Kim et al., 2017; Kim et al., 2018). To prepare human APCs, PBMCs (3×10^6/well) were cultured in 6 well plates in complete RPMI media containing 10% FBS and antibiotics at 37 °C in 5% CO2. The cells were treated with a mixture of IL-4 (20 ng/ml) and GM-CSF (40 ng/ml), and after every three days half of the media was replaced with a fresh media containing equivalent concentrations of IL-4 () and GM-CSF (). On day 7, the cultured cells were collected, washed thrice with RPMI media and then treated with mitomycin-C (100 µg/ml) for 30 min at 37 °C in 5% CO2. Thereafter, the cells were washed twice with RPMI media and the mitomycin-C treated APCs were infected with Sal-HA, Sal-M2e, Sal-NA or *Salmonella* alone for 1 h at 37 °C. The 10 colony forming units (CFU) per cell were used for infection in this study. Uninfected mitomycin-C treated APCs were kept as controls. After 1 h incubation, the cells were washed thrice with sterile phosphate buffered saline (PBS) and then further cultured for 24 h in complete RPMI media with 10% FBS and antibiotics. Thereafter, the cells were collected, washed twice with RPMI media and then coincubated with untreated PBMCs (1: 10 ratio) of the same individual for either 24 or 72 h for analysis of cytokine and T cell proliferation responses, respectively.

2.4. Analysis of T cell proliferation

For analysis of T cell proliferation, APCs infected with either *Salmonella* delivered influenza antigens or *Salmonella* alone were coincubated with the untreated PBMCs (1:10 ratio) of the same individual for 72 h in complete RPMI media containing 10% FBS and antibiotics. Subsequently, the cells were harvested and analysed for the surface $CD3^+$ and $CD3^+CD4^+$ expression markers by flow cytometry (Won et al., 2017). The staining for surface cell CD3 and CD4 markers was performed with PE anti-human CD3 (#130109461) and PerCP-Vio@ 700 labelled CD4 (130113228) antibodies as per the manufacturer's instructions. The percentage of $CD4^+$ T cell population was estimated in the $CD3^+$ gated cells and the results were expressed in percentage of cells showing T cell proliferation. All the antibodies used in this study were purchased from the Miltenyi Biotech, Germany.

The capacity of *Salmonella*-treated APCs to stimulate human T cell proliferation was further assessed by a MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide]-based assay. Briefly, APCs treated with either *Salmonella* delivered influenza antigens or *Salmonella* alone were coincubated with the untreated PBMCs (1:10 ratio) of the same individual in complete RPMI media with 10% FBS and antibiotics at 37 °C in 5% CO2 for 72 h. Thereafter the media was removed and the cells were washed thrice with PBS, followed by the addition of a MTT dye (0.5 mg/ml) for 4 h at 37 °C. The MTT dye was removed and dimethyl sulfoxide solution was added and the plate was further incubated at 37 °C for 30 min. The colorimetric reaction was read at 570 nm with a reference filter at 650 nm in the micro-plate reader.

2.5. Analysis for proinflammatory, Th1 and Th2 cytokine responses by qRT-PCR assay

For analysis of cytokine responses, APCs treated with either *Salmonella* delivered influenza antigens or *Salmonella* alone were coincubated with the untreated PBMCs ($n = 3$; 1:10 ratio) of the same individual for 24 h. Cells were harvested and total RNA isolated from APCs and T cell comixtures was analysed for IL-1β, IL-6, IL-10, IL-12, IL-17, IL-21, IL-23, IFN-γ, TNF-α and TGF-β gene expressions (Supplementary Table 1) by qRT-PCR assay as described previously (Kim et al., 2017).

2.6. Statistical analysis

Statistical analysis was done using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA). One way analysis of variance (ANOVA) was applied to calculate statistical differences. The FACS data were analysed using FlowJo software (Treestar, Inc., San Carlos, CA). $p < .05$ were considered statistically significant.

3. Results

3.1. Vaccinated sera efficiently neutralized H7N9 virus in vitro

Virus neutralization (VN) test reflects the level of functional antibodies that confer protective immunity against influenza infections (Chen et al., 2008). To investigate the ability of sera drawn from the vaccinated chickens at 28th day post-vaccination (Hyoung et al., 2017), vaccinated sera (50 µl) and H7N9 virus ($100TCID_{50}$) mixture incubated at 37 °C for 30 min was applied to the cell monolayers of human lung cells and the cells were further incubated at 37 °C for 48 h. As shown in Fig. 1, the vaccinated sera efficiently neutralized H7N9 virus *in vitro* as indicated by the lack of the cytopathic effects (CPE) (Fig. 1B) in lung cell monolayers. In contrast, the cells incubated with either negative serum plus H7N9 virus or H7N9 virus alone showed CPE (Fig. 1 C&D) in lung cell monolayers. These results thus demonstrate that *Salmonella* delivered HA antigen efficiently induced functional antibodies *in vivo* and, therefore, we reason that *Salmonella*-based influenza vaccines may elicit protective immunity in humans against H7N9 infection.

3.2. APCs presenting Salmonella delivered influenza antigens induced proinflammatory, Th1, Th2 and Th17 cytokine responses in human PBMCs

To investigate whether human APCs can present *Salmonella* delivered influenza antigens to T cells and subsequently activate cytokine production, we coincubated APCs, previously infected with either *Salmonella* alone or *Salmonella* delivered influenza antigens, with untreated PBMCs of the same individual for 24 h *in vitro*. Subsequently, the cells were harvested and analysed for the induction of proinflammatory (IL-1β, IL-6, IL-12, IL-23 and TNF-α), Th1 (IFN-γ), Th17 (IL-17 and IL-23), and Th2 (IL-10 and TGF-β) cytokine responses by qRT-PCR assay (Fig. 2). The induction of cytokines varied depending upon the various factors, including the stimulant being used and the cytokine being examined, atleast in the current experimental set-up. Our results demonstrated that APCs previously treated with either Sal-HA or Sal-M2e induced significantly ($p < .05$) higher cytokine responses in PBMCs compared to the APCs previously treated with either Sal-NA or *Salmonella* alone (Fig. 2). The IL-1β and TGF-β induction

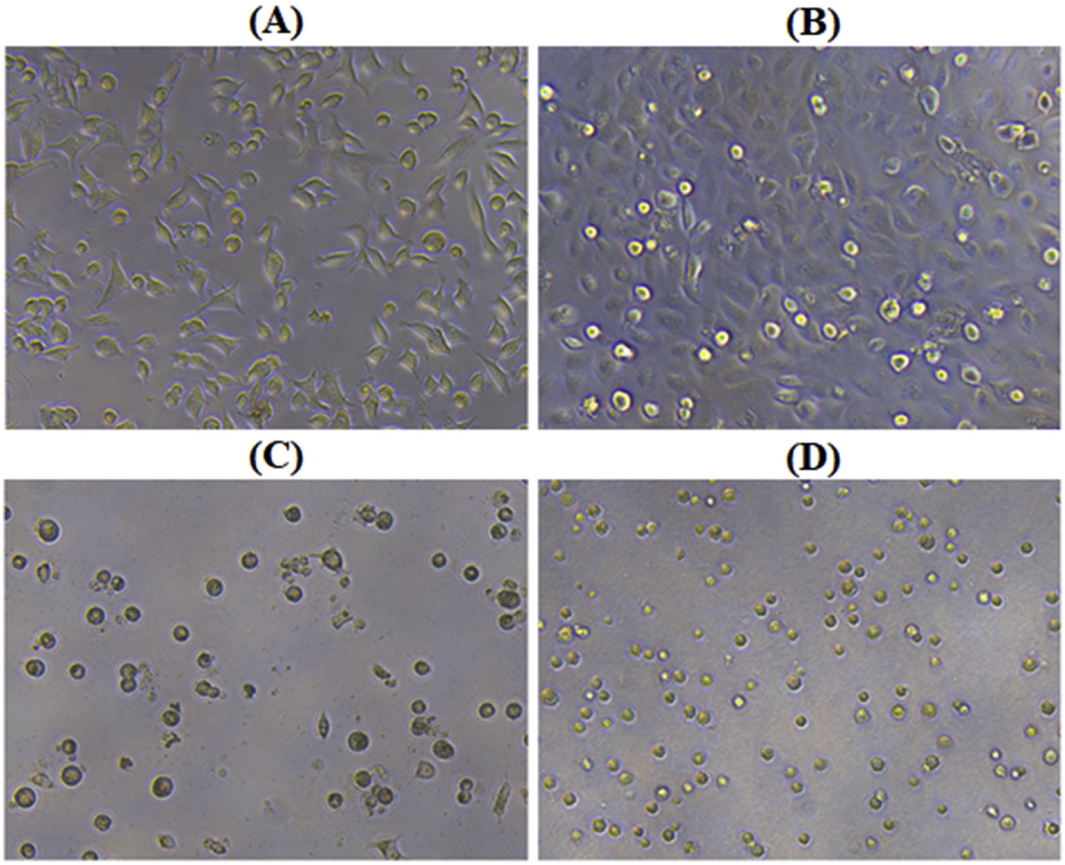

Fig. 1. Neutralization of H7N9 virus *in vitro*. Virus neutralizing ability of the vaccinated chicken sera ($n = 5$) was determined by assaying the capacity of sera to neutralize the cytopathic effects of H7N9 virus in A549 human lung cell line *in vitro*. (A) Cell control. (B) Cells incubated with a mixture of vaccinated serum and 100TCID50 of H7N9 virus. (C) Cells incubated with a mixture of unvaccinated serum and 100TCID50 of H7N9 virus. (D) Cells incubated with 100TCID50 of H7N9 virus only. The experiment was repeated twice.

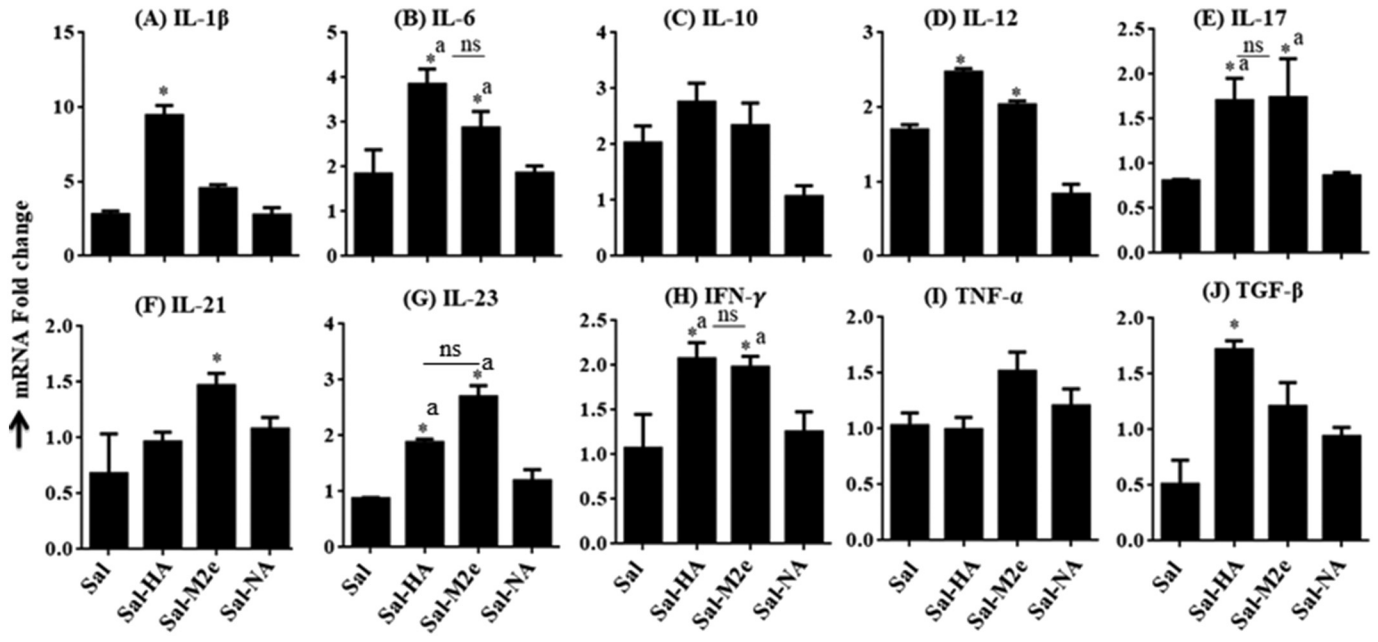

Fig. 2. Cytokine mRNA upregulation in human T cells coincubated with antigen stimulated APCs. APCs ($n = 3$) treated with PBS, *Salmonella* alone (Sal), Sal-HA, Sal-M2e or Sal-NA were cocultured human PBMCs of the same individual for 24 h. RNA was extracted and gene transcription was quantified by qRT-PCR at 24 h post co-culturing of APCs and PBMCs. Results are expressed as fold change of cytokine mRNA transcription in PBMCs induced by activated APCs. GAPDH was used as an internal control and mRNA levels of PBMCs co-cultured PBS treated APCs were used as calibrator. Histograms represent mean cytokine levels and bars represent standard deviation of three individual PBMCs. *$p < .05$, ns = non-significant; a, significant with respect to Sal and Sal-NA.

levels were significantly ($p < .05$) higher in PBMCs cocultured with APCs previously treated with Sal-HA compared to the APCs previously treated with Sal-M2e (Fig. 2 A&J), while IL-21levels were highest in PBMCs cocultured with APCs previously treated with Sal-M2e (Fig. 2F). The mRNA induction levels of IL-6, IL-12, IL-17, IL-23 and IFN-γ were comparable in PBMCs coincubated with APCs previously treated with either Sal-HA or Sal-M2e (Fig. 2B,D,E,G & H), while IL-10 and TNF-α induction levels were nonsignificant in all APCs and PBMCs cocultures (Fig. 2C&I). These results thus clearly suggest that human APCs are capable of processing and presenting *Salmonella* expressing foreign antigens to T cells and can induce diverse cytokine profiles involved in the development of efficient adaptive immune responses.

3.3. Human APCs presenting Salmonella delivered influenza antigens induced proliferation of $CD4^+$ T cells in vitro

To investigate the ability of APCs previously stimulated with *Salmonella* alone or *Salmonella* delivered influenza antigens to stimulate $CD4^+$ T cell responses *in vitro*, we coincubated treated APCs with untreated PBMCs of the same individual for 72 h, followed by analysis of $CD4^+$ T cell proliferations by flow cytometry. Our results indicated that APCs first treated with mitomycin-C and then stimulated with *Salmonella* delivered influenza antigens induced significantly ($p < .05$) higher $CD4^+$ T cell proliferative responses *in vitro* while *Salmonella* alone treatment showed non-significant increase in $CD4^+$ T cell responses (Fig. 3 A&B). Compared to *Salmonella* alone, APCs previously treated with Sal-HA, Sal-M2e or Sal-NA induced significantly ($p < .05$) higher $CD4^+$ T cell proliferative responses in APCs and PBMCs cocultures, with highest responses observed with Sal-HA, followed by Sal-M2e and Sal-NA, which both induced almost comparable $CD4^+$ T cell responses. The T cell proliferative responses were further analysed by a MTT based assay. The results of this experiment demonstrated that *Salmonella*-delivered HA and M2e influenza antigens induced significantly ($p < .05$) higher lymphoproliferative responses than *Salmonella*-NA and *Salmonella* alone treated APCs (Fig. 4 A&B). Our results showed that Sal-NA treated APCs induced some T cell proliferations, but the responses were non-significant compared to the *Salmonella* alone treated APCs.

4. Discussion

The present study was aimed to investigate whether human APCs can process and present *Salmonella* delivered influenza antigens to T cells and subsequent activation of CD4+ T cell responses *in vitro*. Dendritic cells (DCs) are professional APCs with ability to stimulate adaptive immunity through the presentation of foreign antigens, loaded either onto MHC-I or MHC-II class molecules, to T cells. (Benko et al., 2008; Früh and Yang, 1999). The interaction of foreign antigens with APCs in the periphery delivers maturation signals to APCs through up-regulation of co-stimulatory molecules, thereby presenting foreign antigens to T cells in the correct configuration necessary for the elicitation of efficient adaptive immune responses(Benko et al., 2008; Datta et al., 2003). In the present study, we showed that APCs efficiently process and present *Salmonella* delivered influenza antigens to human T cells and subsequently stimulate proliferation and cytokine production in $CD4^+$ T cell responses *in vitro*. Our results are in agreement to the previously published report that shows DCs efficiently process and present live attenuated *Salmonella* mutants compared to the virulent *Salmonella*, which has shown interference with the Ag presentation on the surface of DCs and subsequent T cell activation (Cheminay et al., 2016). It is a well-known fact that $CD4^+$ T cell responses are elicited in response to influenza virus-associated antigens (Waithman and Mintern, 2012). The present study demonstrated that APCs presenting *Salmonella* delivered HA and M2e induced higher $CD4^+$ T cell proliferations *in vitro* compared to the APCs presenting *Salmonella* delivered NA or *Salmonella* antigens alone. The T cells responses are mainly induced against the conserved epitopes and such responses are a key contributor in reducing the disease severity during heterosubtypic infection in animal models (Sridhar, 2016). The role of $CD8^+$ T cells responses in providing cross protective immunity is well established (Seo et al., 2002; Sridhar et al., 2013); however, the role played by $CD4^+$ T cells in mediating heterosubtypic immunity is less clear. We

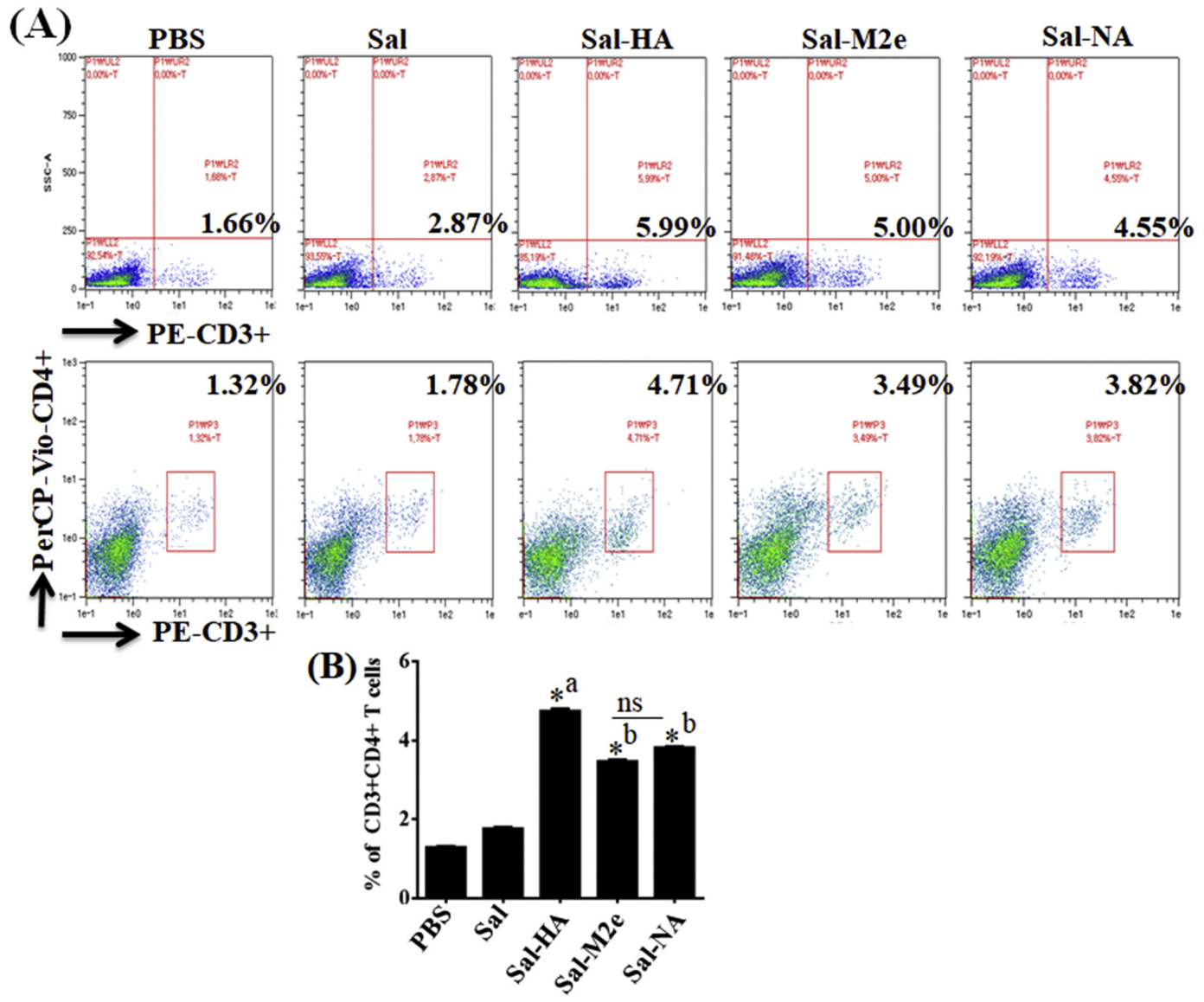

Fig. 3. Flow cytometric analysis of $CD3^+CD4^+$ T cell population in PBMCs and APCs cocultures. APCs (n = 3) treated with PBS, *Salmonella* alone (Sal), Sal-HA, Sal-M2e or Sal-NA were cocultured human PBMCs of the same individual for 72 h for analysis of T cell proliferations by flow cytometry. (A) Representative flow cytometry scatter dot plots for CD3 + and $CD3^+CD4^+$ T cell populations. (B) Histograms representing $CD3^+CD4^+$ T cells. The data are presented as the mean ± s.d. of three individual human PBMCs. *$P < .05$; ns = non-significant; a, significant with respect to Sal, Sal-M2e and Sal-NA; b, significant with respect to Sal.

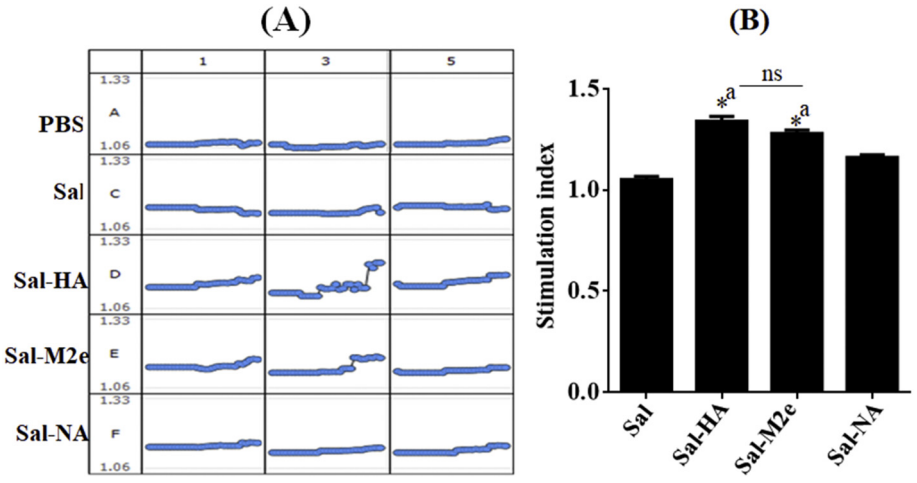

Fig. 4. MTT-based lymphocyte proliferation assay. APCs (n = 3) treated with PBS, *Salmonella* alone (Sal), Sal-HA, Sal-M2e or Sal-NA were cocultured human PBMCs of the same individual for 72 h for analysis of T cell proliferations by MTT-based assay. (A) Kinetics of lymphocyte proliferation in different treatment groups over a period of 3 days. The image was captured using IncuCyte microscopy. (B) Histograms lymphocyte proliferation of each individual treatment. Results are expressed as stimulation indices, defined as proliferation in response to Salmonella alone (Sal) or *Salmonella* delivered influenza antigens antigen relative to the PBS stimulated APCs. The data are presented as the mean ± s.d. of three individual human PBMCs. *P < .05; ns = non-significant; a, significant with respect to Sal and Sal-NA.

previously show that *Salmonella*-based HA vaccine can offer heterosubtypic immunity in chickens possibly through the induction of both CD4+ and CD8+ T cell responses (Hyoung et al., 2017). Our *in vitro* studies, in the present study, provide proof of concept that *Salmonella* delivered vaccine antigens can activate efficient $CD4^+$ T cell responses *in vitro*, and, thus, *Salmonella* system may represent a potential vaccination platform to elicit protective antigen-specific cell mediated immune responses *in vivo*.

Cytokines play an important role in the initiation and maintenance of adaptive immunity (Maldonado-López and Moser, 2001; Matteoli et al., 2008). Cytokines are mainly secreted by $CD4^+$ T cells which have been functionally divided into Th1, Th2 and Th17 subsets based on their production of specific cytokines (O'Garra and Arai, 2000). The Th1 cells mainly secrete IFN-γ, IL-12 and TNF-α, Th2 cells secrete IL-4, IL-10 and TGF-β, while Th-17 cells secrete IL-17 and IL-23 cytokines (Zhou et al., 2009; Ziegler, 2016). The present study demonstrated that APCs treated with either Sal-HA or Sal-M2e induced higher cytokine responses in T cells compared to Sal-NA or *Salmonella* alone treated APCs. This indicates that induction of cytokine responses in T cells seems to be dependent on the type of the antigen being used for the stimulation of APCs. We also observed higher levels of proinflammatory cytokines, IL-1β and IL-6, in T cells induced by APCs treated with either Sal-HA or Sal-M2e compared to other cytokines observed in this study. These proinflammatory cytokines play an important role in the initiation and development of adaptive immune responses through upregulation of costimulatory molecules in APCs, which is prerequisite for efficient elicitation of adaptive immune responses (Matteoli et al., 2008; Santarlasci et al., 2013). The cytokine IL-6 helps in the development of effective and potent mucosal immune responses, and protects the host against bacterial and viral infections (Dienz et al., 2012; Matteoli et al., 2008). Thus, *Salmonella* system, in addition to induction of antigen-specific immunity, may provide non-specific immunity against diverse microbes as recently demonstrated by Kamble et al. (Kamble et al., 2017). The present study further demonstrated that Th1 (IFN-γ and IL-12) and Th2 (IL-10 and TGF-β) cytokine responses were comparable in T cells stimulated with either Sal-HA or Sal-M2e treated APCs, indicating that *Salmonella* system is capable of inducing both humoral and cell mediated immune responses. The present study, thus, provides proof of the concept that *Salmonella* system delivering influenza antigens has potential to activate influenza-specific immune responses, and, therefore, *Salmonella* based vaccines, which are easy to prepare and administer, could be introduced to humans.

In conclusion, we show that human APCs can efficiently present *Salmonella* delivered influenza antigens to T cells and can stimulate diverse profile of cytokine responses involved in development of adaptive immunity. Using *Salmonella* as a vaccine delivery system, antigens would be directly delivered to specific receptors on the surface of DCs, accompanied by pathogen-specific DC maturation and enhancement of adaptive immune responses. Complete understanding how APCs process and present *Salmonella* delivered vaccine antigens would provide valuable information for the development of novel and effective *Salmonella*-based vaccination strategies that will stimulate efficient antigen-specific adaptive immune responses in both animals and humans.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.04.006.

Conflicts of interests

The authors declare no conflict of interest.

Authors' contributions

The work was designed by IAH, KJH and JHL. Experiments and sampling were done by IAH and KJH. Manuscript draft preparation was done by IAH. Statistical analysis was done by KJH and IAH. All authors have read and approved the final manuscript.

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