Graft-*versus*-host disease is the major determinant of humoral responses to the AS03-adjuvanted influenza A/09/H1N1 vaccine in allogeneic hematopoietic stem cell transplant recipients

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ABSTRACT

Background
Responses to influenza vaccines are poorly characterized in immunocompromised patients. The goal of this study was to assess the efficacy of the AS03-adjuvanted influenza H1N1/A/09 vaccine in allogeneic hematopoietic stem cell transplant recipients.

Design and Methods
We enrolled 65 patients and 138 controls in an open prospective study. Controls received one dose and patients 2 doses of the AS03-adjuvanted influenza H1N1/A/09 vaccine at a 3-week interval. Geometric mean titers and seroprotection/seroconversion rates were determined by hemagglutination inhibition before and four weeks after the last immunization. Clinical and biological markers, including immunoglobulins, $CD3^+$, $CD4^+$, $CD8^+$ and naïve $CD4^+$ T-cell counts were assessed in all patients.

Results
Baseline seroprotection rates were low in patients (6.6%) and controls (14.8%). After 2 doses, patients (n=57, 92.3%) achieved similar seroprotection rates (84% *vs.* 87%, *P*=0.65) and antibody titers (305 *vs*. 340, *P*=0.88) as controls (n=131, 93.9%) after one dose. In univariate analysis, transplant-to-vaccination interval less than 12 months, active graft-*versus*-host disease, immunosuppressive drugs, hemoglobin less than 12g/L, lymphopenia less than1G/L, IgG less than 4g/L, IgA less than 0.5g/L, IgM less than 0.5g/L and naive $CD4^+$ T cells less than 150/μL were significantly associated with weaker responses. Multivariate analysis identified transplant-to-vaccination interval and active graft-*versus*-host disease as the most powerful negative predictors of antibody responses (*P*=0.04 and *P*=0.002, respectively). Vaccination was well tolerated in both cohorts.

Conclusions
In allogeneic hematopoietic stem cell transplant recipients, 2 doses of an adjuvanted influenza vaccine elicited comparable responses to a single dose in healthy individuals. However, vaccine responses remained poor in patients with ongoing graft-*versus*-host disease, supporting the need for additional strategies in this high-risk patient population. *(ClinicalTrials.gov Identifier: NCT01022905)*

Key words: influenza A (H1N1), vaccination, allogeneic hematopoietic stem cell transplantation, GvHD, swine flu, antibodies.

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Introduction

Influenza infections that may account for up to 30% of respiratory viral infections after allogeneic hematopoietic stem cell transplantation (HSCT) can cause life-threatening complications.[1-5] Therefore, prevention of influenza in this highly immunocompromised population has received much attention.[6] Although it has been suggested that HSCT recipients may benefit from influenza immunization,[7] it is not yet clear to what extent this vaccination elicits protective responses and whether the recently developed adjuvanted vaccines could further improve its efficacy.

In April 2009, the pandemic outbreak of a new type of influenza A (H1N1) virus[8] exposed HSCT patients to a high risk of morbidity-mortality.[9,10] In September 2009, oil-in-water squalene-based adjuvanted pandemic influenza vaccines were licensed by the European Medicines Agency.[11] However, their safety and immunogenicity had not been tested in immunocompromised patients, and recommendations issued by the European Medicines Agency [12] and national health authorities varied.

The objectives of our study were to assess the immunogenicity and safety profiles of the novel AS03-adjuvanted influenza H1N1/A/09 vaccine in HSCT recipients as compared to healthy individuals and identify the determinants influencing humoral responses. Although the H1N1 pandemic is now over, these questions remain critical to define whether squalene-based adjuvants should be used in future seasonal influenza vaccines[13] and whether additional preventive strategies are needed for particular groups of patients.

Design and Methods

Study design and participants

This study was a single center, prospective, controlled and open-label trial. Participants were recruited in November 2009 as part of a multiple parallel cohort study at the Geneva University Hospital, Switzerland. Eligible patients were adult allo-HSCT recipients who were aged 18 years or older and had received a first allogeneic HSCT from an HLA-identical sibling or an unrelated donor. Exclusion criteria included transplant from a haplo-identical donor or cord blood, patients scheduled to receive donor lymphocyte infusions, platelet counts less than 30×10^9, relapse of the original disease, uncontrolled graft-*versus*-host disease (GvHD), known or suspected allergy to components of the vaccine, ongoing or prior PCR-confirmed H1N1 infection, treatment with intravenous immunoglobulins within six weeks prior to vaccination or life expectancy of less than two weeks. Partners of the recruited patients without chronic disease or treatment likely to affect their immune competence served as healthy controls.

Vaccine and immunizations

According to Swiss national recommendations, patients received 2 intramuscular doses of the AS03-adjuvanted split influenza H1N1/A/09 vaccine (Pandemrix®, GlaxoSmithKline) in the deltoid muscle with a 25-mm needle at a 3-4 week interval while controls received one dose. Each dose of Pandemrix® contained H1N1 antigen (3.75 μg), squalene (10.69mg), DL-α-tocopherol (11.86 mg), polysorbate 80 (4.86 mg).[14] A single vaccine lot was used and all patients were vaccinated in our outpatient clinic.

Samples and data collection

Blood was collected immediately prior to vaccination and at 21-28 days after the last vaccine dose in each group. In addition, patients could give an optional blood sample at 21-28 days after the first dose. Sera were prepared and stored at -20°C until assayed.

Medical information was retrieved through a detailed questionnaire at time of enrollment and through patient medical records. We designed a paper-based Case Report Form for automatic data capturing, processing and transfer into a single database. HSCT recipients were invited to report any influenza-like illness (ILI, defined as an oral temperature of more than 38°C or a history of fever or chills and at least one influenza-like symptom). Symptomatic patients were PCR-screened for H1N1 infection and excluded from the immunogenicity analysis if positive.

Safety monitoring

Safety end points were defined as solicited injection-site (pain, redness, swelling) and systemic (fever, fatigue, headache, myalgia, nausea, anorexia and chills) reactions. Adverse events were recorded in diaries completed by the study subject over the seven days after each immunization. Patients were closely monitored for GvHD occurrence or exacerbation. Serious adverse events (SAE) were defined according to the Swiss regulatory requirements. They were actively searched for and reported until the end of the study on February 28, 2010 and followed up until their resolution. The nature of the SAE and its relation to immunization were assessed by our institution's pharmacovigilance center.

Regulatory requirements

The study was approved by our institutional review board (ID: CER-09-234) and registered at ClinicalTrials.gov (ID: NCT01022905) prior to enrolment. The trial was conducted in accordance with the principles of the Declaration of Helsinki, standards of Good Clinical Practice, and Swiss regulatory requirements. Written informed consent was obtained from all subjects prior to inclusion. Financial support was provided by the Center for Clinical Research, the Louis Jeantet Foundation, and the Center for Vaccinology (Geneva University Hospital and Medical School, University of Geneva).

Laboratory methods

Immunological assessment

Baseline immunological markers assessed in patients prior to vaccination included complete blood counts, immunoglobulin levels (IgG, IgA and IgM by nephelometry) and counts of $CD3^+$, $CD4^+$ and $CD8^+$ T cells. In addition, we determined the number of $CD4^+$ and $CD8^+$ naïve T-cell subsets by measuring the expression of CCR7 and CD45RO with a FACS Calibur cytometer (Becton Dickinson, Mountain View, CA, USA) using the following mAb: CD3-PECy7 (Becton Dickinson), CD4-APC (Miltenyi Biotec, Bergisch Gladbach, Germany), CCR7-FITC (R&D Systems, Abingdon, UK) and CD45RO-PE (Dako, Zug, Switzerland). Naïve T cells were identified by their characteristic expression of high levels of CCR7 which discriminates them from $CD45RO+CCR7^{intermediate}$ central memory T cells and from $CCR7^-$ memory-effector T cells. All routine analyses were performed at our hospital laboratories according to standardized procedures.

Hemagglutination inhibition assay

Sera were decomplemented in a 56°C waterbath for 30 min and treated with the receptor destroying enzyme (RDE, Denka Seiken Co. Ltd., Tokyo, Japan) at a final dilution of 1:4. After an overnight incubation at 37°C, the RDE was inactivated at 56°C for 30 min. HA inhibition (HAI) assays were performed in V-bottom 96-well microtiter plates (Nunc) as described.[15,16] Briefly, sera were subjected to 2-fold serial dilutions (from 1:8 to 1:16384) in PBS and incu-

bated with 4 HA units (titrated daily by an HA test) of the pandemic influenza A/California/7/09 (H1N1) virus (WHO Influenza Collaborating Centre, National Institute for Medical Research (NIMR), London, UK) and 0.4% glutaraldehyde-fixed turkey red blood cells were added at room temperature for 30 min before reading.[17,18] To minimize assay variation, positive and negative controls were used in each plate, paired samples were assessed in the same test, samples were repeated at least twice in independent experiments and plates were read twice by 2 or 3 trained staff and validated using stringent criteria. Results were expressed as the reciprocal of the highest dilution showing a positive HAI. Negative samples were assigned a titer of 1:4 for computational purposes and individual values were log transformed to calculate the geometric mean antibody titers (GMT).

Immunological endpoints

The co-primary immunogenicity end points were measured by the HAI assay according to the conventional criteria used to assess influenza vaccine efficacy: 1) antibody titers prior and after vaccination as described by GMT (± 95%CI) and GMT ratio; 2) the seroprotection rate (defined as a post-vaccination HAI titer more than 1:40); and 3) the proportion of subjects with a seroconversion (defined as a post-vaccination HAI titer more than 1:40 and a 4-fold increase in GMT).

Statistical analysis

Categorical variables were described as counts and percentages. Continuous variables were summarized as medians (interquartile range and ranges). HAI titers are expressed as the reciprocal of the dilution and summarized by the GMT (CI95%). The reverse cumulative distributions (RCD) were obtained by plotting for each possible value of the titer (abscissa) the proportion of subjects with a titer greater than this value.[19] The titers between the strata of categorical variables were compared using the Kruskal-Wallis test. Association between continuous factors and titers were assessed by the Spearman's coefficient of correlation. A multivariate regression model was performed to analyze the association between the potential factors and the titer. As the distribution of the titer was not Gaussian, we modeled log10-transformed titer. The normality of the residuals was checked (Shapiro-Wilks test). The parameter of the linear model indicated the variation on the log10-tranformed titer. To help interpretion, the increase (decrease) in percentage compared to the category of reference (for categorical factors) or corresponding to the increment of one unit (for continuous factors like age) was derived from the regression model. For patient cohort-specific analyses, a procedure of selection was applied because of the limited number of events: only variables with a *P* value less than 0.05 in the univariate analysis were selected in the multivariate model. A multivariate linear model was also performed combining the patients and the controls. The factors common to both groups (age, gender, immunization in 2009) were introduced in the model, along with the group variable. Study size was defined by enrollment capacity and not based on power calculations. The significance level was 0.05. All statistical analyses were performed with S-PLUS 8.0, Insightful Corp. (Seattle, WA, USA).

Results

Baseline characteristics

From November 17 to December 3, 2009, 65 patients and 138 controls were enrolled and vaccinated. Their baseline characteristics are summarized in Table 1. All enrolled patients had an Eastern Cooperative Oncology Group performance status of 0-1 and were in complete remission at the time of vaccination. The median time from transplantation to vaccination was 30 months (range 2-192). Fifteen (23.1%) patients had graft-*versus*-host disease at the time of vaccination or within the prior month. Eleven (17%) patients were receiving 2 or more immunosuppressive treatments (IST) while 4 were on a single IST. Fifteen (23%) patients were on prednisone at a mean dose of 0.44 mg/kg equivalent daily (SD 0.32, 95%CI 0.26-0.62) and 12 (18%) on cyclosporine at a mean dose of 177 mg/day (SD 74.6, 95%CI 131.8-222) as shown in Table 1.

Table 1. Baseline characteristics of patients (n=65) and controls (n=138).

Characteristic, Number of patients, N (%)	HSCT recipients 65 (100)	Controls 138 (100)	*P* value
Age at vaccination, median (range), years	52 (20-72)	50.9 (41.5-63)	0.47
<40 years, n (%)	18 (27.7)	34 (24.6)	
40-60 years, n (%)	34 (52.3)	65 (47.1)	
≥60 years, n (%)	13 (20)	39 (28.3)	
Women, n (%)	28 (43.1)	79 (57.2)	0.08
2009 seasonal influenza immunization (prior to study enrollement), n (%)	55 (84.6)	70 (51.1)	<0.001
Seasonal-to-H1N1 vaccines interval, median (range), days	47 (5-66)	38 (0-85) (32-48)	0.17
Transplantation-to-vaccination interval, median, (range), months	30 (2-192)		
<12 months, n (%)	15 (23)		
≥12 months, n (%)	50 (77)		
Underlying disease, n (%)			
Acute myeloid leukemia	20 (30.7)		
Lymphoma	12 (18.5)		
Chronic myeloid leukemia	9 (13.8)		
Acute lymphoblastic leukemia	5 (7.7)		
Myelodysplastic syndrome	5 7.7)		
Aplastic anemia	4 (6.2)		
Multiple myeloma	4 (6.2)		
Chronic lymphocytic leukemia	3 (4.6)		
Myeloproliferative syndrome	3 (4.6)		
Donor type, n (%)			
HLA-identical sibling	36 (55.4)		
Unrelated donor	29 (44.6)		
T-cell depletion, n (%)			
No	17 (26.2)		
Partial/complete	45 (69.2) / 3 (4.6)		
Conditioning regimen, n (%)			
Myeloablative	44 (67.7)		
Non-myeloablative	21 (32.3)		
Active GvHD*, n (%)			
Acute GvHD ≥grade 2	5 (7.6)		
Chronic GvHD	10 (15.4)		
IST*, n (%)			
Prednisone	15 (23)		
Ciclosporine	12 (18.5)		
Tacrolimus / MMF / ECP	2 (3)		
MMF	2 (3)		
ECP	1 (1.5)		

*ECP: extra-corporeal photopheresis; GvHD: graft-vs-host Disease; IST: immunosuppressive treatment; MMF: mycophenolate mofetil. *Active GvHD and IST present at time of vaccination or within one month prior to vaccination*

Three patients (4.6%) had received chemotherapy within the last six months and 3 (4.6%) had received rituximab (Mabthera®, Roche, Basel, Switzerland) within the last 12 months. None had received donor lymphocyte infusions or alemtuzumab (MabCampath®, Schering AG, Berlin, Germany) during the year before vaccination.

At the moment of vaccination, median neutrophils and platelets counts were normal, but hemoglobin levels were less than 120 g/l in 24 (37%) and lymphocyte counts less than 1G/l in 15 (24%) patients. Median numbers of $CD3^+$, $CD4^+$ and $CD8^+$ T cells were normal though 37 patients had less than 400 /μL $CD4^+$ T cells. Median IgG levels were less than 4 g/L in 4 (7%) patients, and IgA and IgM less than 0.5g/l in 19 (33.3%) and 18 (31.6%) patients, respectively.

Three patients did not agree to a second vaccine dose, 3 contracted an influenza A/09/H1N1 infection and 2 were hospitalized (one for graft-*versus*-host disease and a second for an exacerbation of a chronic obstructive pulmonary disease). Seven controls could not be contacted at the time of the last study visit. Altogether, 57 of 65 (92.3%) patients and 131 of 138 (94.9%) controls completed the study and were included in the per-protocol immunogenicity analyses.

Immunogenicity and clinical efficacy of influenza H1N1/A/09 immunization

Pre-vaccination Ab titers to A/09/H1N1 were available in 61 (94%) patients and 135 (97.8%) controls. At baseline, seroprotection rates were similarly low in patients and controls (6.6%, CI95% 1.8-15.9 *vs.* 14.8%, CI95% 9.3-21.9, respectively; P=0.16) indicating that most had not been exposed to A/09/H1N1 prior to immunization (Figure 1A). A single immunization elicited strong responses in controls who reached seroprotection and seroconverion rates both of 87% (CI95% 80-92.3) and a GMT of 339.9 (CI95% 254.9-453.2). Fourteen patients (14 of 57, 24.6%) provided a single post-dose sample. Their seroprotection (64.3%, CI95% 35.1-87.2), seroconversion (53.8%, CI95% 25.1-80.8) and GMT (69.7, CI95% 31.5-154.6) were significantly lower than those of controls (P=0.04, P=0.007 and P=0.002, respectively). However, after the second dose, patients did reach seroprotection rates (84.2%, CI95% 72.1-92.5), seroconversion (84.2%, CI95% 72.1-92.5) and GMT (305.3, CI95% 182.9-509.5) comparable to those of controls after a single dose (P=0.65, P=0.65 and P=0.88, respectively) (Figure 1A).

As of February 28 2010, 8 (12.3%) patients had reported influenza-like illness symptoms for which 7 patients were

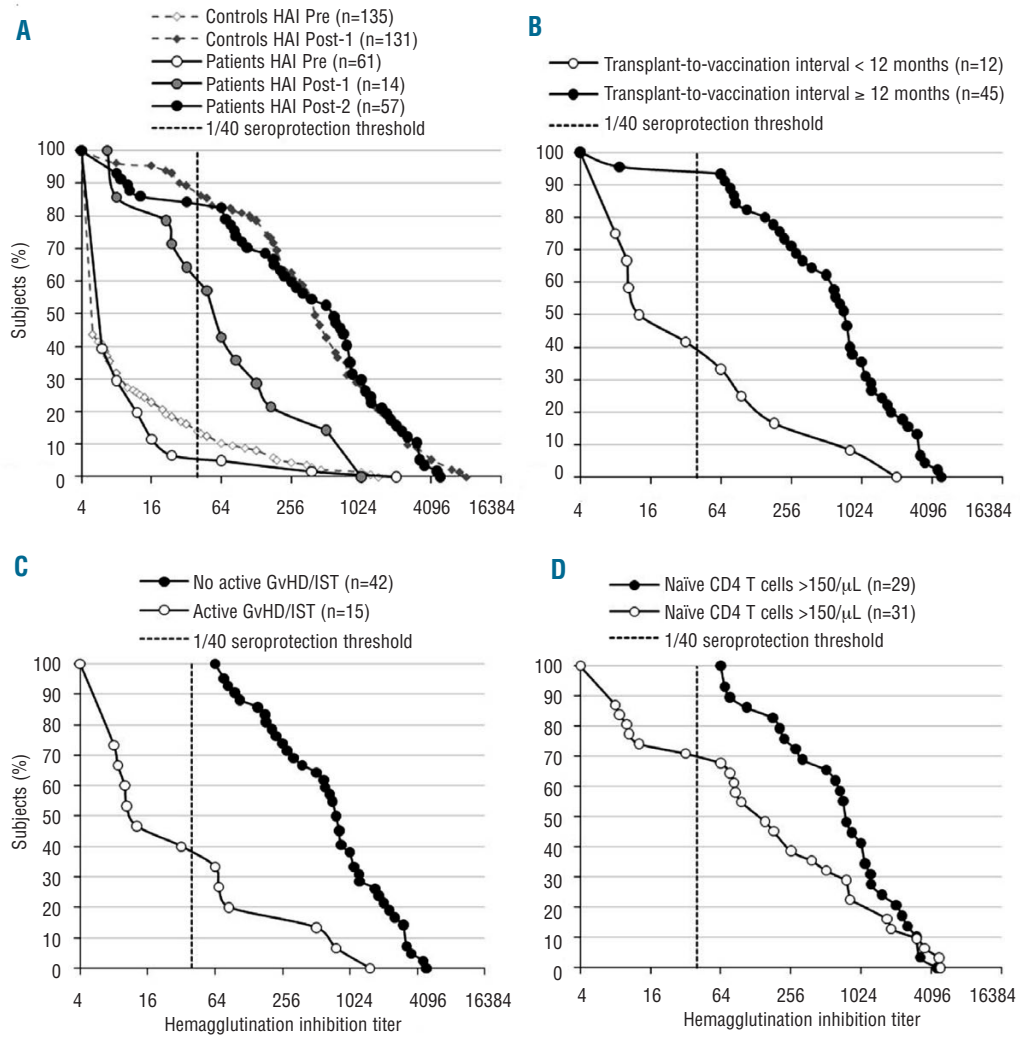

Figure 1. Reverse cumulative distribution of anti-influenza H1N1/A/09 antibody titers in HSCT patients and controls. Blood was collected prior to immunization and 21-28 days after each vaccine dose. Results were expressed as the reciprocal of the highest dilution showing a positive hemagglutination inhibition (HAI). The vertical dotted line represents the seroprotection threshold (HAI titer 1:40). The reverse distribution curves represent the distribution of individual antibody levels: (A) in HSCT patients and controls; (B) in patients transplanted < or ≥ 12 months prior to immunization; (C) in patients with or without active GvHD/immunosuppressive treatment (IST); (D) in patients with naive $CD4^+$ T-cell counts < or ≥150/μL.

treated with oseltamivir. Of the latter, 3 (4.6%) cases had an H1N1/A/09 influenza infection confirmed by PCR and diagnosed at a median of 15 days (range 1-28) after the first immunization. These patients have been extensively described elsewhere.[20]

Parameters influencing vaccine responses

Clinical and biological factors affecting vaccine responses in HSCT recipients and controls were then studied (Table 2). Gender and prior immunization against seasonal influenza had no effect. Age had a strong impact on

Table 2. Univariate analysis of determinants of antibody responses in patients and controls.

Patients and controls			Pre-vaccination N (%)	Pre-vaccination GMT (95%CI)	*P* value	Post-vaccination* N (%)	Post-vaccination* GMT (95%CI)	*P* value
Age								
	Controls	20-39y	34 (25.2)	13.8 (8.2-23.2)	0.08	31 (23.7)	712.2 (450.5-1126.1)	<0.001
		40-60y	63 (46.7)	8.2 (5.7-11.8)		63 (48.1)	414.2 (350.7-665.8)	
		>60y	38 (28.1)	7.9 (5.8-10.8)		37 (28.2)	130.5 (77.4-220.1)	
	Patients	20-39y	16 (26.2)	5.8 (4.4-7.8)	0.84	14 (24.6)	234.5 (72.8-755.2)	0.32
		40-60y	34 (55.7)	8 (5-12.8)		32 (56.1)	424.8 (226.8-795.5)	
		>60y	11 (18)	6.2 (4.5-8.4)		11 (19.3)	163.3 (48-555.4)	
Gender								
	Controls	Men	59 (43.7)	7.2 (5.1-10.2)	0.28	58 (44.3)	228 (145-358.6)	0.08
		Women	76 (56.2)	10.4 (7.6-14.3)		73 (55.7)	413.1 (287.5-593.6)	
	Patients	Men	37 (60.7)	5.9 (5-7)	0.95	34 (59.6)	207.1 (102.3-419.2)	0.10
		Women	24 (39.3)	9.2 (4.8-17.7)		23 (40.4)	541.6 (275.4-1064.9)	
2009 seasonal influenza (prior to study enrollement)								
	Controls	No	66 (49.3)	8.1 (6-10.9)	0.27	64 (49.2)	449 (300.6-669.7)	0.09
		Yes	68 (50.7)	10.4 (7.3-14.9)		66 (50.8)	264 (175-397.9)	
	Patients	No	10 (16.4)	6.4 (4.2-9.6)	0.9	10 (17.5)	275.6 (68.8-1104.4)	0.86
		Yes	51 (83.6)	7.2 (5.2-9.9)		47 (82.5)	312 (179.2-543)	
Patients								
Transplantation-to-vaccination interval	<12 months		13 (21.3)	5.6 (4.4-7.1)	0.98	12 (21.1)	38.4 (12-122.4)	0.0005
	≥12 months		48 (78.7)	7.5 (5.3-10.6)		45 (78.9)	530.6 (336.7-836.2)	
Active GvHD/IST								
	No		45 (73.8)	6.4 (4.9-8.4)	0.05	42 (73.7)	681 (471.5-983.3)	<0.001
	Yes		16 (26.2)	9.1 (4.4-19.1)		15 (26.3)	32.3 (11.9-87.9)	
Hemoglobin levels								
	<120 g/L		21 (34.4)	6.7 (4.3-10.3)	0.99	19 (33.3)	109.8 (38.8-310.7)	0.02
	≥120 g/L		40 (65.6)	7.2 (5-10.4)		38 (66.7)	508.9 (308.1-840.4)	
Lymphocytes counts								
	<1 G/l		15 (24.6)	10.2 (4.7-22.3)	0.03	12 (21.1)	66.1 (18.2-240)	0.007
	≥1 G/l		46 (75.4)	6.2 (4.8-8.1)		45 (78.9)	459.1 (280.4-751.8)	
Ig G levels								
	<4 g/L		4 (6.6)	5.8 (3.5-9.7)	0.91	4 (7)	45.4 (8.8-233.1)	0.03
	≥4 g/L		57 (93.4)	7.1 (5.3-9.6)		53 (93)	352.5 (209.5-593)	
Ig M levels								
	<0.5 g/L		18 (29.5)	6.2 (4.9-7.8)	0.50	18 (31.6)	69.2 (25.4-188.8)	0.0004
	≥0.5 g/L		43 (70.5)	7.4 (5-10.9)		39 (68.4)	605.4 (383.9-954.8)	
Ig A levels								
	<0.5 g/L		19 (31.1)	7.5 (4-14.2)	0.73	19 (33.3)	119 (44.9-315.2)	0.01
	≥0.5 g/L		42 (68.9)	6.8 (5.1-9.1)		38 (66.7)	489 (283.9-842.4)	
CD4⁺ T cells								
	<400/μL		33 (54.1)	7.6 (4.8-11.8)	0.46	29 (50.9)	189.6 (82.1-437.8)	0.16
	≥400/μL		28 (45.9)	6.5 (4.7-8.8)		28 (49.1)	499.8 (292.1-855.3)	
Naive CD4⁺ T cells* **								
	< 150/μL		31 (51.7)	8 (5-12.9)	0.11	27 (48.2)	128 (53.8-304.6)	0.008
	≥ 150/μL		29 (48.3)	5.6 (4.4-7.2)		29 (51.8)	670 (428.9-1046.4)	

*95%CI: 95% Confidence Interval; active GvHD/IST: active graft-vs-host disease (acute ≥grade 2 or chronic extensive) and/or immunosuppressive treatment. *Antibody responses were assessed after one (controls) or 2 (patients) doses of AS03-adjuvanted vaccine; **61 patients/135 controls and 57 patients/131 controls were evaluable for pre-vaccination and post-vaccination HAI respectively; ***one missing.*

responses in the control group with individuals younger than 40 years reaching approximately 5-fold higher GMTs than individuals older than 60 years (P<0.001). This was not observed in HSCT recipients who responded similarly regardless of age. The serological responses of the 12 (21.1%) patients vaccinated during the first year after transplantation were significantly lower than the responses of patients vaccinated after the first year (P<0.001) (Figure 1B).

A marked reduction of Ab responses was also observed in the 28 (49.1%) patients who had a history of graft-*versus*-host disease (P=0.02). Importantly, we found active GvHD (acute GvHD of grade 2 or over, or chronic extensive GvHD) and IST to be the main factors affecting patient response (P<0.001 and P<0.001, respectively). Since 13 patients who suffered from active GvHD were receiving IST, we combined these 2 variables (active GvHD/IST) in our analysis; only 6 of 15 (40%) patients with active GvHD and/or IST reached seroprotection after 2 doses of adjuvanted vaccine (P<0.001) (Table 2 and Figure 1C).

Lymphopenia (less than 1G/l) and hemoglobin less than 12 g/L correlated with lower responses (P=0.007 and P=0.02, respectively). Serological responses were also significantly weaker in patients with IgG less than 4 g/l, IgM less than 0.5 g/l and IgA less than 0.5g/l (P=0.03, P=0.0004 and P=0.01, respectively). Interestingly, patients with naïve CD4$^+$ T cells less than 150/μL had significantly weaker responses than patients with naïve CD4$^+$ T cells more than 150/μL (P=0.008) (Figure 1D) whereas total numbers of CD3$^+$, CD4$^+$ or CD8$^+$ T cells had no impact. Furthermore, T-cell depletion, conditioning regimen (myeloablative regimen (MAC) *versus* reduced intensity conditioning (RIC)), the source of HSC, donor or patient age at transplantation, the number of neutrophils or platelets, the underlying disease or donor type (identical sibling *vs.* unrelated donor) did not have an impact on the responses to vaccination (*data not shown*).

A multivariate analysis including transplant-to-vaccination interval, active GvHD/IST, IgA- and IgM-levels, hemoglobin levels, total lymphocyte and naive CD4$^+$ T-cell counts showed that vaccine responses were first and foremost influenced by active GvHD/IST (P=0.002) and transplant-to-vaccination interval (P=0.04) (Table 3). When both patients and controls were included in the multivariate analysis, GMT remained strongly influenced by active GvHD/IST (P=0.001) resulting in a 97.8% decrease of Ab titers as compared to controls (Table 4). As in the univariate analyses, age had no impact on GMT in patients whereas each additional ten years resulted in a 28.3% decrease of antibody titers in controls (P=0.001) (Table 4).

Safety

Reactogenicity data were available from 133 (96.4%) controls and 63 (97%) patients after dose 1 and 57 (100%) patients after dose 2 (Table 5). Immunization was well tolerated in both cohorts. Overall, 117 of 133 (88%) controls and 55 of 63 (87%) patients reported inflammatory reactions (mostly pain at the injection site) after the first dose. Similar rates (48 of 57, 84.2%) were reported by patients after the second dose. Systemic reactions were limited and fever rarely occurred. Four of 15 patients (26.7%) suffered from exacerbation of graft-*versus*-host disease during follow up, but all had experienced similar fluctuations in the severity of their GvHD in the six months before vaccination. During the study, 3 serious adverse events (SAE) were declared: one patient was hospitalized for exacerbation of GvHD, one for exacerbation of chronic obstructive pulmonary disease and one for respiratory failure due to H1N1 infection. None of these were considered to have been caused by immunization.

Discussion

This prospective study reports that 2 doses of the AS03-adjuvanted influenza H1N1/A/09 vaccine can elicit high levels of seroprotection in allogeneic HSCT recipients comparable to those achieved by healthy individuals after a single dose. However, even 2 doses could not overcome the severe immunosuppression caused by GvHD and its treatment.

Several studies evaluating the immunogenicity of seasonal influenza vaccines have been performed in HSCT recipients.[21-25] However, these were often limited by their small size and confounded by heterogeneous baseline influenza immunity, with pre-vaccination seroprotection rates ranging from 12% to 92%.[21, 23-25] Also, vaccine

Table 3. Multivariate analyses of determinants of antibody responses in patients.

Patients		Estimates (SE)	Effect*	*P* value
Active GvHD/IST	No	0		
	Yes	-0.8 (0.25)	-84.0%	0.002
Lymphocyte count	<1 G/l	0		
	≥1 G/l	0.1 (0.26)	25.6%	0.71
Transplantation-to-vaccination interval	<12 months	0		
	≥12 months	0.52 (0.26)	234.0%	0.049
Ig M	<0.5 g/L	0		
	≥0.5 g/L	0.36 (0.2)	128.9%	0.09
Ig A	<0.5 g/L	0		
	≥0.5 g/L	0.08 (0.2)	21.0%	0.69
Naive CD4$^+$ T cells	< 150/μL	0		
	≥150/μL	0.09 (0.2)	22.7%	0.67
Hemoglobin	< 120 g/L	0		
	≥ 120 g/L	0.04 (0.2)	8.9%	0.86

*Active GvHD/IST: active graft-vs.-host disease (acute ≥grade 2 or chronic extensive) and/or immunosuppressive treatment, Ig: immunoglobulin, SE: standard error. *Antibody responses were assessed after 2 doses of AS03-adjuvanted vaccine.*

Table 4. Multivariate analyses of determinants of antibody responses in patients and controls.

Patients and controls		Estimates (SE)	Effect*	*P* value
Group	Control	0		
	Active GvHD/IST	-1.66 (0.47)	-97.8%	0.001
	No active GvHD/IST	-0.38 (0.44)	-58.1%	0.5
Gender	Men	0		
	Women	0.18 (0.1)	51.5%	0.05
Age per 10 years	in controls	-0.15 (0.04)	-28.3%	0.0011
	in patients	0.0001 (0.07)	0%	0.99
2009 seasonal influenza	No	0		
	Yes	-0.14 (0.1)	-27.9%	0.19

*Active GvHD/IST: active graft-vs-host disease (acute ≥grade 2 or chronic extensive) and/or immunosuppressive treatment, SE: standard error. *Antibody responses were assessed after one (controls) or 2 (patients) doses of AS03-adjuvanted vaccine.*

Table 5. Vaccine related adverse effects within seven days after the first (patients and controls) and second dose (patients).

Adverse reaction, number of patients, N (%) (95%CI)		Patients post-dose 1 N=63 (100%)	Controls post-dose 1 N=133 (100%)	Patients post-dose 2 N=57 (100%)
Any reaction		55 (87.3) (76.5-94.4)	117 (88) (81.2-93)	47 (82.5) (70.1-91.3)
Systemic reactions				
Fever	No	60 (95.2) (86.7-99)	125 (94) (88.5-97.4)	56 (98.2) (90.6-100)
	≥38°C	3 (4.8) (1-13.3)	8 (6) (2.6-11.5)	1 (1.8) (0-9.4)
	≥38.5°C	2 (3.2) (0.4-11)	6 (4.5) (1.7-9.6)	1 (1.8) (0-9.4)
	≥39°C	1 (1.6) (0-8.5)	1 (0.8) (0-4.1)	0 (0) (0-6.3)
Fatigue		29 (46) (33.4-59.1)	47 (35.3) (27.3-44.1)	26 (45.6) (32.4-59.3)
Anorexia		5 (7.9) (2.6-17.6)	14 (10.5) (5.9-17.0)	7 (12.3) (5.1-23.7)
Myalgia		8 (12.7) (5.6-23.5)	7 (5.3) (2.1-10.5)	9 (15.8) (7.5-27.9)
Chills		2 (3.2) (0.4-11)	0 (0) (0-2.7)	2 (3.5) (0.4-12.1)
Headache		10 (15.9) (7.9-27.3)	5 (3.8) (1.2-8.6)	5 (8.8) (2.9-19.3)
Nausea		6 (9.5) (3.6-19.6)	4 (3) (0.8-7.5)	6 (10.5) (4-21.5)
Injection-site reactions				
Pain*	No	15 (23.8) (14-36.2)	19 (14.3) (8.8-21.4)	18 (31.6) (19.9-45.2)
	Mild	24 (38.1) (26.1-51.2)	64 (48.1) (39.4-56.9)	19 (31.6) (19.9-45.2)
	Moderate	16 (25.4) (15.3-37.9)	37 (27.8) (20.4-36.3)	20 (31.6) (19.9-45.2)
	Severe	8 (12.7) (5.6-23.5)	13 (9.8) (5.3-16.1)	3 (5.3) (1.1-14.6)
Redness	No	56 (88.9) (78.4-95.4)	118 (88.7) (82.1-93.5)	50 (87.7) (76.3-94.9)
	1-3cm	5 (7.9) (2.6-17.6)	11 (8.3) (4.2-14.3)	6 (10.5) (4-21.5)
	>3cm	2 (3.2) (0.4-11)	4 (3) (0.8-7.5)	1 (1.8 (0-9.4)
Swelling	No	56 (88.9) (78.4-95.4)	102 (76.7) (68.6-83.6)	53 (93) (83-98.1)
	1-3cm	5 (7.9) (2.6-17.6)	28 (21.1) (14.5-29)	2 (3.5) (0.4-12.1)
	>3cm	2 (3.2) (0.4-11)	3 (2.3) (0.5-6.5)	3 (3.5) (0.4-12.1)

**Mild: no interference with normal activities; Moderate: interference with normal activities; Severe: prevented daily activity or required medical attention. 95%CI: 95% Confidence Interval*

responses were evaluated using different methods, assessing humoral responses to one or several vaccine strains with various immunogenicity end points. As there have been no vaccine efficacy trials in immunocompromised patients, the interpretation of these studies has been challenging.[6,26] The emergence of a novel influenza virus against which little or no pre-existing immunity existed[27] provided an opportunity to assess primary B-cell responses to an adjuvanted influenza vaccine. It also allowed the use of GMT (rather than seroprotection or seroconversion rates) as a primary immunogenicity end point which allowed a more powerful evaluation of the determinants of vaccine responses.

Seasonal influenza vaccine responses have been poor in HSCT recipients, particularly in patients vaccinated early after transplantation and those taking a high dose of immunosuppressive drugs.[21,23,25] Various strategies to enhance influenza vaccine immunogenicity have been reported.[25,28] The potential impact of a 2-dose schedule in HSCT recipients has been controversial. Although some investigators found that it could induce higher responses,[29,30] others failed to reproduce these results,[23,24] leading to the current recommendation to administer a single dose starting at six months post-transplant.[31] Recently, Issa *et al.*[32] observed modest rates of seroprotective titers (51.2%) in HSCT recipients after one dose of vaccine. Our study shows that 2 doses of an adjuvanted vaccine were much more effective than a single dose, confirming the results of De Lavallade *et al.*[22] who demonstrated in a smaller cohort of 22 HSCT recipients that seroprotection was significantly boosted by a second dose of the AS03-adjuvanted vaccine. The very low incidence of confirmed H1N1/A/09 infections (4.6%), all of which occurred within two weeks after the first dose, confirms that 2 doses may have induced a protective immunity against H1N1/A/09 in our patient population.

There have been conflicting data as to the impact of graft-*versus*-host disease.[6] We identified active GvHD as the most powerful predictor for poor Ab responses (P=0.002): even 2 doses of a potent squalene-based adjuvanted vaccine did not overcome the severe immunosuppression induced by GvHD. Although several studies showed little or no impact of GvHD on responses to vaccines,[25,32] we believe that severe GvHD and its corollary of immunosuppressive treatment is likely to exert long-term negative effects on the patient's ability to respond to vaccination,[23] which further supports the need for additional prophylactic strategies in this high-risk patient population. The use of potent adjuvanted vaccines has raised much concern about the risks of immunological side effects. Actually, adverse events in our study were similar to those of healthy controls and we did not observe any triggering or exacerbation of GvHD, similar to other reports.[22,33] Obviously, due to the relatively small size of our cohort, these results must be interpreted with caution.

It is widely accepted that transplant-to-vaccination interval has an important impact on vaccine immunogenicity.[7,21-23,25,34,35] Our results confirm that this remains the case even when more potent adjuvanted vaccines are used. Furthermore, we confirm that the type of conditioning has little impact. As reported by others,[22,32] we found no significant differences in responses between RIC and

MAC patients, probably reflecting similar long-term immune reconstitution after both conditioning regimens.[36]

Ageing is known to affect humoral responses.[37-39] Although increasing age had a profound effect in the control group, we did not observe any impact of age on the patient's capacity to respond to vaccination. This has also been observed by others[22,32] and endorses the hypothesis that allogeneic HSCT may accelerate the physiological ageing of the immune system.

In univariate analysis, we observed a significant association between Ig levels and vaccine seroresponses, particularly for IgM (P=0.0004). During the first two years post-transplant, most B cells are naïve and produce IgM rather than IgG or IgA.[40] In fact, serum IgG levels provide little insight into B-cell reconstitution, as long-lived, radioresistant plasma cells survive most preparative regimens[41] and can produce substantial levels of IgG without providing humoral responses to specific pathogens.[42] Therefore, our findings suggest that IgM-levels may be a better surrogate marker for B-cell reconstitution than IgG levels.

The number of $CD4^+$ T cells was not predictive of the patient's ability to respond to vaccination. As previously reported in a much smaller group of patients,[43] we did find a significant correlation between a higher number of naïve $CD4^+$ T cells and improved responses to the vaccine. The fact that this association was only observed in univariate analysis is not surprising because GvHD severely impedes the capacity of the thymus to produce naïve T cells.[44] Therefore, the reconstitution of the T-cell compartment by naïve T cells that is necessary to restore immunity[43] may never occur in patients having experienced severe GvHD. This observation not only suggests that immune reconstitution may be best monitored by measuring naive $CD4^+$ T cells but also shows that the number of $CD4^+$ T cells, often used as a surrogate marker of immune reconstitution, may be less valid than is currently thought. This may be particularly the case in recipients of T-cell depleted grafts, in whom the T-cell compartment is initially reconstituted mainly through expansion of transfused donor T cells, a process which restores T-cell counts without restoring the patient's immunity.[45]

Our study has several limitations. Only a few patients gave a blood sample after the first dose of vaccine (24.6%) limiting our ability to conclusively deduce that 2 doses of vaccine were indeed required. However, this finding has been recently confirmed by others.[22,32] The impact of monoclonal antibody therapy could not be assessed since only 3 patients had received rituximab prior to vaccination and none had received alemtuzumab. Finally, our conclusion regarding the impact of graft-*versus*-host disease and transplant-to-vaccination interval would have been stronger if the cohort had included more patients with active GvHD or transplanted less than 12 months before vaccination.

In conclusion, our study shows that a 2-dose regimen of the AS03-adjuvanted vaccine is strongly immunogenic, providing further evidence to recommend a booster influenza vaccine dose in HSCT recipients. However, the poor serological responses observed in case of graft-*versus*-host disease support the need to consider additional prophylactic strategies for these high-risk patients.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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