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A single dose of inactivated hepatitis A vaccine promotes HAV-specific memory cellular response similar to that induced by a natural infection

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ABSTRACT

Based on current studies on the effects of single dose vaccines on antibody production, Latin American countries have adopted a single dose vaccine program. However, no data are available on the activation of cellular response to a single dose of hepatitis A. Our study investigated the functional reactivity of the memory cell phenotype after hepatitis A virus (HAV) stimulation through administration of the first or second dose of HAV vaccine and compared the response to that of a baseline group to an initial natural infection. Proliferation assays showed that the first vaccine dose induced HAV-specific cellular response; this response was similar to that induced by a second dose or an initial natural infection. Thus, from the first dose to the second dose, increase in the frequencies of classical memory B cells, TCD8 cells, and central memory TCD4 and TCD8 cells were observed. Regarding cytokine production, increased IL-6, IL-10, TNF, and IFNγ levels were observed after vaccination. Our findings suggest that a single dose of HAV vaccine promotes HAV-specific memory cell response similar to that induced by a natural infection. The HAV-specific T cell immunity induced by primary vaccination persisted independently of the protective plasma antibody level. In addition, our results suggest that a single dose immunization system could serve as an alternative strategy for the prevention of hepatitis A in developing countries.

Abbreviations: HAV, hepatitis A virus; WHO, World Health Organization; SAGE, Strategic Advisory Group of Experts; Anti-HAV, hepatitis A virus antibodies; BMI, body mass index; T0, time point immediately before vaccination; T1, time point immediately before administration of the second dose of the hepatitis A vaccine and 6 months after administration of the first dose of this vaccine to healthy subjects; T2, time point 24 months after administration of the second dose of hepatitis A vaccine to healthy subjects; FIOCRUZ, Oswaldo Cruz Institute; ELISA, enzyme-linked immunosorbent assay; CONEP, National Commission on Ethics in Research; PBMCs, peripheral blood mononuclear cells; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; CFSE, carboxyfluorescein succinimidyl ester; PHA, phytohemagglutinin; LPS, lipopolysaccharide; FSC, forward scatter; SSC, side scatter; PI, proliferation index; IL-2, interleukin 2; IL-4, interleukin 4; IL-6, interleukin 6; IL-10, interleukin 10; IL-17A, interleukin 17A; IFNγ, interferon gamma; TNF, tumor necrosis factor; CBA, cytometric bead array; AH, acute phase in natural infection by the hepatitis A virus.

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1. Introduction

Vaccines against hepatitis A (HAV) were first developed in the 1980s and were licensed for use in the early 1990s [1]. There is only one serotype of HAV, although multiple strains of HAV are found worldwide. Therefore, a vaccine prepared from a strain with a different geographic origin or from a different virus isolate can still protect against infection by hepatitis A [2,3].

Immunization programs for children (≥1 year of age) have been introduced in several countries [4–6]. A two-dose vaccination schedule is generally used at an interval of 6–18 months between the first (primary) and second (booster) doses. The effectiveness of the HAV vaccine is attributed to the reduction in the reported worldwide incidence of acute hepatitis A [4,7–9].

Several middle-income regions throughout the world, including Asia, Latin America, Eastern Europe, and the Middle East, have undergone a transition in the epidemiology of HAV, in which the number of young subjects susceptible to HAV infection has substantially increased. During a meeting of the World Health Organization (WHO) Strategic Advisory Group of Experts (SAGE) on Immunization [10], it was noted that populations in these countries may benefit the most from large-scale HAV vaccination programs. However, one of the main barriers to the introduction of universal hepatitis A immunization of children in developing countries is high vaccine prices. A single dose schedule would remove barriers to the use of these vaccines for public health purposes by reducing the cost associated with immunization. The efficacy and safety of both one- and two-dose inactivated HAV vaccines have already been established [11–15]. At present, two Latin American countries have adopted single dose hepatitis A childhood immunization programs. Argentina was a pioneer in implementing this vaccination schedule in 2005. After two years, with a vaccination coverage of 95%, the incidence of symptomatic viral hepatitis A decreased by >80% in all age groups [16]. Brazil began HAV vaccination in 2014. This decision was based on cost-benefit studies showing that national universal HAV vaccination would have an important impact on the epidemiology of this disease [17].

The duration of immune protection after HAV vaccination has been investigated. Two of the longest follow-up studies in adults reported the persistence of anti-HAV antibodies up to at least 17 years after receiving a monovalent inactivated HAV vaccine (Havrix™, GlaxoSmithKline Vaccines, Belgium) according to a two-dose schedule. Hens and colleagues [44] projected duration of protection of at least 25 years in ≥95% of the vaccines [10,14,15]. However, only humoral responses have been considered when verifying protection; cellular responses have not been assessed. This knowledge would be relevant for the investigation of low responsiveness or non-responsiveness to the HAV vaccine, as this phenomenon is often observed in response to different vaccines, affecting 1–10% of all vaccines [18–20]. Garner-Spitzer and colleagues [26] demonstrated a good correlation between the antibody concentration and the cellular response; specifically, low antibody production was associated with a low antigen-specific response after a booster vaccination. However, no studies have investigated activation of the memory T cell response profile after a single HAV vaccine dose. Here, we investigated antigen-specific memory cell proliferation in seronegative individuals prior to and following immunization with a formalin-inactivated HAV vaccine. For the first time, a single HAV vaccine dose was shown to effectively induce a memory T cell response after stimulation with a wild HAV strain. The T cell response after a single HAV vaccine dose was similar to that observed after both the booster dose and HAV infection.

2. Materials and methods

2.1. HAV vaccination

Twenty-two healthy individuals (21.0 ± 0.45 years) who were seronegative for anti-HAV were randomly selected and immunized with an inactivated hepatitis A vaccine (Merck Sharp & Dohme, West Point, PA, USA, lot 0526F) using a 0–6 month schedule, as previously described [5]. Of these individuals, 12 (54.5%) were female. The vaccine contained 50 U/1.0 mL of inactivated hepatitis A antigen adsorbed to 0.45 mg of aluminum hydroxide (adult dose) and was administered intramuscularly into the deltoid region of the left arm. The mean body mass index (BMI) of the participants was 21.95 ± 0.5, and none of the participants had a history of drug use. To investigate the post-vaccination immune response, vaccinated individuals were monitored three times throughout the study: time 0 (*T*0), before administering the 1st dose of the vaccine; time 1 (*T*1), 6 months after administering the 1st dose and before administering the 2nd dose; and time 2 (*T*2), 24 months after administering the 2nd dose. All vaccinated individuals were monitored for 30 months.

2.2. Acute hepatitis A patients

Twenty outpatients (25.62 ± 2.65 years) with self-limited acute hepatitis A attending the viral hepatitis ambulatory clinic of the Oswaldo Cruz Institute (FIOCRUZ) were recruited as the control group. Of these individuals, 9 (45%) were female. Acute hepatitis A cases were defined as follows: (1) by the use of a commercially available enzyme-linked immunosorbent assay (ELISA) kit for anti-HAV IgM (Abbott, USA); (2) by the observation of clinical features of acute-onset hepatitis within 2–8 weeks in a previously healthy individual; and (3) by the observation of aminotransferase levels that were at least 10-fold higher than the upper limit of normal [21–24]. Patients exhibiting co-infection with other forms of viral hepatitis were not included.

2.3. Blood sample collection and processing

Blood samples (~40 mL) were collected from the vaccinated individuals via venipuncture (Vacutainer™, Becton Dickinson, Franklin Lakes, NJ, USA) at three time points (*T*0, *T*1, and *T*2) and at the onset of acute hepatitis during the clinical examination of the control group. Signed informed consent was obtained from all participants. The study protocol was approved by the National Commission on Ethics in Research (CONEP) and by the institutional review boards of FIOCRUZ (protocol #222-03 and 401-07). The blood samples were centrifuged (224 g), and the plasma was separated and frozen at −20 °C. Peripheral blood mononuclear cells (PBMCs) were separated via centrifugation in Ficoll density gradient medium (30 min at 400 g at 18 °C). Freezing medium (10% dimethylsulfoxide (DMSO), and 90% fetal bovine serum (FBS)) was added, and the cells were stored in liquid nitrogen until the assay was conducted. The plasma and PBMC samples used for the different assays were thawed only once.

2.4. Laboratory tests

2.4.1. Anti-HAV detection

Plasma samples were assayed for total anti-HAV levels using a commercial competitive ELISA kit (Bioelisa HAV-EIA, Biokit, Barcelona, Spain), and a commercial immunoassay (ImmunoComb®II HAV Ab, Orgenics, Israel) was used to retest the samples displaying negative results. The detection limits of the lots used in the Bioelisa HAV-EIA were 75 mIU/mL (lot L-0407), and

67 mIU/mL (lot I-3308), and the ImmunoComb®II HAV Ab assay was used at a limit of detection of 10 IU anti-HAV antibodies/L [25].

2.4.2. Antigen-specific proliferation assay

PBMCs were suspended in RPMI 1640 medium (Sigma-Aldrich, USA) at a concentration of 5×10^6 cells/mL and mixed with an equal volume of a 10 mM working solution of carboxyfluorescein succinimidyl ester (CFSE-FITC; Molecular Probes, Invitrogen, USA), which was diluted to 1/1000 for all analyses. The procedure was performed according to the manufacturer's instructions. PBMCs were suspended in RPMI 1640 medium supplemented with 110 U/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated FBS at a concentration of 2.5×10^6 cells/mL. Non-CFSE-labeled cells were used as a negative control. The HAF-203 HAV strain was propagated in FRhK-4 cells [22]. The virus suspension was filtered through a 0.22 μm filter (Merck Millipore, USA) and used for polyclonal HAV antigen-specific proliferation (viral titer of 10^6 HAV RNA copies/mL). The mitogen inducers phytohemagglutinin (PHA) (L1668, Sigma-Aldrich, USA), and lipopolysaccharide (LPS) (L2762, Sigma-Aldrich, USA) were used at final concentrations of 10 μg/mL, and 1 ng/mL, respectively, to control clonal proliferation. Proliferating cell cultures were set up in duplicate at 5×10^5 cells/well in 96-well flat-bottom culture plates. The plates were incubated in a 37 °C incubator containing 5% CO_2 for 24 h after LPS stimulation, for 72 h after PHA stimulation or for 96 h after HAV stimulation. After incubation, the cells were harvested for use in flow cytometry assays.

2.4.3. Flow cytometric analysis

A minimum of 20,000 live cells were collected from each sample and assessed using a CyAn flow cytometer (DakoCytomation, USA). The data were analyzed off-line using Summit version 6.0 software (DakoCytomation, USA). PBMCs were labeled with αCD4-FITC (clone MT310), αCD8-PerCP (clone DK25), αCD22-FITC (clone 4KB128), αCD45RO-PE (clone UCHL1) (all from DakoCytomation, USA), αCD62L-PE (clone MHCD62L04), αCCR7-APC (CD197) (clone 3D12), and isotypes (all from eBioscience, San Diego, CA, USA) and were then quantified. The memory cell phenotypes were defined as previously described [26,27].

Total mononuclear cells were electronically gated using forward scatter (FSC) and side scatter (SSC) properties; cellular debris and granular cells were excluded. Based on their FSC and SSC properties, proliferating cells were defined as described by others [28–30]. The proliferation index (PI), a measure of the frequency of cells that have gone through more than three divisions (positive proliferation, $CFSE^{low}$), was assessed using a software program (Summit version 6.0) [28–30]. The final PI was calculated as the ratio of the average PI for antigen-stimulated cells to the average PI for unstimulated cells. The highly expressed surface markers on the memory T and B cell subsets activated by antigenic stimulation were considered for off-line software analysis (Figs. 1 and 2).

2.4.4. Cytokine quantification assay

Cytokine production was measured using culture supernatant samples from the proliferation assay. IL-2, IL-4, IL-6, IL-10, IL-17A, interferon–gamma (IFNγ), and tumor necrosis factor (TNF) were analyzed using a BD™ Cytometric Bead Array (CBA) kit (cat. #560484; BD Biosciences, San Jose, CA, USA).

2.5. Statistical analysis

The data are expressed as the mean values ± standard error. One-way analysis of variance (ANOVA) was performed for intergroup comparisons of vaccinated individuals. If a significant difference was found, a pair of variables was assessed using the Wilcoxon matched pairs *t*-test. Differences over time throughout the vaccination schedule and between the vaccinated subjects and the acute hepatitis cases were evaluated using the Kruskal–Wallis test. If a significant difference was found, each pair of variables was assessed using the Mann–Whitney *U*-test. The *R* Project for Statistical Computing (http://www.r-project.org/) was used to perform statistical analysis. The level of significance for all statistical analyses was defined as a P value <0.05.

3. Results

3.1. Humoral immunity

Total anti-HAV antibodies were detected in 16/22 (72.73%) vaccinated subjects after the first dose (T1) and in 22/22 (100%) vaccinated subjects after the second dose (T2). The subjects who did not display total anti-HAV antibody levels above the limit of detection after receiving the first dose (6/22) were considered as poor responders.

3.2. HAV-induced clonal proliferation in vaccinated subjects

HAV-specific cellular response was observed at *T*1. At this time point, the PI was increased by 3.21-fold compared to the PI before vaccination (*T*0). No significant differences were found in the PI between *T*2 and *T*1 (Table 1). A clonal proliferation response to mitogens (PHA and LPS) was apparent at all time points during the follow-up period (PHA: $T0 = 44.61 \pm 12.25$, $T1 = 62.23 \pm 15.64$, and $T2 = 22.25 \pm 9.43$; LPS: $T0 = 39.57 \pm 11.89$, $T1 = 53.27 \pm 13.67$, and $T2 = 31.55 \pm 10.56$).

3.3. Activation of memory cell phenotypes after HAV stimulation

Table 1 shows the frequencies of T and B cell subsets of memory cell phenotypes after vaccination with HAV stimulation. Changes in the frequencies of classical memory TCD4 ($CD4^+CD45RO^+$), TCD8 ($CD8^+CD45RO^+$), and B cells ($CD22^+CD45RO^+$) after vaccination were observed (Fig. 1). The frequency of HAV-specific TCD4 cells was significantly increased at T2 (Table 1). HAV-specific classical effector memory TCD8 cells and effector memory B cells were activated at *T*1 (Table 1).

HAV-specific central memory T cells exhibiting the phenotypes $CD4^+CD62L^+CCR7^+$ and $CD8^+CD62L^+CCR7^+$ were also activated after the first dose (Fig. 2). The frequencies of effector TCD4 ($CD62L^-CCR7^-$) and TCD8 ($CD62L^-CCR7^-$) cells remained unchanged after vaccination (Table 1).

3.4. HAV-specific cytokine production

A significant increase in IFNγ, IL-6, and TNF levels was observed after the first dose. The IL-10 levels were significantly increased after the second dose (Table 1). No change in IL-2, IL-4, or IL-17A levels was detected at any time during follow-up.

3.5. HAV stimulation in poor responders after the first dose

The analysis of HAV-specific stimulation in the group of poor responders showed HAV-specific proliferation ($p = 0.0245$) and HAV-specific central memory TCD4 ($p = 0.0164$) and TCD8 ($p = 0.0170$) cell subset activation after the first dose compared with at *T*0. In this group of individuals, evidence of increase in the frequencies of classical memory $CD4^+CD45RO^+$ cells ($p = 0.0386$) and classical memory TCD8 cells ($CD45RO^+$) ($p = 0.023$) was found. IL-6 ($p = 0.0009$), and IFNγ ($p = 0.0006$) were the only cytokines detected. No difference in the clonal proliferation of memory B cells ($CD22^+CD45RO^+$) was found between *T*1 and *T*0 ($p = 0.6492$).

Table 1
Cellular immune responses after HAV stimulation during vaccination or natural infection: measurements of the proliferation index, memory cell phenotypes, and cytokine production.

	T0 (Naïve)	*T1* (1st dose)	*T2* (2nd dose)	AH (Natural)	*p* value					
					T0 vs T1	*T0 vs T2*	*T1 vs T2*	*T0 vs* AH	*T1 vs* AH	*T2 vs* AH
Proliferation index	0.85 ± 0.06	2.73 ± 0.25	2.84 ± 0.27	2.8 ± 0.81	0.0020	0.0059	Ns	0.008	Ns	Ns
$CD4^+CD45RO^+$ (%)	13.5 ± 3.20	18.1 ± 4.31	23.7 ± 2.64	21.1 ± 1.92	Ns	0.0137	Ns	0.0488	Ns	Ns
$CD8^+CD45RO^+$ (%)	4.6 ± 1.39	10.7 ± 1.92	6.0 ± 1.63	8.9 ± 1.65	0.0137	Ns	0.0371	0.0195	Ns	Ns
$CD22^+CD45RO^+$ (%)	2.7 ± 0.42	8.0 ± 1.71	2.8 ± 0.62	7.3 ± 1.22	0.0488	Ns	0.0156	0.0273	Ns	0.0137
$CD4^+CD62L^+CCR7^+$ (%)	14.32 ± 2.58	31.24 ± 4.88	20.73 ± 3.01	13.0 ± 3.21	0.0020	Ns	Ns	Ns	0.0020	Ns
$CD4^+CD62L^-CCR7^-$ (%)	12.91 ± 3.34	16.27 ± 1.55	17.28 ± 1.51	30.14 ± 3.34	Ns	Ns	Ns	0.0035	0.0038	0.0038
$CD8^+CD62L^+CCR7^+$ (%)	11.5 ± 2.64	24.72 ± 4.41	17.08 ± 2.74	10.15 ± 6.69	0.0028	Ns	Ns	Ns	0.0030	Ns
$CD8^+CD62L^-CCR7^-$ (%)	15.82 ± 1.57	15.28 ± 1.90	17.39 ± 2.92	28.59 ± 4.32	Ns	Ns	Ns	0.0028	0.0030	0.0032
IL-6 (pg/mL)	123.9 ± 70.49	10,043.1 ± 1,151.1	6,664.9 ± 1,751.7	73.8 ± 33.32	0.0020	0.0020	0.0488	0.0001	0.0001	0.0001
IL-10 (pg/mL)	22.02 ± 10.39	55.73 ± 9.33	225.9 ± 33.34	39.5 ± 24.62	Ns	0.0020	0.0039	Ns	Ns	0.0001
TNF (pg/mL)	14.20 ± 6.76	237.89 ± 88.77	143.9 ± 52.89	31.2 ± 16.18	0.0039	0.0020	Ns	Ns	0.0007	0.0022
IFNγ (pg/mL)	2.23 ± 2.03	197.49 ± 50.90	20.81 ± 5.69	159.1 ± 5.77	0.0117	0.0039	0.0273	0.0185	Ns	0.0345

The mean values ± standard error obtained from PBMCs stimulated with HAF-203 before and after vaccination or during natural infection. The proliferation index, the memory T cell ($CD4^+CD45RO^+$; $CD8^+CD45RO^+$; $CD4^+CD62L^+CCR7^+$; or $CD8^+CD62L^+CCR7^+$) and memory B cell ($CD22^+CD45RO^+$) frequencies, and the cytokine levels were detected in culture supernatants after HAF-203 stimulation. T0: non-vaccinated (naïve) samples; T1: samples collected 6 months after the 1st dose of hepatitis A vaccine; T2: samples collected 24 months after the 2nd dose of hepatitis A vaccine; AH: samples collected from acute hepatitis A patients. All cytokine levels are expressed in picograms per milliliter (pg/mL); all data on the numbers of live cells are presented as frequencies (%) from 20,000 events acquired via flow cytometry. Ns = Not significant.

Upon comparing the group of poor responders with the group of individuals who seroconverted after the first dose, the only observed difference was the extent of classical effector memory B cell activation (Table 2).

3.6. The cellular immune response in acute hepatitis A patients is similar to that in vaccinated subjects after HAV stimulation

The memory cell phenotypes observed in patients with acute hepatitis A were the same as those observed in the vaccines after the first vaccine dose, except for the central memory T cell phenotype ($CD62L^+CCR7^+$) (Table 1). Additionally, no significant difference in the production of the anti-viral cytokines IFNγ and IL-10 was observed between these two groups. However, the percentages of effector TCD4 ($CD62L^-CCR7^-$) and TCD8 ($CD62L^-CCR7^-$) cells were elevated only in the acute HAV patients. The memory B cell phenotype and the cytokine levels (IL-6, IL-10, TNF, and IFNγ) observed in the vaccines after receiving the second vaccine dose significantly differed from those detected in the patients with natural HAV infection (Table 1).

4. Discussion

In this study, we showed that the extent of functional clonal proliferation after the administration of a single dose of HAV vaccine was sufficient to sensitize the host immune system and to induce HAV-specific immune response. Progressive clonal PBMC proliferation 2–4 weeks after the first dose and 1–4 weeks after the booster dose has previously been demonstrated [18,19,26]. However, we demonstrated for the first time that the memory T cell profile is activated following administration of the first HAV vaccine dose. Increases in the frequencies of classical effector memory B and T cells ($CD45RO^+$) and in the proportions of the TCD4 and TCD8 subsets of HAV-specific cells expressing the homing receptors CD62L and CCR7 (central memory T cells) were detected six months after administering the first dose (*T1*). Notably, the central memory T cell phenotype ($CD62L^+CCR7^+$) remained activated after administering the booster dose (*T2*). Limited data are available on the activation of central memory cells after HAV vaccination. Garner–Spitzer and colleagues [26] administered a booster dose to individuals who were previously immunized with an inactivated HAV vaccine. They observed an increase in the frequency of HAV-specific TCD4 cells expressing CD62L but not in the frequency of classical memory B or T cells in adults below 60 years of age.

Effector cells ($CD62L^-CCR7^-$) have been proposed as precursors of memory cells ($CD45RO^+$) [27]. Thus, when *naïve* T cells are activated, they mature into classical memory T cells and differentiate into central memory T cells and subsequently into T effector cells [31,32]. In agreement with this understanding, the results obtained from our *in vitro* assays suggest that once central memory cells are activated by HAV using only one dose of HAV vaccine, these cells activate effector cells *in vivo* and prevent infection upon subsequent HAV exposure [33–35].

The cellular response observed in vaccinated subjects at *T1* was similar to the HAV-specific memory lymphocyte activation ($CD45RO^+$) observed in patients with acute HAV infection (control group). Previous studies revealed that classical memory T cells subjected to antigen-specific activation proliferate approximately 2 weeks after viral infection and remained at a constant level thereafter to confer T cell-mediated immunological protection for the rest of the subject's life [27,31,36]. As expected, the numbers of effector TCD4 and TCD8 cells ($CD62L^-CCR7^-$) were not altered after HAV vaccination but were significantly elevated in the acute HAV patients. Studies explain that these effector T cells are increased after pathogen exposure as a primary response to contribute to

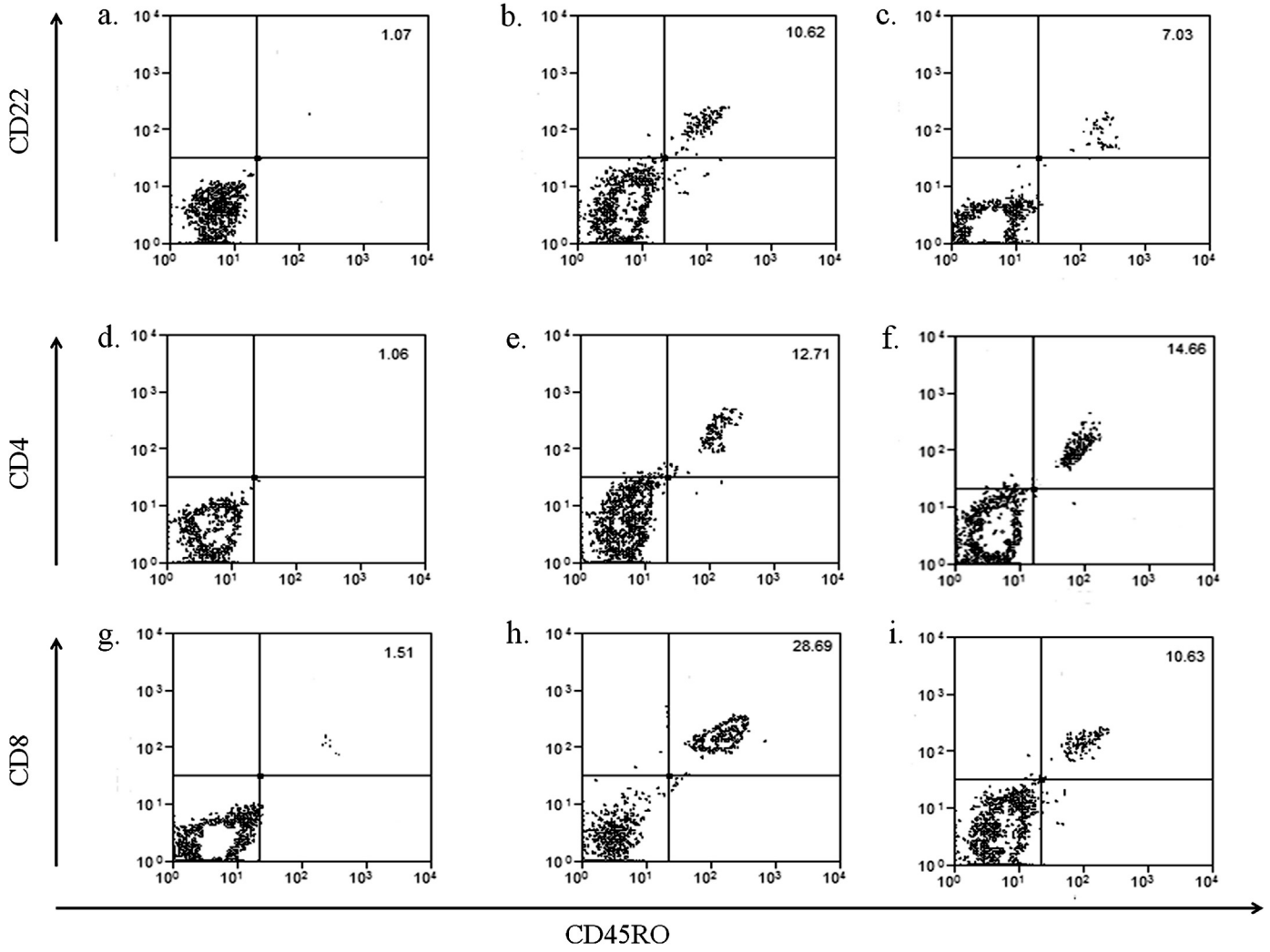

Fig. 1. Representative dot plots of HAV-specific classical memory B, TCD4 and TCD8 cells**.** Fluorescence-activated cell sorting (FACS) plots showing high CD22/CD45RO expression in PBMC-gated cells (memory B cells) after HAF-203 stimulation before vaccination (a), 6 months after the 1st dose (b), or 24 months after the 2nd dose (c). FACS plots showing high CD4/CD45RO expression in PBMC-gated cells (memory TCD4 cells) after HAF-203 stimulation before vaccination (d), 6 months after the 1st dose (e), or 24 months after the 2nd dose (f). FACS plots showing high CD8/CD45RO expression in PBMC-gated cells (memory TCD8 cells) after HAF-203 stimulation before vaccination (g), 6 months after the 1st dose (h), or 24 months after the 2nd dose (i).

Table 2
Comparison between poor responders and good responders after HAV stimulation following vaccination with the 1st dose.

Immunological parameter	6 months after the 1st dose (*T*1)		*t*-test
	Poor responders	Good responders	Good *vs* Poor*p* value (95% CI)
Anti-HAV IgG (IU anti-HAV antibodies/L)	≤10	>10[a]	0.0189 (35.33–55.55)
Proliferation index	2.24 ± 0.33	2.92 ± 0.32	Ns
$CD4^+CD45RO^+$ (%)	19.44 ± 7.25	17.33 ± 6.04	Ns
$CD8^+CD45RO^+$ (%)	10.33 ± 3.61	8.44 ± 2.44	Ns
$CD22^+CD45RO^+$ (%)	2.22 ± 0.42	10.33 ± 2.09	0.0306 (1.46–15.07)
$CD4^+CD62L^+CCR7^+$ (%)	30.84 ± 1.9	31.49 ± 8.37	Ns
$CD8^+CD62L^+CCR7^+$ (%)	20.83 ± 1.17	27.31 ± 7.38	Ns
IL-6 (pg/mL)	9,864.0 ± 1,871	10,163 ± 1,062	Ns
IL-10 (pg/mL)	56.58 ± 12.68	55.16 ± 14.04	Ns
IFNγ (pg/mL)	265.35 ± 60.8	152.24 ± 72.60	Ns
TNF (pg/mL)	272.37 ± 165.26	214.90 ± 11.58	Ns

Immunological parameters after HAV stimulation in the poor and good responders during vaccination. The mean values ± standard error obtained in PBMCs stimulated with HAF-203 before and after administering the 1st dose of inactivated HAV vaccine. The proliferation index, the frequency of memory T cells ($CD4^+CD45RO^+$; $CD8^+CD45RO^+$; $CD4^+CD62L^+CCR7^+$; or $CD8^+CD62L^+CCR7^+$) and memory B cells ($CD22^+CD45RO^+$), and the levels of cytokines were detected in culture supernatants after HAF-203 stimulation and were compared using the t-test. T0: non-vaccinated (naïve) samples; T1: samples collected 6 months after the 1st dose of hepatitis A vaccine; all cytokine levels are expressed in pg/mL; all data on the numbers of cells are presented as frequencies (%) from 20,000 events acquired via flow cytometry. pg/mL = Picograms per milliliter. Ns = Not significant.

[a] The "good responders" group was determined using Bioelisa HAV-EIA (Biokit, Barcelona, Spain).

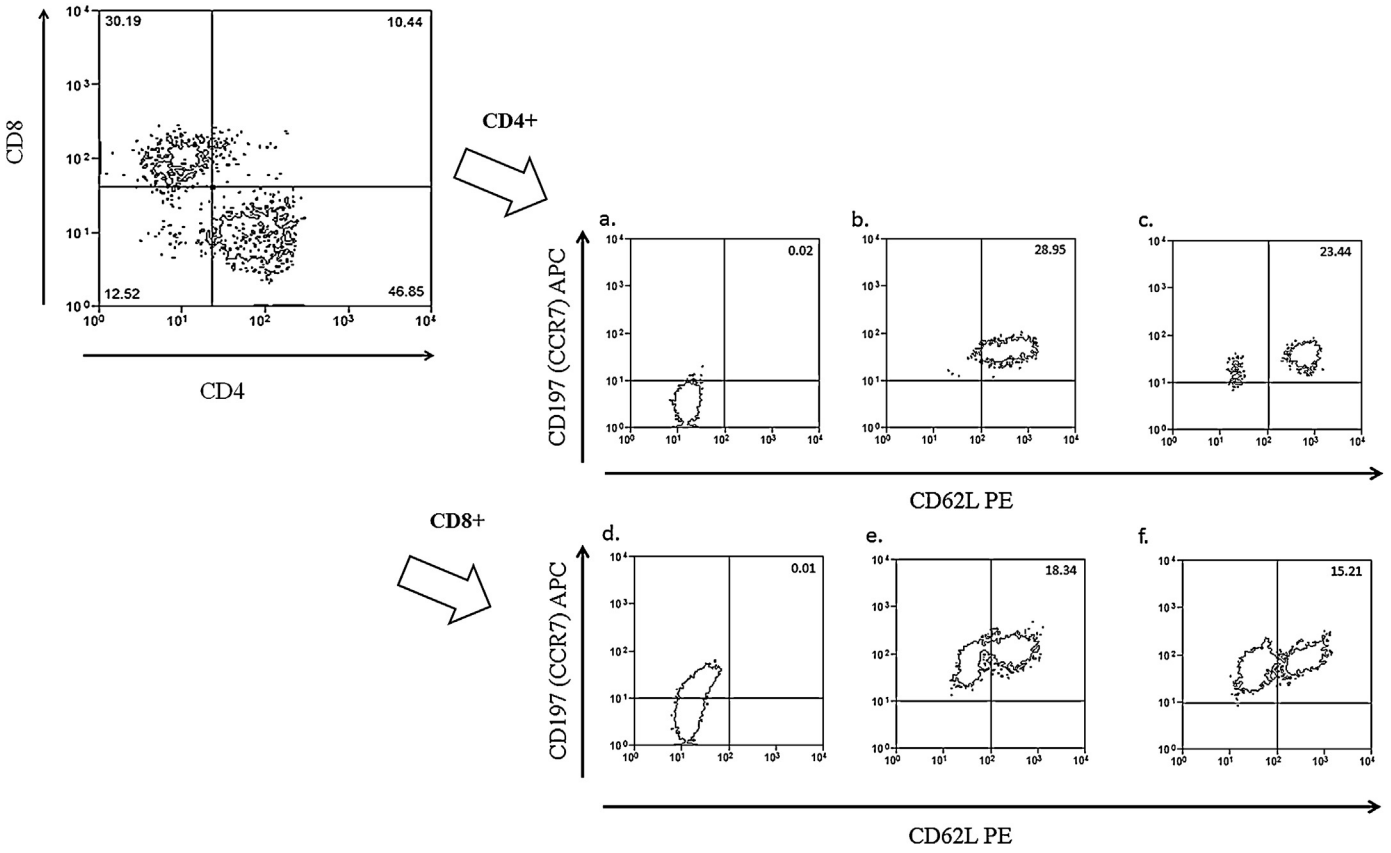

Fig. 2. Representative dot plots of HAV-specific central memory TCD4 and TCD8 cells. The dot plot strategy has been applied according to the highest levels of CD4-FITC and CD8-PerCP labeling to select $CD62L^+/CCR7^+$ T lymphocytes according to the flow cytometric parameters and using Summit version 6.0 software. (a) FACS plots showing central memory TCD4 cells after HAF-203 stimulation before vaccination (a), 6 months after the 1st dose (b), or 24 months after the 2nd dose (c). FACS plots showing central memory TCD8 cells after HAF-203 stimulation before vaccination (d), 6 months after the 1st dose (e), or 24 months after the 2nd dose (f).

successful viral clearance and to generate a set of precursors that can form the memory pool [27,31,36]. In addition, our results showed that central memory T cell ($CD62L^+CCR7^+$) proliferation could not be observed during acute HAV infection, as these cells do not exhibit proliferative potential for 6–12 months following virus exposure [27,31,36].

A significant increase in IFNγ, IL-6, and TNF levels was noted after HAV stimulation in PBMC samples obtained at T1, as previously described by Cederna and colleagues [18] IL-2, IL-10, and IFNγ production has also been described following the administration of a booster HAV vaccine dose [26]. HIV-infected children immunized with an inactivated HAV vaccine may display undetectable IL-2, IL-4, IL-5, IL-10, and IFNγ levels [37]. Similar IL-10 and IFNγ levels were observed at T1 and during the acute phase of HAV infection. These results reinforce the contribution of these cytokines to the protection provided by the HAV vaccine. Alternatively, a significant decrease in IL-6 and TNF levels after HAV stimulation was observed in acute hepatitis patients compared with the vaccines following the first or second vaccine dose. These results can be explained by the attempts of the immune system to control the inflammatory process during HAV infection, as has been described in the literature [38,39]. The decrease in these cytokines may also be associated with progression of the resolution of acute hepatitis A infection or with the sequestration of immune events to the intrahepatic compartment, which is the major disease site, as has been described by others [38–41].

Individuals exhibiting an undetectable humoral response after HAV vaccination have also been reported in other studies [18,19,26]. Antibody levels ranging from 10 to 33 IU/mL using different assays have been proposed as the threshold for protection from HAV infection in humans [10]. However, clinical experience suggests that protection following vaccination may be present even in the absence of detectable anti-HAV antibodies using standard immunoassays [10]. In this study, these subjects, termed poor responders, were investigated for their cellular immunity. Although HAV-specific memory B cells were not significantly activated after the first dose, the clonal expansion of HAV-specific memory T cells was observed. In other studies, poor responders with ongoing T cell activation have been observed after HAV [18,19] or hepatitis B vaccination [42].

The indication for a booster dose of inactivated HAV vaccine was based on early projections of waning antibody levels. However, long-term follow-up studies showed that 1 dose of HAV vaccine induces immunological memory and in most cases, the production of anti-HAV antibodies that persist for 4–11 years [15,16]. The results obtained herein extend these findings and suggest that the adoption of a single dose immunization system can serve as an alternative strategy for the prevention of hepatitis A in developing countries. Moreover, it has been argued that the natural booster response induced by wild virus circulation in endemic areas could ensure persistent protection [43].

In summary, the findings of this study demonstrate that a single dose of inactivated HAV vaccine may induce the proliferation of HAV-specific PBMCs, classical memory B and T cells, and central memory T cells and the production of cytokines after wild HAV strain stimulation in susceptible young adults. Additionally, a relevant improvement in terms of the HAV-specific cellular immune response was not observed after administering a booster dose of inactivated HAV vaccine. These findings suggest that once activated, the immune system is able to produce a specific cellular

response. Based on this evidence, protection against HAV infection may be guaranteed by a single dose of HAV vaccine in children in endemic areas.

Conflicts of interest

The authors declare no competing interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.06.099

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