In Kidney Transplant Patients, Alemtuzumab but Not Basiliximab/Low-Dose Rabbit Anti-Thymocyte Globulin Induces B Cell Depletion and Regeneration, Which Associates with a High Incidence of De Novo Donor-Specific Anti-HLA Antibody Development

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In Kidney Transplant Patients, Alemtuzumab but Not Basiliximab/Low-Dose Rabbit Anti-Thymocyte Globulin Induces B Cell Depletion and Regeneration, Which Associates with a High Incidence of De Novo Donor-Specific Anti-HLA Antibody Development

Marta Todeschini,*‡ Monica Cortinovis,*‡ Norberto Perico,*‡ Francesca Poli,‡ Annalisa Innocente,‡ Regiane Aparecida Cavinato,*,‡ Eliana Gotti,‡ Piero Ruggenenti,‡ Flavio Gaspari,*‡ Marina Noris,*‡ Giuseppe Remuzzi,*‡ and Federica Casiraghi*,‡

In this single-center matched-cohort study, we evaluated the phenotype of repopulating B cells and its correlation with donor-specific anti-HLA Ab development and long-term graft function in 16 renal transplant recipients and 32 age- and gender-matched controls induced with alemtuzumab or basiliximab (Bas)/low-dose rabbit anti-thymocyte globulin (rATG), respectively. Alemtuzumab, but not Bas/rATG, profoundly depleted peripheral B cells in the first 2 mo posttransplantation. Early posttransplant, naive B cells were significantly depleted, whereas Ag-experienced and memory B cells were partially spared. Transitional B cells transiently increased 2 mo posttransplant. At month 6 posttransplant, pregerminatal center B cells emerged, a process promoted by increased BAFF serum levels. Thereafter, B cell counts increased progressively, mainly due to expansion of naive B cells. Conversely, Bas/rATG did not modify the B cell phenotype throughout the follow-up period. Alemtuzumab was associated with a higher incidence of de novo DSA compared with Bas/rATG. DSA development was predicted by changes in the B cell compartment and correlated with worse long-term graft function. Thus, alemtuzumab-induced B cell depletion/reconstitution may promote chronic humoral responses against the graft. The Journal of Immunology, 2013, 191: 2818–2828.

Improved immunosuppression strategies allowed the reduced incidence and severity of acute rejections after kidney transplantation, but they failed to significantly affect the long-term outcome of the graft (1). Side effects of chronic maintenance immunosuppression, including increased patient cardiovascular morbidity and mortality, and late immunoreactivity against the allograft are the major causes of graft loss in the long-term. Thus, novel antirejection regimens have been implemented to yield similar short-term results, limiting the long-term sequelae of chronic immunosuppression and promoting a tolerance-permissive environment (2).

Induction therapy with alemtuzumab (Campath-1H), a humanized monoclonal anti-CD52 Ab inducing near-immediate and profound T and B lymphocyte, NK cell, and monocyte depletion, allowed successful transplantation in the setting of minimal maintenance immunosuppression. However, alemtuzumab treatment was associated with an increased risk for the development of donor-specific anti-HLA Abs (DSA) and with an excess risk for early Ab-mediated rejection, particularly in a calcineurin inhibitor–free immunosuppressive regimen (3–5). The above data converge to suggest that, in the clinical setting of minimal maintenance immunosuppression, alemtuzumab-induced lymphocyte depletion might result in activation of the humoral response toward alloantigens. In contrast, more recent investigations showed that, in kidney transplant patients, lymphocyte depletion induced by alemtuzumab was followed by a transient increase in transitional B cells, including B cells with phenotypic characteristics of regulatory B cells, as well as long-term dominance in naive B lymphocytes (6, 7). A B cell repertoire similar to that described to be prominent in the peripheral blood of operationally tolerant renal allograft recipients (8).

More conventional induction therapy with rabbit anti-thymocyte globulin (rATG) has a limited risk for acute humoral rejection (9), but it has major side effects: acute cytokine release syndrome, increased opportunistic infections, including CMV reactivation, and posttransplant lymphoproliferative disease (10, 11). Induction therapy with very low-dose rATG combined with the mAb anti–IL-2R basiliximab (Bas), given with the aim of inhibiting the activity of T cells surviving the lymphocytolytic effect of rATG, prevented acute graft rejection in kidney transplant patients as effectively as did full-dose rATG monotherapy but with remarkably fewer side effects (12, 13) and negligible risk for acute humoral rejection.

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Altogether, the above observations suggest that the risk for Ab-mediated graft rejection associated with lymphocyte-depleting induction therapy in kidney transplantation varies greatly, according to the specific regimen adopted. We hypothesized that such differences may depend on the different effects on B cell depletion and lymphopenia-induced proliferation of B cell subsets. To formally test this hypothesis, we conducted a single-center, matched-cohort study in 16 consecutive patients induced with alemtuzumab and in 32 gender- and age-matched controls induced with Bas/rATG to evaluate the profile of the different circulating B cell subsets in the early and late posttransplant period, the development of de novo DSA and its impact on long-term graft dysfunction, and the correlations between changes in specific B cell subpopulations and the development of de novo DSA.

Materials and Methods

Patients and immunosuppressive therapies In this single-center, matched-cohort study, we evaluated the longitudinal profile of B cell subsets, de novo DSA development over a 2-6 year follow-up, and their impact on long-term graft function in 16 consecutive patients given induction therapy with alemtuzumab and in 32 gender- and age-matched reference patients given Bas/rATG. The two studies were performed consecutively. For both cohorts, the same inclusion/exclusion criteria were used: age 18–70 y, non-HLA identical to the donor, negative cross-match test, and current panel reactive Abs < 10%. All subjects were managed by the same transplant physicians, according to the same standardized protocol of monitoring and immunosuppressive treatment. Thus, the two groups of patients differed only with respect to their immunosuppressive regimens. Protocols were approved by the Ethics Committee, Azienda Ospedaliera Papa Giovanni XXIII, Bergamo, Italy, and written informed consent was obtained from all participating patients according to the regulations of Helsinki. All data were used according to the standard regulations of the Nord Italia Transplant network for data registration and use and for the preservation of patients’ anonymity and privacy. Patients. Study patients received a single i.v. administration of 30 mg alemtuzumab at the time of surgery (ClinicalTrials.gov identifier: NCT00309270). On day 1, they were randomized (1:1) to either low-dose sirolimus or low-dose cyclosporine combined with 750 mg twice daily of mycophenolate mofetil (MMF). The dose of sirolimus was titrated to target trough concentrations of 5–10 ng/ml [by HPLC (14)]. Cyclosporine was initially infused i.v. and was then given per os at doses titrated to target trough blood concentrations of 120–220 ng/ml in the first month posttransplant and 70–120 ng/ml thereafter [by HPLC (15)]. Five hundred milligrams of methylprednisolone was administered before alemtuzumab infusion and continued for two additional days of posttransplantation (250 and 125 mg, respectively). Then, oral prednisone (75 mg) was administered, which was progressively tapered and discontinued after day 7 postsurgery. Sirolimus and cyclosporine trough levels and B cell counts measured through the 24-mo follow-up of the original 16 patients enrolled in this study, as well as glomerular filtration rate (GFR) values measured through 30-mo follow-up, were reported previously (16, 17) Reference patients. Since the day of transplant, reference patients received five consecutive rATG (thymoglobulin) infusions at a daily dose of 0.5 mg/ kg (12, 13). The dose of rATG was reduced or temporarily withdrawn when WBC or platelet counts decreased to <2,000/μl or <50,000/μl, respectively. None of the patients experienced lymphopenia or thrombocytopenia, and all received five doses of rATG. Twenty milligrams of Bas was infused i.v. before transplant and 4 d later. Maintenance immunosuppression with low-dose cyclosporine and MMF, as well as steroid treatment, was the same as used in alemtuzumab patients. Notably, the study arm with maintenance immunosuppression with low-dose sirolimus/MMF after induction therapy with Bas/rATG was planned in the initial study design. However, the first four renal transplant recipients randomized to the low sirolimus/MMF maintenance immunosuppressive regimen developed acute allograft rejection. The sirolimus/MMF arm was interrupted, and all patients were given cyclosporine maintenance immunosuppression. Follow-up and graft outcome GFR was measured by plasma clearance of iothanol (18) at 6 and 12 mo posttransplant and at yearly intervals thereafter. At the same time points, 24-h urine samples were collected for evaluation of urinary protein excretion.

Peripheral B lymphocyte counts and phenotype The absolute count of B cell populations was determined using a single-platform method (Multitest four-color Abs and Trucount tubes; BD Bioscience, San Jose, CA). PBMC samples were incubated with different fluorochrome-conjugated murine mAbs against human CD3, CD19, CD27, CD45, CD24, and CD38 (BD Bioscience and BioLegend). The samples were analyzed using a FACSComp flow cytometer (BD Bioscience) and FlowJo software. For each marker, blank samples with isotype-matched control Abs were analyzed.

PBMCs were collected pretransplant at 15–30 d, and at 2, 6, 12, and >24 mo posttransplant. Results obtained from PBMCs collected from 24–48 mo after transplant were pooled and considered >24 month posttransplant.

BAFF and alemtuzumab assays Soluble BAFF was measured in serum samples of kidney transplant recipients and healthy volunteers using a commercially available ELISA (R&D Systems, Minneapolis, MN), following the manufacturer’s instructions. Alemtuzumab serum levels also were assessed by ELISA (Supplemental Fig. 1). Anti-HLA Ab determination Anti-HLA Abs in the subjects’ sera collected before surgery and at years 1 and 2 posttransplant were tested with a bead-based screening assay (19, 20). Briefly, we used the LABCopt Mixed kit (One Lambda), which simultaneously detects HLA class I and class II Abs with MicroBeads coated with purified Ags. The Single Ag kit (One Lambda) was also used to identify HLA specificities. Results above a cut-off value of 2000 mean fluorescence intensity were considered positive. The tests were carried out according to the manufacturer’s instructions, and the analysis was performed with One Lambda software (HLA Visual Version 2.2).

Statistics The repeated-measures ANOVA test was used for comparison of variables over time. Categorical values were compared using the Fisher exact t test, and continuous variables were compared using the Student two-tailed t test. Individual GFR slopes (GFR changes over time) were calculated by linear-regression analysis. Computation of the area under the receiver operating characteristic (ROC) curve for B cell counts and phenotypes was performed, and the sensitivity and specificity of these parameters for DSA positivity were determined. All calculations were made using MedCalc 12.3.0.0 (MedCalc Software, Mariakerke, Belgium). The p values < 0.05 were considered statistically significant.

Results At the time of transplant, the characteristics of patients (alemtuzumab) and reference patients (Bas/rATG) were similar (Table I). Characteristics were also similar between patients induced by alemtuzumab and randomized to cyclosporine or sirolimus maintenance therapy (Supplemental Table I).

B cell depletion after alemtuzumab or Bas/rATG induction Alemtuzumab induced a profound B cell depletion during the first 6 mo posttransplant; subsequently, B cells recovered to pretransplant levels and, in the long-term, progressively increased to levels higher than those observed in reference patients (Fig. 1). No differences were observed between patients given cyclosporine or sirolimus maintenance immunosuppression after alemtuzumab-mediated B cell depletion, indicating that B cell reconstitution was not influenced by maintenance immunosuppressive drugs. Bas/rATG induction therapy did not modify B cell counts during the >24 mo follow-up period (Fig. 1).

Phenotype of repopulating B cells in alemtuzumab and Bas/ rATG cohorts The phenotype of repopulating B cells was characterized based on Bm1–Bm5 (21) and IgD-CD27 (22, 23) classifications (Figs. 2A, 3A) in the alemtuzumab and Bas/rATG kidney transplant cohorts. In the early posttransplant period, alemtuzumab significantly depleted naive Bm1 and Bm2 cells, as documented by both lower cell
counts (Fig. 2B) and percentages (Fig. 2C) of these B cell subsets at 15–30 d and 2 mo posttransplant compared with pretransplant values. Alemtuzumab also significantly depleted Bm2, early Bm5, and late Bm5 B cells, as documented by decreases in cell counts at 15–30 d and 2 mo for early and late Bm5 cells) posttransplant (Fig. 2B), although, during the first month posttransplant, the relative percentage increased significantly during the late posttransplant period (Fig. 2C), whereas cell counts and percentages of Bm2, early Bm5, and late Bm5 B cells normalized to pretransplant levels. At 6 mo posttransplant, the percentages of Bm3+4 cells also had normalized, whereas their counts increased from 2 to 6 mo after transplant compared with baseline levels, although not to a statistically significant extent. In contrast, both cell counts and percentages of Bm2 B cells underwent a significant increase compared with pretransplant values; Bm2 B cells represented nearly 20% of the peripheral B cells at 6 mo posttransplant (Fig. 2B, 2C).

At 6 mo posttransplant, naive Bm1 cells remained significantly depleted in alemtuzumab patients, as reflected by cell counts and percentages that were lower than pretransplant values (Fig. 2B, 2C), whereas cell counts and percentages of Bm2, early Bm5, and late Bm5 B cells normalized to pretransplant levels. At 6 mo posttransplant, the percentages of Bm3+4 cells also had normalized, whereas their counts increased from 2 to 6 mo after transplant compared with baseline levels, although not to a statistically significant extent. In contrast, both cell counts and percentages of Bm2 B cells underwent a significant increase compared with pretransplant values; Bm2 B cells represented nearly 20% of the peripheral B cells at 6 mo posttransplant (Fig. 2B, 2C).

In the long-term posttransplant, cell counts of Bm1 and late Bm5 cells were significantly higher than pretransplant values, whereas Bm2, Bm3+4, and early Bm5 cell counts returned to pretransplant values (Fig. 2B, 2C). Of note, Bm2 cell counts in the long-term were higher than pretransplant levels and were significantly higher than in the Bas/rATG group.

According to IgD/CD27 B cell classification, alemtuzumab significantly depleted all B cell subsets, as documented by IgD/CD27 B cell counts in the first 2 mo posttransplant that were lower than pretransplant numbers (Fig. 3B). The relative percentages of naive and unswitched memory B cells were also reduced in the early posttransplant period (Fig. 3C), whereas the relative percentages of switched memory B cells increased significantly; at 15–30 d posttransplant, they accounted for 70–80% of the residual IgD/CD27 B cells (Fig. 3C). The percentages of double-negative B cells did not undergo significant changes, with the exception of a transient increase at 2 mo posttransplant (Fig. 3C). At 6 mo posttransplant, all IgD/CD27 B cell subsets had returned to pretransplant values (Fig. 3B, 3C).

Changes in both Bm1–Bm5 and IgD/CD27 B cell phenotypes induced by alemtuzumab were similar in the cyclosporine and sirolimus groups (Supplemental Table I).
In reference patients given Bas/rATG, B cell counts did not change appreciably (Figs. 2, 3). Specifically, B cell subsets defined based on Bm1–Bm5 classification and can be used to identify multiple subsets, including Bm1 (virgin naive B cells, IgD⁺CD38⁻), Bm2 (activated naive B cells, IgD⁺CD38lo), Bm2' (pregerminal center cells, IgD⁺CD38hi), Bm3+4 (centroblasts and centrocytes germinal center cells, IgD⁺CD38hi), and Bm5 (memory B cells, IgD⁺CD38lo) cells. Bm5 memory cells, which express levels of CD38 ranging from moderately positive to negative, can be divided into early Bm5 (CD38hi) and late Bm5 (CD38⁻) cells (21). An inference from this model is that, in peripheral lymphoid organs, naive Bm1 lymphocytes, after Ag encounter, become Bm2 cells and then develop into germinal center founder Bm2' cells. These cells can differentiate into centroblast Bm3 and centrocyte Bm4 cells (Bm3+4) and, later, into early memory Bm5 cells and late memory Bm5 cells or plasma cells. For the duration of this process, cells representing each subset are released into the blood. Longitudinal profile of Bm1, Bm2, Bm2', Bm3+4, and early and late Bm5 cell counts (cells/µL) (B) and percentages (on total CD3⁺CD19⁺ B cells) (C) from baseline to >24 mo after transplant in kidney transplant recipients given either alemtuzumab or Bas/rATG induction therapy. Data are mean ± SEM. *p < 0.05 versus time 0, $p < 0.05$ versus 12 and >24 mo posttransplant. $p < 0.05$ versus Bas/rATG group at the same time point.

**CD52 expression of B cell subsets**

We wondered whether differences in the expression of CD52, the target Ag of alemtuzumab, may account for the differences in B cell composition observed early after transplantation. The expression of CD52 Ag was evaluated on Bm1–Bm5 and IgD/CD27 B cell subsets before alemtuzumab administration. As shown in Fig. 4A and 4B, ~12% of Bm3+4 B cells did not express the CD52 Ag, resulting in a significantly lower percentage of CD52⁻ Bm3+4 B cells compared with the other Bm1–Bm5 B cell subsets. Because Bm3+4 cells represent 3% of the entire B cell population (see gating strategy in Supplemental Fig. 2A), the proportion of CD52⁻ Bm3+4 cells in the overall B cell population is <0.5%. Bm3+4 cells expressed CD52 Ag at a lower intensity compared with the other Bm1–Bm5 B cell subsets (Fig. 4B, 4C). A lower percentage of CD52⁺ cells was also observed in early Bm5 B cells, although the difference did not reach statistical significance (Fig. 4A). In the IgD/CD27 analysis, a lower percentage of switched memory B cells expressed the CD52 Ag than did all of the other
subsets (Fig. 4A), although they expressed CD52 at the same intensity as the other IgD/CD27 subsets (Fig. 4C). These findings indicate that the increase in the relative percentage of Bm3+4 and early Bm5 cells among Bm1–Bm5 B cell subsets and of switched memory B cells in the IgD/CD27 population were the result of alemtuzumab sparing of CD52 Bm3+4, early Bm5, and switched memory B cells. In addition, a lower expression intensity of the CD52 Ag by CD52+ Bm3+4 B cells conferred upon them resistance to alemtuzumab depletion.

Alemtuzumab induces a transient increase in transitional B cells

Recently, human circulating transitional B cells, identified as CD24^highCD38^high cells (24), were reported to be increased after alemtuzumab induction in kidney transplant recipients (6, 7). Transitional B cells were present at a very low frequency (<2%) in the peripheral blood of kidney transplant recipients of the two cohorts pretransplant. As shown in Fig. 5, alemtuzumab induced an early expansion of transitional B cells, as documented by increased cell counts and percentages during the first 2 mo posttransplant (to a similar extent in cyclosporine and sirolimus groups; Supplemental Table I); subsequently, beginning at 6 mo posttransplant, both cell counts and percentages returned to pretransplant levels and remained unchanged until >24 mo. Bas/rATG induction therapy did not modify cell counts or percentages of transitional B cells (Fig. 5).

Alemtuzumab-induced B cell depletion increases serum BAFF levels, which correlates with transitional and Bm2^+ B cell expansion

The homeostatic expansion of B cells is regulated primarily by the cytokine BAFF; under conditions of B cell lymphopenia induced by B cell–depleting agents, such as alemtuzumab and rituximab, BAFF availability increased markedly (25, 26). In agreement with these studies, we found very high levels of BAFF in serum samples from alemtuzumab-treated patients at 1 and 2 mo posttransplant compared with pretransplant values, as well as with posttransplant BAFF values in Bas/rATG-treated reference patients (Fig. 6A). In the alemtuzumab group, BAFF levels were higher at all time points in patients given maintenance immunosuppressive therapy consisting of either cyclosporine or sirolimus (Supplemental Fig. 3). To establish whether increased BAFF availability accounted for changes in the midterm posttransplant of Bm1–Bm5 and IgD/CD27 B cells, as well as transitional B cells, we performed linear-
regression analyses of BAFF levels and B cell percentages in the combined kidney transplant cohorts (Fig. 6B). Intriguingly, the peak BAFF serum levels at 1 mo posttransplant correlated inversely with the percentages of Bm1 B cells at 6 mo, whereas they correlated positively and significantly with the percentages of transitional B cells 2 mo after transplantation and with the percentages of Bm2 cells at 6 mo posttransplant (Fig. 6B), indicating a close relationship between early posttransplant BAFF availability and the expansion of transitional and Bm2 B cells. BAFF serum levels did not correlate with the percentages of the other Bm1–Bm5 or IgD/CD27 B cells (Fig. 6B).

Circulating levels of alemtuzumab decline rapidly early posttransplantation

For 13 of the 16 patients given alemtuzumab, serum samples were available at both early (1 h, 12 h, 7 d) and late (1, 2, 6, 12, 24 mo) time points posttransplantation, and alemtuzumab levels were tested by ELISA. As shown in Fig. 7, as early as 1 h after Ab infusion, alemtuzumab serum levels reached 1300 ng/ml and declined to ∼1000 ng/ml after 12 h. Seven days after Ab administration, serum levels were reduced further and became negligible (<80 ng/ml) at 1 mo. From this point onward, alemtuzumab was no longer detectable in any of the serum samples, excluding a possible association between circulating levels of the anti-CD52 Ab and changes in B cell phenotype in the mid- and long-term period after alemtuzumab administration.

De novo DSA development after alemtuzumab or Bas/rATG induction therapy and long-term graft functional changes

None of the patients or reference patients had DSA in serum samples at the time of transplantation. The incidence of de novo DSA at 1 y posttransplantation was significantly higher in alemtuzumab patients than in Bas/rATG-treated reference patients (p = 0.011, Fig. 8A). In the alemtuzumab group, 50% of the patients positive for DSA had Abs against HLA class I Ags, and 50% had Abs against HLA class II Ags (antidonor DQ, Fig. 8B). DSA development was not influenced by maintenance immunosuppressive therapy with cyclosporine or sirolimus (Supplemental Table I). In the Bas/rATG group, 25% of the reference patients positive for DSA had Abs against HLA class I, 50% had Abs against HLA class II, and 25% had Abs against HLA class I and II Ags (Fig. 8B). At year 2 posttransplant, the frequency of subjects positive for DSA was still significantly greater among patients compared with reference patients (p = 0.010, Fig. 8A). All individuals who developed DSA by 1 y posttransplant maintained positivity for these Abs at 2 y postsurgery. None of the patients or reference patients experienced acute humoral rejection.

In alemtuzumab-treated patients, 24-h urinary protein excretion at 2 and 4 y posttransplant was significantly higher (Table I) and the rate of GFR decline (over 4-y follow-up) was significantly faster (p = 0.0284, Fig. 8C) compared with Bas/rATG reference patients. The analysis of two cohorts with regard to calcineurin inhibitor...
treatment showed that the rate of GFR decline during 4 y of follow-up was significantly faster in subjects who had developed de novo DSA 1 y posttransplant compared with DSA-negative individuals (Fig. 5D). Among subjects without DSA at 1 y, the GFR slope was similar following alemtuzumab and Bas/rATG induction ($p = 0.0974$). All of the other specified characteristics of subjects, with and without de novo DSA at 1 y posttransplant, were comparable, with the exception of a trend toward worse proteinuria at 48 mo posttransplant in DSA-positive patients compared with subjects who did not develop de novo DSA (Table II).

**Relationship between B cell depletion/reconstitution and de novo DSA development**

We investigated whether early posttransplant changes in the B cell compartment could predict DSA development in the combined cohorts of kidney transplant recipients. ROC curve analyses were used to evaluate the ability of B cell counts during the first month posttransplantation (i.e., B cell nadir) to predict de novo DSA development at 1 y posttransplantation. B cell counts < 4/μL at 15–30 d posttransplant significantly predicted de novo DSA development (Fig. 9A). To exclude the possibility that these results were biased by the more profound depletion induced by alemtuzumab, we used only patients treated with alemtuzumab. Even in this cohort alone we confirmed that low B cell counts in the first month posttransplant (<2/μL) was associated significantly with de novo DSA development at 1 y ($p = 0.0249$).

Considering ROC curve analysis with B cell subsets, switched memory B cell percentages > 55.9% during the first month after surgery were associated significantly with DSA at 1 y posttransplant (Fig. 9B). In turn, naive B cell percentages < 21.9% in the first month posttransplant predicted de novo DSA development at 1 y (Fig. 9C).

With regard to changes in the B cell compartment in the late posttransplant period (i.e., the significant increase in naive B cells), we found that percentages of naive Bm2 and IgD⁺CD27⁺ cells in the long-term were higher in patients who had developed DSA (Fig. 10), and both correlated with the degree of early B cell lymphopenia at 15–30 d ($r = −0.6908$, $p = 0.0011$; $r = −0.7624$, $p = 0.0001$; respectively).

**Discussion**

In this study, we showed that the phenotype of B cells repopulating the peripheral blood in kidney transplant recipients after alemtuzumab, but not Bas/rATG, induction underwent significant variations that can be summarized in three phases: in the early posttransplant period, the few remaining B cells were composed primarily by Ag-experienced Bm3+4 and switched memory B cells. The second phase (midterm posttransplant, 2–6 mo) was characterized by a transient increase in transitional B cells, followed by the emergence of Bm2⁺ B cells, processes that are mediated by the increase in serum levels of BAFF. The third phase in the long-term (>24 mo posttransplant) was characterized by a progressive increase in circulating B cell numbers, primarily due to the expansion of B cells with a naive phenotype. Both early and late changes in the B cell compartment were associated with an increased incidence of de novo DSA at 1 y posttransplantation. De novo DSA development at 1 y posttransplant was coupled with worse long-term graft function, consistent with the notion that a substantial period of time is required before damage to the
kidney induced by DSA becomes clinically apparent (27), eventually leading to poor long-term graft outcome (27–30).

The alloantibody response in alemtuzumab-treated patients was attributed to a population of depletion-resistant effector/memory T cells that homeostatically expanded in the first month after transplantation and could provide B cell help (31). However, this memory T cell subset homeostatically expanded in rATG-treated patients as well (31), but it was not associated with an increased risk for acute humoral rejection. Thus, the memory T cell hypothesis does not fully explain the higher DSA development that we found in kidney transplant recipients given alemtuzumab compared with reference patients receiving Bas/rATG induction therapy. In contrast, we provide evidence that early changes in the phenotype of circulating B cells in alemtuzumab-treated patients correlated with the development of DSA posttransplantation. A previous report in patients with multiple sclerosis showed that even multiple doses of alemtuzumab failed to significantly decrease CD27+ memory B cell percentages 1 mo after administration (32), confirming our findings of a partial resistance of memory B cells to alemtuzumab-mediated depletion. Higher percentages of switched memory IgD CD27+B cells predicted the late development of DSA, indicating a causal link between this B cell subset and DSA production. Of note, by transferring purified B cell subsets in immunized SCID/SCID mice, human switched memory CD27+B cells were able to produce IgG

### Table II. Characteristics and baseline and immunological parameters of the subjects according to de novo DSA development at 1 y posttransplant

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No DSA (n = 36)</th>
<th>DSA (n = 12)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristic</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Recipient age (y)</td>
<td>46 ± 14</td>
<td>46 ± 12</td>
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<td>Recipient gender (%male)</td>
<td>68</td>
<td>50</td>
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<td>Living donor (%)</td>
<td>11</td>
<td>8.3</td>
<td>1.000</td>
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<td>HLA-A mismatch</td>
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<td>1.44 ± 0.63</td>
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<td>1.50 ± 0.52</td>
<td>0.3121</td>
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<tr>
<td>HLA-DR mismatch</td>
<td>1.14 ± 0.56</td>
<td>1.31 ± 0.48</td>
<td>0.2783</td>
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<tr>
<td>Clinical and immunological parameters</td>
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<tr>
<td>Cold ischemia time (h)</td>
<td>16 ± 3</td>
<td>17 ± 3</td>
<td>0.6878</td>
</tr>
<tr>
<td>Delayed graft function (%)</td>
<td>19</td>
<td>17</td>
<td>1.000</td>
</tr>
<tr>
<td>Acute rejection episodes (%)</td>
<td>17</td>
<td>17</td>
<td>1.000</td>
</tr>
<tr>
<td>Cyclosporine C0 at month 6 (ng/ml)</td>
<td>114 ± 47</td>
<td>105 ± 36</td>
<td>0.5509</td>
</tr>
<tr>
<td>Cyclosporine C0 at month 12 (ng/ml)</td>
<td>106 ± 55</td>
<td>74 ± 25</td>
<td>0.0622</td>
</tr>
<tr>
<td>Cyclosporine C0 at month 48 (ng/ml)</td>
<td>77 ± 35</td>
<td>90 ± 52</td>
<td>0.3295</td>
</tr>
<tr>
<td>Sirolimus C0 at month 6 (ng/ml)</td>
<td>7.1 ± 0.9</td>
<td>8.3 ± 3.5</td>
<td>0.3944</td>
</tr>
<tr>
<td>Sirolimus C0 at month 12 (ng/ml)</td>
<td>10.7 ± 2.5</td>
<td>8.3 ± 2.2</td>
<td>0.1482</td>
</tr>
<tr>
<td>Sirolimus C0 at month 48 (ng/ml)</td>
<td>10.1 ± 2.7</td>
<td>7.4 ± 1.8</td>
<td>0.2560</td>
</tr>
<tr>
<td>Proteinuria at month 48 (g/24 h)</td>
<td>0.51 ± 0.65</td>
<td>1.51 ± 3.16</td>
<td>0.0595</td>
</tr>
<tr>
<td>T cell counts before transplant (cells/μl)</td>
<td>1049 ± 378</td>
<td>1246 ± 260</td>
<td>0.1842</td>
</tr>
<tr>
<td>T cell counts during month 1 (cells/μl)</td>
<td>335 ± 409</td>
<td>200 ± 353</td>
<td>0.2485</td>
</tr>
<tr>
<td>T cell counts at month 12 (cells/μl)</td>
<td>997 ± 497</td>
<td>715 ± 585</td>
<td>0.1246</td>
</tr>
</tbody>
</table>
exclude immature/transitional B cells in addition to pregerminal center B cells among the CD27+ B cells was reexamined 1 y posttransplant. (B) ROC curve analysis shows that a naive IgD− CD27+ B cell percentage < 21.9% in the first month posttransplant predicted de novo DSA development at 1 y posttransplant. (C) ROC curve analysis shows that a naive IgD+ CD27+ B cell percentage < 55.9% during the first month post-transplant predicted de novo DSA development at 1 y posttransplant. (A) According to the ROC curve analysis, B cell counts < 4 cells/μL during the first month post-transplant predicted positivity for de novo DSA at 1 y posttransplant.

Abs against pneumococcal surface protein A, a T cell–dependent Ag (33). Similarly, the CD27+ B cell subset isolated from the peripheral blood of sensitized patients was able to produce alloantibodies in vitro (34). In tetramer-binding studies, a higher frequency of HLA tetramer–positive B cells was found in sensitized subjects (35); these cells were able to produce alloantibodies when stimulated in vitro. These and previous findings led us to hypothesize that, in the presence of alloantigens, spared switched memory B cells could be rapidly converted to plasmablasts and to Ab-secreting cells, leading to DSA development.

From the second month posttransplant, B cells started repopulating the peripheral blood, reaching basal levels at 6 mo post-transplant. The first B cell subtype that transiently dominated the B cell compartment during the early B cell expansion was transitional B cells, followed by Bm2+ B cells. Consistently, a transient increase in transitional B cells, as well as Bm2+ B cells, was reported recently in alemtuzumab-treated kidney transplant recipients 6 mo post-transplantation (6) and in patients with multiple sclerosis (32). The peripheral population of Bm2+ cells could include immature/transitional B cells in addition to pregerminal center B cells. The expression of CD27 on Bm2+ cells allows the distinction of the more immature B cells (CD27+) from pre-germinal center cells (CD27+) (36). Findings from our cohort of alemtuzumab-treated patients that transitional B cells (peaking at 2 mo) and Bm2+ B cells (peaking at 6 mo) had a completely different regeneration profile and that, on average, 60% of Bm2+ B cells were positive for CD27 expression (data not shown), suggest that the Bm2+ B cell subtype represented activated B cells committed to germinal center maturation rather than a population of immature/transitional B cells.

The homeostatic expansion of B cells is regulated primarily by the cytokine BAFF. Serum BAFF levels increased after B cell depletion induced by alemtuzumab, probably as a result of reduced receptor-mediated clearance. Similar to previous studies (25, 32), we found abnormally high levels of BAFF in serum from patients given alemtuzumab induction during the nadir of B cell lymphopenia.

In addition to controlling B cell proliferation, BAFF is the limiting factor for which immature B cell clones compete for their survival and selection. Under lymphoreplete conditions in which BAFF is limiting, immature B cells with lower BCR avidity and specificity are eliminated at the transitional stage (37, 38). However, under lymphopenic conditions in which BAFF is in excess, selective stringency is relaxed, and all clones, including those with autoreactive specificities, may be allowed to mature (39–41). Interestingly, BAFF-dependent rescue of self-reactive B cells from deletion also was proposed as the mechanism responsible for autoimmune disease development in patients receiving alemtuzumab (42). Intriguingly, in our kidney transplant recipients, BAFF levels correlated positively with the percentages of transitional B cells and Bm2+ B cells, as reported in patients with Sjögren’s syndrome (43) and chronic graft-versus-host disease (44), suggesting that, in vivo, these B cell subsets depend on BAFF availability. Moreover, in vitro BAFF promoted the conversion of Bm1 cells to Bm2/Bm2+ (43).

In vitro evidence demonstrates that BAFF promotes the survival of activated B cells by lowering BCR-activation thresholds and by inducing the upregulation of antiapoptotic protein expression (41, 45). This finding, together with an increased incidence of DSA at 1 y posttransplantation in our alemtuzumab-treated patients, led us to formulate the following hypothesis: transitional B cells emerging after B cell lymphopenia are constantly exposed to kidney graft Ags, and those B cells bearing a BCR specific for the donor alloantigens receive sufficient BCR signaling to upregulate BAFF receptor expression. In a BAFF-enriched environment, these cells are allowed to survive and to enter the germinal center for T cell help–mediated maturation and differentiation into donor-specific Ab-producing cells.

Changes in peripheral B cell subsets after alemtuzumab normalized 1 y posttransplant; thereafter, the B cell compartment be-
came dominated by naive B cells, the B cell subtype that started repopulating the periphery as late as 6 mo after transplantation. The delayed expansion of naive B cells was reported after alemtuzumab induction in both kidney transplant recipients (6) and in patients with multiple sclerosis (32), as well as in patients with rheumatoid arthritis after rituximab treatment (46) and after myeloablation in the setting of hematopoietic stem cell transplantation (44, 47). We speculate that the late naive B cell expansion in the peripheral blood of alemtuzumab-treated patients is the result of time needed for both new B cell generation (48) and for expression of the BAFF receptors BR3 and TACI on their cell surface. Indeed, during the maturation process, newly forming B cells exiting the bone marrow progress through the transitional stage before becoming naive B cells. During this maturation course, the cell surface expression of BR3 and TACI increases progressively, reaching the highest level at the final stage of mature naive B cells (49, 50). Although signaling through the BR3 receptor is crucial for the survival of all preimmune B cell subsets from the transitional stage to the naive stage, TACI signals negatively regulate naive B cell survival (51). This suggests that the complex interplay of intracellular signals activated by positive (BR3) and negative (TACI) BAFF receptors on naive B cells can eventually contribute to delaying and prolonging their response to the increased circulating BAFF levels that we documented after alemtuzumab-induced B cell lymphopenia. Increases in both total peripheral B cell numbers and naive B cell percentages were described recently as the main immunologic features of transplant recipients who developed spontaneous tolerance to kidney allografts (8). Actually, in our study, we found that the percentages of naive B cells in the long-term were higher in subjects who had previously developed DSA, and they correlated with the degree of early B cell lymphopenia at 15–30 d, questioning the relevance of the long-term enhanced naive B cells as a biomarker of the state of tolerance, at least in patients given alemtuzumab induction therapy.

In conclusion, we documented that B cells re-emerging after alemtuzumab-induced lymphopenia showed a dysregulated phenotype both early and late posttransplant; this phenomenon did not occur in Bas/RATG-treated reference patients. Induction therapy with alemtuzumab was associated with a higher incidence of de novo DSA development and inferior graft function, and changes in B cell phenotype correlated with de novo DSA development. Thus, in kidney transplant recipients, B cell depletion and regeneration promote chronic humoral immune responses against graft alloantigens that contribute to DSA production and, eventually, long-term graft dysfunction. These data could provide useful insights for improving the choice of induction therapy that limits the risk for B cell–induced late graft loss, at least in kidney transplantation.

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Disclosures
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References


