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Immune memory at 17-years of follow-up of a single dose of live attenuated hepatitis A vaccine

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ABSTRACT

Background: In recent years, hepatitis A virus (HAV) infection has declined considerably in China, associated with wide deployment of HAV vaccines and improvement in socio-economic indicators. Towards the elimination of HA in the country, we assessed the duration and characteristics of immunity conferred by the widely used, locally manufactured HAV vaccine.
Methods: This is a longitudinal cohort study that followed recipients of a live attenuated HAV vaccine 17 years after the initial administration. Blood samples were collected from participants pre- and two-week post-booster HAV vaccine dose. Serum anti-HAV antibody was measured by ELISA method. Memory B and T cells were determined by ELISPOT and Flow Cytometry assays, respectively.
Results: A robust anamnestic response was observed two-week post-challenge. Both HAV-specific memory B cell and T cells remained, and responded quickly when re-encountering HAV. The magnitude of recall responses was present, regardless of the status of the serum anti-HAV antibody pre-booster.
Conclusions: We demonstrated long-term immunity from the live attenuated HAV vaccine, including antibody persistence and immunological memory. Considering the conditions that make elimination of infectious diseases feasible, following polio, hepatitis A could be targeted for elimination in China.

1. Introduction

Hepatitis A (HA) is an acute illness caused by the hepatitis A virus (HAV). Transmission is primarily via the fecal–oral route through person-to-person contact or ingestion of contaminated food or water. The incidence of hepatitis A is strongly correlated with socioeconomic indicators [1,2]. Populations around the world are classified as having high, intermediate, low, or very low levels of HA endemicity based on seroprevalence. Until recently, China was characterized as a high endemicity country [3]. In 1992, two live attenuated hepatitis A vaccines were successfully developed in China through serial passages of the viruses in cell cultures [4]. Universal HAV immunization plus a catch-up campaign for children aged 1–15 years was considered to be feasible and HAV vaccination was integrated into the national Expanded Programme of Immunization (EPI) in 2008 [5].

During the past two decades, China has transitioned from a high to intermediate to low HA endemicity, associated with the wide deployment of HAV vaccines, economic development and changing lifestyles. The national annual incidence rate of HA declined from 56/100,000 person/years in 1991, 6/100,000 person/years in 2007 and 2/100,000 person/year in 2014; a reduction of 90% and 67% on annual incidence was observed between the period of pre- and post-introduction of HAV vaccine into the EPI [6]. Countrywide in 2014, HAV vaccine coverage was 18% among those aged 15–29 years, 64% in children 7–14 years and 91% in children 1–7 years of age [7].

With the decline in HA endemicity in China [8,9], there is the concern for the possibility of outbreaks due to waning vaccine immunity [10] and the lack of boosting from exposure to natural

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infection. It becomes critical to ensure a lifelong protection against hepatitis A virus. Clinical experience suggests that protection following vaccination might be present even in the absence of detectable anti-HAV antibodies, since memory T and B cells as well as long-lived plasma cells maintain at relatively constant numbers in the absence of the eliciting antigen for virtually a lifetime [11]. A previous study showed a good persistence of antibody following a single dose of live attenuated HAV vaccine eight years after the initial HAV vaccination [12]. In this study we assessed immunologic memory 17 years after the initial HAV vaccination and immune response following a booster dose.

2. Materials and methods

2.1. Study cohort and study design

Participants in this study were those included in a randomized trial conducted between 1996 and 1999 [13,14]. In that trial, 3,515 HAV susceptible children aged 1–12 years (mean age 5.4 years) who resided in 24 villages, were enrolled and assigned randomly to either receive a single dose of the HA vaccine or to serve as controls. To assess antibody persistence after the completion of the trial, serological follow up was performed irregularly, alternating between villages.

In this present study, recipients of HAV vaccine residing in 2 villages without either natural infection (defined as an anti-HAV antibody higher than 10,000 mIU/mL [15]) or vaccine booster (defined as either receiving traceable additional dose of HAV vaccine, or 2-fold increase of anti-HAV titers compared to that from last measurement during the 17-year serological follow-up) were selected as participants. A booster dose of HAV vaccine was administered to each participant immediately after collection of a blood sample. A second blood sample was obtained two weeks after the booster. This study was reviewed and approved by the Institutional Review Board of the Institutes of Biomedical Sciences, Fudan University. Written inform consent was obtained from each participant.

PBMC were isolated from fresh blood (10 mL) using Vacutainer cell preparation tubes (BD Biosciences, Mountain View, CA). Serum was isolated by centrifuge at 1600 rpm for 10 min, and stored at −70 °C for anti-HAV antibody assay. Peripheral blood mononuclear cells (PBMC) were isolated on Ficoll density gradients (Axis-Shield Diagnostics Ltd, Scotland) and resuspended in R-10: RPMI 1640 plus 10% FCS (heat inactivated at 56 °C for 30 min) supplemented with penicillin, streptomycin, all from Gibco (Life technology, USA). Fresh cells were used in all assays.

2.2. Vaccine and HAV antigen

The live attenuated HAV vaccine (the details of development are described elsewhere [4,16]) used both for the initial and booster doses was produced by Pukang Biotechnological Co. Hanzhou, China, with the titer of $10^{7.0}$ $TCID_{50}$ (Tissue Culture Infecting Dose).

Bulk virus (consisting of a mixture of incomplete viral particles and broken down proteins with various peptides fragments that resulted from sonication to release the live viruses replicated in the cell) with a concentration of 14 μg/mL (3000 EU/mL) that was used as antigen-specific stimulator for the measurement of T and B cells was kindly provided by the Institute of Medical Biology, the Chinese Academy of Medical Sciences.

2.3. Total anti-HAV antibodies assay

The Abbott Architect HAVab-IgG Microparticle Enzyme Immunoassay (MEIA) system (Abbott Laboratories, Chicago, USA) was employed to quantify anti-HAV antibodies in reference to W1041 anti-HAV immunoglobulin (CLB, Amsterdam, the Netherlands) [17] using non-linear regression standard curves.

2.4. Memory B cell assay: ELISPOT

The frequency of HAV Ag-specific memory B cells was measured by ELISPOT assay using ELIspot^{PLUS} for human IgG kit (MABTECH, Sweden) according to the manufacturer's instructions. Briefly, PBMC were isolated from fresh blood (10 mL) of 47 participants vaccinated 17 years ago. After pre-stimulation with a mixture of R848 and rhIL-2, PBMCs were stimulated with the prepared HAV antigens or unrelated antigen.

2.5. Memory T cell assay: Staining and flow cytometry

PBMCs were seeded into 96-well plate at a concentration of 1×10^6 cells/100 μL in R-10 and cultured at 37 °C in a 5% CO_2 incubator in the presence of HAVAg (3000 EU/mL) or medium as a negative or PMA (0.1 μg/mL, Sigma, USA) and Ionomycin (1 μg/mL, Sigma) as a positive control. Anti-CD28 antibodies (0.1 μg/mL, Miltenyi Biotec, USA) were added to all wells. Brefeldin A (0.1 μg/mL, BD Biosciences, USA) were added to all wells 6 hours before test. After 24 h of culture, the cells were washed and stained with anti-CD3 Pacific Blue^{TM} (Biolegend, San Diego), anti-CD4 APC (eBioscience, San Diego), anti-CD8a PerCp-Cy5.5 (eBioscience) and anti-CD45RO FITC (BD Biosciences monoclonal antibodies (MAbs) for 30 min in the dark on ice and washed in staining buffer (PBS containing 2% FBS), and then fixed and permeabilized using 4% paraformaldehyde (PFA, Sinopharm Chemical Reagent Co., Ltd, China) and 0.2% Triton X-100 (Genview, China) containing 2% FBS. The cells were further stained with anti-human IFN-γ APC/Cy7 (Biolegend) and IL-2 PE (eBioscience) for 60 min in the dark on ice, washed. Cell acquisitions were performed using LSR Fortessa flow cytometer (BD Biosciences, USA). For each analysis, 20,000 events were acquired in the T cell gate. Samples were first run using single fluorochrome-stained preparations for color compensation. The cytometric analysis was performed using FlowJo (TreeStar, Inc., Ashland, OR) and data are shown as% of positive cells.

To enhance the comparability of results from different measurement runs, firstly, standard operation procedure (SOPs) was established and carried out throughout the study of memory B and T cell. Secondly, isolation of PBMC and enrichment *in vitro* of all samples from 31 participants were conducted in the same runs at each time point of pre- and post-booster. Thirdly, the same positive and negative controls were implemented. Finally, to avoid the observer variations, for the measurement of memory B, ELISPORT reader was applied, while for measurements of memory T, all samples from pre- and post-booster were frozen till batch processing with flow cytometry after fixing and staining.

2.6. Statistical analysis

Anti-HAV antibody seropositive rate (SPR), geometric mean concentrations (GMCs) and their 95% confidence intervals were calculated. Antibody titers were logarithmically converted to allow assessment of GMCs. To understand the potential differences on the performances of humoral and cellular immune responses, participants were classified into anti-HAV positive and negative groups by a cutoff of 20 mIU/mL [18,19]. For continuous outcomes comparisons, the Student *t* test or the Mann–Whitney U test were undertaken, and for dichotomous outcomes, the Chi square or Fisher exact test were implemented. The GraphPad Prism 6.0 (GraphPad, San Diego, CA) was applied for statistical analysis. A p -value < .05 was considered statistically significant.

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3. Results

3.1. Characteristics of participants and anti-HAV antibody level

In total, fifty-one previous recipients of HAV vaccine living in the selected two villages were identified from the longitudinal cohort database and a blood sample was collected from each. Excluding 4 vaccinees who met the definition of either history of natural infection or having received booster dose of HAV vaccine, 47 participants included in the analysis. Among these, 29 (62.0%) participants were anti-HAV positive at 17-years after the initial vaccination. The GMCs of anti-HAV serum IgG was 64.8 mIU/mL and 7.6 mIU/mL in anti-HAV positive and negative groups respectively ($p < .05$) (Table 1). There was no statistical difference in gender and age between the anti-HAV positive and negative groups. During the study, 31 out of 47 agreed to receive the booster HAV vaccine, as well as the subsequent procedures. No statistically significant differences in distribution of gender, mean age and proportion of anti-HAV positive were detected between the 47 vaccinees who participated in the 17-year follow-up and the 31 vaccinees who received the booster dose. Two weeks after the booster dose, anti-HAV antibody was detected in 29/31 (94%) participants. Both the anti-HAV positive and negative groups at 17 years showed a significant increase of anti-HAV antibody level following the booster dose (Table 1).

3.2. Memory B cells: Longevity

It was observed that the quantities of IgG-secreting cells stimulated with HAV Ag were significant higher ($p < .0001$) than the unstimulated control (Fig. 1A, B), while the magnitude of HAV-specific memory B cell responses were not correlated to the concentration of anti-HAV IgG in the serum ($p = .18$) (Fig. 1C). More surprisingly, significant differences in the frequency of HAV-specific memory B cells between anti-HAV positive and negative groups were not detected (Fig. 1D, E).

3.3. Memory B cell: recall responses after a booster

Following the confirmation of existing HAV-specific memory B cells, we assessed whether these memory cells could play an important role when encountering HAV Ag. As shown in Fig. 2A, a dramatic increase of serum anti-HAV antibody was observed, which represented a typical anamnestic response. The GMCs increased from 65 mIU/mL to 1832 mIU/mL in the anti-HAV positive group and from 8 mIU/mL to 663 mIU/mL in the negative groups. Although the antibody titer in the anti-HAV negative group was significantly lower than that in anti-HAV positive group pre-challenge ($p < .05$) (Fig. 2B), there was no significant difference in antibody titer between the two groups post-challenge ($p > .05$). After validating the ELISPOT measurement (Fig. 2C), the proliferation of anti-HAV IgG-secreting B cells *in vivo* post-challenge was remarkable in comparison with that pre-challenge ($p < .0001$) (Fig. 2D). The proliferation of anti-HAV IgG-secreting B cells *in vivo* post-challenge was not associated with the serum anti-HAV antibody titer pre-challenge ($p = .91$) (Fig. 2E) and the magnitude of proliferation of anti-HAV IgG-secreting B cells *in vivo* post-challenge was almost equivalent in the anti-HAV negative and positive groups ($p > .05$) (Fig. 2F).

3.4. Memory T cells: Persistence

The cytokine-secreting T cells from the PBMCs, particularly for the frequency of $CD4^+/CD8^+$ memory T cells secreting IFN-γ/IL-2, were detected by measuring the following marker combinations: $CD3^+CD4^+CD45RO^+$ for $CD4^+$ memory T cells (CD4TM); and $CD3^+CD8^+CD45RO^+$ for $CD8^+$ memory T cells (CD8TM) (Fig. 3A). After stimulation with or without HAV Ag for 24 hours, PBMCs from the 47 vaccinees were measured by intracellular staining (ICS). Fig. 3B showed the results of IFN-γ and IL-2 in $CD8^+$ and $CD4^+$ T memory subsets. The frequencies of T cells expressing IFN-γ, IL-2, and dual IFN-γ/IL-2 post- stimulation *in vitro* were statistically higher than those unstimulated in both CD8TM ($p < .01$, $p < .0001$, $p < .01$) and CD4TM ($p < .001$, $p < .05$, $p < .05$), though the frequency of dual IFN-γ/IL-2 was relative low and did not reach the level of 0.1% of positive cells. Similar to the findings from B cell, significant differences on the magnitude of cytokines expressed by either the HAV-specific $CD8^+$ T memory cells or the $CD4^+$ memory subsets were not observed between anti-HAV positive and negative groups (Fig. 3C). Again, there was no correlation between the magnitude of cytokine expressed by HAV-specific memory T cells and serum anti-HAV antibody ($p > .05$, data not shown).

3.5. Memory T cells: Recall responses after the booster

Whether the HAV-specific memory T cells can display recall responses when re-encountering the same antigen is critical. PBMCs from the 31 participants were obtained two weeks after the challenge, and HAV-specific T cell responses were measured by flow cytometry analysis. The gating strategy of IFN-γ/IL-2 secretion in $CD8^+$ and $CD4^+$ T cell subsets is showed in Fig. 4A. The percentages of IFN-γ, IL-2, and dual IFN-γ/IL-2 stimulated with HAV Ag *in vitro* were notably higher than those unstimulated in both $CD8^+$ ($p < .01$, $p < .001$, $p < .01$) and $CD4^+$ ($p < .001$, $p < .01$, $p < .01$) T cells (Fig. 4B). Overall, the magnitude of cytokines expressed by both $CD8^+$ ($p < .01$, $p > .05$, $p < .01$) and $CD4^+$ ($p < .05$, $p < .05$, $p < .05$) T cell subsets escalated dramatically after the booster dose, in comparison with that pre-challenge (Fig. 4C). When looking fur-

Table 1
Characteristics of participants, by anti-HAV antibody level.

Characteristics	Anti-HAV negative	Anti-HAV positive	P value
Before booster dose			
No. of participants	18	29	
Gender (Male, %)	12 (66.7)	22 (75.9)	>.05
Age at initial injection	3.7	3.7	>.05
Anti-HAV level (GMCs, 95%CI; mIU/mL)	7.6 (5.5–10.5)	64.8 (48.3–86.9)	<.05
After booster dose[*]			
No. of participants	13	18	
Gender (Male, %)	9 (69.2)	14 (77.8)	>.05
Age at initial injection	3.9	4.1	>.05
Anti-HAV level before booster (GMCs, 95%CI; mIU/mL)	8.0 (2.8–16.2)	67.6 (20.7–349.0)	<.05
Anti-HAV level after booster (GMCs, 95%CI; mIU/mL)	661.3 (205.0–2133.0)	1832.1 (1349.0–2488.2)	>.05

[*] Characteristics of 31 subjects receiving the challenge dose.

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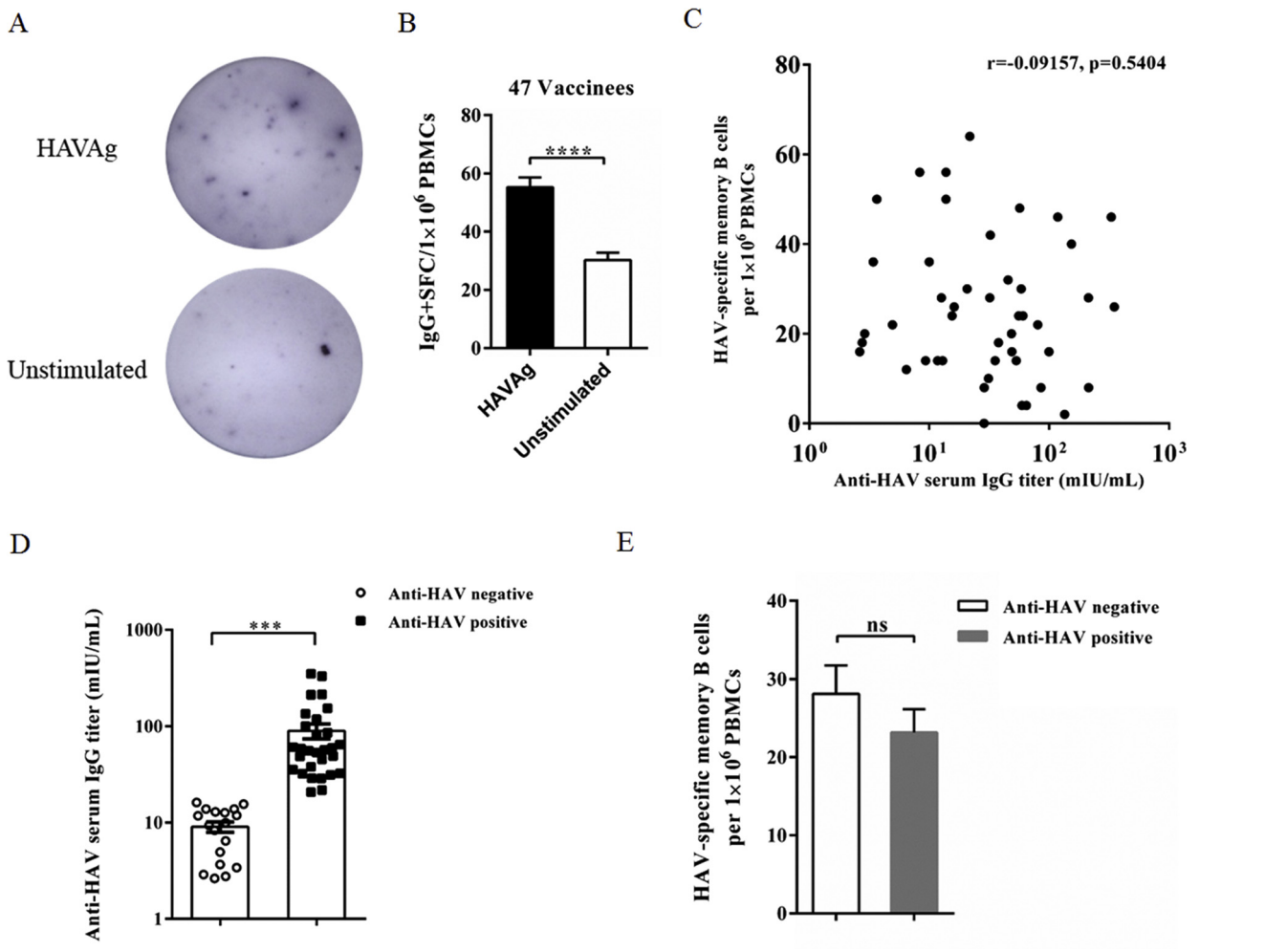

Fig. 1. Longevity of HAV-specific memory B cells in vaccinees immunized 17 years ago. Memory B cells were measured by IgG ELISPOT assay using PBMCs from the 47 vaccinees immunized 17 years ago, first pre-stimulated with a mixture of R848 and rhIL-2 for 3 days, then stimulated with HAV Ag for 24 h or irrelevant antigen. Each spot represents an Ab-secreting cell, (A) one vaccinated individual was shown. (B) The statistical analysis results of 47 vaccinees. (C) Correlation of HAV-specific memory B cells with anti-HAV serum IgG titer. (D) According to the levels of anti-HAV serum IgG, we divided vaccinees into two groups, the anti-HAV negative group (<20 mIU/mL) and the anti-HAV positive group (≥20 mIU/mL). (E) Comparison of HAV-specific memory B cells in anti-HAV negative group with positive group. Data are presented as mean ± SEM. ns, not significant. $^{**}p < .01$, $^{***}p < .001$ and $^{****}p < .0001$ (unpaired Student's *t* test).

ther at the status of serum anti-HAV antibody pre-challenge, similar to the overall profile, cytokines production of IFN-γ, IL-2, and dual IFN-γ/IL-2 increased slightly after the challenge dose, both in the anti-HAV negative and positive groups. Substantial differences on the magnitude of cytokines expressed either by the HAV-specific $CD8^+$ or the $CD4^+$ T cells between the two groups were not found (Fig. 4D).

4. Discussion

In this study, we assessed immunity at 17-years after a single dose of live attenuated HAV vaccine. Although the positive rate of ELISA antibody declined from 95% to 62% over 17-years, as expected, antigen specific memory B and T cell responses remained. The recall responses after a booster suggest that the existence and functions of HAV-specific memory B cells are independent of the status of the serum anti-HAV antibody. The persistence of memory T-cells and rigorous response to HAV Ag stimulation 17 years after initial vaccination indicate that HAV-specific memory $CD4^+$ and $CD8^+$ T cells remain, even though some of participants' serum anti-HAV antibody were not measurable by means of a conventional ELISA method. Critically, both HAV-specific $CD4^+$ and $CD8^+$ T cell were able to respond quickly when re-encountering HAV and secreted abundant cytokines; this response was also presented regardless of the status of the serum anti-HAV antibody. It is encouraging to note that when encountering the antigen again, antigen-specific memory B and T cells were capable of mobilizing rapid responses, which was demonstrated by the ability to promptly mount an anamnestic antibody response to a booster dose and the secretion of specific cytokines. Surprisingly, the magnitude of memory response observed, no matter coming from memory B or T cells, had no association with the level of circulating anti-HAV antibody that existed 17 years after the initial injection.

When assessing the performance of a vaccine, duration of immunity after initial vaccination is paramount. Ideally, one dose should provide lifelong protection against the targeted disease. Theoretically, live attenuated viral vaccines which comprise weakened versions of the pathogens can mimic the natural course of natural infection in humans, and should induce persisting immune responses for several decades, if not lifelong [20]. Given the results of this study which indicates a first and second line of adaptive immunity [21], we speculate that HAV vaccination provides a long-term, probably lifetime protection against infection. The HAV wild type strains continue to circulate in the environment

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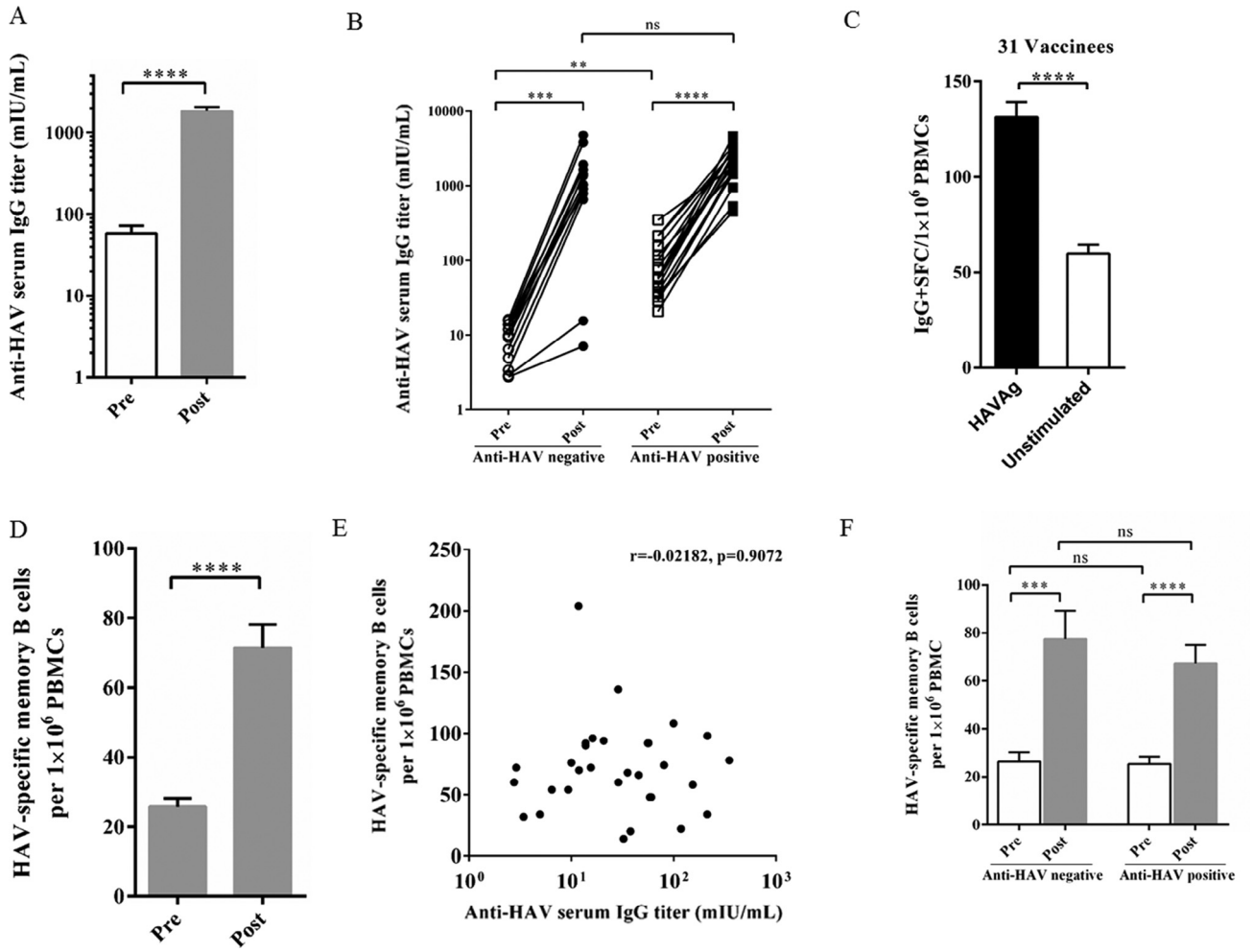

Fig. 2. Memory B cell recalled responses after boosting. Thirty-one of the 47 vaccinees received another dose of live attenuated HAV vaccine after 17 years, and blood samples were obtained 2 weeks after the boost, then serum antibody titers and memory B cell recalled responses were detected. (A) The level of anti-HAV serum IgG before the boost (Pre) and after the boost (Post). (B) The level of anti-HAV serum IgG in anti-HAV positive group and negative group before and after the boost. PBMCs pre-stimulated with a mixture of R848 and rhIL-2 for 3 days, then stimulated with HAV Ag for 24 h after the boost, (C) the numbers of IgG-secreting B cells were shown. (D) Comparison of HAV-specific memory B cells before and after the boost. (E) Correlation of HAV-specific memory B cells with anti-HAV serum IgG titer after the boost. (F) HAV-specific recalled B cell responses in anti-HAV positive group and negative group before and after the boost. Data are presented as mean ± SEM. ns, not significant. $^{**}p < .01$, $^{***}p < .001$ and $^{****}p < .0001$ (unpaired Student's *t* test).

and it is essential to monitor the persistence of immunity in HAV vaccinees during the coming decades. The risk of bias induced by natural booster cannot be ruled out completely, though measures were put in place in this study to screen out participants with natural exposure.

There is existing controversy on the nature of the China-made live attenuated hepatitis A vaccine based on the H2 strain. As a live vaccine, several characteristics based on the oral polio vaccine, generally serve as criteria to assess live attenuated vaccines [22]. Firstly, the H2 strain was excreted in very low amounts from vaccinees. It was reported that fecal viral shedding could be detected only in cell cultures of concentrated fecal specimens [23]. Secondly, oral administration of H2 strain, which mimics the natural infection route, does not elicit an immune response [24]. Thirdly, the possibility of human-to-human transmission of H2 virus failed to be documented through a close contact study [25]. Fourthly, as a rule, soon after the initial injection, anamnestic reactions to live attenuated viral vaccines are minimal, as preexisting antibodies mostly neutralize the vaccine load before replication *in vivo* [20]. However, a booster dose of the H2 strain demonstrated a secondary immune response in the first year after initial vaccination. The antibody level elicited is comparable to that induced by inactivated vaccine [26,27]. Despite the above mentioned issues, paradoxically, IgG1 and IgG3 are the predominant subclass 7 months after a single dose of H2 strain [28]. The current consensus is that, the presence of IgG1 and IgG3 is strongly associated with the presence of IFN-γ, which is the signature of Th1 cytokine. The Th1 cells are primarily responsible for cell-mediated immunity or DTH [29]. Since studies on natural viral infections have shown that anti-viral IgG antibodies were predominantly of the IgG1 and/or IgG3 subclass, and thus, immune mechanism might be speculated through the measurement of IgG subclass [30]. In this study, a significant increase in the secretion of IFN-γ by $CD3^+$ T cell *in vivo* was measured after a booster. Furthermore, the IFN-γ secretion from $CD3^+$-$CD8^+$ subset was remarkably higher than that from $CD3^+CD4^+$ subset, which satisfied the characteristic of live attenuated vaccine to mediate a cellular based response.

As showed in this study, the level of anti-HAV antibodies was not correlated to frequency of both $CD4^+$ and $CD8^+$ T cell in the circulation. This fits the dogma that T cells fade over time while B plasma cells remain for much longer [31]. Even so, the fact that B cells in each individual vaccinee responded to the HAV vaccine differently might due to differential assistance from $CD4^+$ T cells with genetic variations such as preferential HLA haplotypes. Besides the

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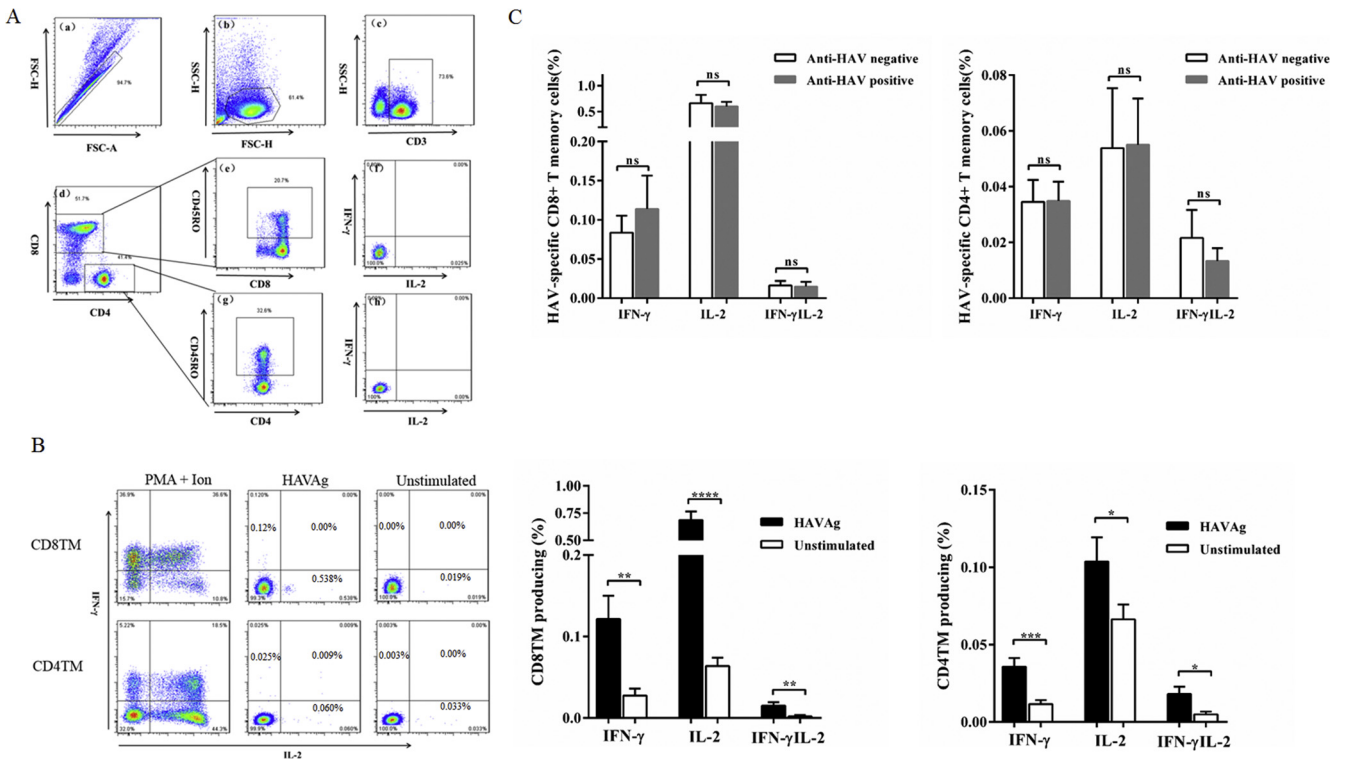

Fig. 3. Persistence of HAV-specific memory T cells to HAV Ag. HAV-specific T cells were detected in all 47 vaccinees by intracellular staining. (A) Flow cytometry analysis of a representative PBMC sample stimulated with HAV Ag for 24 h. Dot plots show the gating strategy to identify $CD4^+$ memory T cells (CD4TM, $CD3^+CD4^+CD45RO^+$) or $CD8^+$ memory T cells (CD8TM, $CD3^+CD8^+CD45RO^+$). (B) The frequencies of CD8TM and CD4TM expressing IFN-γ, IL-2, and dual IFN-γ/IL-2 were shown (bottom). (C) Comparison of IFN-γ, IL-2, and dual IFN-γ/IL-2 production between the anti-HAV negative group and positive group in HAV-specific CD8TM and CD4TM. Data are presented as mean ± SEM. ns, not significant. $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$ and $^{****}p < .0001$ (unpaired Student's *t* test).

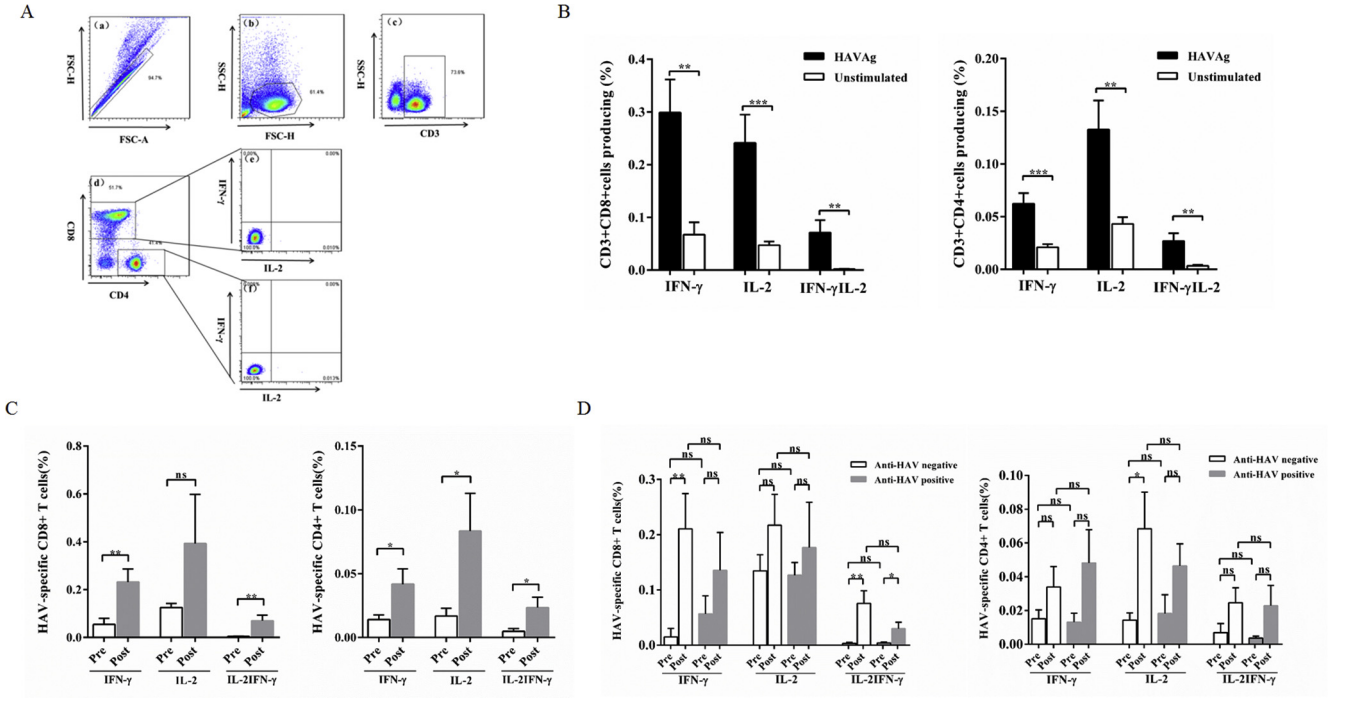

Fig. 4. T cell recalled responses after boosting. PBMCs from 31 boosted subjects were obtained 2 weeks after the boost, and HAV-specific T cell responses were measured by flow cytometry analysis. (A) Gating strategy of IFN-γ-secreting and IL-2-secreting T cell subsets after the boost. (B) The frequencies of $CD8^+$ and $CD4^+$ T cell secreting IFN-γ, IL-2, and dual IFN-γ/IL-2 were shown after the boost. (C) Comparison FN-γ, IL-2, and dual IFN-γ/IL-2 in $CD8^+$ and $CD4^+$ T cell before and after the boost. (D) Comparison of IFN-γ, IL-2, and dual IFN-γ/IL-2 production between the anti-HAV negative group and positive group in HAV-specific $CD8^+$ and $CD4^+$ T cell before and after the boost. Data are presented as mean ± SEM. ns, not significant. $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$ and $^{****}p < .0001$ (unpaired Student's *t* test).

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B cell recall responses, HAV-specific T cells are also an important factor that contributes to protection. Since both $CD4^+$ and $CD8^+$ T cells undergo proliferation, retraction and few of them survived as long term memory cells. Memory T cells could be divided into two subpopulations, central memory T cell (Tcm) and effector memory T cell (Tem) subsets [32]. Tcm can rapidly proliferate, respond and produce mainly IL-2 against the same pathogen once it enters; while Tem mainly secreted cytokines such as IFN-γ or IL-4, can react to the infection quickly [32,33]. A functional T cell recall response appears to play a critical role against HAV infection, particularly in those anti-HAV antibody negative vaccinees. These sera-negative individuals started with lower frequency of both $CD4^+$ and $CD8^+$ T cells, but could rapidly respond *in vitro* stimulation with the same antigen. More importantly, an antigen booster prompted the primed T cells to produce higher level of cytokines in either of these two group (Fig. 4C), but magnitude from these negative individuals was much greater than those in positive individuals as seen in Fig. 4D, suggesting T cells in these "negatives" are better responders than those "positives". Similar results have been also observed in previous studies [34]. For this reason, a booster injection for those individuals may not be necessary. Strong connection of anti-viral infection and antigen specific $CD8^+$ T cell has been demonstrated in Yellow Fever and Vaccinia vaccinations [35,36]. This study demonstrated that functional $CD8^+$ T cells maintains at a high frequency in circulation and can achieve robust recall responses once re-encountering antigen, and thus provide a second line of defense, in addition to circulating antibody.

In summary, a single dose of the live attenuated HA vaccine showed good B cell and T cell immune memory and likely provides long-term protection. The success of vaccines against smallpox and polio demonstrates the possibility of disease elimination and even eradication either at the international or national level. The criteria that make elimination of Hepatitis A in China achievable are present [37]. These include biologic feasibility (effective vaccines can provide lifelong protection and continuous improvement of sanitation), adequate public health infrastructure, sufficient funding and sustained political/societal will. Moreover, with the lack of animal reservoir, human beings are the only hosts for HAV in the natural world. In this regards, our results further support the feasibility of hepatitis A elimination using wide-scale vaccination in the country [38]. Considering limited health resources, hepatitis A might be set as a target for elimination following polio in China.

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Conflict of interest

No competing financial interests exist.

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