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ORIGINAL ARTICLE

Alterations of naïve and memory B-cell subsets are associated with risk of rejection and infection in heart recipients

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Abstract

Rejection and infection are relevant causes of mortality in heart recipients. We evaluated the kinetics of the maturation status of B lymphocytes and its relationship with acute cellular rejection and severe infection in heart recipients. We analyzed B-cell subsets using 4-color flow cytometry in a prospective follow-up study of 46 heart recipients. Lymphocyte subsets were evaluated at specific times before and up to 1 year after transplantation. Higher percentages of pretransplant class-switched memory B cells (CD19+CD27+IgM-IgD- >14%) were associated with a 74% decrease in the risk of severe infection [Cox regression relative hazard (RH) 0.26, 95% confidence interval (CI), 0.07–0.86; $P = 0.027$]. Patients with higher percentages of naïve B cells at day 7 after transplantation (CD19+CD27-IgM+IgD+ >58%) had a 91% decrease in the risk of developing acute cellular rejection (RH 0.09; 95% CI, 0.01–0.80; $P = 0.02$). Patients with infections showed a strong negative correlation between baseline serum B-cell–activating factor (BAFF) concentration and absolute counts of memory class-switched B cells ($R = -0.81$, $P = 0.01$). The evaluation of the immunophenotypic maturation status of B lymphocytes could prove to be a useful marker for identifying patients at risk of developing rejection or infection after heart transplantation.

Introduction

Rejection and infection are among the main causes of mortality in heart recipients [1]. However, reliable markers to detect patients at risk for developing these complications are lacking [2]. A better understanding of the immunological mechanisms triggered during the post-transplantation period could reveal new markers. The kinetics of functionally distinct T- and B-cell subsets in peripheral blood and its relationship with the development of complications such as acute cellular rejection and severe infection has been poorly investigated in heart transplantation [3–9]. Monitoring of peripheral blood B-cell subsets has proved useful for identifying patients at risk of complications in other models of immunodeficiency, such as primary immunodeficiencies [10,11]. The hypothesis of this study is that evaluation of the immunophenotypic maturation status of B cells could enable us to identify patients at risk of rejection or infection in heart recipients. Our main objective was to define the kinetics of and alterations affecting the immunophenotypic maturation status of B cells in heart recipients and

their relationship between these parameters and transplant-related complications.

Material and methods

We performed a prospective follow-up study between March 2006 and March 2010. The study population comprised 46 consecutive adult heart recipients (group A), 33 age-matched patients who had undergone open-heart surgery without immunosuppression (surgical controls, group B), and 36 healthy individuals (healthy controls, group C).

Group A. This group comprised 30 men and 16 women (mean age, 55 years; range, 23–68 years) undergoing transplantation for ischemic heart disease (47.8%), dilated cardiomyopathy (19.6%), valvular heart disease (19.6%), and other types of heart disease (13%). All patients received induction therapy with the interleukin-2 receptor antagonist daclizumab. Maintenance immunosuppression included mycophenolate mofetil, prednisone, and either cyclosporine ($n = 4$) or tacrolimus ($n = 42$). Prophylaxis for infections included oral trimethoprim-sulphamethoxazole during the first year, oral nystatin during the first month, and oral norfloxacin during the first month. Itraconazole was indicated in patients with risk factors for invasive aspergillosis [12]. Universal prophylaxis with intravenous ganciclovir was administered to all recipients in the early post-transplant period. Patients with IgG hypogammaglobulinemia (defined as serum IgG<600 mg/dL) at development of severe infection received replacement therapy with nonspecific intravenous immunoglobulin (IVIG). None of the patients were treated with IVIG before transplantation or before the diagnosis of infection.

Group B. Surgical controls were selected from the cardiovascular surgery department at the time of the indication for open-heart surgery. This group was included to evaluate the effect of open-heart surgery on the lymphocyte subsets assessed in the early post-transplantation period. The group comprised 19 men and 14 women (mean age, 53 years; range, 26–75 years) who underwent valvular surgery (45.5%), valvular surgery+aortic operations (24.2%), aortic operations (9.1%), valvular surgery+coronary artery bypass (6.1%), aortic operations+coronary artery bypass (6.1%), and other procedures (9.1%).

Group C. Healthy controls were selected after completion of a questionnaire. Given the lack of normal values, this group was included as a reference of normality for the lymphocyte subsets in the baseline study. The group comprised 29 men and 7 women (mean age, 50 years; range, 27–62 years).

To rule out causes of alterations in the distinct lymphocyte subsets, we excluded heart recipients and surgical controls with chronic infections (hepatitis B, hepatitis C, HIV), opportunistic infections, presurgery immunosuppressive or immunomodulatory therapies, active substance abuse, or ongoing treatment for malignancy within 6 months prior to surgery. None of the patients studied underwent plasmapheresis before or after transplantation.

Transplant candidates with a history of primary immunodeficiency ($n = 0$), protein-losing enteropathy (diagnosed by fecal examination, $n = 0$), or nephrotic-range proteinuria (>3 g/24 h, $n = 0$) were not included. Patients who died early (during the first week, $n = 3$) were not included.

Immunological monitoring

Measurements for heart recipients were made before transplantation at inclusion on the waiting list and after transplantation at 7 days, 30 days, 3 months, 6 months, and 12 months. Measurements for surgical controls were made before surgery and at 7 days after surgery.

Lymphocyte phenotype analysis

Four-color flow cytometry of peripheral blood lymphocytes was performed within 2 hours of collection. The monoclonal antibodies used were directly conjugated with the fluorochrome fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), or allophycocyanin (APC). We investigated B-cell subsets using the FITC/PE/PerCP/APC combination of CD27/IgD/CD19/IgM. Lymphocyte staining was carried out using a whole-blood lysis technique, according to the recommended methodology. Stained samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, California, USA) with Cellquest Pro software (Becton Dickinson). Total CD19 + B cells are expressed as a percentage of total lymphocytes. Percentages of the functionally distinct subpopulations are expressed as a percentage of total CD19 lymphocytes, respectively. Absolute values of lymphocyte subsets were obtained by a method which combines the percentage of positive cells obtained by flow cytometry and the absolute cell count obtained by automated hematology.

To investigate possible mechanisms explaining the impact of different levels of selected B-cell subsets on development of infection, we evaluated specific humoral immunity parameters before and after heart transplantation at the same time as the cellular immunity tests. These parameters were as follows: total IgG, IgA, IgM, and IgG subclasses (performed by nephelometry, Beckman-Coulter, California, USA); and specific antibodies including T-cell–dependent antibodies (anti-tetanus toxoid, performed by ELISA: Binding Site, Birmingham, UK), T-cell–independent antibodies (anti-pneumococcal polysaccharide, anti-PPS,

Binding Site, Birmingham, UK), anti-CMV antibodies (ELISA, Enzygnost, Siemens, Marburg, Germany), anti-varicella zoster antibodies, and anti-*Haemophilus* influenza type-B antibodies (ELISA, Binding Site, Birmingham, UK).

As for development of rejection, post-transplant (3 months) proliferative responses of CD19 + B cells were evaluated in a subgroup of 12 patients. The technique used was carboxyfluorescein diacetate succinimidyl ester (CFSE)-based cytometric analysis of peripheral blood mononuclear CD19 + B cells proliferating in response to phytohemagglutinin. We also tested serum concentrations of B-cell–activating factor (BAFF) at the same time as the lymphocyte subsets (ELISA, Human BAFF/BLyS/TNFSF13B Quantikine test, R&D Systems Inc., Minneapolis, USA). As B cells can enhance IFN-gamma production by T cells, the concentration of this cytokine was also measured using ELISA at 30 days after transplantation (Human interferon-gamma high sensitivity test, Gen Probe, Diaclone SAS, Besançon, France) [13].

Clinical follow-up and definition of transplant-related complications

According to the protocol, patients were followed up prospectively during the first year after transplantation. Visits were every 2 weeks during the first 3 months after discharge, every month from 4 to 6 months, and every 3 months from 6 months to 1 year after transplantation. The clinical variables of heart recipients according to the presence of rejection and infection are presented in Table 1.

Definition of acute cellular rejection

Acute cellular rejection episodes were defined according to the 2005 revised ISHLT grading system. We only investigated treated episodes graded as 1R or greater (grade 2–4 according to the 1990 grading system) [14]. Routine surveillance based on endomyocardial biopsies was

Table 1. Demographic and medical characteristics of heart transplant recipients with and without post-transplant complications.

Variables*	Rejection (n = 11)	No Rejection (n = 35)	P-Value†	Infection (n = 22)	No Infection (n = 24)	P-Value†
Age, years	57.3 ± 6.3	54.1 ± 12.1	0.37	57.7 ± 7.6	52.6 ± 12.7	0.11
Male sex	66	69	0.50	59	71	0.54
Weight, kg	71 ± 13	66 ± 10	0.47	69 ± 11	66 ± 11	0.41
Ischemic etiology	72.7	40	0.08	63.6	33.3	0.08
NYHA class IV	49	73	0.29	59	75	0.35
LVEF	24 ± 11	22 ± 10	0.61	20 ± 10	24 ± 10	0.29
Urgent transplant	30.7	27.2	0.54	45.4	12.5	0.021
Pre-HT renal failure	7.7	21.2	0.41	18	16.7	0.59
Pre-HT infection	7.7	12.1	0.56	13.6	8.3	0.66
Donor age, years	36 ± 13	37 ± 12	0.79	37 ± 14	36 ± 10	0.69
Donor weight, kg	68 ± 19	70 ± 14	0.65	75 ± 12	64 ± 16	0.025
Shared HLA antigens	1.3 ± 1.49	1.41 ± 0.84	0.78	1.41 ± 1.27	1.35 ± 0.81	0.86
Ischemia time, minutes	223 ± 61	233 ± 63	0.65	248 ± 62	213 ± 57	0.053
ECC, minutes	158 ± 54	145 ± 49	0.45	156 ± 54	142 ± 47	0.34
Time in ICU, days	16 ± 16	15 ± 18	0.98	18 ± 15	12 ± 18	0.31
Severe allograft dysfunction	30.8	24.2	0.72	31.8	20.8	0.51
CMV donor (+) receptor (–)	0	12	0.56	14.3	4.2	0.33
Post-HT diabetes mellitus	50	61	0.73	50	35	0.37
Acute Cellular Rejection	–	–	–	27.3	29.2	0.90
Early infection (<3 months)	46.2	39.4	0.75	–	–	–
Death	9.1	14.3	0.55	18.2	8.3	0.41
Tacrolimus, day 7, ng/mL	9.35 ± 4.33	9.88 ± 5.07	0.77	10.1 ± 4.8	9.3 ± 4.9	0.64
Mycophenolate, day 7, ug/mL	4.05 ± 4.08	2.59 ± 3.22	0.29	3.5 ± 3.8	2.7 ± 3.3	0.53
Tacrolimus, day 30, g/mL	13.6 ± 5.26	11.7 ± 4.05	0.25	11 ± 3.02	13.5 ± 5.3	0.07
Mycophenolate, day 30, ug/mL	2.69 ± 2.15	2.37 ± 2.49	0.72	2.21 ± 2.11	2.65 ± 2.63	0.56
Serum IgG, day 30 (<630 mg/dl‡)	80	41.2	0.06	71	30.4	0.015

*Data are presented as mean ± SD; categoric data as percentage.

†Student's t-test or chi-square test as indicated.

‡Median value of day 30 IgG in heart recipients.

CMV, cytomegalovirus; ECC, extracorporeal circulation; HT, heart transplant; ICU, intensive care unit; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association.

performed according to the following protocol: first month, weeks 2, 3 and 4; second and third months, every other week; fourth month to sixth month, every month; sixth month to 1 year, every 3 months. To evaluate the presence of antibody-mediated rejection (AMR), the preliminary categories for the reporting of AMR of the ISHLT working formulation for pathologic diagnosis of AMR in heart transplantation were taken into account [15]. In the pretransplant evaluation, all heart recipients were analyzed for the presence of anti-HLA cytotoxic antibodies by complement-dependent cytotoxicity assay (panel reactive antibody screening [panel of 30 lymphocytes]). Flow-cytometric technology for anti-HLA antibody screen with HLA class-I and HLA class-II antigen coated micro-beads was also performed.

Definition of severe infection

Severe infections were those requiring intravenous drug therapy in the hospital during the first year after transplantation. Infections were defined according to standard criteria proposed by the Centers for Disease Control and Prevention [16]. Minor infections, superficial incisional surgical site infection, and catheter-related infections were not considered infectious episodes in this study, as the focus was on severe infections without a clearly identified predisposing factor.

CMV antigenemia testing was performed weekly from 2 to 4 weeks, every other week from 1 month to 3 months, and monthly from 3 months to 12 months after transplantation. Patients who had positive persistent detectable CMV antigenemia in whole blood samples (with 2 or more positive samples) during follow-up were considered to have active CMV infection. The first day the CMV antigen was detected was considered to be the day of CMV infection.

Statistical analysis

Comparisons of means between 2 sample populations were made using the *t*-test. Overall differences in the mean of the subsets between more than 2 groups were assessed using an analysis of variance. Associations between categorical variables were determined using the chi-square test. For the functionally distinct subsets, a cut-off was identified using the median value, and the patients were stratified in 2 groups. Pearson correlation coefficients calculated the positive or negative correlation between variables. Immunological and clinical variables that were found to be significantly associated with the prevalence of acute cellular rejection or severe infection in the comparison of means or in the comparison of categorical variables were included in survival analysis as these were assessed before clinical events occurred. Survival analysis was performed using the Cox regression method. Those variables that were found to be significant in the univariate models were entered in bivariate regression models. Kaplan–Meier curves were plotted to illustrate free survival of acute cellular rejection and severe infection. *P*-values less than 0.05 were considered significant. *P*-values close to 0.1 were considered to indicate a tendency toward significance.

The study protocol was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki and approved by our hospital research ethics committee (IRB approval number: Project FIS 050839). Patients gave their informed consent for blood analysis.

Results

Baseline immunophenotypic characteristics of heart recipients

Pretransplantation percentages of functionally distinct lymphocyte subsets in heart recipients were compared with those of healthy controls and used to define the peripheral blood baseline immunophenotypic B-cell maturation status, which was characterized by a lower unswitched memory B-cell percentage (CD19+CD27+IgM+IgD+) (Figure 1).

Kinetics of lymphocyte subsets during the first year after transplantation

We assessed the kinetic changes that occurred in the B-cell lymphocyte subsets by comparing the values observed at each post-transplant study point with the pretransplant values. On day-7 after transplantation, a significant increase in total CD19 + B-cell percentages was observed (Figure 2). Significantly lower absolute counts of total CD19 + B cells were observed at 6 months and 1 year after transplantation (Figure 2). Expression of CD27 on CD19 + B cells

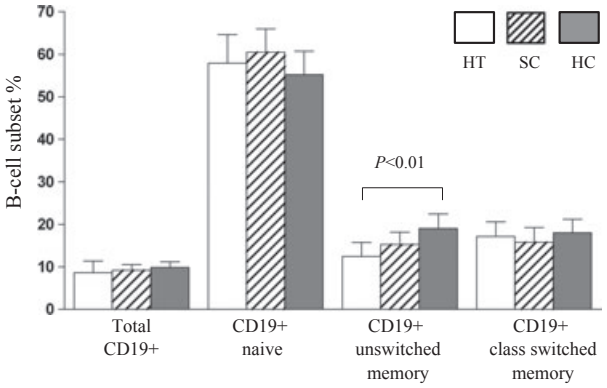

Figure 1 Baseline immunophenotypic characteristics of heart recipients. HT: heart transplantation, HC: healthy controls, SC: surgical controls. Lymphocyte subsets are expressed as percentages of CD19+ B cells.

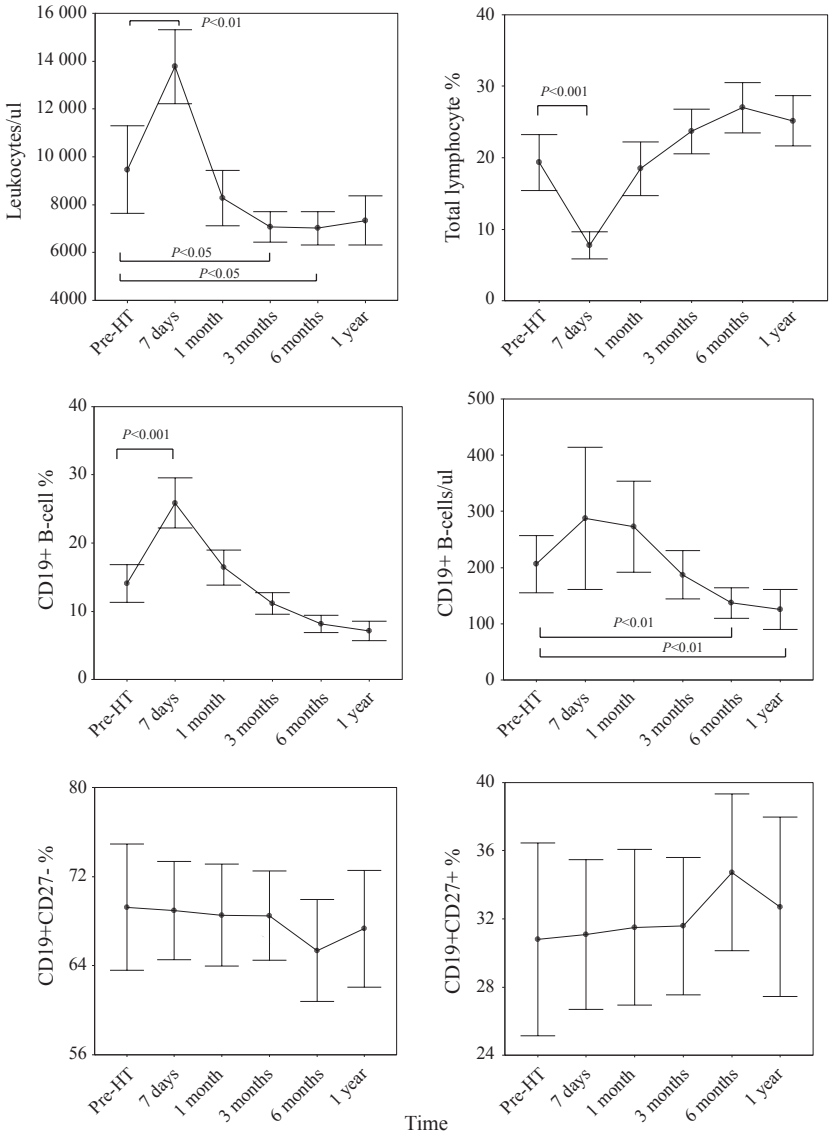

Figure 2 Kinetics of total B cells and CD27+ expression by B cells during the first year after transplantation. Mean values of all 46 transplant recipients are presented. Pre-HT: before heart transplantation. Error bars mark the error observed in the average of the different lymphocyte subsets during follow-up.

remained stable throughout follow-up (Figure 2). As for memory status, significantly higher absolute counts of naïve B cells (CD19+CD27-IgM+IgD+) were observed at 1 month after transplantation. Significantly higher absolute counts and percentages of unswitched memory cells were observed at 1 and 6 months, respectively (Figure 3). We compared the percentages observed in heart recipients with surgical control values at day 7: CD19 + total B-cell percentages were significantly higher (11 ± 7.1 vs. 7 ± 4%, $P = 0.003$), and unswitched memory B-cell percentages tended to be higher in heart recipients (15 ± 9.6 vs. 11 ± 6.8%, $P = 0.12$).

Association between B-cell immunophenotypic alterations and development of acute cellular rejection

During follow-up, 11 patients (24%) developed acute cellular rejection that required treatment [ISHLT grade 2 ($n = 6$), 3 ($n = 3$), and 4 ($n = 2$)]. Mean time to development of rejection was 87 days (range, 7–267 days).

Patients with rejection (R) showed sustained lower percentages of naïve B cells from day 7 up to 6 months (Table 2 and Figure 4) than patients who did not experience rejection (NR). Absolute naïve B-cell counts were lower from day 7 to 3 months in patients who developed

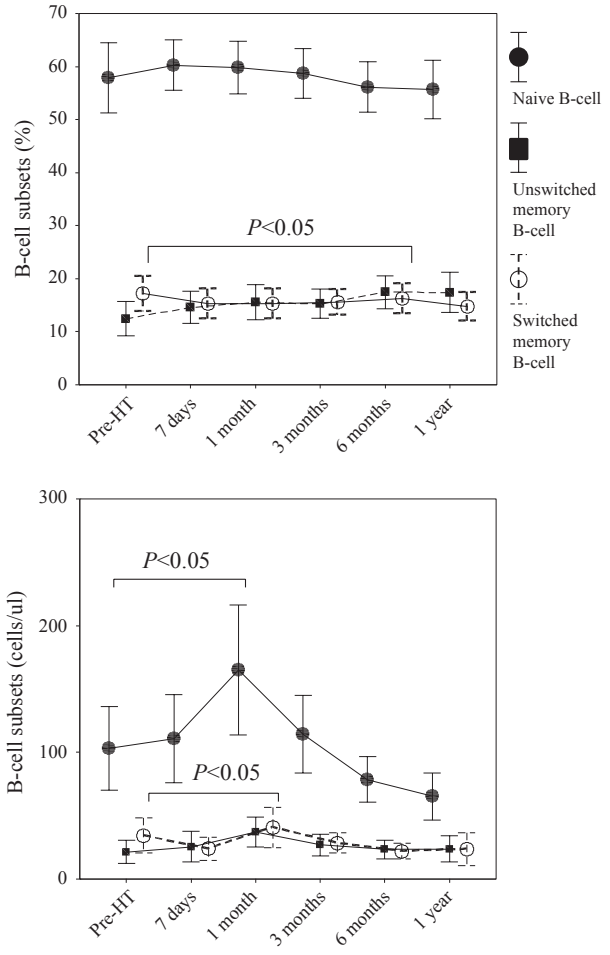

Figure 3 Kinetics of CD19+ B-cell subsets during the first year after transplantation. Pre-HT: before heart transplantation. Solid error bars (with black circles): percentage or absolute counts of naïve B cells; solid error bars (with black boxes): unswitched memory cells; dotted error bars (with white circles): class switched memory B cells. Comparison of each study point with the pre-HT.

rejection although the difference was not significant. In this group, higher percentages of class-switched memory B cells were observed at 3 months (R 20.4 ± 7.1 vs. NR 14.0 ± 7.5%, $P = 0.021$), as were higher percentages and absolute counts of unswitched memory cells at 6 months (R 25.3 ± 8.7 vs. NR 15.1 ± 9.3%, $P = 0.004$; R 40 ± 29 vs. NR 17 ± 10 cells/uL, $P = 0.021$, respectively). To evaluate how these B-cell populations changed before and after treatment for an episode of acute cellular rejection, we compared the levels obtained in the scheduled studies that were performed before with those observed after the rejection episode. We observed a tendency toward a decrease in naïve B-cell percentages and absolute counts (16 ± 4.9 vs. 12 ± 3.9%, $P = 0.15$; 82 ± 52 vs. 52 ± 21 cells/uL; $P = 0.075$) with a concomitant increase in the percentage of unswitched memory B cells (6 ± 1.9 vs. 11 ± 3.3%, $P = 0.07$).

When we stratified patients using the median value of the distinct B-cell subsets, patients with higher percentages of naïve B cells at day 7 (>58%) had a 91% decrease in the risk of developing acute cellular rejection (Table 3 and Figure 5). This association remained significant after adjustment for the presence of IgG levels >630 mg/dL (median value) at day 30 that was found to be the only variable significantly associated with the development of rejection in the univariate Cox regression analysis (Table 1 and Table 3).

One patient developed AMR 7 days after transplantation (pAMR2). During follow-up, 2 patients developed ventricular dysfunction, which was treated with methylprednisolone boluses at 2 and 7 months after transplantation, respectively. No data were available on acute cellular rejection from the endomyocardial biopsies at the time of these clinical events. Biopsies revealed neither histopathologic nor immunopathologic features of AMR (pAMR0). These patients did not exhibit significant differences in the distinct B-cell subsets compared with patients who did not develop any type of rejection during follow-up (data not shown).

Four patients had pretransplant positive (>30%) anti-HLA cytotoxic antibodies (by flow-cytometry: anti-HLA class I, $n = 3$; anti-HLA class II, $n = 1$). Prospective pretransplant cross-matches were negative in all these cases. The mean values of the distinct lymphocyte subsets, as well as the BAFF concentrations, were similar in patients with positive anti-HLA antibodies compared with the rest of the cohort (naïve B cells [55 ± 25 vs. 59 ± 18%, $P = 0.79$], memory class switched B cells [19 ± 7 vs. 16 ± 9%, $P = 0.65$], BAFF [1599 ± 634 vs. 1600 ± 1099 pg/mL, $P = 0.84$]).

Association between B-cell immunophenotypic alterations and development of severe infection

During follow-up, 22 (48%) patients experienced severe infectious complications. The distribution of infectious agents recovered (by culture or antigenemia) from the first severe infectious episode was as follows: cytomegalovirus ($n = 8$), *Pseudomonas aeruginosa* ($n = 5$), *Corynebacterium sp* ($n = 2$), *Enterococcus sp* ($n = 1$), *Stenotrophomonas maltophilia* ($n = 1$), *Aspergillus fumigatus* ($n = 2$), *Aspergillus nidulans* ($n = 1$), *Mucor circinelloides* ($n = 1$), and *Candida tropicalis* ($n = 1$). Eighty-two percent of the primary events of severe infection occurred during the first 3 months. Patients who were free of severe infectious complications tended to have higher mean percentages and absolute counts of pretransplant class-switched memory B cells than recipients who developed infections (Table 2).

Table 2. Distribution of distinct B-cell subsets in heart recipients with and without post-transplant complications.

Variables*	Rejection (n = 11)	No Rejection (n = 35)	P-Value†	Infection (n = 22)	No Infection (n = 24)	P-Value†
Baseline study						
CD19 + CD27-IgM+IgD+%	55 ± 20	59 ± 18	0.71	62 ± 17	54 ± 19	0.25
CD19 + CD27-IgM+IgD+ cells/uL	137 ± 65	95 ± 75	0.31	116 ± 80	87 ± 67	0.40
CD19 + CD27 + IgM+IgD+%	12 ± 7	12 ± 10	0.98	12 ± 8	13 ± 10	0.69
CD19 + CD27 + IgM+IgD+ cells/uL	40 ± 30	16 ± 13	0.17	16 ± 11	28 ± 26	0.22
CD19 + CD27 + IgM-IgD-%	19 ± 11	16 ± 9	0.59	14 ± 7	20 ± 10	0.066
CD19 + CD27 + IgM-IgD- cells/uL	55 ± 45	27 ± 22	0.25	25 ± 18	44 ± 38	0.13
Day 7						
CD19 + CD27-IgM+IgD+%	49 ± 12	64 ± 15	0.008	61 ± 16	59 ± 14	0.62
CD19 + CD27-IgM+IgD+ cells/uL	82 ± 52	120 ± 93	0.22	117 ± 73	106 ± 97	0.76
CD19 + CD27 + IgM+IgD+%	17 ± 11	14 ± 9	0.46	14 ± 9	14 ± 11	0.88
CD19 + CD27 + IgM+IgD+ cells/uL	32 ± 38	23 ± 27	0.55	24 ± 27	26 ± 33	0.86
CD19 + CD27 + IgM-IgD-%	18 ± 10	14 ± 8	0.25	15 ± 9	16 ± 9	0.80
CD19 + CD27 + IgM-IgD- cells/uL	31 ± 24	21 ± 22	0.39	25 ± 27	23 ± 19	0.87

*Data are presented as mean ± standard deviation.
†Student's *t*-test.

When we stratified patients according to the median value of baseline class-switched memory B-cell percentages, patients with higher levels (>14%) had a 74% decrease in the risk of developing severe infection after transplantation (Table 3).

As we previously reported that post-transplant IgG measurement is useful for identifying the risk of developing severe infections, we analyzed both parameters. No significant correlation was observed between pretransplant memory class-switched B-cell percentages and IgG levels at baseline and at 1 month after transplantation [R = 0.19 (P = 0.34) and R = 0.304 (P = 0.1), respectively]. Interestingly, bivariate Cox regression analysis revealed that baseline assessment of class-switched memory B cells (>14%) added information to IgG levels above the median value (>630 mg/dl) at 1 month after transplantation as a protective factor of infection. In this analysis, only infectious events occurring after the first month of transplantation were included (Table 3). The association between higher percentages of class-switched memory B cells and development of severe infection remained significant after adjustment for distinct clinical variables in bivariate Cox regression analysis (Table 3).

Distribution of patients according to the level of baseline memory class-switched B cells, naïve B cells at day 7 and development of clinical complications is presented in Figure 6.

Patients with recurrent bacterial infections (at least 2 episodes of culture-positive bacterial infections, n = 17) had significantly lower percentages of pretransplant class-switched memory B cells (11.7 ± 6.9 vs. 19.7 ± 9.3%; P = 0.0014) and tended to have lower absolute counts (25 ± 22 vs. 42 ± 24 cells/uL; P = 0.18).

During the 12-month follow-up period, 18 patients (39%) developed CMV infection, with a mean CMV antigenemia value of 16 cells. The mean time until development of CMV infection was 114 ± 77 days after transplantation (interval, 14–302 days). CMV-infected patients tended to have lower numbers of unswitched memory B cells at 3 months after transplantation than uninfected recipients (19 ± 12 vs. 32 ± 28 cells/uL, P = 0.09, respectively). Nine patients with CMV infection required intravenous antiviral treatment (n = 9, 19.5%). The mean CMV antigenemia value in these patients was 37 cells. Patients who required antiviral therapy had lower percentages and numbers of pretransplant class-switched memory B cells (11 ± 48 vs. 19 ± 10% [P = 0.015] and 17 ± 16 vs. 41 ± 32 cells/uL [P = 0.036], respectively). In Cox regression analysis, patients with pretransplant percentages of class-switched memory B cells above 14% had an 86% lower risk for developing CMV infection that required treatment (P = 0.04). Serum BAFF concentrations were significantly higher in these patients at 7 days (955 ± 531 vs. 622 ± 289 pg/mL, P = 0.044), 3 months (1719 ± 868 vs. 1110 ± 545 pg/mL, P = 0.012), and 1 year after transplantation (3295 ± 3062 vs. 1249 ± 575 pg/mL, P = 0.007). Ten heart recipients who developed severe infection were found to have IgG hypogammaglobulinemia at diagnosis of infection and started IVIG replacement therapy. No significant changes in the distinct B-cell subsets were observed compared with patients who did not receive IVIG (data not shown).

Nine patients were bridged to transplant on ventricular assist devices. We found no significant differences in the levels of B-cell subsets in these patients during follow-up (data not shown).

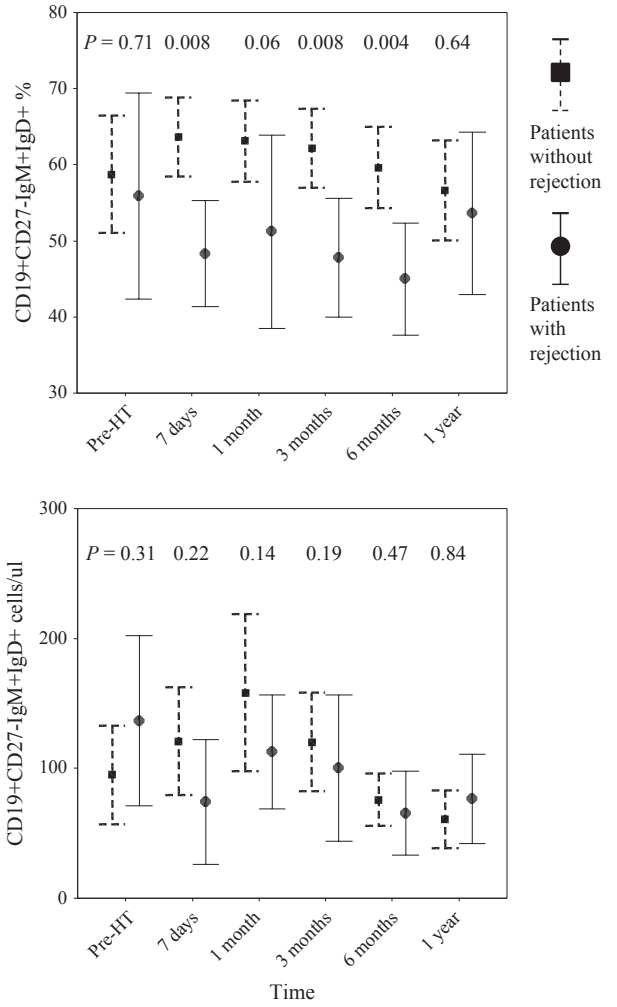

Figure 4 Time course of naïve B cells at different times before and after heart transplantation Pre-HT: before heart transplantation. Dotted error bars (with black boxes) mark the error observed in the average percentage or absolute counts of naïve B cells in patients without acute cellular rejection, while solid error bars (with black circles) mark the same information in patients with rejection.

Naïve/memory B-cell ratio

We also analyzed the potential role of the naïve (CD19+CD27-IgM+IgD+%)/memory class-switched (CD19+CD27+IgM-IgD-%) B-cell ratio. In the baseline study, patients who developed severe infections had a higher naïve/memory B-cell ratio (6 vs. 4, $P = 0.031$). A lower naïve/memory B-cell ratio was observed in heart recipients who developed acute cellular rejection than in patients without rejection at 7 days (3.5 vs. 8, $P = 0.015$), 30 days (4 vs. 8, $P = 0.030$), 3 months (3 vs. 7, $P = 0.004$), and 6 months (3 vs. 6, $P = 0.019$). In the Cox regression analysis, a higher naïve/memory B-cell ratio (i.e., higher than a median value of 2.4) tended to increase the risk of severe infection after transplantation (RH 3.17, 95% CI 0.97–10.38, $P = 0.05$). A lower day-7 naïve/memory B-cell ratio (i.e., less than a median value of 3.5) was not significantly associated with the risk for development of rejection (RH 0.44, 95% CI 0.11–1.78, $P = 0.25$).

Table 3. Cox regression analysis of factors associated with acute cellular rejection and severe infection after heart transplantation.

Factors	RH (95% CI)	*P*-value
Associated with overall acute cellular rejection development		
Univariate		
Day 7 CD19+CD27-IgM+IgD+ (>58%)	0.09 (0.01–0.80)	0.02
Day 30 IgG > 630 mg/dl.	0.21 (0.04–0.99)	0.04
Bivariate		
Day 7 CD19+CD27-IgM+IgD+ (>58%)	0.10 (0.01–0.84)	0.03
Day 30 IgG > 630 mg/dl.	0.51 (0.10–2.59)	0.06
Associated with overall severe infection development		
Univariate		
Baseline CD19 + CD27 + IgM-IgD- (>14%)	0.26 (0.07–0.86)	0.027
Day 30 IgG > 630 mg/dl	0.31 (0.12–0.81)	0.01
Urgent transplantation	2.96 (1.26–6.95)	0.013
Donor weight	1.04 (1.001–1.08)	0.04
Bivariate		
Baseline CD19 + CD27 + IgM-IgD- (>14%)	0.24 (0.07–0.79)	0.02
Day 30 IgG > 630 mg/dl	0.32 (0.09–1.05)	0.06
Baseline CD19+CD27+IgM-IgD- (>14%)	0.28 (0.08–0.94)	0.039
Donor weight	1.02 (0.98–1.06)	0.36
Baseline CD19+CD27+IgM-IgD- (>14%)	0.29 (0.09–0.95)	0.04
Urgent transplantation	0.27 (0.09–0.84)	0.02

RH, relative hazard; 95% CI, 95% confidence interval.

Humoral and cellular immune status according to levels of B-cell subsets

We evaluated distinct humoral and cellular immunity parameters to evaluate the potential mechanisms underlying the impact of B-cell subsets on the outcomes investigated.

Post-transplant naïve B cells, proliferative B-cell responses, BAFF, interferon-gamma, and rejection

Post-transplant nonspecific CD19+ B-cell, CD19+IgD+, and CD19 + IgM+ proliferative responses were similar in a subgroup of patients with higher percentages of naïve B cells at day 7 ($n = 6$) and in a subgroup of patients with lower percentages of naïve B cells ($n = 6$) (Figure 7). Serum BAFF levels were significantly higher after rejection than before rejection (1238 ± 290 vs. 621 ± 272 pg/mL, $P = 0.012$). Patients with higher absolute counts of naïve B cells at day 7 (above the median value) had lower concentrations of serum BAFF at day 7 (546 ± 241 vs. 943 ± 416 pg/mL, $P = 0.032$). Patients with higher percentages or absolute

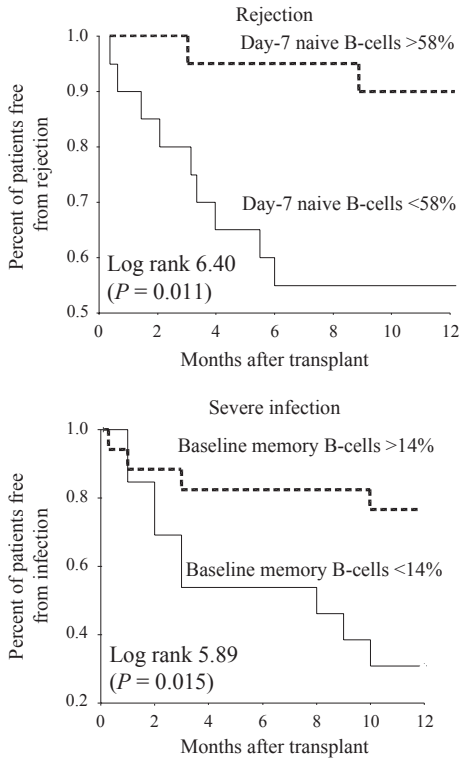

Figure 5 Kaplan–Meier curves for acute cellular rejection-free and severe infection-free survival in heart recipients. Baseline: Before heart transplantation.

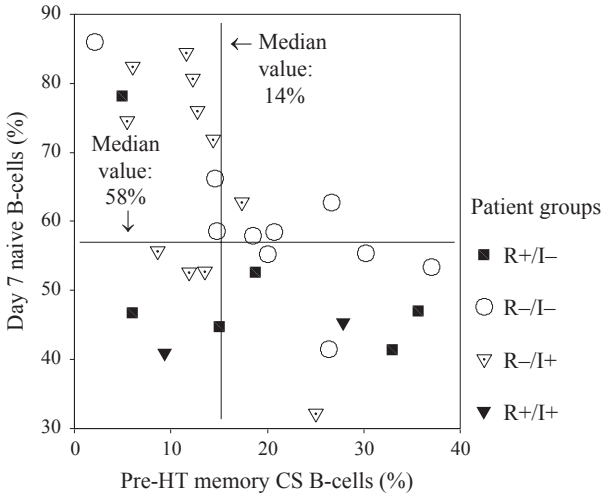

Figure 6 Distribution of patients according to the level of baseline memory class-switched B cells, day 7 naïve B cells and clinical complications. Pre-HT: preheart transplantation. CS: Class-switched. Patients who developed severe infections (I+) were more frequent among patients who had lower pretransplant levels of memory class-switched B cells (<14%). Prevalence of rejection episodes (R+) was higher in heart recipients with lower CD19 + naïve percentages at day 7 (<58%).

counts of naïve B cells at day-7 had concentrations of interferon-gamma at day 30 that were similar to those of patients with lower values (data not shown).

Class-switched memory B cells at baseline and infection

Heart recipients with lower baseline memory class-switched B cells were found to have significantly lower pretransplant levels of anti-CMV titers and tended to have lower concentrations of IgG2 subclass, IgG3 subclass, anti-tetanus toxoid, and anti-PPS antibodies (Table 4). During follow-up, patients with lower baseline class-switched memory B cells had significantly lower levels of anti-PPS (day-90, 10.9 ± 5.3 vs. 17 ± 9 mg/dL; $P = 0.024$). We observed a tendency towards lower concentrations of anti-CMV antibodies [day 7, 11123 ± 9213 vs. 17694 ± 12587 titer dilutions ($P = 0.13$); day 30, 9940 ± 8359 vs. 16184 ± 15293 titer dilutions ($P = 0.17$); and day 90, 11662 ± 9268 vs. 21435 ± 17987 titer dilutions ($P = 0.065$)]. Patients who experienced severe infections showed strong negative correlations between baseline serum BAFF concentrations and baseline absolute counts of total B cells, memory unswitched and class-switched B cells ($R = -0.79$, $P = 0.02$; $R = -0.83$, $P = 0.01$ and $R = -0.81$, $P = 0.01$, respectively); these correlations were not observed in patients who did not develop infections ($R = 0.3$, $P = 0.62$; $R = -0.50$, $P = 0.39$; and $R = -0.1$, $P = 0.87$, respectively).

Baseline serum BAFF levels were significantly higher in heart recipients with bacterial infections (2232 ± 1066 vs. 1145 ± 632 pg/mL, $P = 0.007$). Day-90 serum BAFF concentrations were higher in patients who developed CMV infection (1719 ± 868 vs. 1118 ± 555 pg/mL, $P = 0.035$).

Discussion

Long-term success of heart transplantation is limited by various forms of graft rejection [1]. To date, the specific mechanisms initiating and mediating these immune responses remain unclear. Correlates of immune-mediated protection against rejection have not been defined. While the role of T cells in cellular rejection has been explored, the function of B cells in acute cellular rejection has received less attention. The increasing interest in the role of B cells in the alloimmune response is driven by emerging evidence suggesting that B cells might mediate cellular rejection [17–19]. Successful biomarker development depends on a series of steps that originates in the discovery phase (identification of potential pathogenic pathways) and culminates in the clinical validation of an appropriately targeted biomarker. The hypothesis of our study was that the evaluation of the selected B-cell subsets could enable us to identify patients at risk of acute cellular rejection or

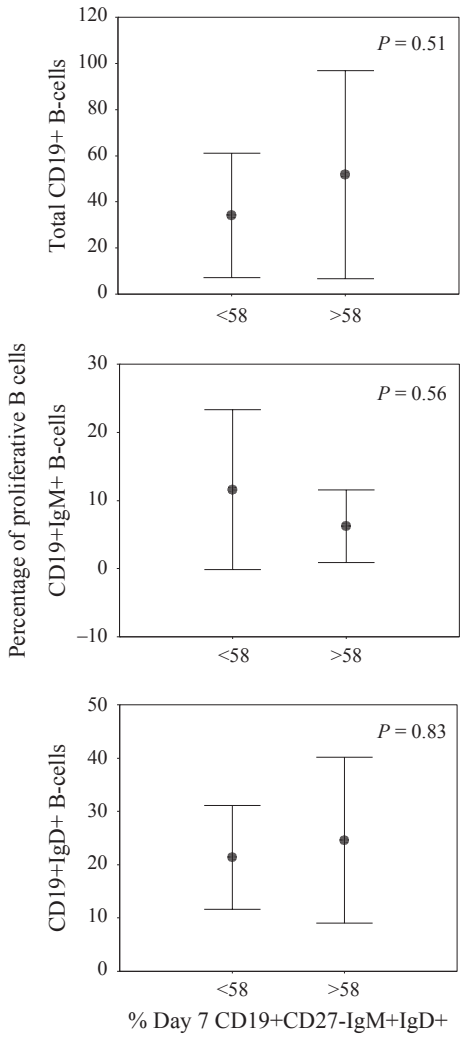

Figure 7 Non-specific B-cell proliferative responses according to the level of naïve B cells at day 7. Pre-HT: before heart transplantation. Dotted error bars mark the error observed in the average percentage of proliferative B-cell responses in patients stratified according to the median value of naïve (CD19+CD27-IgM+IgD+) B cells. Carboxyfluorescein diacetate succinimidyl ester–based cytometric analysis of peripheral blood mononuclear CD19+ B cells proliferating in response to phytohemagglutinin was performed at 3 months after transplantation.

infection. To our knowledge, we present the first report of an association between the immunophenotypic maturation status of peripheral blood B lymphocytes and development of acute cellular rejection.

The fact that higher naïve B-cell percentages were observed during the first 6 months after transplantation and lower memory B-cell levels were observed at several study points in patients who did not experience rejection suggests that this homeostatic B-cell pattern might protect against rejection. Decreased naïve/memory B-cell ratio has been described in pediatric kidney recipients experiencing acute rejection [20]. Naïve B cells are considered unable to activate naive T cells [21]. Our observations suggest that a higher proportion of naïve over memory B cells might protect against acute cellular rejection. This finding also adds support to the observation that B cells may act as regulators of an alloimmune response [22]. In addition to their recognized role in antibody production, B lymphocytes can be implicated in regulatory mechanisms [23–25]. The presence of alterations in the maturation status of B cells independently of the presence of pathogenic antibodies has been described in other human models of T cell–mediated disease [26]. A bias toward differential expression of B cell–related genes has been suggested in tolerant and rejection-free recipients [27,28]. The coexistence of humoral and cellular rejection has been documented elsewhere [29], as has the presence of B cells in cardiac tissue after transplantation [30]. In addition, the finding of complement deposition in endomyocardial biopsy specimens in some patients with acute cellular rejection further suggests the potential role of B cells [29]. We observed that naïve B-cell percentages at day 30 correlated negatively with the rejection grades observed in the first acute rejection episode (data not shown).

In our study, which was performed in a small number of patients, naïve B-cell percentages but not absolute B-cell numbers correlated with reduced acute cellular rejection in heart recipients. Absolute numbers were affected by a higher dispersion of total lymphocyte values and by the use of 2 separate methodologies. We used dual-platform flow

Table 4. Pre transplant antibody titers according to levels of Class-Switched Memory B Cells.

	Pre transplant% of CS memory B cells		
Variables	<14 (n = 23)	>14 (n = 23)	P-value*
IgG (mg/dL)	1033 ± 444	1202 ± 287	0.24
IgA (mg/dL)	317 ± 157	343 ± 157	0.68
IgM (mg/dL)	71 ± 13	66 ± 10	0.78
IgG1 (mg/dL)	633 ± 358	726 ± 155	0.42
IgG2 (mg/dL)	294 ± 169	420 ± 222	0.15
IgG3 (mg/dL)	40 ± 24	59 ± 24	0.09
IgG4 (mg/dL)	56 ± 62	83 ± 67	0.36
IgG anti-tetanus toxoid (mg/dL)	0.7 ± 1.01	2.09 ± 2.75	0.12
IgG anti-PPS (mg/dL)	12 ± 10	20 ± 19	0.09
IgG anti-CMV (titer dilution)	11323 ± 10265	23114 ± 16421	0.02
IgG anti-HIB (mg/L)	2.12 ± 2.64	2.47 ± 2.97	0.78
IgG anti-varicella zoster (mIU/mL)	651 ± 239	693 ± 204	0.65

*Student's *t*-test.
CMV, cytomegalovirus; CS memory B cells, class-switched memory B cells (CD19+CD27+IgM-IgD-); HIB = type B *Haemophilus influenza*; HT, heart transplant; PPS, pneumococcal polysaccharide. Data are presented as mean ± SD.

cytometry, which directly measures the percentage of lymphocytes that are positive for a specific marker. In order to calculate the absolute B-cell subset counts, a complete blood cell count was performed to determine the absolute number of cells. As this method is prone to measurement errors, patients with high naïve B-cell percentages but lower absolute counts may in fact have spuriously low absolute counts if the total lymphocyte count is decreased by concurrent infections or other transplant-related phenomena (Figure 2). On the other hand, we acknowledge that naïve B-cell percentages might be an artifact of other B-cell subset changes (e.g., an increase in circulating memory B cells). However, the tendency of lower naïve B-cell percentages was clear at several study points in patients who developed acute cellular rejection during the first 3 months after transplantation (the time when most rejection episodes were diagnosed). It has been previously demonstrated that immune monitoring of the percentages of these B-cell subsets are useful for identifying patients at risk of having clinical complications in other models of disease [10]. The value of lymphocyte subset percentages over the absolute count has been seen even in HIV infection [31]. Taking these observations into account, we suggest that B-cell subset percentages might be more reliable than absolute counts as a potential biomarker of clinical outcomes in heart recipients.

We did not aim to determine the specificity of the immune responses evaluated in this study. Such testing is necessary to assess whether our observations of the kinetics of naïve or memory B cells indicate allograft rejection or represent surrogate markers for the development of this complication. We performed additional analyses to investigate possible correlations between B-cell subset levels and functional B-cell–related parameters. BAFF has recently attracted attention as a potent cytokine involved in B-cell stimulation. To date, little is known of the role of BAFF in transplantation. Our serial studies revealed that BAFF levels were higher after development of acute cellular rejection. Interestingly, patients with lower naïve B-cell numbers at day 7 also had higher levels of BAFF at day 7. In experimental studies, the binding of BAFF to BAFF-R expressed by CD4 + T cells costimulates T-cell activation and allo-proliferation leading to cardiac allograft rejection [25]. The potential role of B-cell subsets and BAFF in the pathogenesis of acute cellular rejection warrants further investigation.

As for the association between immunophenotypic abnormalities and risk of severe infection, an interesting finding was the presence of higher levels of pretransplant memory class-switched B cells in patients who remained free of severe infections in the post-transplant period. We previously demonstrated that higher pretransplant IgG concentrations were associated with a lower risk of severe infection [32]. This hazard profile suggests that enhanced baseline humoral immunity provides these patients with greater protection from infections. We further investigated whether the pretransplant B-cell maturation status reflects individual baseline differences between patients. We observed that lower pretransplant levels of class-switched memory B cells were associated with baseline and post-transplant lower concentrations of distinct humoral immunity parameters that might explain the increased risk for development of infection that we observed in this subgroup of patients. In our study, assessment of the B-cell maturation status added information to post-transplant IgG determination as a marker of risk for infection. As it is unlikely that any single marker could explain the risk of infection in the transplant setting, the use of functional combined markers is advisable. Decreased memory B-cell values with IgG hypogammaglobulinemia associated with the risk of infection have recently been reported in bone marrow recipients [33,34]. These patients deserve a more careful follow-up and might be candidates for prolonged prophylactic measures or replacement IVIG therapy. The potential role of BAFF as a biomarker of infection in heart recipients warrants further investigation. High serum levels of BAFF can predict the outcome of chronic infections [35].

Our study is limited by its small sample size, which prevented us from performing a multivariate analysis to identify factors protecting against the complications analyzed. However, we did not find that clinical parameters helped to identify the risk of acute cellular rejection. In the case of severe infection, the bivariate analysis revealed that higher percentages of memory class-switched B cells were independently associated with the risk of infection.

When lower baseline memory class-switched B-cell percentages and day-7 lower naïve B-cell percentages indicate an increased probability of infection and rejection, respectively, they could be considered a surrogate marker for these complications in heart recipients. It is noteworthy that the changes in these subsets were observed mostly before or early after transplantation in patients who subsequently developed the complications investigated. Most infectious and rejection-associated complications appear more than 1 week after transplantation, thus providing us with the opportunity to improve monitoring of high-risk patients. In summary, baseline memory class-switched B-cell levels and day-7 naïve B-cell percentages should be evaluated as potential biomarkers in clinical practice.

Assessment of B-cell subsets might be an easily administered test for identifying patients at risk of developing these complications. Further studies with larger numbers of patients are needed to validate these observations.

Authorship

NL, ES and JC: Designed research, performed research, analyzed data, and wrote article. AG, LC, MJ, JN and MR:

Performed research. JP and JF-Y: Designed research, performed research. EF-C: Designed research.

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