Transplant Immunology 53 (2019) 51–60

Contents lists available at ScienceDirect

Transplant Immunology

journal homepage: www.elsevier.com/locate/trim

Kinetics of antibody response to influenza vaccination in renal transplant recipients

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ARTICLE INFO

Keywords:
Kidney transplant
Immunosuppression
Influenza
Vaccination
Antibody

ABSTRACT

Annual vaccination is routinely used in organ transplant recipients for immunization against seasonal influenza. However, detailed analysis of the kinetics of vaccine-induced immune responses in this population is lacking. In this study, we investigated the kinetics of vaccine strains-specific antibody responses to trivalent influenza vaccine in a group of renal transplant recipients and a control group. First, we found that the geometric mean hemagglutination inhibition titer against all 3 vaccine strains in the transplant cohort was significantly low when compared to control subjects. Next, whereas the control group sera showed significantly higher HA-specific IgG and isotype IgG1 antibodies at all four time points, a similar increase in the transplant group was delayed until day 28. Interestingly, within the transplant group, subjects receiving belatacept/MMF/prednisone-based regimen had significantly lower levels of total IgG and HA-specific IgG when compared to tacrolimus/MMF/prednisone-based regimen. Even though IgG-ASC response in both cohorts peaked at day 7 post-vaccination, the frequency of IgG-ASC was significantly low in the transplant group. Taken together, our studies show delayed kinetics and lower levels of influenza vaccine-specific antibody responses in renal transplant recipients and, more importantly, indicate the need to probe and improve current vaccination strategies in renal transplant recipients.

1. Introduction

Annually, influenza virus infection causes highly contagious and acute respiratory disease that poses serious public health problems resulting in significant morbidity and mortality, worldwide [1,2]. Vaccination and use of antiviral drugs are the two well recognized strategies for prevention and treatment of influenza virus infection [2]. However, the problems associated with the choice of vaccine strains against annual epidemics of influenza have prompted researchers and health agencies to focus not only on improvement of vaccines for defined age groups but also, more importantly, for immunocompromised populations [3,4]. Notably, severe or fatal disease during the 2009 A(H1N1)pdm09 outbreak occurred primarily in individuals with risk factors and underlying medical conditions [5–7]. Among high-risk groups, while a number of investigations have focused on aged and pediatric populations, very few studies looked at the effects of clinically-induced immunosuppression on immunity to influenza. As a result, the impact of clinically-induced immunosuppression on immunity to influenza viruses is largely unknown.

Organ transplant recipients rely on a long-term immunosuppressive regimen for successful graft maintenance and function [8]. As a result, this population is likely to be more susceptible to seasonal influenza virus infection. Specifically, with reference to renal transplantation patient population, some studies have addressed the safety and efficacy of influenza vaccination. For example, studies using trivalent influenza vaccine and vaccination against A(H1N1)pdm09 among healthy and renal transplant cohorts have yielded outcomes ranging from comparable responses [9,10] to significantly impaired responses [11–16]. Although, the results for these studies show that influenza vaccination is safe in renal transplant recipients, vaccine responses in these studies varied based on the time of vaccination relative to transplantation [17], type of immunosuppressive regimen used [13,18–20], or the graft

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https://doi.org/10.1016/j.trim.2019.01.001
Received 25 September 2018; Received in revised form 11 January 2019; Accepted 15 January 2019
Available online 18 January 2019
0966-3274/ Published by Elsevier B.V.

function [21] during the course of the study. Notably, majority of these studies investigated vaccine specific responses at baseline and 3–4 weeks post-vaccination. Because many of these studies did not examine the kinetics of immune response at early time points after vaccination, potential differences in the induction as well as magnitude and maintenance of vaccine-specific immune response in renal transplant cohorts maintained on different immunosuppressive regimen are not well established.

2. Objective

In this study, we determined the kinetics (days 7, 14, 28, and 90 post-vaccination) of vaccine strain-specific hemagglutination inhibition (HI) titer, seroconversion rates, hemagglutination (HA)-specific IgG and IgG1, HA binding rates of antibodies, and frequency of IgG-antibody secreting cells (ASC) induced in response to 2007-08 trivalent influenza vaccine (TIV) in renal transplant recipients and control subjects.

3. Materials and methods

3.1. Study population, vaccination, and blood collection

As described in Table 1, this study included 12 patients (8 females and 4 males) who have received a renal transplant between the ages of 34 and 58, and 8 age-matched control subjects (5 females and 3 males) between the ages of 33 and 58. Both groups were vaccinated with the 2007-08 trivalent vaccine (Fluzone, Sanofi Pasteur), which included the H1N1 strain A/Solomon Island/3/2006, the H3N2 strain A/Wisconsin/67/2005, and the Influenza B strain B/Malaysia/2506/2004. A volume of 72 mL of blood was collected in BD vacutainer tubes (Becton Dickinson) on days 0, 7, 14, 28, and 90 post-vaccination for separation of serum. The transplant group received renal grafts 7 months to 8 years prior to immunization and was receiving a stable immunosuppressive regimen throughout the course of the study. The immunosuppressive regimen consisted of belatacept/mycophenolate mofetil/prednisone ($n = 5$) hereafter referred to as B/M/P, tacrolimus/mycophenolate mofetil/prednisone ($n = 6$) hereafter referred to as T/M/P, and sirolimus/mycophenolate mofetil/prednisone ($n = 1$) hereafter referred to as S/M/P (Table 1). Briefly, MMF daily dosage in 12 subjects ranged from 500 mg–2000 mg. Prednisone daily dosage in 12 subjects was either 5 or10 mg. Belatacept dosage in 5 subjects was 5 mg/kg. Tacrolimus level at study enrollment in 6 subjects ranged from 4.2–12.2 ng/dL and the target levels were 5–8 ng/dL. Sirolimus level in one subject at study enrollment was 4.2 ng/dL and the target level was 5–10 ng/dL. The study was conducted according to the protocol approved by the Emory University Institutional Review Board (IRB) and Centers for Disease Control and Prevention-Reliance IRB (#5445). There were no vaccine related complications in either study groups during the course of the study.

3.2. HI assay

HI assays were performed with pre- and post-vaccination serum specimens as previously described [22] using 0.5% turkey erythrocytes. Serum samples were first treated with receptor destroying enzyme overnight. Then non-specific agglutinins were removed by serum adsorption with packed turkey erythrocytes. Twenty five μL of serial 2-fold diluted sera were incubated with 4 HAU/25 μL of virus and 50 μL 0.5% turkey erythrocytes for 30 min. The HI titer was defined as the reciprocal of the last dilution of serum that completely inhibited hemagglutination. All viruses used in HI assays were propagated in 9–11 day old embryonic chicken eggs.

3.3. ELISA

As described previously [23], total serum IgG was measured by a sandwich ELISA. Briefly, Immulon 2 HB microtiter plate (Thermo Fisher Scientific, IL) wells were coated with 100 μL of 2 μg/mL unlabeled Goat F(ab′)2 Anti-Human IgG (SouthernBiotech, AL) for 3 h. The corresponding wells for human IgG standards were washed with cold PBS and 100 μL of human IgG standard (1000, 500, 375, 250, 188, 125, 94, 63, 47, 31, 16, and 0 ng/mL) (Thermo Fisher Scientific, IL) were added, followed by overnight incubation at 4 °C. For detection of HA-specific IgG [23] and IgG1 by indirect ELISA, Immulon 2 HB microtiter plate was coated with 100 μL of 2 μg/mL unlabeled Goat F(ab′)2 Anti-Human IgG (Southern Biotech, AL) for 3 h. Unbound coating antibodies were washed with PBS and 100 μL of purified human IgG standard (Thermo Fisher Scientific, IL) or purified human IgG1 standard (Athens Research and Technology, GA) were added. For HA-specific antibody detection, the plate was coated with 100 μL of mixture of histidine tagged recombinant hemagglutinin (rHA) from 2007 to 08 northern hemisphere vaccine component (1 μg/mL of rHA from A/Solomon Islands/3/2006 (H1N1), 1 μg/mL of rHA from A/Wisconsin/67/2005 (H3N2), 0.5 μg/mL of globular head HA1 domain rHA from B/Brisbane/60/2008 (B/Victoria lineage) followed by overnight incubation at 4 °C. Next day, the plate was washed with a wash buffer (PBS containing 0.05% (v/v) Tween® 20 (Sigma, SO)). Sera were 5-fold diluted in antibody 'Diluent buffer' [(PBS containing 2% (w/v) BSA (Sigma, SO) and 0.05% (v/v) Tween® 20 (Sigma, MO)] and added to each well followed by incubation at room temperature for 1 h. The plates were washed with wash buffer and horseradish peroxidase-labeled goat anti-human anti-IgG (Kirkegaard & Perry Laboratories, MD) or mixture of 4 HRP conjugated mouse anti human IgG1 monoclonal antibodies (Clone #: 4E3 and HP6001 from Southern Biotech, AL and Clone #: HP6069 and HP6070 from Life Technologies Corporation, CA) was added to corresponding wells. Fifty μL of SureBlue™ TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories, MD) was added to each well and the reaction was stopped with 50 μL of TMB stop solution (Kirkegaard & Perry Laboratories, MD). Plates were read at 450 nm with a SPECTRAmax plate spectrophotometer (Molecular Devices, CA). The total serum IgG and HA-specific IgG and IgG1 concentrations were calculated

Table 1
Description of study subjects and immunosuppression regimen at the time of vaccination.

	Control group	Renal transplant group
Number	8	12
Age range	33–58	34–58
Gender	5F, 3 M	8F, 4 M
Immunosuppressive regimen	-	B/M/P; belatacept/ mycophenolate mofetil /prednisone ($n = 5$) T/M/P; tacrolimus/ mycophenolate mofetil /prednisone ($n = 6$) S/M/P; sirolimus/ mycophenolate mofetil /prednisone ($n = 1$)
Time from transplant to vaccination	-	7 months-8 years
ESRD	None	Congenital obstructive uropathy (n = 1), Diabetes mellitus type 2 ($n = 2$), Focal segmental glomerulonecrossis ($n = 3$), Hypertensive nephrosclerosis (n = 1), Interstitial nephritis (n = 1), SLE-lupus nephritis (n = 1), Chronic pyelonephritis/reflux (n = 1), Urate nephropathy (n = 1), Polycystic kidney disease (n = 1)
Vaccine related complications	None	None

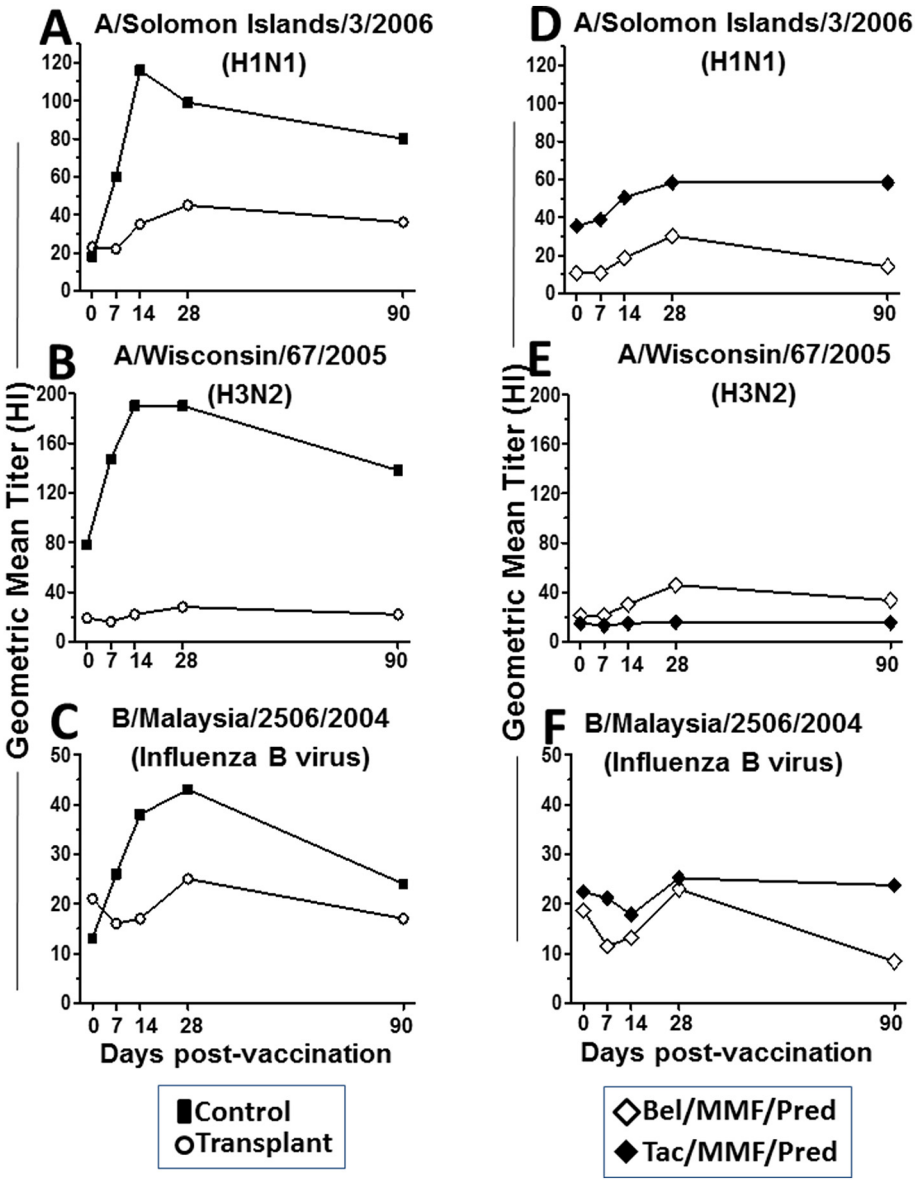

Fig. 1. Serum HI titer in control and transplant groups vaccinated with TIV 2007–08. Sera collected from control group (filled square), transplant patients (open circle), transplant patients receiving B/M/P (open diamonds) and T/M/P regimen (closed diamonds) at baseline and different time points post-vaccination were assayed for HI titer against 2007–08 vaccine strains: H1N1, A/Solomon Island/3/2006 (A, D); H3N2, A/Wisconsin/67/2005 (B, E); Influenza B virus, B/Malaysia/2506/04 (C, F), as described in the 'Methods' section. Data represent GMT values for control and transplant subjects at days 0, 7, 28, 90 post-vaccination.

according to the corresponding standard curve using SoftMax Pro v5 software (Molecular Devices, CA).

3.4. Antibody-binding assay

As described previously [24], antibody binding to rHA was measured by Bio-Layer Interferometry (BLI) using an Octet RED96 system (PALL Fortebio LLC, Menlo Park, CA). Briefly, rHA containing a C-terminal His-Tag was diluted in 1× kinetics buffer (PBS with 0.02% Tween 20, 0.005% sodium azide, and 100 μg/mL BSA) and bound to Anti-Penta His biosensors. Antibody binding to the bound rHA was measured as wavelength shift (in nanometers). The system software was used for data analysis and human sera from the study groups and ferret sera raised against vaccine strains were all pre-treated with RDE as described previously [24].

3.5. IgG-ASC ELISPOT assay

Briefly, as described previously [25], PBMC were isolated using Vacutainer tubes (Becton Dickinson), washed and re-suspended in R10 supplemented culture media (RPMI/10%FCS/ PenStrep/L-glutamine/ BME). For ELISPOT assay, 96-well filter plates (Millipore, MA) were coated with 1/20 diluted influenza vaccine or goat anti-human Ig (10 μg/mL) (Caltag, CA) in PBS. Plates were washed with PBS and blocked with R10 for 2 h at 37 °C. The cultured PBMC were washed and added to ELISPOT plates followed by incubation for 6 h at 37 °C. Plates were then washed with PBS and incubated overnight with anti-hu-IgG-biotin (Caltag, CA) followed by incubation with avidin-D-HRP (Vector Laboratories, CA) and developed using AEC substrate (Sigma, MO). Developed plates were analyzed for ASC using an ELISPOT counter (Cellular Technology, OH). The data are represented as frequency of IgG-ASC per million PBMC.

3.6. Statistical analysis

For serology data of HI titer, ELISA-IgG concentration, and HA binding rate, Log2 transformed values were used as dependent variables and summarized as geometric mean concentrations. Means and differences in means were estimated using repeated measures linear mixed models [26,27]. Back-transforming model-estimated means yielded geometric mean values and back-transforming differences from day 90, 28, 14 and 7 to day 0 means yielded geometric mean ratios (from day 0 to day 7, 14, 28 and 90 fold-rise). Models contained indicator variables representing serum draw days (90, 28, 14 and 7) vs. day 0, indicator variables representing treatment (control vs. transplant and T/M/P vs. B/M/P) groups and product terms representing interaction between serum draw and treatment group (control vs. transplant and T/M/P vs. B/M/P). Interaction terms allowed us to estimate fold-rises by treatment group, and test for differences in fold-rises between control and transplant group. For HA binding, similar models were used. However, as these data are expressed as percentages, we used Log10 transformation. Back-transforming model-estimated means yielded geometric mean percentages (GMP) and back-transforming differences from day 90, 28, 24 and 7 to day 0 means yielded GMP ratios. He et al. [28] likewise used Log10 transformed percentages of antigen-specific cells, and models for correlated data to estimate GMP and GMP fold-rise for cell-mediated responses to influenza vaccine. Analyses were performed using SAS software version 9.3 (SAS Institute Inc., Cary, NC) or GraphPad Prism. For IgG-ASC analysis, Two-tailed *t*-test was used. p values of < 0.05 were considered to be statistically significant.

4. Results

4.1. Kinetics of vaccine strain-specific HI antibody titer in transplant recipients and age-matched control subjects

Sera were collected from transplant recipients and age-matched control subjects on days 0, 7, 14, 28, and 90 relative to vaccine administration. Serum HI assay [22] was performed against all 3 viral strains represented in the 2007-08 trivalent vaccine. As shown in Fig. 1 (left panels), control group showed a noticeably increasing HI-GMT by day 7 against all three vaccine strains in the vaccine. The peak HI-GMT in the control group was seen on day 14 against A/H1N1 (Fig. 1A) and A/H3N2 (Fig. 1B), and by day 28 against Influenza B virus (Fig. 1C). In the transplant cohort, the increase in vaccine-specific HI-GMT was less evident and, more importantly, the peak response was delayed until day 28 (Fig. 1A, B, and C). The control subjects had significantly higher HI-GMT than transplant recipients against all vaccine strains (Fig. 1, left panels and Table 2). Specifically, HI-GMT titer against A/H1N1 in the control group was increased ~5-fold higher by day 14, whereas transplant patients only had a ~2-fold increase during their peak HI-GMT on day 28 post-vaccination (Fig. 1A and Table 2). The HI-GMT against A/H3N2 in control subjects were 2-fold higher by day 14, while no such change was observed in the transplant group (Fig. 1B and Table 2). Similarly, while the HI-GMT against influenza B virus in the control group increased by 3-fold, there was no considerable increase of HI-GMT in the transplant cohort (Fig. 1C and Table 2). Although the baseline HI-GMT against A/H1N1 was comparable for the two groups, the control group had higher (~3 fold) baseline HI-GMT against A/H3N2 vaccine strain. However, the transplant cohort had 2-fold higher baseline HI-GMT titer against influenza B virus compared to their counterpart. Comparison of HI-GMT within the control group to their baseline values showed significant increase in fold-rise against all 3 vaccine strains and at all 4 time points post vaccination (Table 2). Nevertheless, the increase in fold-rise in the transplant group was significant only at day 28 and against 2 vaccine strains (A/H1N1 and A/H3N2) (Table 2).

The overall reduction in HI-GMT in transplant group prompted us to analyze HI-GMT difference among different immunosuppressive regimens. However, we analyzed the HI-GMT only between the B/M/P and T/M/P regimens since there were relatively higher number of study subjects under these two groups. Notably, the T/M/P group had > 2-fold higher baseline HI-GMT titer against A/H1N1 when compared to the B/M/P group. Nonetheless, in the B/M/P group, when compared to baseline HI-GMT, the increase in fold-rise was significantly high by day 28 post-vaccination (Fig. 2D and Table 2). However, the increase in HI-GMT fold-rise over the baseline values was not significant in the T/M/P group at any given time point post-vaccination (Fig. 1D and Table 2). Although the baseline HI-GMT against A/H3N2 was comparable for the two groups (B/M/P and T/M/P), the B/M/P group had significantly higher HI-GMT at day 28 post-vaccination (Fig. 1E and Table 2). Additionally, when compared to baseline titer, the T/M/P group failed to show any appreciable increase in HI-GMT at any given time point post-vaccination. The differences in HI-GMT against influenza B virus for both T/M/P and B/M/P groups were not significantly different when compared to their baseline titers (Fig. 1F and Table 2). Furthermore, analysis of HI-GMT between the transplant and control groups showed a difference in HI-GMT although only against the A/H3N2 vaccine strain (Table 3). However, within the two transplant groups, the differences in HI-GMT between the B/M/P and T/M/P regimens were not significantly different (Table 3). Overall, HI-GMT in the transplant cohort against 2007-08 TIV viral strains was significantly low with a delay in response when compared to controls.

Analysis of the HI-GMT for seroconversion (convalescent titer greater than or equal to 40) in response to vaccination showed differences between the control and the transplant groups. Specifically, the A/H1N1 elicited highest incidence of seroconversion in the control group (4/8; 50%), and to a lesser extent in the transplant group (3/12; 25%) (Table 4). Incidence of seroconversion against the A/H3N2 strain was not considerably different between the control (1/8; 12.5%) and transplant group (1/12; 8.3%) (Table 4) although post-vaccination GMT was much higher in the control group (Fig. 1B). The difference in seroconversion between the two groups was also seen against influenza B vaccine strain with a few (2/8; 25%) control subjects showing seroconversion, but none in the transplant group (0/12; 0%) (Table 4).

4.2. Kinetics of total serum IgG, HA-specific IgG and IgG1 responses in the sera of transplant recipients and control subjects

The lower HI-GMT in transplant patients could be a consequence of the immunosuppressive regimen that can lower antigen-specific antibody secretion and, therefore, affect the host's immunity against specific pathogens in transplant patients. In order to take a closer look at the humoral immunity and to test this hypothesis, we analyzed the amounts of influenza HA-specific antibodies in the serum of control and transplant subjects. As shown in Fig. 2A, when compared to control group, the mean total serum IgG level at all time points was low in the transplant group. Also, when compared to baseline IgG levels, while total IgG concentration in the control group did not show any significant variations over the course of vaccination, day 7 sera from the transplant group showed lower levels of IgG (Fig. 2A, Table 5). Since HA-specific antibody induction forms the basis for inactivated influenza vaccines, we quantified the antibody response to the HA antigen using recombinant HA (rHA) antigens from all three influenza strains of 2007–08 northern hemisphere vaccine [A/Solomon Islands/3/2006 (H1N1), A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004 (B/Victoria lineage)]. Both groups had about equivalent rHA-binding IgG antibodies at baseline (day 0) (Fig. 2B). In response to vaccination, the rHA-specific mean IgG level in the control group showed a significant increase at days 7, 14, 28, and 90 when compared to baseline (day 0) time point (Fig. 2B, Table 5). However, the increase in HA-IgG level in the transplant group was delayed and reached significantly higher concentrations by day 28 post vaccination (Fig. 2B, Table 5). In a study by Garcon et al., IgG1 was shown to be the most predominant IgG

Table 2
HI-GMT fold-rise in control and renal transplant subjects after influenza vaccination.

Vaccine strain	Baseline (Day 0) comparison	Control group			Transplant group		
		Point estimate	95% CI (Low-High)	*p* value	Point estimate	95% CI (Low- High)	*p* value
A/Solomon Islands/3/2006	Day 7	3.29	1.7–6.3	< 0.001*	0.98	0.5-1.6	0.956
	Day 14	6.30	3.2–12.0	< 0.001*	1.49	0.8-2.5	0.133
	Day 28	4.37	1.0–18.2	0.043*	1.97	1.1-3.3	0.013*
	Day 90	4.64	2.3–9.1	< 0.001*	1.59	0.9-2.7	0.094
A/Wisconsin/67/2005	Day 7	1.87	1.2–2.9	0.006*	0.91	0.6-1.3	0.629
	Day 14	2.42	1.5–3.7	< 0.001*	1.15	0.8-1.6	0.426
	Day 28	2.42	1.5–3.7	< 0.001*	1.47	1.0-2.1	0.034*
	Day 90	2.32	1.4–3.6	< 0.001*	1.14	0.7-1.6	0.474
B/Malaysia/2506/2004	Day 7	2.09	1.2–3.3	0.003*	0.77	0.5-1.1	0.191
	Day 14	3.37	2.0–5.4	< 0.001*	0.81	0.5-1.2	0.311
	Day 28	3.45	2.1–5.6	< 0.001*	1.22	0.8-1.8	0.310
	Day 90	2.13	1.2–3.5	0.004*	0.88	0.5-1.3	0.553
Vaccine strain	Baseline (Day 0) comparison	B/M/P group Point estimate	95% CI (Low - High)	*p* value	T/M/P group Point estimate	95% CI (Low - High)	*p* value
A/Solomon Islands/3/2006	Day 7	0.99	0.4–2.0	0.992	1.09	0.5–2.1	0.783
	Day 14	1.73	0.8–3.5	0.127	1.41	0.7–2.7	0.284
	Day 28	2.81	1.3–5.7	0.006*	1.63	0.8-3.1	0.134
	Day 90	1.64	0.7–3.5	0.197	1.63	0.8–3.1	0.134
A/Wisconsin/67/2005	Day 7	1.00	0.6–1.6	1.000	0.89	0.5–1.4	0.611
	Day 14	1.41	0.8–2.3	0.168	1.00	0.6–1.5	1.000
	Day 28	2.14	1.3–3.5	0.004*	1.06	0.6-1.6	0.793
	Day 90	1.32	0.7–2.2	0.296	1.03	0.6–1.6	0.893
B/Malaysia/2506/2004	Day 7	0.64	0.1–3.9	0.601	0.94	0.6–1.4	0.778
	Day 14	0.70	0.4–1.1	0.140	0.89	0.5–1.3	0.585
	Day 28	1.23	0.7–1.9	0.370	1.12	0.7–1.7	0.585
	Day 90	0.68	0.4–1.1	0.132	0.94	0.6–1.4	0.778

* *p* values of < 0.05.

subclass specific to influenza antigens [29]. In addition, IgG1 responses was shown to predominate over other IgG subclasses during antibody responses to natural infections and vaccinations [30]. Therefore, we determined the amount of anti-rHA IgG1 antibodies produced by both groups post-vaccination. When compared to baseline (day 0), the control group showed a significant increase in rHA-specific IgG1 levels at days 7, 14, 28, and 90 post-vaccination (Fig. 2C, Table 5). However, despite similar increase in rHA-IgG1 in the transplant group, the peak response was delayed until day 28 (Fig. 2C, Table 5). While the increase in rHA-IgG and rHA-IgG1 antibody levels at different time points post-vaccination were significantly different in the control and transplant groups when compared to their respective base line levels, the differences in the levels of total IgG, rHA-IgG, or rHA-IgG1 between control and transplant groups at any given time point was not statistically significant (Supplemental Data Table).

Within the transplant cohort, mean IgG concentration for B/M/P group was relatively low when compared to T/M/P group at base line as well as at all other time points post-vaccination (Fig. 2D and Table 5). Although the mean values for T/M/P group at baseline (day 0) was > 2-fold high when compared to B/M/P group, there was a significant increase in mean rHA-IgG and rHA-IgG1 in the B/M/P group at day 28 post-vaccination (Fig. 2E, F and Table 5). However, the differences in total Ig G, rHA-IgG, or rHA-IgG1 between T/M/P and B/M/P groups at any given time point was not statistically significant (Supplementary Data Table).

4.3. Kinetics of HA binding rate of serum antibodies in the transplant and control groups

Since the induction of HA-specific antibody (Day 0–14) was found to be relatively low and delayed in transplant patients (Fig. 2B and Table 5), we determined whether there was any differences in the quality of HA-specific antibodies by measuring the HA-specific antibody binding using label-free biolayer interferometry [24]. When compared to baseline values, sera from the control group showed antibodies with a significant increase in GMP-binding rates to H1-rHA (A/Solomon Islands/3/2006) at all time points post-vaccination (Fig. 3A, Table 6). Although a similar increase over baseline values in the control group was also seen against H3-rHA (A/Wisconsin/67/2005), the differences were significant only at Days 14 and 90 post-vaccination (Fig. 3B, Table 6). However, in the transplant group and between the two immunosuppressive regimens (T/M/P and B/M/P), when compared to baseline values, none of the post-vaccination sera showed any significant changes in GMP-binding rate against either strains (Fig. 3, Table 6). Finally, comparison of GMP binding rate between the control and transplant groups showed significant difference in HA binding rate for both strains at day 14 post-vaccination (Fig. 3, Table 7). However, the difference in GMP binding rate between the T/M/P and B/M/P groups were not significant at any given time post-vaccination (Table 7).

4.4. Kinetics of IgG-ASC response in the transplant recipients and control groups

The differences in the kinetics of HI titer (Fig. 1) and HA-specific IgG responses (Fig. 2) in the serum specimen impelled us to query the quality of B cells in the two cohorts. Accordingly, we determined the kinetics and proportions of IgG antibody secreting B cells in the control and transplant cohorts. Using a previously described ELISPOT assay for characterization of IgG-ASC in PBMC [25], we analyzed the IgG-ASC response at baseline and days 7, 14, 28, and 90 post-vaccination. As shown in Fig. 4A, despite variations in the magnitude of response with in the two cohorts, the peak IgG-ASC response in both cohorts was seen at day 7 post-vaccination. The IgG-ASC response in both cohorts at all other time points remained below the level of detection (Fig. 4A). More importantly, the proportion of IgG-ASC in the transplant group at day 7

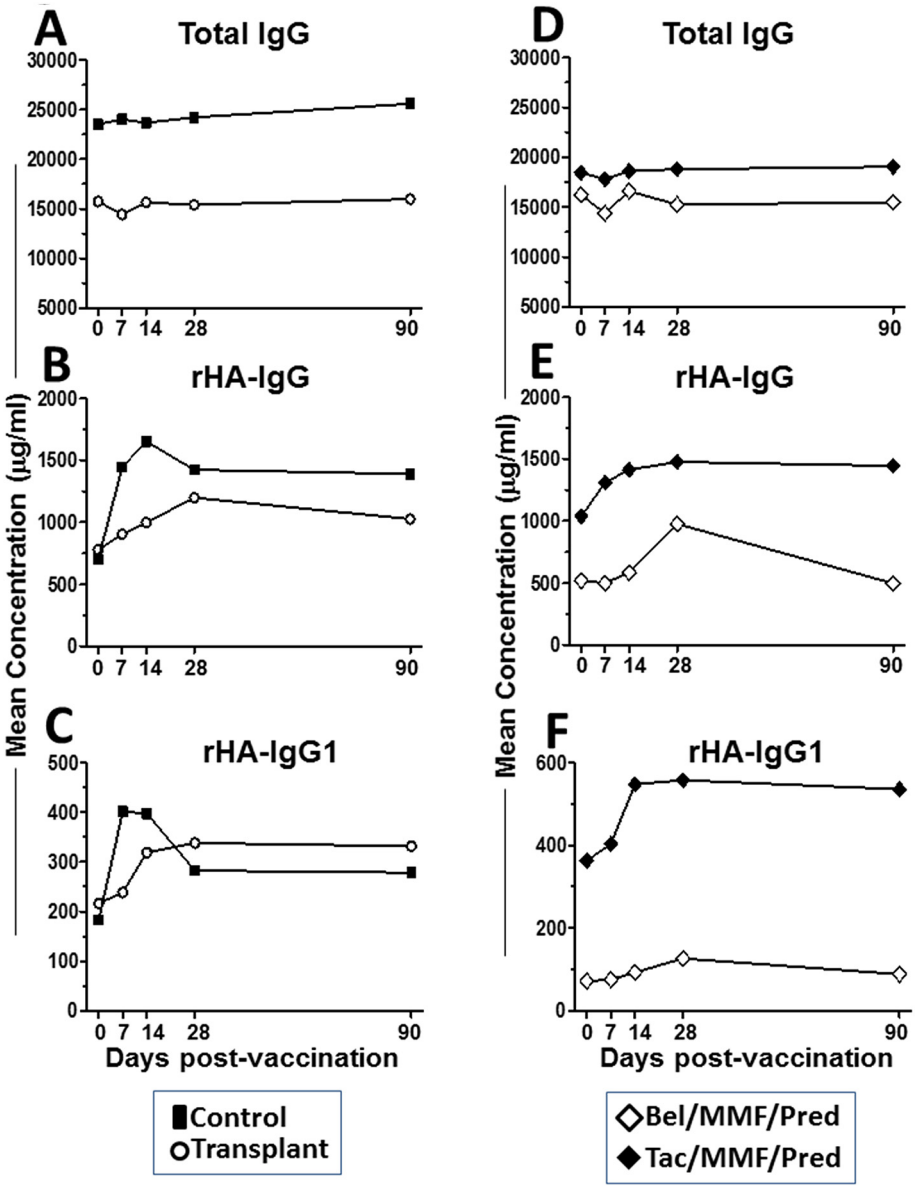

Fig. 2. Serum total IgG, rHA-specific IgG, rHA-specific IgG1 in control and transplant groups vaccinated with TIV 2007–08. Sera collected from control group (filled square), transplant patients (open circle), transplant patients receiving B/M/P (open diamonds) and T/M/P regimen (closed diamonds) at baseline and different time points post-vaccination were assayed for total IgG (A, D), rHA-IgG (B, E), and rHA-IgG1 (C, F), as described in the 'Methods' section. Data represent mean concentration (µg/mL) of serum antibody for control and transplant subjects at days 0, 7, 28, 90 post-vaccination.

post vaccination was significantly low when compared to the control group (Figs. 4A & B). Specifically, while the control group showed higher proportion (7/8; 87%) of IgG-ASC, a significantly lower proportion (7/12; 58%) of subjects in the transplant group showed responses that were above the limits of detection (Fig. 4B). Lastly, analysis of IgG-ASC responses with in the transplant cohort with detectable IgG-ASC response (7/12; 58%) showed that four subjects receiving T/M/P regimen and three subjects receiving B/M/P regimen showed detectable IgG-ASC response.

5. Discussion

Annual vaccination is recommended for transplant recipients for protection against seasonal influenza infection. Previous studies in renal transplant recipients have shown vaccine efficacy ranging from comparable [9,10] to a reduction [11,12,16,31] in protective immune parameters when compared with control cohorts. However, detailed kinetics of antibody response including time required for peak responses and maintenance of influenza strain-specific antibody levels have not been addressed in detail. In the present study, we determined serum HI titer, HA-specific IgG levels and their HA binding rate as well as proportions of IgG-ASC at four different time points (days 7, 14, 28, and 90) post-vaccination. We found significant differences in the magnitude; proportions of seroconversion, and kinetics of HI titer between transplant cohort and healthy controls. The differences in B cell responses in the transplant subjects were also evident by the ELISA (total IgG, rHA-specific IgG and IgG1), in vitro HA-binding assay, and IgG-ASC ELISPOT assay. Even though none of the study participants developed influenza infection during the 90 day follow up, it is possible that influenza strains prevalent at sub-clinical levels during the study season may have contributed to toward the changes in humoral response seen in some study participants.

Antibody response to vaccination is considered as one of the critical immune correlates for protection against infection. A typical four-fold

Table 3
Comparison of control and transplant subjects for changes in HI-GMT at different time points after influenza vaccination.

Vaccine strain	Control vs transplant group comparison	Point estimate	95% CI (Low-High)	*p* value
A/Solomon Islands/3/2006	Day 0	0.79	0.1–3.3	0.743
	Day 7	2.65	0.6–11.0	0.171
	Day 14	3.34	0.8–13.9	0.094
	Day 28	2.18	0.5–9.1	0.269
	Day 90	2.32	0.5–9.8	0.241
A/Wisconsin/67/2005	Day 0	4.15	1.1–15.5	0.036*
	Day 7	8.49	2.2–31.8	0.003*
	Day 14	8.73	2.3–32.7	0.003*
	Day 28	6.82	1.8–25.6	0.006*
	Day 90	8.47	2.2–32.0	0.003*
B/Malaysia/2506/2004	Day 0	0.67	0.2–1.9	0.442
	Day 7	1.83	0.6–5.1	0.240
	Day 14	2.78	0.9–7.8	0.053
	Day 28	1.90	0.6–5.3	0.213
	Day 90	1.62	0.5–4.6	0.350
Vaccine strain	T/M/P vs B/M/P group comparison	Point estimate	95% CI (Low-High)	*p* value
A/Solomon Islands/3/2006	Day 0	3.30	0.3–27.7	0.241
	Day 7	3.63	0.4–30.4	0.209
	Day 14	2.70	0.3–22.6	0.325
	Day 28	1.92	0.2–16.1	0.511
	Day 90	3.29	0.3–27.9	0.246
A/Wisconsin/67/2005	Day 0	0.69	0.1–4.5	0.679
	Day 7	0.62	0.1–4.0	0.586
	Day 14	0.49	0.1–3.2	0.422
	Day 28	0.34	0.1–2.2	0.236
	Day 90	0.54	0.1–3.5	0.488
B/Malaysia/2506/2004	Day 0	0.95	0.1–5.8	0.956
	Day 7	1.46	0.2–8.9	0.651
	Day 14	1.20	0.2–7.3	0.825
	Day 28	0.87	0.1–5.3	0.868
	Day 90	1.31	0.2–8.0	0.744

* *p* values of < 0.05.

Table 4
Seroconversion in response to TIV in control subjects and transplant recipients.

TIV (2007–08) vaccine strains	Control group	Transplant group
A/Solomon Islands/3/2006 (H1N1)	4/8 (50%)	3/12 (25%)
A/Wisconsin/67/2005 (H3N2)	1/8 (12.5%)	1/12 (8.3%)
B/Malaysia/2506/2004 (Influenza B virus)	2/8 (25%)	none

rise in serum HI titer over baseline value and a post-vaccination HI titer ≥ 40 (seroconversion), as well as a HI titer of 40 is estimated to provide 50% protection against homologous influenza viruses in humans [32–34]. In our study, considering all the participants, the baseline HI-GMT for A/Solomon Islands/3/2006 was comparable between the healthy controls and transplant group whereas the values for the other two vaccine strains (A/Wisconsin/67/2005 and B/Malaysia/2506/2004) varied between the two groups. Upon vaccination, we found that control group showed a steady increase in HI titer against all three vaccine strains by day 14 post-vaccination and maintained the HI-GMT above the baseline values. However, although HI-GMT in the transplant group showed higher levels compared to base line value, when compared to control group, there was a difference in the magnitude of response. Moreover, whereas peak HI-GMT in the control group was seen by day 14 post-vaccination, the transplant group showed a delay until day 28 post-vaccination. In addition, although the incidence of seroconversion in the two groups was variable and ranged from none to 50%, when compared to the control group, the transplant group showed a much lower proportion of seroconversion against all three vaccine strains. Notably, despite differences in both magnitude and kinetics of HI-GMT responses between control and transplant groups, the changes were significantly different only against A/H3N2 vaccine strain. Higher baseline values seen in the control group may have contributed to this difference between the two groups. Furthermore, recent studies have raised concerns on repeat influenza vaccination on vaccine efficacy with different age groups and populations [35,36]. In our study, 6/12 study participants in the transplant group received influenza vaccine during 2006-07. The influenza vaccine recommended for 2006-07 and 2007-08 shared the same H3N2 and influenza B virus components (A/Wisconsin/67/2005/H3N2 and B/Malaysia/2506/2004) but however had variation in the H1N1 strain (2006-07, A/Solomon Islands/3/2006 Vs 2007-08, A/New Caledonia/20/99). As a result, it is possible that the differences seen in our study may also be due to prior immunization status of study participants and/or the differences in vaccine strains recommended for the earlier vaccination season.

Decrease in the magnitude and kinetics of HI-GMT in transplant cohort raises the possibility of alterations in antibody levels and/or antibody secreting B cells due to differences in the immunosuppressive treatment protocols (Table 1) prescribed for the transplant group. In fact, our total serum IgG ELISA studies supports the possible impact of immunosuppression on B cell response in the transplant cohort. Moreover, immunosuppressive effects were also seen on vaccine-specific B cell response since the transplant group showed lower magnitude as well as a delay in HA-IgG response. Fittingly, our serology data supports earlier findings from Cowan et al. reporting a similar reduction in magnitude of the seroresponse (anti-influenza IgG) by ELISA and rate of seroconversion by micro-neutralization assay in renal transplant subjects [16]. Also, in our study, IgG1 isotype antibody that forms the major bulk of IgG response in humans showed an early peak (day 7) in controls but however was not only low but also delayed (day 28) in the transplant group. Considering numerous reports linking MMF usage to decreased influenza vaccine responsiveness in renal transplant subjects [13,18] and that all the transplant subjects in our study received a MMF-based immunosuppressive regimen (Table 1), it is possible that MMF use may play a role in lowering vaccine-induced antibody responses. Indeed, as shown in Tables 1, 50% of study subjects in the transplant cohort received tacrolimus (calcineurin inhibitor), MMF, and steroid-based immunosuppressive regimen which was shown previously by Quitana et al. to impact seroconversion to A(H1N1)pdm09 influenza vaccination in renal transplant recipients [37].

Vaccine-induced differences in serum antibody levels within the control groups when compared to baseline were also reflected in the quality of HA-specific antibodies. Specifically, when compared to baseline value, HA-binding rate was significantly high after vaccination. In fact, the control group showed peak HI-GMT, concentration of antibody, as well as HA binding rate by 2 weeks post vaccination. However, sera from the transplant group failed to show higher binding rates when compared to their base line values. Although sera from control group showed peak GMP binding rate by day 14, the response rate was more pronounced against H1-rHA. Failure to achieve higher HA binding rates and the delay in reaching peak responses in the transplant cohort indicates a possibility for differential/suboptimal priming of B cell responses in the face of immunosuppression. The problems associated with the source and availability of a reliable recombinant HA for B/Malaysia/2506/2004 restricted our efforts to verify HA binding rate against all three relevant vaccine strains.

Our studies show significant changes in quantity and quality of antibody response to influenza vaccination in transplant subjects when compared with control group. This alteration in antibody response could be the result of the differences in the proportion of vaccine-induced antibody secreting cells in the transplant cohort. Previously, it was shown that IgG-ASC can be detected in the PBMC in response to influenza vaccination [25]. Also, a study by Cowan et al. demonstrated a significant reduction in frequency of influenza-specific ASC in renal transplant subjects when compared to healthy controls [16]. Indeed, in our study, analysis of PBMC collected at various time points showed that IgG-ASC were detectable only at day 7 post-vaccination.

Table 5
Fold-rise for IgG in control and renal transplant groups after influenza vaccination.

IgG	Baseline (Day 0) comparison	Control group			Transplant group		
		Point estimate	95% CI (Low-High)	*p* value	Point Estimate	95% CI (Low-High)	*p* value
Total IgG	Day 7	0.98	0.9–1.1	0.791	0.91	0.8–0.9	0.021*
	Day 14	0.98	0.9–1.1	0.763	0.99	0.9–1.0	0.859
	Day 28	1.03	0.9–1.0	0.413	0.97	0.9–1.0	0.555
	Day 90	1.06	0.9–1.0	0.156	1.01	0.9–1.0	0.710
rHA-IgG	Day 7	2.02	1.5–2.6	< 0.0001*	1.05	0.8-1.3	0.667
	Day 14	2.48	1.8–3.2	< 0.0001*	1.14	0.9-1.4	0.241
	Day 28	2.19	1.6–2.8	< 0.0001*	1.29	1.0-1.6	0.025*
	Day 90	2.20	1.6–2.9	< 0.0001*	1.19	0.9-1.5	0.126
rHA-IgG1	Day 7	1.85	1.3–2.4	< 0.0001*	1.04	0.8-1.3	0.709
	Day 14	2.25	1.6–3.0	< 0.0001*	1.27	0.9-1.6	0.052
	Day 28	1.91	1.4–2.5	< 0.0001*	1.39	1.0-1.7	0.008*
	Day 90	1.97	1.4–2.7	< 0.0001*	1.28	0.9-1.6	0.052
IgG	Baseline (Day 0) comparison	B/M/P group Point estimate	95% CI (Low-High)	*p* value	T/M/P group Point estimate	95% CI (Low-High)	*p* value
Total IgG	Day 7	0.90	0.7–1.0	0.102	0.94	0.8–1.0	0.299
	Day 14	1.02	0.9–1.1	0.740	0.98	0.8–1.1	0.753
	Day 28	0.93	0.8–1.0	0.311	1.01	0.9–1.1	0.776
	Day 90	1.04	0.9–1.1	0.555	1.01	0.9–1.1	0.821
rHA-IgG	Day 7	0.95	0.7–1.2	0.751	1.14	0.8–1.4	0.275
	Day 14	1.10	0.8–1.4	0.487	1.18	0.9–1.5	0.172
	Day 28	1.34	1.0–1.7	0.034*	1.26	0.9-1.6	0.066
	Day 90	1.12	0.8–1.5	0.417	1.24	0.9–1.6	0.084
rHA-IgG1	Day 7	1.04	0.7–1.4	0.779	1.05	0.7–1.3	0.710
	Day 14	1.33	0.9–1.8	0.060	1.22	0.9–1.6	0.145
	Day 28	1.58	1.1–2.1	0.004*	1.27	0.9-1.6	0.082
	Day 90	1.30	0.9–1.8	0.108	1.24	0.9–1.6	0.114

* *p* values of < 0.05.

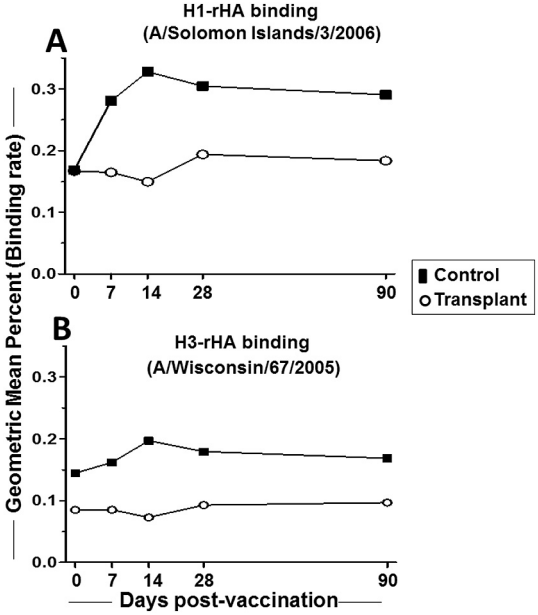

Fig. 3. HA binding rate of serum from control and transplant groups vaccinated with TIV 2007–08. Sera collected from control (filled square) and transplant patients (filled circle) at baseline and different time points post-vaccination were assayed for HA binding rate against H1-rHA (A) and H3-rHA (B), as described in the 'Methods' section. Data represent GMP values for control and transplant subjects at days 0, 7, 28, 90 post-vaccination.

Specifically, while the IgG-ASC response was seen in majority of the control group of subjects (7/8; 87%), nearly half of the transplant group failed to show detectable IgG-ASC. Considering the fact that transplant subjects were under a triple therapy immunosuppressive regimen (Table 1), it is possible that one or more of the immunosuppressive drugs could have adversely impacted the priming, proliferation or survival of B cells. However, despite reduction in IgG-ASC responses in both arms of triple immunosuppressive therapy (T/MP and B/M/P) when compared to control group, comparison of T/M/P and B/M/P regimen for differences in proportions of IgG-ASC at day 7 post-vaccination did not show any significant differences between the two forms of immunosuppression (data not shown).

With regard to immunosuppressive regimens and their impact on vaccine-specific HI-GMT and IgG responses, despite limitation with number of subjects under each immunosuppressive regimen in our study, we compared the responses between the T/M/P and B/M/P groups. Interestingly, when compared to baseline values, a significant increase in HI-GMT was seen against two vaccine strains (A/H1N1 and A/H3N2) in the B/M/P group at day 28 post-vaccination. However, there was no such increase in HI-GMT over baseline values in the T/M/P group at any given time point post-vaccination. This may be due to the high level of baseline HI-GMT representing higher magnitude of preexisting/cross reactive immunity against A/H1N1 in the T/M/P group. Alternatively, since both HI-GMT against two vaccine strains and HA-specific IgG levels in the B/M/P group, when compared to baseline level, is significantly high at 28 post-vaccination, it is possible that belatacept-based immunosuppressive regimen despite low antibody levels (Figs. 1 and 2) does not however interfere with induction of influenza vaccine-induced HI and IgG responses. Although, an earlier study by Cordero et al. found a decrease in influenza-specific serological responses in solid organ transplant recipients treated with mTOR inhibitors [20], we were unable to draw any conclusions on mTOR-based immunosuppressive regimen (R/M/P group) due to the limitation in number of study subjects. Moreover, in addition to differences in the types of immunosuppressive medications used, as shown in other studies [38–41], various other underlying diseases and comorbidities may be contributing toward the alterations in vaccine-induced immune response.

Our study with 12 transplant subjects and 8 healthy controls reveals

Table 6
Comparison of HA binding rate in control and renal transplant groups after influenza vaccination.

HA	Baseline (Day 0) comparison	Control group			Transplant group		
		Point estimate	95% CI (Low-High)	*p* value	Point estimate	95% CI (Low-High)	*p* value
H1-HA	Day 7	1.66	1.2–2.2	0.002*	1.01	0.7-1.3	0.930
	Day 14	1.94	1.4–2.6	< 0.001*	0.88	0.6-1.1	0.416
	Day 28	1.80	1.3–2.4	< 0.001*	1.16	0.8-1.5	0.249
	Day 90	1.83	1.3–2.5	0.001*	1.09	0.8-1.4	0.524
H3-HA	Day 7	1.11	0.8–1.5	0.460	1.00	0.7–1.2	0.992
	Day 14	1.36	1.0–1.8	0.045*	0.85	0.6-1.0	0.212
	Day 28	1.24	0.9–1.6	0.159	1.09	0.8–1.3	0.488
	Day 90	1.39	1.0–1.9	0.040*	1.14	0.8-1.4	0.283
HA	Baseline (Day 0) comparison	B/M/P group Point Estimate	95% CI (Low-High)	*p* value	T/M/P group Point Estimate	95% CI (Low-High)	*p* value
H1-HA	Day 7	0.94	0.8–1.0	0.309	1.07	0.9–1.2	0.212
	Day 14	0.89	0.7–1.0	0.087	1.02	0.9–1.1	0.725
	Day 28	1.04	0.9–1.1	0.466	1.04	0.9–1.1	0.396
	Day 90	1.00	0.8–1.1	0.966	1.03	0.9–1.1	0.507
H3-HA	Day 7	0.92	0.8–1.0	0.245	1.06	0.9–1.1	0.341
	Day 14	0.90	0.7–1.0	0.122	0.99	0.8–1.1	0.964
	Day 28	0.97	0.8–1.1	0.748	1.05	0.9–1.1	0.384
	Day 90	0.94	0.8–1.0	0.450	1.10	0.9–1.2	0.107

* *p* values of < 0.05.

Table 7
Comparison of HA binding rate in control and transplant groups at different time points after influenza vaccination.

HA	Control vs transplant group comparison	Point estimate	95% CI (Low-High)	*p* value
H1-HA	Day 0	1.01	0.5–1.7	0.961
	Day 7	1.66	0.9–2.8	0.064
	Day 14	2.21	1.2–3.8	0.006*
	Day 28	1.57	0.9–2.6	0.097
	Day 90	1.70	0.9–2.9	0.058
H3-HA	Day 0	1.69	0.8–3.5	0.146
	Day 7	1.89	0.9–3.9	0.082
	Day 14	2.70	1.3–5.5	0.010*
	Day 28	1.93	0.9–4.0	0.074
	Day 90	2.06	0.9–4.2	0.053
HA	T/M/P vs B/M/P group comparison	Point estimate	95% CI (Low-High)	*p* value
H1-HA	Day 0	1.02	0.7–1.2	0.957
	Day 7	0.99	0.8–1.4	0.300
	Day 14	1.12	0.8–1.4	0.313
	Day 28	0.99	0.7–1.2	0.976
	Day 90	1.02	0.8–1.3	0.824
H3-HA	Day 0	0.88	0.6–1.1	0.400
	Day 7	1.01	0.7–1.3	0.910
	Day 14	0.98	0.7–1.3	0.896
	Day 28	0.95	0.7–1.2	0.745
	Day 90	1.03	0.7–1.3	0.814

* *p* values of < 0.05.

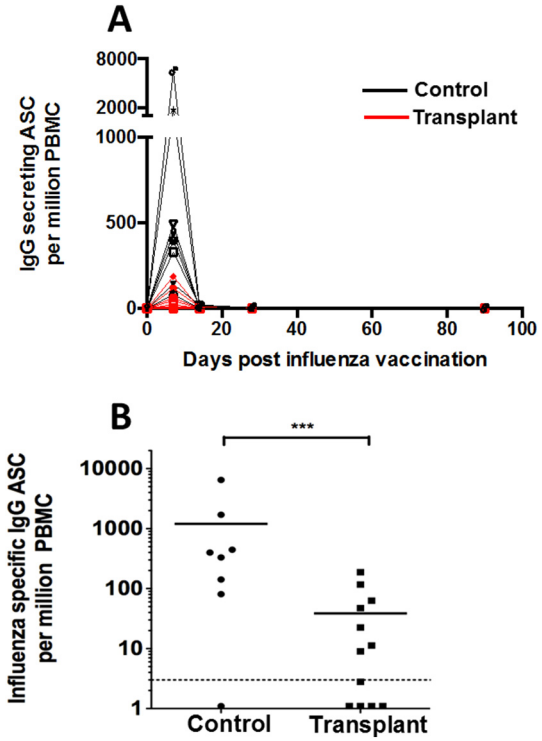

Fig. 4. Ig-ASC in control and transplant groups vaccinated with TIV 2007–08. PBMCs collected from control and transplant patients at baseline and different time points post-vaccination were analyzed for proportions of Ig G-ASC as described in 'Methods' section. Fig. 4A shows proportion of IgG-ASC at different time points post-vaccination. Fig. 4B shows proportion of IgG-ASC in control group and transplant subjects at day 7 post-vaccination along with limit of detection of the ELISPOT assay. Hatched horizontal line indicates limit of detection of the ELISPOT assay. Data represent influenza-specific IgG-ASC per million PBMC.

important differences in the magnitude and kinetics of humoral response between a renal transplant cohort and control group. Notably, the delay in peak antibody response seen in the transplant group suggests the possibility for differences in magnitude and duration for generation of vaccine-induced plasmablasts. Although a negative correlation between the types of immunosuppressive medications and seroconversion rate has been reported [13,19,21], the small sample size in our study makes it difficult to assess whether any specific immunosuppressive regimen in the transplant group contributed to the observed decrease in vaccine-induced immune responses. Further studies focusing on higher number of subjects under each arm (control and transplant group) as well as studies including a higher numbers of transplant subjects receiving the same form of immunosuppression are needed to confirm our findings and identify immunosuppressive agents that specifically alter vaccine-specific responses. Since booster vaccination [42] and intradermal vaccination [43] studies in renal transplant subjects have yielded better outcomes, additional mechanistic studies

are needed to better understand the kinetics of vaccine-induced cellular and humoral immunity in the face of clinically-induced immunosuppression. In addition, such studies could be helpful in identification of effective vaccination regimen for boosting B cell-mediated immune responses in transplant recipients.

Acknowledgements

We would like to acknowledge the Association of Public Health Laboratories (APHL) (8515 Georgia Avenue, Suite 700, Silver Spring, MD 20910) for the Emerging Infectious Disease (EID) Fellowship awarded to AKT. We also would like to acknowledge Battelle Institute (2987 Clairmont Rd., Suite 450, Atlanta, GA 30329), Atlanta Research and Education Foundation (AREF) (Atlanta VA Medical Center, 1670 Clairmont Road, Decatur, GA. 30033, U.S.A.), and the Institutional Research and Academic Career Development Award (IRACDA) for the Fellowships in Research and Science Teaching (FIRST) (Whitehead Biomedical Research Bldg., Suite 648, Atlanta, GA 30323) awarded to JSL. We thank members of the Immunology and Pathogenesis Branch in the Influenza Division, Centers for Disease Control and Prevention, for providing constructive comments on this research. This study was supported by the Emory Transplant Center (NIH Contract # NO1-AI-50025-02) and CDC intramural funding.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention/the Agency for Toxic Substances and Disease Registry.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.trim.2019.01.001.

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