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# Antibody but not memory B-cell responses are tuned-down in vertically HIV-1 infected children and young individuals being vaccinated yearly against influenza

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ABSTRACT

Yearly immunization against seasonal influenza is highly recommended for HIV-1 infected individuals but evaluating the success of vaccination by serological markers may not be fully informative in this population. Recently, it has been hypothesized that the generation of long-lasting immune responses may depend on whether similar antigens challenge the immune system frequently and intermittently. In the present study, in order to search for additional correlates of vaccine-induced protective immunity and to further dissect this theory, both humoral and memory B-cell responses to the trivalent 2012–2013 seasonal influenza vaccination has been evaluated by strain-specific (separately for H1N1, H3N2 and B strain) standard hemagglutination inhibition (HI) assay and B-cell enzyme-linked immunosorbent spot (ELISpot) in a cohort of vertically HIV-1 infected children and young individuals as compared to age-matched healthy controls. A high number of HIV-1 infected individuals had protective antibody levels prior to vaccination and showed low seroconversion rates after vaccination as compared to healthy controls. On the contrary, similar frequencies of influenza-specific memory B-cells were detected by B-cell ELISpot in both groups suggesting that an adequate B-cell response has been elicited. Data from the H1N1 strain, which is recurrent in seasonal influenza vaccines since 2009, pointed out decreasing antibody but not memory B-cell responses for HIV-1 infected patients being vaccinated for a greater number of years. Further investigations are required to standardize the influenza-specific B-cell ELISpot and to understand whether it could be used routinely as an additional tool to evaluate response to influenza vaccination in immune-compromised individuals being vaccinated yearly.



## 1. Introduction

High incidence of influenza infection has been reported among HIV-1 infected individuals [1–3] with several studies recording greater hospitalization rates [4], prolonged illness [5] and increased mortality [6]. During HIV-1 infection, CD4+ T-cells and age have been shown to be important for a successful response to influenza infection and vaccination [7–11]. In the context of vertically HIV-1 infected children or young individuals starting antiretroviral therapy (ART) early in life, high levels of CD4+ T-cells have been recorded [12]. Yet, these individuals are at risk of unsuccessful immunization due to B-cell defects [13,14]. Yearly immunization against seasonal influenza is highly recommended for HIV-1 infected individuals [15]. However, evaluating the success of influenza vaccination in immune-compromised populations by antibody fold increase may not be fully informative [13,16]. Interestingly, it has recently been hypothesized that the generation of long-lasting cellular immune responses may depend on whether similar antigens challenge the immune system frequently and intermittently [17]. This may be the case of yearly administration of influenza vaccines (containing same or drift variant strains) to HIV-1 infected patients. The aim of the present study was to search

*Abbreviations:* HI, hemagglutination inhibition; ELISpot, enzyme-linked immunosorbent spot.

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for additional correlates of successful influenza vaccination and to further dissect this theory. In line with this, both humoral and memory B-cell responses to the trivalent 2012–2013 seasonal influenza vaccination (separately for the H1N1, H3N2 and B strains) has been evaluated by strain-specific standard hemagglutination inhibition (HI) assay and B-cell enzyme-linked immunosorbent spot (ELISpot) in a cohort of vertically HIV-1 infected children and young individuals receiving seasonal influenza vaccines yearly as compared to age-matched healthy controls for whom seasonal influenza vaccination is not routinely performed.

## 2. Materials and methods

### 2.1. Subjects

Totally, 23 HIV-1 negative individuals/healthy controls (abbreviated as HC) and 65 ART-treated HIV-1 infected patients (abbreviated as HIV) were enrolled between September 2012 and November 2012 at the Unit of Immune and Infectious Diseases of the Bambino Gesù Children's Hospital, Rome, Italy. As the Centers for Disease Control and Prevention (CDC) points out, some children 6 months through 8 years of age may respond differently to flu vaccination and may require 2 doses of vaccine (http://www.cdc.gov/flu/protect/children.htm). Therefore, HIV and HC were further subdivided into two groups: data from 12 HIV and 9 HC with age between 2 and 8 years were included in the first group (abbreviated as $2 \leq yy \leq 8$) while 53 HIV and 14 HC with age between 8 and 28 years were included in the second group (abbreviated as $yy > 8$). Restrictions for inclusion were the presence of flu-like symptoms or concomitant diseases at the time of enrollment. Written informed consent was obtained from all subjects or parents/legal guardians before enrollment and the ethical committees of the Bambino Gesù Children's Hospital approved the study. Characteristics of all subjects are summarized in Table 1.

### 2.2. Vaccination and sample preparation

All subjects received one dose of the Inactivated Influenza Vaccine Trivalent Types A and B (Split Virion) VAXIGRIP® (sanofi pasteur). The strains for the 2012–2013 season are: A/California/7/2009 (H1N1)pdm09-like strain (abbreviated as H1N1), A/Victoria/361/2011 (H3N2)-like strain (abbreviated as H3N2) and B/Wisconsin/1/2010-like strain (abbreviated as B). Vaccination was given during October and November 2012. Blood was collected prior to vaccination (abbreviated as T0) and after 21 days from vaccination (abbreviated as T1). Peripheral Blood Mononuclear Cells (PBMCs) and sera were purified from Ficoll (LiStarFish) EDTA (ethylenediaminetetraacetic acid)-treated and non-treated blood and temporarily frozen until further analyses.

### 2.3. Hemagglutination inhibition (HI) assay

The antibody titers to the H1N1, H3N2 and B influenza strains in sera from HIV and HC were evaluated separately by HI assay. The virus strains used in the HI assay were A/California/7/2009 (H1N1)pdm09-like strain, A/Victoria/361/2011 (H3N2)-like strain and B/Wisconsin/1/2010-like strain according to the 2012–2013 influenza vaccine formulation. The HI assay was performed as previously described [18]. The HI antibody titers were expressed as the reciprocal of the highest serum dilution at which hemagglutination was prevented. A 4-fold increase in the reciprocal of the titer from vaccination to 21 days after vaccination was considered as a positive response to the vaccine as it corresponds to seroconversion while antibodies with an HI titer above 1:40 were considered as protective (http://www.gmp-compliance.org/guidemgr/files/021496EN.PDF) [13].

### 2.4. PBMC cultures and B-cell enzyme-linked immunosorbent spot (B-cell ELISpot)

PBMCs collected at T0 and T1 from HIV and HC were thawed and policlonally activated *in vitro* in complete RPMI medium (Invitrogen) supplemented with 2.5 μg/mL CpG type B (Hycult biotech), 20 ng/mL IL-4 (Peprotech) and 20 ng/mL IL-21 (ProSpec). Cells were harvested after 5 days of culture at 37 °C. ELISpot 96-well filtration plates (Millipore) were coated as previously described [19] with the addition of purified H1N1, H3N2 and B influenza inactivated virus particles and subsequently loaded with $2 \times 10^5$ cells/well. Plates were then processed as previously described [19]. Membranes were punched out with an Eli.Punch device and developed spots were scanned with an Eli.Scan and counted with the ELISpot Analysis Software V5.1 (all from A.EL.VIS).

### 2.5. Statistical analysis

The 95% confidence intervals (CI) of the proportion of individuals with an HI titer ≥ 1:40 or with an HI fold increase ≥4-fold in the different groups was calculated with the Wilson procedure without a correction for continuity. Fisher's exact test was applied to analyze the proportion of individuals with an HI fold increase ≥4-fold among groups, Mann–Whitney test and Spearman correlation were used for all other analyses. A $p$ value <0.05 was considered as statistically significant while a $p$ value between 0.06 and 0.09 was considered as indicating a statistical trend. The GraphPad Prism software for Windows was used to perform the analyses.

## 3. Results

### 3.1. HIV-1 viral load, CD4+ T-cell counts and seroprotection prior to and after 2012–2013 seasonal influenza vaccination

Totally, 10/65 corresponding to 15% of the HIV-1 infected individuals (abbreviated as HIV) was having a viremic blip at the time of vaccination (Table 1). However, this did not relate to any of the parameters analyzed, *i.e.* H1N1, H3N2 and B HI titers, HI fold increase as well as data from the B-cell ELISpot, as confirmed by Spearman correlation ($p > 0.05$). Moreover the CD4+ T-cell counts were similar in the viremic and aviremic patients in both the HIV age groups (from 2 to 8 years abbreviated as $2 \leq yy \leq 8$ and from 8 to 28 years as $yy > 8$) ($p > 0.05$). The HI titers before and after 2012–13 seasonal vaccination were measured; reverse cumulative distribution curves and the absolute percentage of seroprotected individuals (HI titers ≥ 1:40) are reported in Fig. 1A and Table 2. Following national guidelines, HIV-1 infected individuals are being vaccinated yearly against seasonal influenza (Table 1) therefore HI titers to H1N1 and H3N2 ≥ 1:40 were commonly detected at T0 in the HIV groups (75% to over 90% of individuals) (Fig. 1A). No correlation was found between the CD4+ T-cell counts at vaccination and the different levels of anti-H1N1, H3N2 or B influenza antibodies observed at T0 and at T1 in both the HIV groups ($p$-values > 0.05).

### 3.2. Anti-H1N1, H3N2 and B influenza antibodies in the serum of HIV and HC

In order to evaluate the response to the 2012–2013 seasonal influenza vaccination, the seroconversion rates (HI fold increase ≥4-fold after 21 days from vaccination), were used for further analyses. At T1, a significantly lower % of HIV-1 infected individuals seroconverted after the additional H1N1 and H3N2 challenge in both age groups as compared to healthy controls (abbreviated as HC). In the $2 \leq yy \leq 8$ age group, 50% of HIV-1 infected children with 95% CI 25.4–74.6% and 25% with 95% CI 8.9–53.2% respectively, responded to H1N1 and H3N2 as compared to HC (77.8% with