ORIGINAL ARTICLE

Dynamic changes of B-cell compartments in kidney transplantation: lack of transitional B cells is associated with allograft rejection

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SUMMARY

B cells play an important role in the immune responses which affect the outcomes of kidney allografts. Dynamic changes of B-cell compartments in clinical kidney transplantation are still poorly understood. B-cell subsets were prospectively monitored using flow cytometry for 1 year in 98 kidney transplant recipients. Data were correlated with immunosuppression and clinical outcomes. An increase in the total population of B lymphocytes was observed during the first week after transplantation. The level of $IgM^{high}CD38^{high}CD24^{high}$ transitional B cells reduced significantly up until the third month, with partial repopulation in the first year. Lower numbers of transitional B cells in the third month were associated with higher risk of graft rejection. $IgM^{+}IgD^{+}CD27^{-}$ naive B cells did not change within follow-up. $IgM^{+}CD27^{+}$ nonswitched memory B cells and $IgM^{-}CD27^{+}$ switched memory B cells increased on post-operative day 7. $IgM^{-}CD38^{high}CD27^{high}$ plasmablasts showed similar kinetics during the first post-transplant year, similar to transitional B cells. In conclusion, sensitized kidney transplant recipients as well as those with either acute or chronic rejection within the first post-transplant year exhibited lower levels of transitional B cells. Therefore, these data further support the hypothesis that transitional B cells have a protective role in kidney transplantation.

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Key words
B cells, kidney transplantation, rejection, transitional B cells

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Introduction

B-cell compartments play a key role in alloimmune antibody-mediated responses. Donor-specific anti-HLA antibodies are recognized as an important risk factor, particularly in organ transplantation [1–3]. Additionally, B cells are also believed to be involved in the regulation of cellular effector mechanisms against donor HLA antigens [4–7].

Transitional B cells, naive B cells, nonswitched and switched memory B cells, and plasmablasts are the main subpopulations of B-cell compartments, which can be detected in peripheral blood. Each of these subpopulations plays a specific role in organ transplantation. While memory B cells and plasmablasts appear to be important in early acute antibody-mediated rejection [4], the role of naive and transitional B cells along with the impaired maturation of plasma cells are believed to be involved in

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operational tolerance in kidney and liver transplantation [5,6]. Similarly, naive B-cell-related gene expression patterns have been found to be upregulated in rejection-free kidney grafts [7]. The role of regulatory B cells contained in a subset of transitional B cells has been extensively studied. Their inhibitory effect on alloresponses is mediated by an overproduction of IL-10, the regulation of functions of immune cells through the release of granzyme B, TGFβ and the expression of death-inducing ligands, and the production of other cytokines involved in the suppression of immune responses [8–13].

Immunosuppressive therapy can affect proportions of different cell subsets in peripheral blood of patients immediately after kidney transplantation. We have recently demonstrated that corticosteroids and rabbit antithymocyte globulin (rATG) increase the number of CD163-positive M2-like monocytes, while the percentage of nonclassical 'proinflammatory' $CD14^+CD16^+$ peripheral monocytes is downregulated during the first week after transplantation [14]. It is believed that calcineurin inhibitors inhibit peripheral regulatory T cells [15], while induction therapy with rATG increases the proportion of these cells [16]. Even though the action of inductive agents on T-cell function is well known, their effects on the constitution of B-cell compartments are still poorly understood.

Therefore, the aim of this study was to prospectively characterize the kinetics of B-cell peripheral blood compartments in kidney transplantation using CD45, CD19, IgD, CD27, CD38, CD24, IgM, and CD21 as lineage-specific markers. Another aim of the study was to characterize the potential changes of specific B-cell subpopulations with respect to induction immunosuppressive therapy using rATG or basiliximab at specific time points and clinical settings.

Materials and methods

Patients

A total of 98 patients in receipt of renal transplants from deceased donors between October 2013 and June 2014 were enrolled in the study and followed for 12 months. The demographic features are summarized in Table 1. All patients received immunosuppression therapy according to the center's protocol. In addition to standard triple maintenance therapy—based on tacrolimus, mycophenolic acid, and prednisone—patients received induction therapy according to immunological risk. First, kidney transplant recipients with historic *CDC-based* panel reactive antibodies (PRA) < 20% (n = 24) received 20 mg of basiliximab (Simulect®; Novartis, Basel, Switzerland) on days 0 and 4, while all others received rATG (n = 74) (Thymoglobulin®; Genzyme Corporation, Cambridge, MA) within the first week at an intended cumulative dose of 5 mg/kg.

Kidney graft biopsies were performed based on clinical indications or as defined by the protocol at 3 months after transplantation. Rejection was diagnosed according to the Banff 2013 classification [17]. Acute rejection occurred in 17 patients (12 patients with T-cell-mediated rejection; 4 with antibody-mediated rejection, 1 patient with mixed acute cellular, and antibody-mediated rejection). Chronic rejection was detected in three other patients (1 patient with chronic T-cell-mediated rejection, 1 with chronic antibody-mediated rejection, and another patient with mixed chronic cellular and antibody-mediated rejection). One patient had mixed chronic and acute T-cell-and antibody-mediated rejection. Individual rejection episodes of these patients are summarized in Table S1. For the purposes of the analysis, patients exhibiting all types of rejection (borderline changes were not defined as rejection) were characterized as a single rejection group. Three patients died prior to the end of the study in months 7, 10, and 11. All patients signed their informed consent, and the study protocol

Table 1. Baseline characteristics of the patient population in our prospective observational study.

Number of patients	98	
Gender (male/female)	68/30	
Recipient age (years)*	60	[19; 81]
Donor age (years)*	55	[2; 76]
Induction therapy (rATG/basiliximab) (n)	74/24	
1st/2nd/3rd transplantation (n)	84/13/1	
Preformed HLA/DSA	31/14	
PRA max (%)*	6	[0; 97]
PRA actual (%)*	2	[0; 94]
HLA mismatch*	3	[0; 6]
CIT (h)*	15,2	[1; 9; 27]
Dialysis vintage (years)*	2,2	[0; 9; 4]
Cause of renal failure		
Glomerulonephritis	30	
Tubulointerstitial nephritis	17	
Vascular diseases	9	
Diabetes mellitus	20	
Polycystic kidney	12	
Other causes	10	

*Median [min; max].

PRA, historical panel reactive antibodies, measured every 3 months before transplantation. The highest number (PRA_{max}) and actual PRA were considered for each patient; CIT, cold ischemia time.

was approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine (No: 1081/12).

Flow cytometry and isolation of peripheral blood mononuclear cells

Peripheral blood was collected in a test tube with ethylenediaminetetraacetic acid (EDTA) before transplantation, and at 1 week, 3 months, and 1 year after transplantation. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient. For isolation, Lymphoprep (Axis-Shield, Oslo, Norway) was used and cells were washed twice in PBS (phosphate-buffered saline).

Isolated PBMCs (100 μl) were incubated for 20 min in the dark with the appropriate monoclonal antibodies. The panels of monoclonal antibodies contained CD45-KromeOrange (clone J.33), CD19-ECD (clone J3-119), CD27-PC7 (clone 1A4CD27), CD21-PacificBlue (clone BL13), CD38-APC-AF750 (clone LS198-4-2), CD24-APC (clone ALB9; Beckman Coulter, Brea, CA), IgD-PerCP-Cy5.5 (clone IA6-2), and IgM-FITC (clone G20-127; BD Biosciences, San Diego, CA). One ml of optimized PBS (CellWash; Beckman Coulter, Brea, CA, USA) was added after incubation. Samples were measured using a Cyan ADP 9C flow cytometer (Beckman Coulter), and the data were analyzed using KALUZA software (Beckman Coulter). The gating strategy is shown in Fig. 1. Flow cytometry analysis was performed with at least 100 events in the gate. Absolute numbers of B lymphocyte subsets were calculated from the absolute numbers of leukocytes analyzed using the Sysmex hematology analyzer (Sysmex Corporation, Kobe, Japan). Absolute numbers of all subpopulations are shown as numbers of cells $\times$ 10^6/l.

Statistical analysis

Statistical analysis was performed using GRAPHPAD INSTANT3 software (GraphPad Software, La Jolla, CA, USA). Data normality was assessed using the Kolmogorov–Smirnov test. The frequency of missing data for each B subpopulation ranged from 0% to 4.5%. Missing values were imputed as medians of particular B subpopulations in the whole sample at particular time points. The Friedman test for repeated measures of nonparametric data in combination with a post-test (Dunn's Multiple Comparisons Test) was used to compare specific B subpopulations at each time point. The Mann–Whitney test and Bonferroni's post-test correction were used to compare unpaired data (rATG/basiliximab groups; groups divided according to HLA or groups according to PRA max.). Differences were regarded as statistically significant where $P < 0.05$. Results are presented as medians with interquartile ranges. To evaluate whether absolute numbers of particular B-cell subpopulations could be used to discriminate between rejection-free patients and patients with rejection, we used receiver operating characteristic (ROC) curves and area under the curve (AUC) calculations using SIGMAPLOT software (Systat Software, San Jose, CA, USA).

Results

$CD19^+$ B lymphocytes

The absolute number of $CD19^+$ B lymphocytes increased within the first post-transplant week in comparison with levels observed prior to transplantation ($P < 0.01$) (Fig. 2a). After three months, B-cell counts dropped to initial values and remained unchanged until the end of follow-up.

$IgM^+IgD^+CD27^-$ naive B cells

The subpopulation of $IgM^+IgD^+CD27^-$ cells corresponded to the compartment of naive B lymphocytes (Fig. 2b). This population of B cells did not show any changes after transplantation in comparison with baseline pretransplant data.

Memory IgM^+ and IgM^- B cells

The population of memory B cells was split according to the expression of surface IgM in the two groups (IgM^+CD27^+ nonswitched memory B cells and IgM^-CD27^+ switched memory B cells). For both nonswitched and switched memory B cells, an increase in the absolute number was observed ($P < 0.001$) on postoperative day 7 (POD 7). At month 3, their numbers decreased to baseline and remained unchanged until the end of follow-up (Fig. 2c and d).

$IgM^-CD38^{high}CD27^{high}$ plasmablast

The distribution of $IgM^-CD38^{high}CD27^{high}$ B cells corresponded to the dynamics of plasmablasts (Fig. 2e). During the first week after transplantation, there was a decrease in absolute numbers of these cells ($P < 0.001$). Subsequently, the gradual repopulation of these cells was observed during follow-up.

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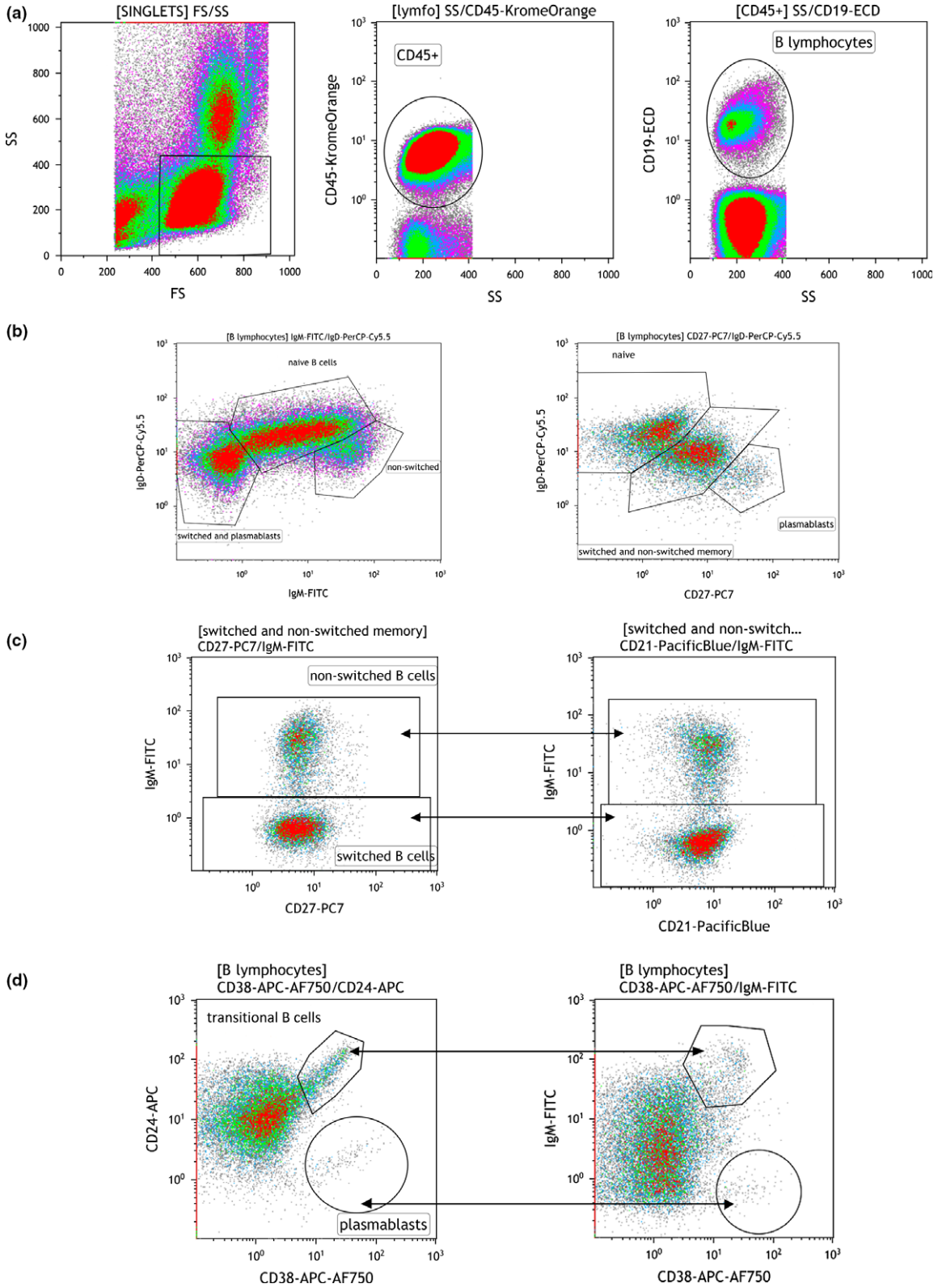

Figure 1 Gating strategy for the determination of human peripheral blood B lymphocyte subpopulations. (a) Definition of B cells via distribution profiles of SS/FS, $CD45^+$, and $CD19^+$. (b) Determination of mature naive cells as $IgM^+IgD^+CD27^-$ B cells. (c) Memory cells are defined by the expression of $CD27^+$ and divided into IgM^-CD27^+ or IgM^-CD21^+ switched memory B cells and IgM^+CD27^+ or IgM^+CD21^+ nonswitched B cells. (d) Transitional B cells represent the $CD24^{high}CD38^{high}$ subset with same corresponding population of $IgM^{high}CD38^{high}$ cells; plasmablasts are IgM^-CD38^{high} B cells or $CD24^-CD38^{high}$ B cells.

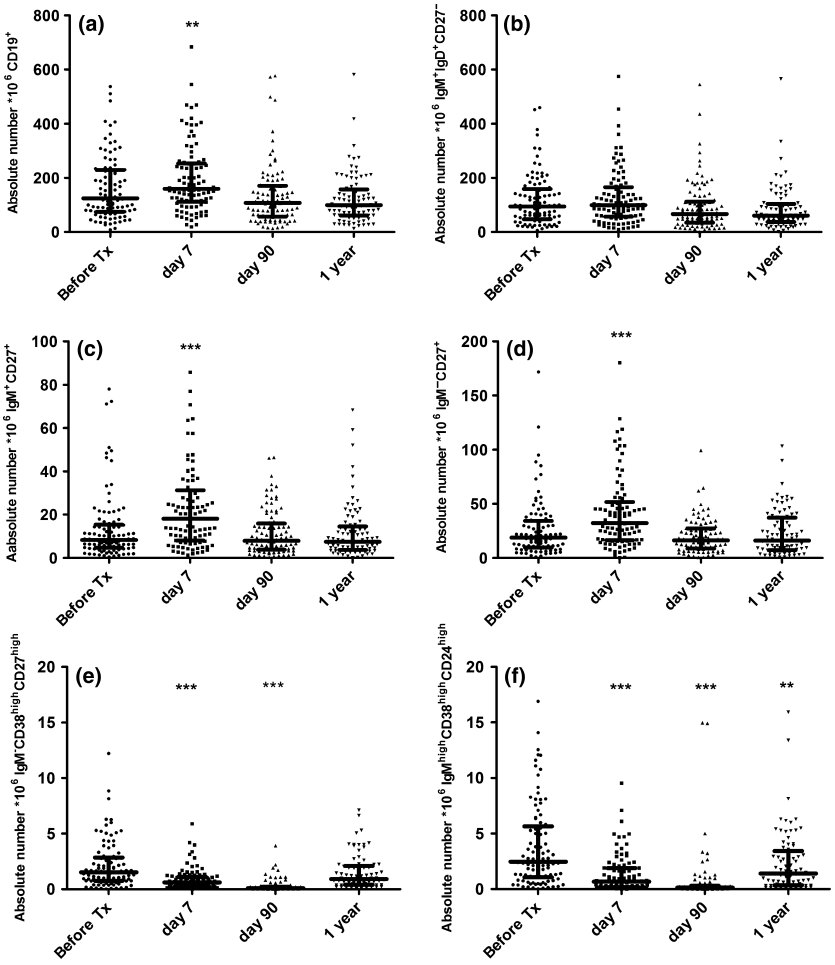

Figure 2 Dynamic changes of B-cell subpopulations in patients with kidney allograft transplantation. Figures demonstrate changes in absolute numbers of each subset of B lymphocytes at specific time points (before transplantation, one week, 3 months, and 1 year after transplantation). Absolute numbers are calculated as the number of cells $\times$ 10^6/l in peripheral blood. There was a redistribution of $CD19^+$ B lymphocytes (a). $IgM^+IgD^+CD27^-$ naive B cells did not significantly change for all time points (b). Increased numbers of IgM^+CD27^+ nonswitched memory B cells (c) and IgM^-CD27^+ switched memory B cells (d) were evident on day 7 after Tx. Decreased $IgM^-CD38^{high}CD27^{high}$ plasmablasts (e) and $IgM^{high}CD38^{high}CD24^{high}$ transitional B cells (f) persisted for 3 months after transplantation. Statistically significant differences (**$P < 0.01$; ***$P < 0.001$) are related to the initial level before transplantation.

$IgM^{high}CD38^{high}CD24^{high}$ transitional B cells

On POD 7, the absolute number of IgM^{high}-$CD38^{high}CD24^{high}$ transitional B cells decreased ($P < 0.001$) (Fig. 2f). The reduction of transitional B cells lasted until month 3 ($P < 0.001$). The restoration of these cells was observed in the first post-transplant year. However, their numbers remained lower in comparison with baseline ($P < 0.01$).

Pretransplant sensitization and post-transplant B-cell compartments

Next, we analyzed the possible association of pretransplant HLA antibodies with specific post-transplant B-cell compartments. There were no differences in B-cell subpopulations at any time point (including pretransplant) between patients with and without pretransplant HLA antibodies (data are not shown).

Furthermore, we divided patients according to pretransplant PRA into three groups (<20%, 20–50%, >50%). Patients with PRA > 50% had significantly lower numbers of $IgM^{high}CD38^{high}CD24^{high}$ transitional B cells on POD 7 than nonsensitized patients ($P < 0.05$) (Fig. 3). According to PRA groups, we did not find any further differences in other B-cell subpopulations at any time point.

The effect of induction therapy on B-cell compartments

The possible roles of depletive (rATG, $n = 74$) and nondepletive (basiliximab, $n = 24$) inductive agents were further evaluated (Fig. S1). Interestingly, the only difference was observed in the proportion of IgM^-CD27^+ switched memory B cells. On the 7th post-operative day, the increase of this population was observed in rATG but not in basiliximab ($P < 0.01$; Fig. S1).

Rejection and B-cell compartments

To find out whether the absolute number of particular B-cell subpopulations was sufficient to discriminate

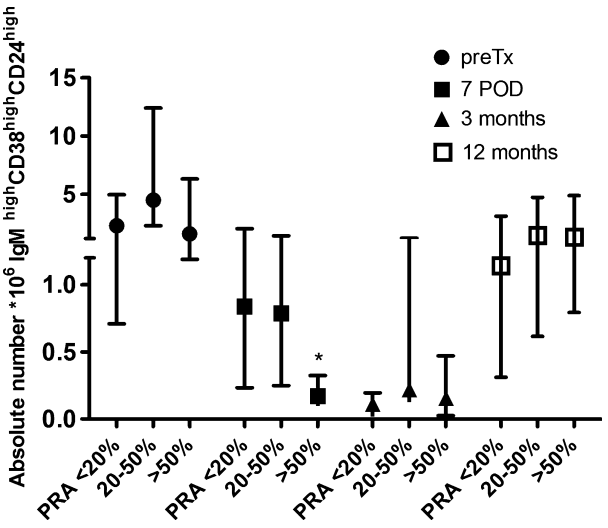

Figure 3 Distribution of $IgM^{high}CD38^{high}CD24^{high}$ transitional B cells according to PRA_{max}. Recipients with PRA > 50% (n = 19) had statistically and significantly lower transitional B-cell counts than low risk patients (PRA < 20%, n = 65; PRA 20–50%, n = 14) on day 7 (*P < 0.05). PRA, pretransplant panel reactive antibodies.

between rejection and rejection-free patients within the first post-transplant year, we performed receiver operating characteristic curve analysis (ROC) of these subpopulations before transplantation (Tx), on day 7, and at 3 and 12 months. According to ROC curve analysis, the only predictor of graft rejection was lower numbers of transitional B cells at 3 months (AUC: 0.66, SE = 0.075; cut-off = 0.0968 at 77.9% sensitivity and 66.7% specificity) (Fig. 4a). Patients with absolute numbers of transitional B cells at 3 months below the cut-off point (defined by ROC analysis) displayed statistically significantly higher incidence of rejection (Fig. 4b). Interestingly, ROC analysis performed separately for acute TCMR or AMR, showed similar association for transitional B cells at 3 months with both types of rejection (TCMR AUC: 0.64, SE = 0.077; cut-off = 0.093 at 72.2% sensitivity and 66.7% specificity; AMR AUC = 0.65, SE = 0.19; cut-off = 0.094 at 75% sensitivity and 65.7% specificity) (Fig. S2a, b).

Discussion

Undoubtedly, B-cell lineage is involved in many transplant reactions. Several groups have evaluated B-cell compartments after kidney transplantation using different techniques, resulting in the publication of inconsistent results [18–22]. Kinetics within B-cell compartments and their possible role in post-transplant outcomes thus remain poorly understood. In our prospective study, the increase of peripheral B lymphocytes on day 7 together with the increase of both populations of memory B cells was observed. For $IgM^{-}CD38^{high}CD27^{high}$ plasmablasts and $IgM^{high}CD38^{high}CD24^{high}$ transitional B cells, we observed a significant decrease until the third month after transplantation and consequent gradual repopulation during the first year. This rebound might be associated with the transient effect of induction therapy given within first post-transplant days. Additionally, we demonstrated that recipients with higher numbers of transitional B cells exhibited lower incidence of graft rejection. Transitional cells were the only group from the B-cell lineage associated with rejection.

Circulating B cells contain almost one-third of the cells that express CD25 [23]. However, both anti-CD25 monoclonal antibody basiliximab and rATG had no depletory effects on the total population of B-cell lymphocytes, even though, conversely, numbers of these cells increased on POD 7. Concurrently, polyclonal rATG contains several antibodies against B-cell-specific and nonspecific surface proteins including CD19, CD20, CD40, CD80, CD30, CD38, CD95, and the plasma cell-specific marker, CD138 [24,25]. Therefore, in addition to its anti-T-cell action, rATG may play an important role in the regulation of B-cell responses. The effect of both rATG and basiliximab on the redistribution of B-cell subpopulations was similar in our study. Contrary to our previous studies in which T cell [16] and monocyte [14] populations were examined, B cells seem to be less sensitive to a regimen of induction therapy.

Although induction immunosuppression prevents T-cell activation and thereby indirectly affects signaling for the activation and development of naive B cells [4], the absolute number of $IgM^{+}IgD^{+}CD27^{-}$ naive B cells in the post-transplantation period did not alter in comparison with the level detected before transplantation [20]. In recent clinical studies, operationally tolerant patients (defined as stable graft function in the absence of immunosuppression for one year) exhibited higher levels of naive B cells in comparison with patients receiving immunosuppression [5,26,27]. Moreover, in a recent study, a decrease in naive B-cell subsets before transplantation was shown to be related to patients who are at higher risk of acute rejection [28]. Interestingly, in our study such an association was not found. Naive B cells have been shown to be the most affected subpopulation when using anti-CD20 therapy with rituximab which affect long term composition of B-cell compartment [29,30]. Clearly, the possible role of naive

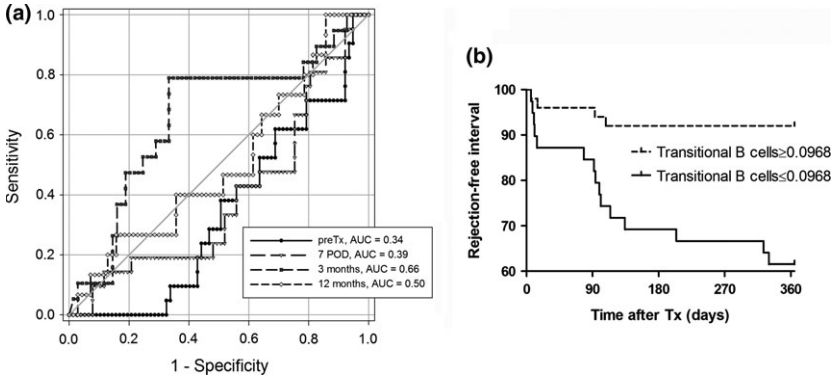

Figure 4 The prognostic value of absolute numbers of $IgM^{high}CD38^{high}CD24^{high}$ transitional B cells 3 months after kidney allograft transplantation. (a) Receiver operator characteristic (ROC) curves based on absolute numbers of $IgM^{high}CD38^{high}CD24^{high}$ transitional B cells before Tx, on day 7 and at 3 and 12 months after Tx. (b) Kaplan–Meier rejection-free survival estimates with transitional B-cell numbers at 3 months after Tx above (high) or below (low) the cut-off point defined by ROC analysis (Log-rank $P = 0.0007$) are given.

B cells in the alloimmune response needs to be further validated.

Memory B cells may be immediately transformed into antibody-secreting cells during the early phase of antibody-mediated rejection in sensitized recipients [4]. In this respect, the upregulation of both nonswitched $IgM^{+}CD27^{+}$ memory B cells (corresponding to spleen-marginal zone B cells) [31] and switched $IgM^{-}CD27^{+}$ B cells on POD 7 might represent the initiation of the humoral response against donor HLA antigens. Moreover, higher amounts of these cells are also linked to chronic antibody-mediated rejection and graft loss [28,32,33]. The expansion of memory B cells is not exclusively related to allogenic responses. Also, recipients with concomitant CMV viremia in the post-transplantation period had higher levels of memory B cells and fewer naive B cells in comparison with stable patients [33].

The main source of donor-specific antibodies (DSA) related to both acute and chronic rejection is long-lived plasma cells [34]. Recently, an *in vitro* study has shown that B cells from operationally tolerant kidney transplant recipients exhibited a defective final differentiation into plasma cells and higher propensity for cell apoptosis than those from patients with stable graft function [5]. In our study, peripheral blood precursors of plasma cells (plasmablasts) were found to be downregulated during the first week after transplantation. Furthermore, their differentiation has recently been found to be suppressed *in vitro* by calcineurin inhibitors, including tacrolimus [35] used as a standard immunosuppression in our patients as well.

Previous studies have shown a significant decrease of $IgM^{high}CD38^{high}CD24^{high}$ transitional B cells after transplantation [18,36]. Chung *et al.* [18]. describe the significant decrease of transitional B cells at 1 month. Similarly, Heidt *et al.* [36]. observe decline regardless of the subsequent occurrence of rejection. In our prospective monitoring study, the decline of transitional cells persisted for 3 months, after which partial repopulation of these cells at 1 year was observed. The population of transitional B cells partially overlaps B regulatory cells with potential protective effects [10]. Interestingly, sensitized recipients (PRA > 50%) exhibited lower levels of these cells on POD 7. It is likely that the presence of anti-HLA antibodies is also associated with reduced numbers of protective transitional B cells. Other observations on the association of transitional B cells with operational tolerance seem to be in keeping with this hypothesis [5,26,37,38]. In our study, we found that patients who suffered from rejection, whether it be acute or chronic, had lower absolute numbers of $IgM^{high}CD38^{high}CD24^{high}$ transitional B cells at month 3. Interestingly, the induction strategy, either with basiliximab or rATG, had no effects on transitional B cells numbers at month 3 (Fig. S1). Both agents are known to affect the either function or numbers of peripheral T cells rather than B cells. Rituximab, anti-CD-20 monoclonal antibody, given in two patients as part of antirejection therapy, decreased the peripheral B cells number to zero at 3 months and therefore rituximab-treated patients were not involved in the ROC analyses from 3 months. Whether rituximab therapy protects from chronic rejection remains to be solved, however, the relative increase of transitional and memory-like B cells at one year after rituximab induction therapy [30] favors this hypothesis.

Our findings further support recent observations that increased frequencies of transitional B cells are associated with protection from rejection [39]. In conclusion, nonsensitized recipients and rejection-free patients exhibited distinct changes in B-cell compartment favouring transitional B cells as early as within the first 3 months after kidney transplantation.

Authorship

AS, IS and OV: contributed to the design of the study. VS, PH, IS and OV: participated in the writing of the manuscript. VS, AS, MR, EC, JS and EH: participated in the performance of the research. VS, PH, IT and OV: participated in data analysis.

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Conflicts of interest

O.V. received speaker's fees from Sanofi and Novartis; J.S., E.H., and I.S. received speaker's fees from Novartis.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. The effect of Thymoglobulin and Basiliximab on absolute numbers of B-cell subpopulations.

Figure S2. ROC analysis for the risk of T cell-mediated rejection (a) or antibody-mediated rejection (b) based on absolute numbers of $IgM^{high}CD38^{high}CD24^{high}$ transitional B cells before Tx, on day 7 and at 3 and 12 months after Tx.

Table S1. Rejection episodes.

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