MAJOR ARTICLE

Effect of Immunosuppression on T-Helper 2 and B-Cell Responses to Influenza Vaccination

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Background. Influenza vaccine immunogenicity is suboptimal in immunocompromised patients. However, there are limited data on the interplay of T- and B- cell responses to vaccination with simultaneous immunosuppression.

Methods. We collected peripheral blood mononuclear cells from transplant recipients before and 1 month after seasonal influenza vaccination. Before and after vaccination, H1N1-specific T- and B-cell activation were quantified with flow cytometry. We also developed a mathematical model using T- and B-cell markers and mycophenolate mofetil (MMF) dosage.

Results. In the 47 patients analyzed, seroconversion to H1N1 antigen was demonstrated in 34%. H1N1-specific interleukin 4 (IL-4)–producing $CD4^+$ T-cell frequencies increased significantly after vaccination in 53% of patients. Prevaccine expression of H1N1-induced HLA-DR and CD86 on B cells was high in patients who seroconverted. Seroconversion against H1N1 was strongly associated with HLA-DR expression on B cells, which was dependent on the increase between prevaccine and postvaccine H1N1-specific IL-4^+CD4^+ T cells ($R^2 = 0.35$). High doses of MMF (≥2 g/d) led to lower seroconversion rates, smaller increase in H1N1-specific IL-4^+CD4^+ T cells, and reduced HLA-DR expression on B cells. The mathematical model incorporating a MMF-inhibited positive feedback loop between H1N1-specific IL-4^+CD4^+ T cells and HLA-DR expression on B cells captured seroconversion with high specificity.

Conclusions. Seroconversion is associated with influenza-specific T-helper 2 and B-cell activation and seems to be modulated by MMF.

Keywords. immunosuppression; cellular response; cytokines; computational model; influenza; vaccine; B cells; T cells; cytokine profile.

Infection with influenza viruses in organ transplant recipients is associated with greater risk of hospitalization and mortality [1, 2]. The primary means to prevent influenza in this population is annual influenza vaccination [3]. In immunogenicity studies, transplant recipients show suboptimal seroconversion rates against influenza [4, 5]. Factors associated with reduced humoral immunity include the use of mycophenolate mofetil (MMF), vaccination within the first year after transplantation, and receipt of a lung transplant [4, 6–9]. In particular, MMF dosage >2 g/d is a strong predictor of vaccine failure [9–11].

Protective immunity against influenza involves a multitude of interactions between the innate and adaptive immune system [12, 13]. Neutralizing antibody against influenza is well known to protect against infection [14]. Seroprotection and seroconversion are frequently used as surrogates of vaccine protection in the general population. Although neutralizing antibodies play an important role in prevention of infection, influenza-specific T-cell responses add to the protection and may be cross-reactive to provide broad protection against drifted strains of influenza [15]. Whereas T-helper (Th) 1 and cytotoxic T-cell response clear viral infected cells, Th2 responses stimulate antibody

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production. The type of immunity stimulated by influenza vaccination likely defines the degree of protection.

Previous studies of cellular immunity against influenza after transplantation have focused on production of interferon (IFN) γ (a Th1 cytokine) from T cells and have not shown an association with humoral immunity [16,17]. Limited data exist for the transplant population and the interaction of influenza-specific Th2 cells and B cells.

We hypothesized that influenza vaccine immunogenicity after transplantation is likely to be related to the interplay of Th2 and B cells and that immunosuppression is an important regulator. To test these hypotheses, we analyzed humoral and cellular immunity in a cohort of solid organ transplant recipients and healthy controls that were given influenza vaccine. Peripheral blood mononuclear cells (PBMCs) were stimulated with influenza antigen and underwent flow cytometric analysis for intracellular IFN-γ as a marker for Th1 response, interleukin 4 (IL-4) as a marker of Th2 response, and HLA-DR and CD86 expression as activation markers for B-cell response. We also developed a computational model to investigate the influence of MMF on the interplay of T and B cells.

MATERIALS AND METHODS

Patient Population

From the original trial comparing intradermal versus intramuscular influenza vaccine, adult solid organ transplant recipients were randomly selected to participate in a cellular immunity substudy (n = 47) [6]. No significant differences in humoral immunity between intradermal and intramuscular vaccine were seen [6]. All patients received nonadjuvanted 2010–2011 influenza vaccine containing the following influenza strains: A/California/7/2009 (H1N1), A/Perth/16/2009 (H3N2), and B/Brisbane/60/2008 in either a high-dose (18 μg per antigen) intradermal (Intanza; Sanofi-Pasteur) or a standard-dose (15 μg per antigen) intramuscular preparation (Vaxigrip; Sanofi-Pasteur). Patients had whole blood collected before and 4 weeks after vaccination.

Eleven healthy volunteers were separately recruited and received standard intramuscular vaccination with the 2011–2012 vaccine (Vaxigrip, Sanofi-Pasteur). The vaccine viruses in the 2011–2012 vaccine were identical to those in the 2010–2011 vaccine. The study protocols were approved through the institutional research ethics board, and written informed consent was obtained from all participants.

Humoral Response to Vaccine

Serum samples underwent hemagglutination inhibition (HAI) assay for the H1N1 antigen contained in the vaccine, as described elsewhere [18]. Definitions of vaccine immunogenicity were based on recommendations for annual licensure of influenza vaccine (European Medicines Agency document CHMP/VWP/164653/2005). Seroconversion was defined as a ≥4-fold rise in titer from the prevaccine titer and a postvaccine titer ≥1:40. However, if prevaccine antibodies are present, the definition of *seroconversion* may also represent a booster effect or a cross-reactive immune response. *Seroprotection* was defined as a titer of ≥1:40.

PBMC Isolation and Stimulation

The PBMCs were isolated from whole blood using a Ficoll-based method, cryopreserved until use, and then stimulated with formalin-inactivated, partially purified A/California/7/2009 (H1N1) (NIBSC, NYMC-X179A), the same strain as in the vaccine. A final concentration of 0.3 μg/mL was used to stimulate cells for 16 hours at 5% carbon dioxide. Cells were stimulated in Roswell Park Memorial Institute containing 5% fetal calf serum and 1% Glutamax (R5; all Sigma) at a concentration of 2×10^5 cells per well.

Flow Cytometry

Experiments were performed on a FACSCanto II flow cytometer (BD) and analyzed with FlowJo software (version 10.0.5; TreeStar). Supplementary Figure 1 shows the overall gating strategy (Supplementary Figure 1). Medium alone served as negative control and was subtracted from samples stimulated with influenza antigen. LIVE/DEAD staining was performed (near-IR; Invitrogen). Markers for identifying T-cell subsets were CD3 (Pacific Blue), CD4 (phycoerythrin–cyanine 7), and CD8 (characterized as the CD4-negative gate). Intracellular cytokine staining was performed according to protocols published elsewhere [19]. IFN-γ (Alexa Fluor 488) and IL-4 (allophycocyanin) were used as key representatives for Th1 and Th2 cytokine production, respectively. All reagents, including perm and fixation buffers and antibodies, were from eBioscience. Isotype controls have previously been used to establish the assays. Markers for B-cell subsets were CD20 (Alexa Fluor 488) and CD27 (PE), with $CD20^+CD27^-$ as naive B cells and $CD20^+CD27^+$ as memory B cells. HLA-DR (Pacific Blue) and CD86 (allophycocyanin) served as activation markers (Biolegend or eBioscience).

Cytokine Profile

Cell-free supernatants from H1N1-stimulated PBMCs were collected after 24 hours and used for cytokine analysis. A 17-plex Luminex-based cytokine profiling kit was used (Eve Technologies), including fractalkine, IFN-α, IFN-γ, growth regulated oncogene, monocyte chemoattractant protein 3 (MCP-3), interleukin 13, soluble CD40-L, interleukin 9, interleukin 1β, interleukin 2, IL-4, interleukin 5, interleukin 6, interferon gamma induced protein 10, MCP-1, macrophage inflammatory protein 1α (MIP-1α), and tumor necrosis factor α.

Cytokine Profile Analysis

GeneSpring GX version 12 (Agilent Technologies) was used for cluster and principal component analysis of the cytokines measured in H1N1-stimulated PBMCs. Nonstimulated samples were subtracted from stimulated PBMCs. Percentile shift was used as normalization algorithm, and baseline transformation was performed to the median of all samples. Hierarchical clustering was done using Euclidean as the similarity measure and centroid linkage as the linkage rule. Principal component analysis was used to detect major trends in the experimental conditions, and cluster analysis of variance was used to compare antibody response clusters.

Mathematical Model

We constructed a phenomenological ordinary differential equation (ODE) model, as described in detail in the Supplementary Information and Supplementary Table 1. Numerical simulations were performed in MATLAB (R2014a; MathWorks), with the CVODE ODE integrator from the sundialsTB toolbox [20]. Parameter estimation was performed using the MEIGO toolbox [21], and confidence intervals were determined using the HYPERSPACE toolbox [22].

Statistical Analysis

Statistical analyses were performed using SPSS Statistics (version 20.0; IBMIL) and GraphPad Prism (version 4.0; GraphPad Software). Data are shown as medians and interquartile ranges. Categorical variables were analyzed using a χ^2 test, and continuous nonnormal distributed data (Shapiro–Wilk test) were analyzed using a Mann–Whitney *U* test or, if paired, a Wilcoxon matched-pairs rank test. All tests were 2 tailed.

RESULTS

Impact of MMF on Humoral Immunity

We enrolled 51 transplant recipients for cellular immune assays. Of these, 2 were lost to follow-up and 2 did not have sufficient T cells in the postvaccine sample for analysis. Of the remaining 47 patients, the median age was 53.4 years (range, 21–77 years), and 24 of 47 (51%) were kidney transplant recipients (Table 1). Patients were under a combination of immunosuppressive drugs. All transplant recipients had received prior-year influenza vaccine and had not previously had microbiologically proven influenza infection. By HAI assay, the prevaccine seroprotection rate to influenza A/H1N1 was 46.8% (22 of 47). The postvaccine seroprotection rate was 68.1% (32 of 47) for influenza A/H1N1, but only 16 of 47 (34%) demonstrated seroconversion. Transplant recipients receiving MMF at ≥2 g/d showed significantly lower geometric mean antibody titers than those receiving <2 g/d (geometric mean titer, 43.1 vs 128.4; $P = .03$). The median trough level of tacrolimus and cyclosporine in patients receiving MMF at ≥2 g/d MMF not significantly different from that in with those receiving <2 g/d (7.85 vs 6.5 μg/mL and 68 vs 187 μg/mL, respectively). In lung transplant recipients compared with non–lung transplant recipients seroconversion rates did not differ significantly for H1N1 ($P = .35$).

Table 1. Characteristics of Transplant Recipients and Healthy Volunteers

Characteristic	Transplant Recipients (n = 47)	Healthy Volunteers (n = 11)	*P* Value
Age, median (IQR), y	53.4 (16)	33 (10)	<.05
Sex, male/female, No. (%)	33/14 (70.2/29.8)	7/4 (63.6/36.4)	NS
Time between transplantation and immunization, median (range), y	4.34 (0.26–22.34)	. . .	. . .
Type of vaccine, No. (%)			
Standard intramuscular	26 (55)	11 (100)	. . .
High-dose intradermal	21 (44.7)	. . .	. . .
Seroconversion, No. (%)			
Influenza A/H1N1	16 (34.0)	11 (100)	<.05
Influenza A/H3N2	12 (25.5)	11 (100)	<.05
Influenza B	7 (14.9)	7 (63.6)	<.05
Prevaccine titer (GMT)			
Influenza A/H1N1	26	66	.06
Influenza A/H3N2	19	55	.01
Influenza B	16	12	NS
Postvaccine titer (GMT)			
Influenza A/H1N1	82	345	.02
Influenza A/H3N2	43	206	.001
Influenza B	29	34	NS
Type of graft, No. (%)			
Kidney	24 (51.1)	. . .	. . .
Lung	15 (31.9)	. . .	. . .
Heart	4 (8.5)	. . .	. . .
Liver	4 (8.5)	. . .	. . .
Immunosuppression, No. (%)[a]			
Prednisone	35 (74.5)	. . .	. . .
Tacrolimus	36 (76.6)	. . .	. . .
Cyclosporin	9 (19.1)	. . .	. . .
MMF	34 (72.3)	. . .	. . .
Sirolimus	5 (10.6)	. . .	. . .

Abbreviations: GMT, geometric mean titer; IQR, interquartile range; MMF, mycophenolate mofetil; NS, not significant.

[a] The median doses for prednisone and MMF were 5 mg/d and 2 g/d, and the median trough levels for tacrolimus, cyclosporin, and sirolimus were 7.4, 78, and 8.2 μg/mL.

H1N1-Specific T-Cell Responses After Vaccination

In transplant recipients, H1N1-specific IL-4$^+$CD4$^+$ T cells showed a significant increase from before to after vaccination

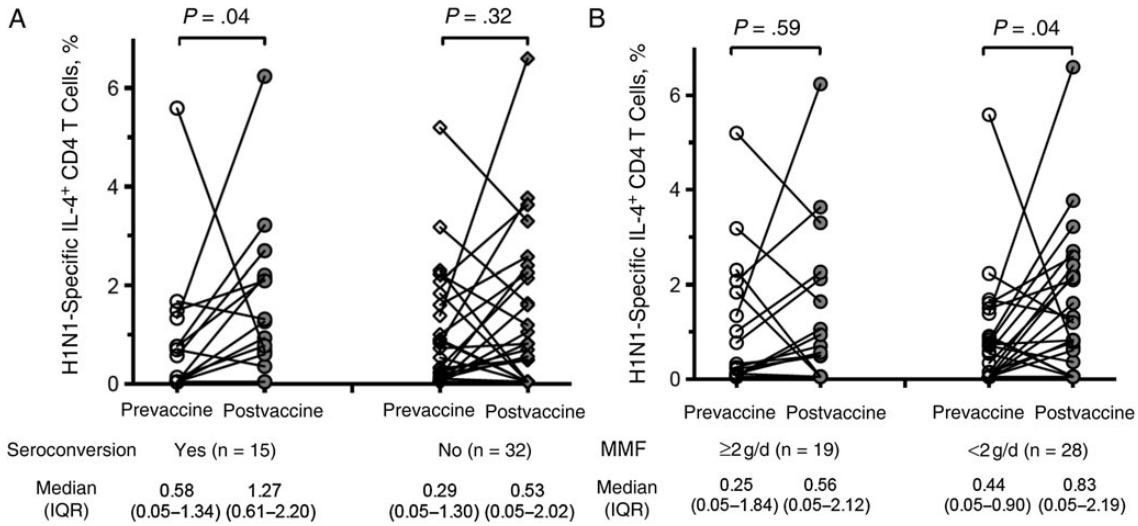

Figure 1. H1N1-specific interleukin 4 (IL-4)$^+$CD4$^+$ T-cell dynamics during vaccination. *A*, Frequency of H1N1-specific IL-4$^+$CD4$^+$ T cells in patients who seroconverted to influenza A/H1N1 versus those who did not. Patients who seroconverted had a greater rise in the frequency of IL-4$^+$CD4$^+$ T cells after vaccination ($P = .04$). *B*, Frequency of H1N1-specific IL-4$^+$CD4$^+$ T cells in patients who received mycophenolate mofetil (MMF) at ≥2 g/d versus those who received <2 g/d. Abbreviation: IQR, interquartile range.

(median, 0.32%–0.78%; $P = .04$) (Supplementary Figure 2*A*). Of the 47 patients, 25 (53.2%) had an increase in H1N1-specific IL-4$^+$CD4$^+$ T cells after vaccination. This increase was driven primarily by seroconverting patients. We observed a significant induction of postvaccine IL-4$^+$CD4$^+$ T cells only in persons with seroconversion (Figure 1*A*). Patients who were receiving MMF at ≥2 g/d had a lower, nonsignificant increase in their IL-4$^+$CD4$^+$ T cells, whereas those receiving lower MMF doses had a significant increase ($P = .04$; Figure 1*B*). The frequency of H1N1-specific IFN-γ^+CD4$^+$ and IFN-γ^+CD8$^+$ T cells did not show a significant increase after vaccination (Supplementary Figure 2*B*). IFN-γ^+ T-cell frequencies were not significantly associated with seroconversion and did not show any association with MMF dose (data not shown). However, the prevaccine-postvaccine ratio of IL-4$^+$CD4$^+$ to IFN-γ^+CD4$^+$ T cells was significantly higher in patients with seroconversion, suggesting differences in expansion dynamics of T-cell subsets (median, 1.7-fold to 1.0-fold; $P = .047$). Lung transplant recipients showed a median prevaccine IL-4$^+$CD4$^+$ T-cell frequency of 0.33% versus 0.35% in non–lung transplant recipients ($P = .26$). Similarly, the postvaccine IL-4$^+$CD4$^+$ T-cell frequency was 0.62% versus 0.86% ($P = .58$) in lung transplant recipients versus recipients of other transplant types.

H1N1 Stimulated B-Cell Activation After Vaccination

We measured B-cell activation markers after stimulation with H1N1 antigen. In transplant recipients, HLA-DR expression in all B cells (measured by mean fluorescence intensity) did not significantly increase after vaccination. However, baseline HLA-DR expression was significantly greater before vaccination in patients who eventually went on to seroconversion. This was true for both the naive B-cell subset (CD20$^+$CD27$^-$; $P < .001$) as well as memory B cells (CD20$^+$CD27$^+$; $P < .001$) (Figure 2*A*). Table 2 provides an overview comparison of patients in different subsets (Table 2).

H1N1-inducible CD86 expression did not significantly change after vaccination. However, CD86 expression was significantly greater in patients who seroconverted specifically in the naive B-cell subset (Figure 2*B*). In addition, after vaccination, patients receiving MMF at ≥2 g/d had reduced H1N1-stimulated CD86 expression on naive B cells compared with those receiving <2 g/d ($P = .05$) (Figure 2*C*). Prevaccine (baseline) levels of CD86- and HLA-DR expression were predictors of seroconversion (area under the curve for HLA-DR, 0.843). For HLA-DR (CD86) expression, the highest sensitivity and specificity were 76.5% (70.6%) and 84.6% (76.9%), respectively (Table 3). We further explored the role of IL-4$^+$CD4$^+$ T cells on B-cell activation, a process partially regulated by T-helper cells and Th2 cytokines, such as IL-4. In patients who seroconverted, the increase in H1N1-specific IL-4$^+$CD4$^+$ T cells after vaccination was directly correlated to HLA-DR expression on B cells ($P = .02$; Figure 2*D*).

H1N1 Induced Cytokine Profile in Transplant Recipients

Cytokine profiles were measured in supernatants collected from postvaccine samples in which PBMCs were stimulated with H1N1 antigen. We explored the differences in cytokine profiles with or without seroconversion using a heat map and principal component analysis (Figure 3*A*). Based on the expression profile of 28 immune markers (including 17 cytokines), the cluster analysis indicated 3 predominant groups. These 3 clusters

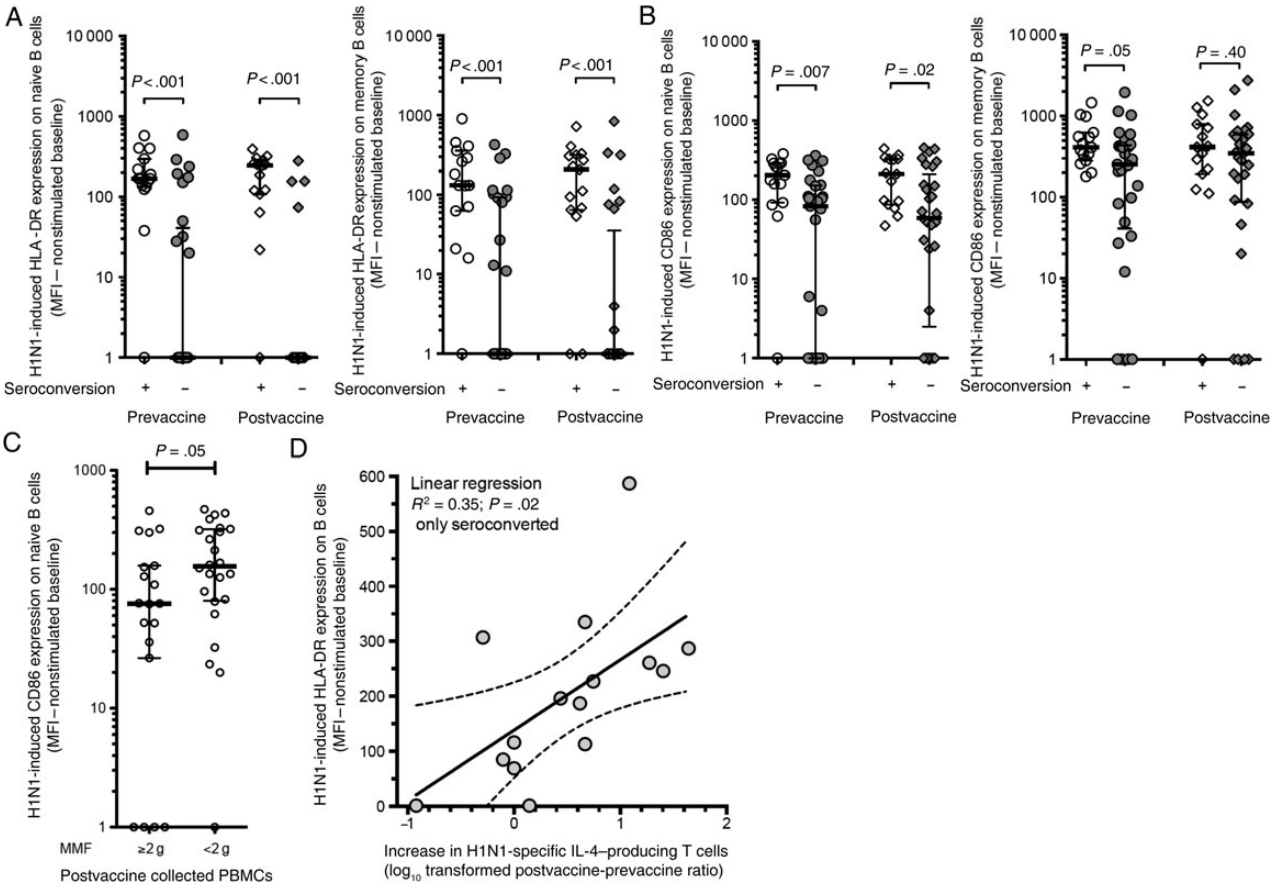

Figure 2. H1N1-induced B-cell activation during vaccination. The background (nonstimulated) expression of HLA-DR and CD86 was subtracted. The median background was 95.9 AU for HLA-DR and 43.7 AU for CD86 (also see Table 2). *A,* Expression of H1N1-induced HLA-DR expression on naive and memory B cells before vaccination was greater in patients who seroconverted (n = 15) than in those who showed no seroconversion to influenza A/H1N1 (n = 32). *B,* Expression of H1N1-induced CD86 expression on naive and memory B cells was greater in patients who seroconverted (n = 15) than in those who showed no seroconversion to influenza A/H1N1 (n = 32). *C,* Expression of H1N1-induced CD86 expression on naive B cells after vaccination based on mycophenolate mofetil (MMF) dose. Patients receiving MMF at ≥2 g/d had significantly less expression of CD86. In *A–C,* bars and whiskers represent indicate median values with interquartile ranges; Mann–Whitney *U* tests were used to identify significant differences. *D*, Regression analysis of fold change in interleukin 4 (IL-4)$^+$ T-cell frequencies and their impact on HLA-DR expression on B cells in patients with seroconversion (n = 15). Abbreviations: MFI, mean fluorescence intensity; PBMCs, peripheral blood mononuclear cells.

represent patients without seroconversion but with seroprotection (cluster 1), patients without seroconversion or seroprotection (nonresponders) (cluster 2), and patients with both seroconversion and seroprotection (cluster 3). Figure 3*B* shows the mean expression of each marker in the 3 clusters. Cluster 1 showed a relatively high expression of Th2 cytokines and low expression of Th1 cytokines; this was associated with high pre- and postvaccine HAI antibody titers to H1N1, even though these patients did not seroconvert. Cluster 3 identified a population of patients with a significant increase in HAI titers after vaccination; in particular the B-cell activation markers were significantly up-regulated in these patients. Two-dimensional principal component analysis indicated that HLA-DR expression and Th2 cytokines may be responsible for this clustering (data not shown).

Use of Phenomenological Mathematical Model to Predict Vaccine Response

We developed a dynamic mathematical model of the following structure (Figure 4*A*). An assumption for the model was that IL-4–producing CD4$^+$ T cells activate B cells and up-regulate HLA-DR expression in a positive feedback loop. This interaction results in antibody production from B cells. MMF serves as a dose-dependent inhibitor of this feedback loop. The model describes the evolution over time of the percentage of H1N1-specific IL-4$^+$CD4$^+$ T cells, HLA-DR expression on B cells, and serum antibody (HAI) titers as a result of H1N1 vaccination.

Using this model, day 30 (postvaccine) values for each variable as well as dynamics for these variables after vaccination can be predicted in patients who seroconverted versus those that

Table 2. B-Cell Activation Markers Before and After Vaccination in B-Cell Subsets According to Seroconversion Status

Vaccine	HLA-DR, MFI[a]				CD86, MFI[a]			
	Seroconversion		No Seroconversion		Seroconversion		No Seroconversion	
	Prevacccine	Postvacccine	Prevacccine	Postvacccine	Prevacccine	Postvacccine	Prevacccine	Postvacccine
All ($CD20^+$)	203 (125–321)	196 (85–287)	1 (1–81)	1 (1–1)	276 (145–330)	235 (101–419)	105 (1–213)	142 (64–274)
Naive ($CD20^+CD27^-$)	168 (133–296)	246 (108–287)	1 (1–41)	1 (1–1)	204 (92–289)	211 (87–328)	83 (1–151)	59 (3–209)
Memory ($CD20^+CD27^+$)	132 (63–358)	208 (65–318)	1 (1–94)	1 (1–36)	409 (282–623)	414 (192–790)	253 (41–436)	348 (88–596)

Abbreviation: MFI, mean fluorescence intensity.

[a] MFI values are shown in pH1N1 overnight-stimulated B-cell subsets (background subtracted). Data represent median values (interquartile range).

did not. The model was calibrated to conservatively predict seroconversion, and based on our current data it achieves a sensitivity of 70% and specificity of 100%, as determined by leave-one-out cross-validation (see Supplementary Information for details and Supplementary Table 1 for the estimated model parameters). Figure 4*B* shows the predicted dynamics of the IL-4^+CD4^+ T-cell frequency, HLA-DR expression on B cells, and H1N1 antibody HAI titer starting from patient baseline values at day 0 with modulation by actual MMF dosages. These simulation results illustrate a strong dependency of seroconversion on sufficient prevaccine HLA-DR expression levels. Simulations with hypothetical low (Figure 4*C*) and high (Figure 4*D*) MMF dosages demonstrate an inhibitory effect of MMF that is especially pronounced at high dosages (≥2 g/d).

Healthy Volunteer Response to Vaccine

Humoral and cellular vaccine responses were also evaluated in 11 healthy volunteers. Healthy volunteers were significantly younger than the transplant recipients and did not receive immunosuppressive drugs. All volunteers had received influenza vaccine in prior years and did not have a history of microbiologically proven influenza infection. A significant increase in HAI titers to H1N1 after immunization (tumor necrosis factor, 66–345; $P = .009$) was observed (100% seroprotection and seroconversion to H1N1). Geometric mean titers were significantly higher in healthy controls than in transplant recipients (83 vs 345; $P = .02$). Similar to transplant recipients, healthy volunteers also showed a significant increase in the frequency of H1N1-specific IL-4^+CD4^+ T cells after vaccination. However, unlike transplant recipients, healthy volunteers did show an increase in IFN-γ^+CD4^+ T cells after vaccination (Supplementary Figure 2*B*). H1N1-induced B-cell activation markers were high in healthy volunteers and were similar to those in transplant recipients (data not shown); healthy volunteers also had no significant changes in HLA-DR- and CD86 expression from before to after vaccination (data not shown). The postvaccine cytokine profile showed that healthy controls had significantly greater levels of fractalkine, IFN-γ, MCP-3, interleukin 1β, interleukin 6, and MIP-1α (Supplementary Figure 2*C*). The results of the cytokine profile are shown in Supplementary Table 2. These findings should be interpreted in the context of the significantly younger age of the healthy volunteers compared with the transplant recipients.

DISCUSSION

We performed a detailed analysis of H1N1-induced B- and T-cell responses to assess factors associated with successful seroconversion after influenza vaccination in the organ transplant population. We show that Th2 cytokines are associated with H1N1-induced B-cell activation in terms of HLA-DR (antigen presentation) and CD86 (costimulatory signaling) expression,

Table 3. ROC Curve Statistics for Prevaccine HLA-DR and CD86 Expression on All B Cells for the Prediction of Successful Seroconversion

Variable	AUC, Mean (SE)	*P* Value	95% CI	Best Cutoff Value, MFI	Sensitivity, %	Specificity, %
HLA-DR	0.843 (0.060)	<.001	.72–.97	123	76.5	84.6
CD86	0.700 (0.083)	.01	.58–.89	213	70.6	76.9

Abbreviations: AUC, area under the curve; CI, confidence interval; MFI, mean fluorescence intensity; ROC, receiver operating characteristic; SE, standard error.

as well as antibody secretion. MMF reduced IL-4$^+$CD4$^+$ T-cell frequencies and B-cell activation. The type of graft (lung vs nonlung transplant) did not have an effect on T-cell responses. We used several analytical techniques to formulate sensitive and specific predictive models to discriminate vaccine responders from nonresponders.

The influenza vaccine stimulates the Th1- and Th2-pathway in order for B-cell differentiation to occur [23]. One of our key findings was the increase in H1N1-specific IL-4$^+$CD4$^+$ T cells after vaccination. IL-4 is involved in humoral immunity and, to our knowledge, has not previously been explored in the context of vaccination of transplant recipients. IL-4 (originally termed *B-cell stimulatory factor 1*) is a cytokine produced by Th2 cells and has been shown to increase HLA-DR expression on resting B cells, thereby increasing production of immunoglobulin G (IgG) [24, 25]. Th2 responses have also been shown to be important in mice immunized with a universal influenza vaccine [26].

We did not observe a significant rise in IFN-γ$^+$CD8$^+$ T-cell frequency after vaccination in transplant recipients, contrary to that seen in the healthy volunteer cohort. The frequency of H1N1-specific IFN-γ$^+$CD4$^+$ and IFN-γ$^+$CD8 T cells was consistent with findings in previous studies of influenza vaccine [27]. These results were also similar to results shown by Cowan et al [28], who showed a significantly greater increase in IFN-γ responses in healthy controls compared with kidney transplant recipients. Another study in lung transplant

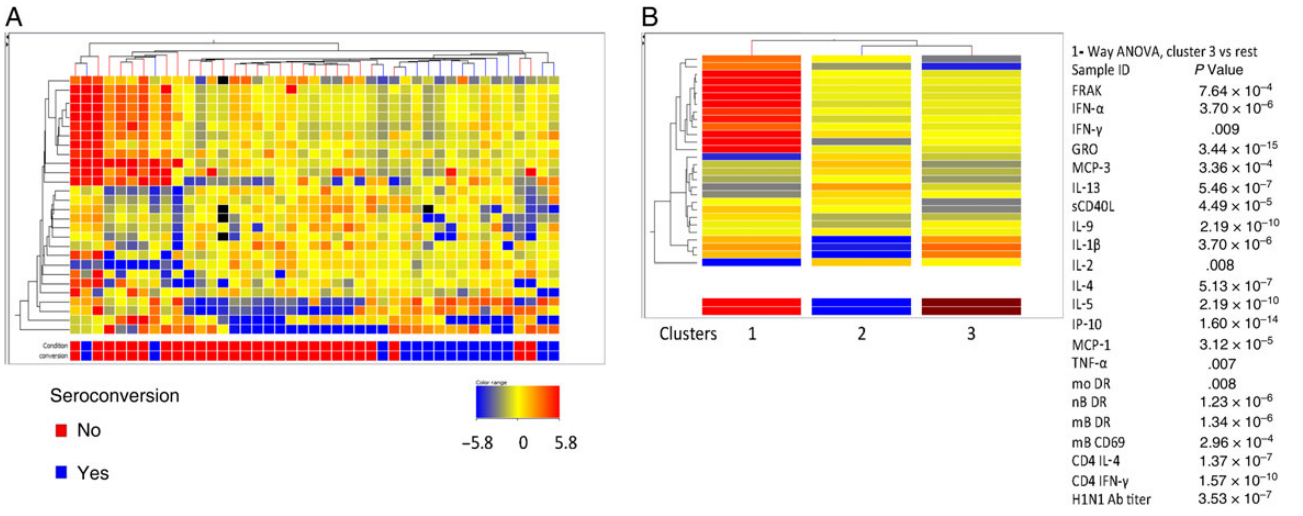

Figure 3. H1N1-induced cytokine profile. *A*, Heat map of cytokines, antibody titers and B-cell activation markers in H1N1-stimulated peripheral blood mononuclear cells of the total postvaccine transplant cohort. Blue represents seroconversion (n = 15); red, no seroconversion (n = 28; for 4 samples, no cytokine profile was available). Nonstimulated background samples were subtracted before the normalization algorithm. Relative changes in a marker are indicated by change in color code (blue, maximum 5.8-fold down-regulation; red, maximum 5.8-fold up-regulation). Markers analyzed (top to bottom): fractalkine (FRAK), interleukin 13 (IL-13), interleukin 9 (IL-9), interleukin 5 (IL-5), CD4$^+$ T-cell (CD4) interferon (IFN) γ, CD4 interleukin 4 (IL-4), IL-4, memory-phenotype B-cell (mB) CD69, interleukin 1β (IL-1β), growth regulated oncogene (GRO), soluble CD40L (sCD40L), H1N1 antibody (Ab) titer, IFN-α, interleukin 2 (IL-2), IFN-γ, tumor necrosis factor (TNF) α, interleukin 6 (IL-6), macrophage inflammatory protein 1α (MIP-1α), monocyte chemoattractant protein 3 (MCP-3), naive-phenotype B-cell (nB) CD69, interferon gamma induced protein 10 (IP-10), mB CD86, nB CD86, monocyte (mo) CD86, nB HLA-DR expression (DR), mB DR, mo DR, and MCP-1. *B*, Cluster 1 represents patients without seroconversion but with seroprotection (*left*); cluster 2, patients without seroconversion or seroprotection (*middle*); and cluster 3, patients with both seroconversion and seroprotection (*right*). Relative changes are indicated by changes in color code (see *A*). Markers analyzed (top to bottom): FRAK, mo DR, IL-13, CD4 IFN-γ, IL-9, IL-5, CD4 IL-4, IL-1β, IL-4, mB CD69, sCD40L, H1N1 antibody titer, GRO, IFN-α, IFN-γ, IL-6, MIP-1α, MCP-3, IL-2, TNF-α, mo CD86, nB CD86, mB CD86, nB CD69, MCP-1, nB DR, mB DR, and IP-10. One-way analysis of variance (ANOVA; performed with GeneSpring software) was calculated to indicate that a cytokine is differentially expressed in ≥1 of the 3 groups analyzed; ANOVA used the mean of the sum of squared deviates as an aggregate measure of variability for the selected group of genes, applying parametric testing for equal group sizes and independent samples, without post hoc testing. Principal component (PC)1, PC2, and PC3 had weights of 54.6%, 31.9% and 13.5% respectively.

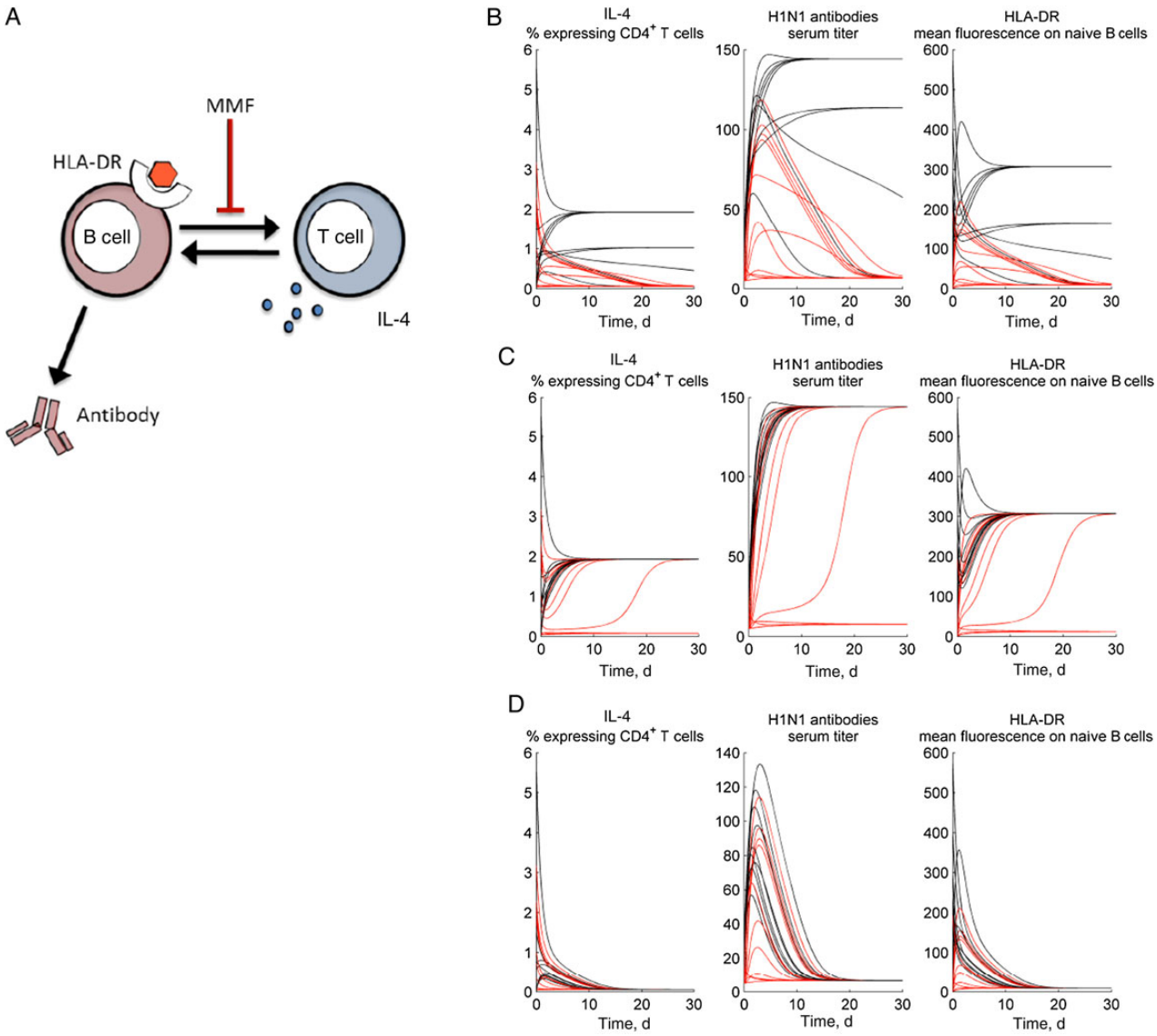

Figure 4. Mathematical model. *A,* Overview of the structure of the phenomenological mathematical model. Interleukin 4 (IL-4)–producing T cells and HLA-DR–expressing B cells activate each other in a mutual feedback loop that is inhibited by mycophenolate mofetil (MMF). Antibody production is modeled in a HLA-DR expression–dependent manner. *B–D,* Dynamics of the ordinary differential equation model for IL-4$^+$ T-cell frequency, HLA-DR expression on naive B cells, and H1N1 antibody hemagglutination inhibition titer starting from patient baseline values on day 0 for nonseroprotected patients and distinguishing between patients who seroconverted (*black*) and those who did not (*red*). Simulations were performed with patient-specific MMF dosage (*B*) and 2 hypothetical situations where patients receive no MMF (*C*) or a 3-g daily dose (*D*).

recipients also did not show an increase in influenza vaccine–related IFN-γ after influenza vaccination [17]. Previous studies in transplant recipients have not found a relationship between IFN-γ and humoral responses. For example, in a kidney transplant cohort, although an increase in IFN-γ was seen after vaccination, this was not associated with humoral responses [16]. The study by Cowan et al [28] also did not show a correlation between IFN-γ T-cell responses and influenza-specific IgG responses. Virus-specific CD8$^+$ T cells typically recognize peptides derived from internal components of the virus [29]. Thus, during influenza replication, cytotoxic T cells could respond to a broader spectrum of possible expressed epitopes and proteins in comparison to strain-specific antibodies to the viral hemagglutinin (HA) and neuraminidase (NA) glycoproteins [30]. However, inactive vaccines do not replicate, and therefore they contain only a limited spectrum of proteins, namely HA and NA glycoproteins; the induced CD8$^+$ T-cell response may not directly correlate with an antibody response. Nevertheless, if an IFN-γ response develops, it could promote the induction of HA-specific neutralizing antibodies and may

in fact help with broadening responses to heterologous influenza viruses [31, 32].

In the nontransplant literature, the ratio of Th2 to Th1 cytokines has been suggested to be associated with vaccine responses in the elderly. Similar to our study, McElhaney et al [33] measured interleukin 10 as a marker of Th2 responses and showed a significant rise after vaccination in PBMCs of elderly persons stimulated with H1N1. In our cohort we observed a significant increase of IL-4–producing $CD4^+$ T cells in patients with seroconversion. Our cytokine profile also indicated significant differences in Th2 cytokines in patients with seroconversion and seroprotection compared with nonresponding patients.

We also explored B-cell immunity using HLA-DR and CD86 as markers of activation. HLA-DR acts as a ligand for the T-cell receptor resulting in antigen presentation and further stimulation of T cells. In addition, CD86 acts as a costimulatory molecule on B cells and interacts with CD28 on T cells [34]. To our knowledge, these markers have not previously been explored in the context of immunization of organ transplant recipients. The prevaccine expression of these markers was high in patients who seroconverted, suggesting a role for these markers in prediction of seroconversion. Indeed, receiver operating characteristic curve analysis showed that baseline HLA-DR and CD86 expression had high sensitivity and specificity in predicting seroconversion. The high responsiveness of memory B-cell subsets before vaccination may be partially explained by prior illness or previous vaccination. All transplant recipients and healthy volunteers had previously received influenza vaccine.

General effects on IgG serum concentration in transplant recipients treated with MMF have been described elsewhere [35]. Several studies have now shown that high doses of MMF reduce the immunogenicity of influenza vaccination [9–11]. Our cohort also had reduced responses by HAI assay in patients receiving ≥2 g/d. MMF and mycophenolic acid have been shown to inhibit B-cell activation and proliferation and plasma cell formation [36, 37]. Our study provides insight into a possible mechanism. We found that high doses of MMF had a deleterious impact on IL-4^+CD4^+ T-cell frequencies and was associated with reduced HLA-DR expression on B cells.

Our results are consistent with those of a previous study, which showed a dose-dependent reduction of HLA-DR expression on B cells with increasing mycophenolate [38]. In addition, the ODE model predicts high, sustained antibody titers in most patients who seroconverted and low antibody titers (with possibly high transient titers) in those who did not. Without MMF, the model predicts that most patients who did not seroconvert in the study would develop high antibody titers at day 30, and with a hypothetical MMF dosage of 3 g/d, it predicts that none of the patients would seroconvert. Large uncertainties in estimated model parameters, however, indicate that more data (patients and time points) and possibly model extensions will be required to draw final conclusions on the predictive power of this modeling approach.

Our study has some limitations. Almost all of our patients and healthy volunteers had been previously vaccinated, which may have influenced responses. We used only certain signature cytokines for the Th1 and Th2 response, and it is possible that other cytokines may behave differently. In addition, although the trivalent influenza vaccine contains 2 A strains and 1 B strain, we used influenza A/H1N1 as a model for vaccine responses. It is possible that other strains of influenza such as A/H3N2 and B strains induce differing Th1 and Th2 profiles. We also had different organ types represented in our population, though this allowed us to provide a broad overview of cellular immunity to vaccine in the transplant recipient population. An important point is that our healthy control group was also significantly younger than the transplant cohort. It has been well described that aging is an important factor for reduced humoral responses [33], and the comparative immunological responses in transplant and healthy individuals should be interpreted in this context.

In summary, Th2 responses seem to be key regulators of influenza vaccine response in transplant recipients. B-cell activation markers before immunization have the potential to predict future humoral responses to vaccine. MMF is a key regulator of these responses at the cellular level. Tailoring immunosuppression to influence the vaccine response via up-regulation of a Th2 cytokine profile may be a future strategy to improve outcomes of vaccination.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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