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# Patients with drug-free long-term graft function display increased numbers of peripheral B cells with a memory and inhibitory phenotype

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**Several transplant patients maintain stable kidney graft function in the absence of immunosuppression. Here we compared the characteristics of their peripheral B cells to that of others who had stable graft function but were under pharmacologic immunosuppression, to patients with chronic rejection and to healthy volunteers. In drug-free long-term graft function (DF) there was a significant increase in both absolute cell number and frequency of total B cells; particularly activated, memory and early memory B cells. These increased B-cell numbers were associated with a significantly enriched transcriptional B-cell profile. Costimulatory/migratory molecules (B7-2/CD80, CD40, and CD62L) were upregulated in B cells; particularly in memory $CD19^+IgD^-CD38^{+/-}CD27^+$ B cells in these patients. Their purified B cells, however, responded normally to a polyclonal stimulation and did not have cytokine polarization. This phenotype was associated with the following specific characteristics which include an inhibitory signal (decreased FcγRIIA/FcγRIIB ratio); a preventive signal of hyperactive B-cell response (an increase in BANK1, which negatively modulates CD40-mediated AKT activation); an increased number of B cells expressing CD1d and CD5; an increased BAFF-R/BAFF ratio that could explain why these patients have more peripheral B cells; and a specific autoantibody profile. Thus, our findings show that patients with DF have a particular blood B-cell phenotype that may contribute to the maintenance of long-term graft function.**

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Organ transplantation is the treatment of choice for life-sustaining terminal organ deficiencies. Nevertheless, prevention of rejection requires nonspecific immunosuppression, which increases the risk of complications such as infections,[1] malignancies, and side effects.[2] Moreover, immunosuppression poorly influences chronic rejection, the main cause of graft loss in the long term.[3] Thus, a major goal in transplantation is to induce tolerance.

Tolerance to allografts was first described by Billingham *et al.*[4] more than 50 years ago. The definition of this 'true' tolerance has been proposed as a well-functioning graft lacking histological lesions of rejection, in the absence of immunosuppression in an immunocompetent host accepting a second graft of the same donor, while able to reject a third-party graft. In humans, we reported on a state of long-term acceptance of mismatched kidney allografts after immunosuppressive drug withdrawal after kidney transplantation.[5] Because several key elements of transplant tolerance in rodents cannot be demonstrated in humans, this state has been referred to as drug-free long-term graft function (DF). For example, graft biopsies are often unavailable, particularly given that this state of clinical tolerance has been observed by chance and not in the context of planned tolerance-inducing protocols and that these patients are usually incompliant and refuse invasive investigative procedures.[5] The mechanisms of

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this state of DF remain unknown. In particular, no significant alteration in their T-cell phenotype or function has been reported.[6,7] In a previous paper, we showed that these patients were characterized by a higher number of peripheral B cells compared with both patients with stable graft function under immunosuppression and patients with histological signs of antibody-mediated chronic rejection.[7] In this paper, we analyzed the peripheral B cells of these patients in terms of transcription and phenotype, as well as their capacity to respond to short *in vitro* activation.

As these patients may not display an 'optimal' response to alloantigens and rarely develop an alloimmune response,[5] we hypothesized that regulation and/or control of this response may take place and that B cells could be involved in this process of tolerance. Although B cells are primarily known for their ability to differentiate into antibody-producing cells, they display additional functions by producing cytokines and functioning as antigen-presenting cells.[8] Recent evidence indicates that regulatory B cells can enhance tolerance,[9–12] may control organ-specific inflammation driven by Th1, Th2, or Th17 cell responses, or may directly interact with other immune cells.[10,13–15]

## RESULTS

### DF patients display high numbers of B cells and particularly Bm2, EBm5/Bm5 memory B cells

We used the Bm1–Bm5 classification system to identify B-cell developmental stages in the blood of the patients. Bm3/Bm4 were not included in the analysis because they were absent in the blood. We first confirmed that DF patients displayed a significantly higher number (frequency, absolute value) of peripheral B cells (Figure 1a and b). This increase in circulating B cells was mainly due to a significant increase in $IgD^+CD38^+$ (corresponding to activated Bm2 cells) (Figure 1c) and $IgD^-CD38^{+/-}$ in absolute values (corresponding to EBm5/Bm5 memory B cells that also express the CD27 marker) (Figure 1d and e). No difference was observed for the frequencies of other B-cell sub-populations. Despite a small trend toward an increased number in DF patients, only very low numbers of Bm2′ cells and transitional B cells were detected (limit of flow-cytometry sensitivity). Similarly, no statistically significant difference was found with respect to healthy volunteers (HV). Total B cells of DF patients displayed a significantly higher level of CD80 ($P<0.05$), CD86 ($P<0.01$), CD40 ($P<0.05$), and CD62L ($P<0.05$) compared with other transplant patients (Figure 2a–d). A significant difference toward HV was only found for CD80. We then analyzed the mean fluorescence intensity (MFI) of these markers within the gated $CD19^+IgD^-CD38^{+/-}$ population. CD40 MFI was significantly increased in $IgD^-CD38^{+/-}$ memory populations from DF patients compared with the patients with stable graft function and HV (Figure 2e). Altogether, these data show that DF patients display a high number of circulating B cells expressing an activated-memory phenotype with expression of costimulatory molecules.

### DF patients display a B cell-enriched gene profile

We analyzed data from our previous pangenomic microarrays[16] according to the B-cell signatures described in the literature.

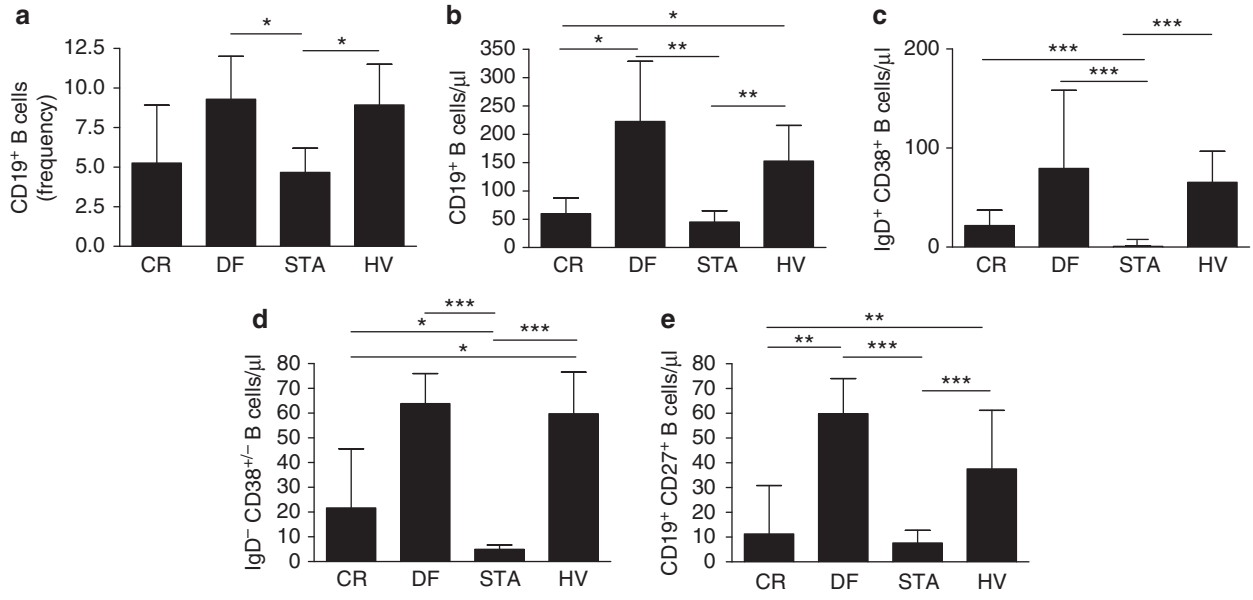


**Figure 1 | Drug-free long-term graft function (DF) patients display a higher number of peripheral B cells.** DF patients display a significantly higher frequency (**a**) and absolute value (**b**) of total peripheral B cells. The increase of B cells in DF patients is mainly due to a significant increase in $IgD^+CD38^+$ (activated Bm2 cells) (**c**) and $IgD^-CD38^{+/-}$ (EBm5/Bm5 memory B cells) (**c**). This was confirmed by an increase of the number of memory $CD19^+CD27^+$ B cells in DF patients (**e**). Differences were defined as statistically significant when $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***). CR, patients under standard immunosuppression with deteriorating kidney graft function; HV, healthy volunteers; STA, kidney recipients with stable graft function under standard immunosuppression.

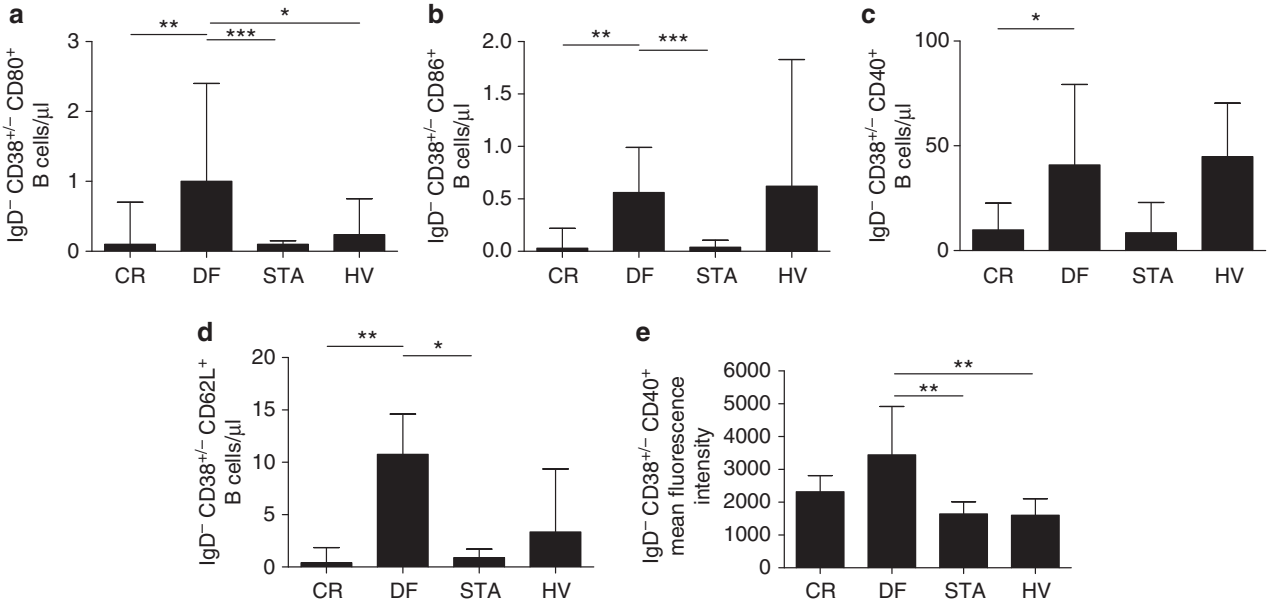


**Figure 2 | DF patients display a higher number of memory B cells that express markers of costimulation.** Drug-free long-term graft function (DF) patients display a significantly higher level of IgD$^-$CD38$^{+/-}$CD80$^+$ memory B cells (**a**), IgD$^-$CD38$^{+/-}$CD86$^+$ memory B cells (**b**), IgD$^-$CD38$^{+/-}$CD40$^+$ memory B cells (**c**), and IgD$^-$CD38$^{+/-}$CD62L$^+$ memory B cells (**d**). Moreover, IgD$^-$CD38$^{+/-}$ memory B cells from DF patients exhibit a significant higher level of CD40 markers at their surface (mean fluorescence intensity) (**e**). Differences were defined as statistically significant when $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***).

Gene set enrichment analysis was used to identify enrichment of genes related to B-cell pathways.[17] Among the genes significantly enriched (false discovery rate $<25\%$, $P<5\%$) in DF patients compared with patients with stable graft function, seven major sets of genes were related to B-cell pathways (Table 1 and Supplementary Figures SA–G).[18–21] Genes that contributed to the enrichment result were selected and their biological function analyzed using Gominer software.[22] Altogether, DF patients are characterized by a significant regulation of several B-cell-associated subsets with upregulation of genes related to cell cycle (CCNA2, CCND2, BIRC5, CDC2, CDKN3, CKS2, PCNA), proliferation (CCNA2, CDC20, BUB1), development, and maturation. These findings largely reflect the 'abnormal' larger pool of B cells (due to proliferation and increased cell cycling) in DF patients.

### B cells from DF patients display an inhibitory profile

We analyzed transcript level and/or surface expression of molecules reported to be involved in inhibitory/regulatory B-cell profiles. CD32 is a complex cluster of the low-affinity FcγReceptor (FcγR). Although FcγRIIA (CD32a) transduces an activator signal, FcγRIIB (CD32b) contains an immunoreceptor tyrosine-based inhibition motif, transducing an inhibitory signal upon co-ligation with the B-cell receptor. Because the CD32a/CD32b ratio is a relevant index of B-cell activation/inhibition, we analyzed this in the blood of our patients. We found that DF patients had a significantly decreased CD32a/CD32b ratio compared with patients with chronic rejection ($P<0.05$) in total blood, and a trend for a decreased CD32a/CD32b ratio was also observed at the level of CD19+ B cells (reported to the CD19 gene) (Figure 3a and b). Moreover, the significant increased CD32b mRNA in total peripheral blood mononuclear cells fits with a trend to higher expression (at the protein level by flow cytometry both in absolute value and in MFI) of CD32b in DF patients (Figure 3c–e).

Because the B-cell scaffold protein with ankyrin repeats 1 (BANK1) was highlighted as a leader gene in our previous study,[23] we looked at its expression in blood from our patients. BANK1 is a negative modulator of CD40-mediated AKT activation preventing hyperactive B-cell responses.[24] We found a significant accumulation of BANK1 transcripts in the blood of DF patients compared with stable patients ($P<0.01$) and other controls ($P<0.01$, $P<0.05$) (Figure 4a). The same tendency was observed when the BANK1 molecule was analyzed in purified CD19$^+$ B cells, suggesting that this increased expression was not only due to a higher number of B cells but a higher expression of BANK1 in B cells from DF patients (Figure 4b). Finally, we looked at the surface expression of CD1d and CD5 among CD19$^+$ cells, two molecules potentially involved in a regulatory phenotype.[9,11] DF patients had a significantly higher number of B cells expressing CD5 and CD1d compared with stable patients ($P<0.05$), both in frequency and absolute values (Figure 4c and d).

Altogether, these data suggest that the B cells of DF patients display an inhibitory profile.

### DF patients are characterized by a transcriptional profile favouring B-cell survival

Because of the increased B-cell number in DF patients, we looked at key molecules involved in B-cell survival:

**Table 1 | Description of gene sets (GS) related to B-cell pathways identified by gene set enrichment analysis in the blood of patients with drug-free long-term graft function compared with patients with stable function under immunosuppression**

| Gene set name | Number of genes | NES | NOM *P*-val | FDR *q*-val | Gene set description | References |
|---|---|---|---|---|---|---|
| BASSO_REGULATORY_HUBS | 61 | 1.80 | 0.006 | 0.21 | Genes highly interconnected in the reconstructed regulatory networks from expression profiles in human B cells. | Basso *et al.*[18] |
| HOFFMANN_BIVSBII_BI_TABLE2 | 100 | 1.73 | 0.005 | 0.19 | Genes with at least fivefold change in expression between pre-BI and large pre-BII cells. | Hoffmann *et al.*[19] |
| TARTE_PLASMA_BLASTIC | 160 | 1.71 | 0.014 | 0.20 | Genes overexpressed in mature plasma cells isolated from tonsils and mature plasma cells isolated from bone marrow as compared with polyclonal plasmablastic cells. | Tarte *et al.*[21] |
| ZHAN_MM_CD138_PR_VS_REST | 22 | 1.70 | 0.014 | 0.21 | 50 top ranked overexpressed genes in each of the seven subgroups of multiple myeloma in CD138-enriched plasma cells from 414 newly diagnosed patients. | Zhan F *et al.*, Blood 2006 |
| GREENBAUM_E2A_UP | 15 | 1.54 | 0.014 | 0.24 | Genes upregulated at least threefold in the E2A-deficient cell lines. | Greenbaum *et al.*[20] |
| HOFFMANN_BIVSBII_LGBII | 60 | 1.53 | 0.013 | 0.24 | Genes with at least fivefold change in expression between large and small pre-BII cells. | Hoffmann *et al.*[19] |
| TARTE_PC | 38 | 1.59 | 0.015 | 0.25 | Genes overexpressed in polyclonal plasmablastic cells, mature plasma cells isolated from tonsils and mature plasma cells isolated from bone marrow as compared with B cells purified from peripheral blood and tonsils. | Tarte *et al.*[21] |

The size is the number of genes in the gene set after filtering out those genes not in the expression data set. The normalized enrichment score (NES) is the degree to which a gene set is overrepresented, normalized across analyzed gene sets. The nominal *P*-value (NOM *P*-val) is the statistical significance of the enrichment score. The false discovery rate (FDR) is the estimated probability that the normalized enrichment score represents a false-positive finding.

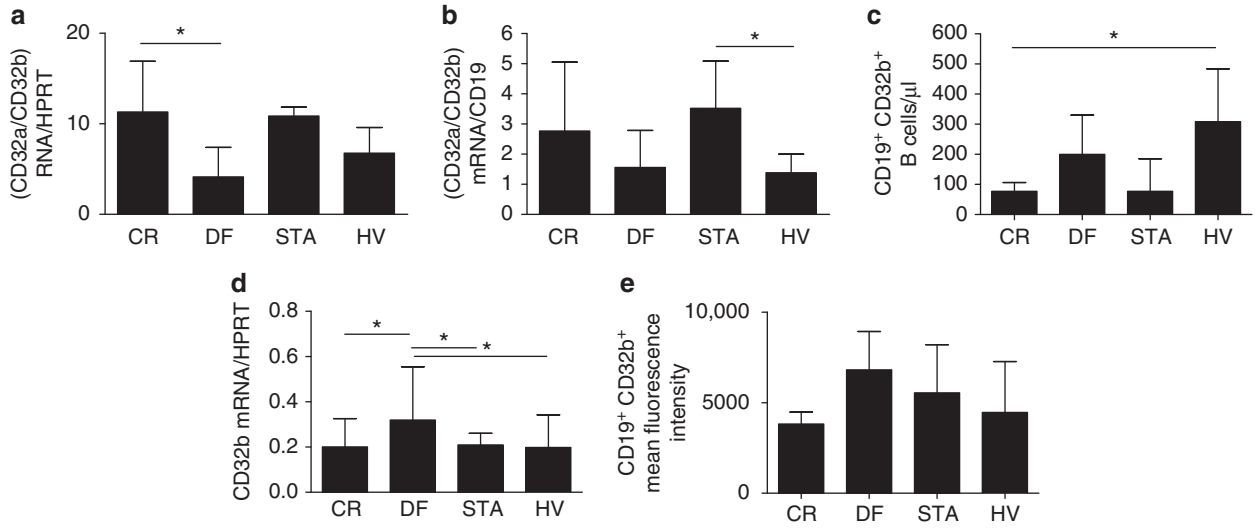


**Figure 3 | DF patients display B cells with an inhibitory profile.** (**a**) Peripheral blood mononuclear cells (PBMCs) from drug-free long-term graft function (DF) patients display a significantly decreased CD32a/CD32b FcγRIIA/FcγRIIB transcript ratio both at the level of total PBMC (**a**) and at the level of $CD19^+$ B cells (**b**) compared with patients with chronic rejection and healthy volunteers, respectively. Among total PBMC, DF patients express higher number of $CD19^+$ $Fc\gamma RIIB^+$ (CD32b) B cells as shown by flow cytometry (**c**) and a significant increase in FcγRIIB (CD32b) mRNA expression (**d**). Finally, B cells from DF patients tend to express more FcγRIIB (CD32b) at their surface (mean fluorescence intensity) (**e**). Differences were defined as statistically significant when $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***).

transmembrane activator and CAML interactor (TACI), B-cell-activating factor receptor (BAFF-R), and B-cell maturation protein (BCMA). Although BCMA and BAFF-R are involved in B-cell survival, TACI is a negative regulator of B-cell survival.[25,26] BAFF–BAFF-R interactions were shown to be crucial for maintaining normal peripheral B-cell numbers[27] and instrumental for survival of memory B cells.[28,29] We found that DF patients displayed an increased BAFF-R/BAFF ratio compared with patients with stable graft function ($P<0.01$), healthy individuals ($P<0.05$), and patients with chronic rejection ($P<0.05$) that was correlated to a significant upregulation of the TACI/BAFF signaling pathway ($P<0.05$) (Figure 4e and f).

Together, these data suggest that DF patients are characterized by a transcriptional profile favouring B-cell survival that may contribute to their high B-cell number and transcriptional profile.

### B cells from DF patients respond normally to *in vitro* polyclonal activation

B cells from three DF patients, five patients with stable graft function under immunosuppression, five HV and five

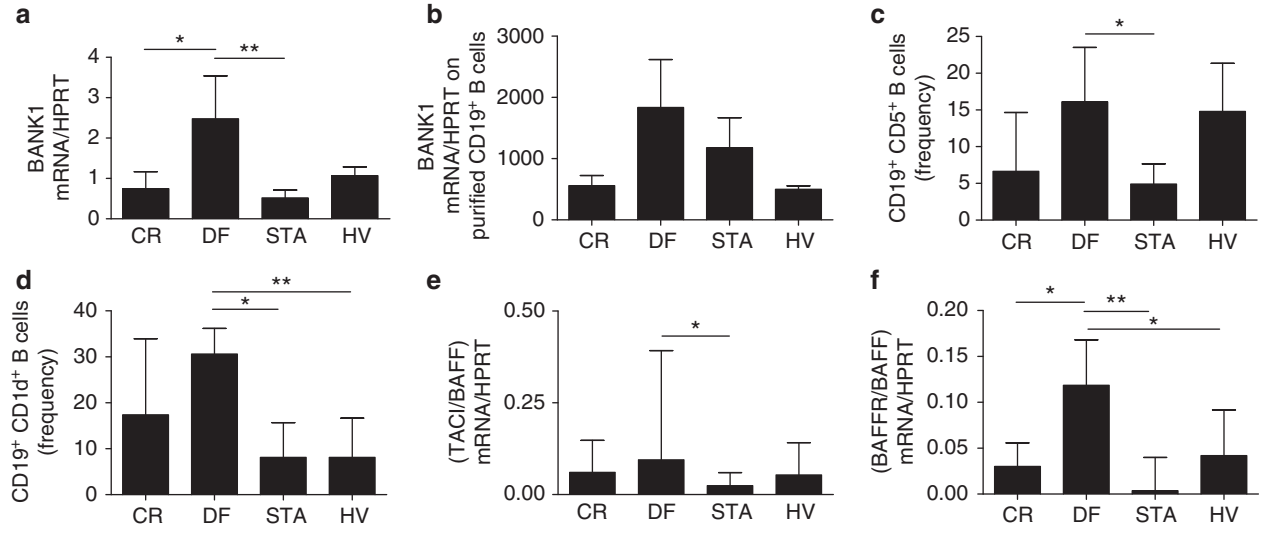


**Figure 4 | DF patients display B cells with an inhibitory profile and profile of B cell survival.** (**a**) Blood from drug-free long-term graft function (DF) patients is characterized by a significant accumulation of BANK1 transcripts, a negative modulator of CD40-mediated AKT activation, thereby preventing hyperactive B-cell responses. (**b**) The same tendency is observed in purified $CD19^+$ B cells (**b**). DF patients display a significantly higher number of B cells expressing the CD5 (**c**) and CD1d markers (**d**). DF patients are characterized by an upregulation of the BAFF-R/BAFF ratio (**e**) and an upregulation of the TACI/BAFF signaling pathways (**f**). Differences were defined as statistically significant when $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***).

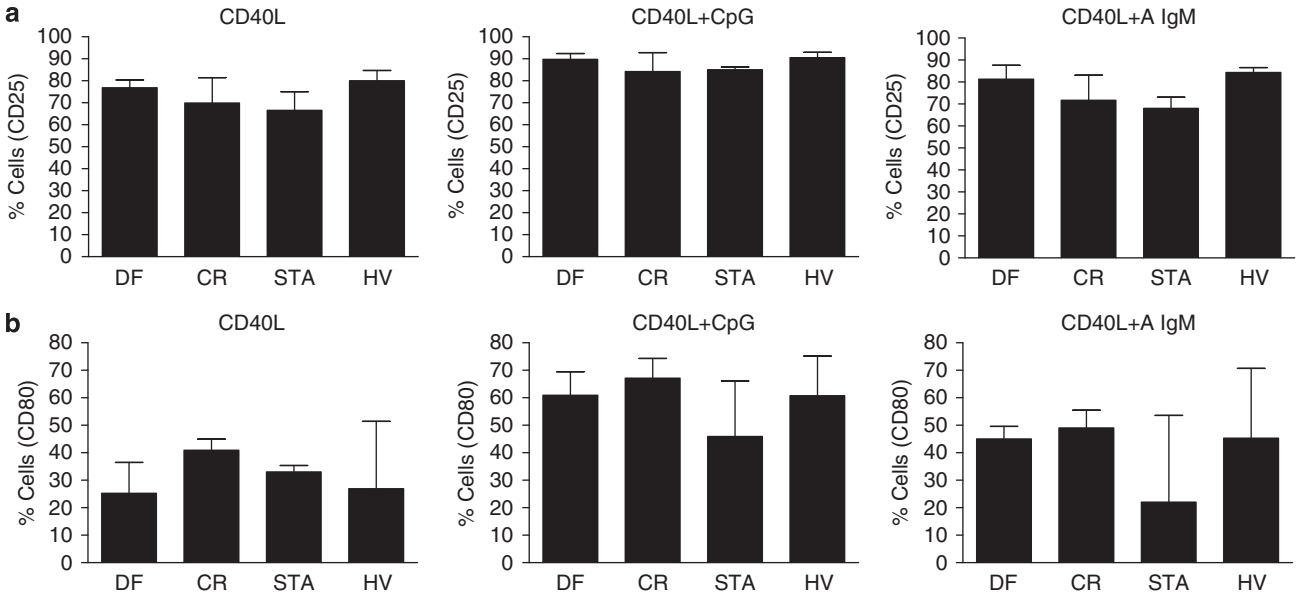


**Figure 5 | B cell activation.** Cultured blood B cells were incubated with anti-CD25 (**a**) or anti-CD80 (**b**) to assess B-cell activation after 40 h culture with CD40L alone, CD40L and CpG or CD40L and antihuman immunoglobulin M (IgM).

patients with chronic rejection were isolated and activated using different stimuli (CD40 ligand, CD40 ligand + anti-IgM, CD40 ligand + CpG) over 4 time points (0, 16, 24, 40 h, following stimulation). CD25 and CD80 expression was analyzed (MFI and frequency) to control B-cell activation (Figure 5). We analyzed by Luminex xMAP technology the level of interleukin (IL)10, transforming growth factorβ, IL4, IL6, tumor necrosis factorα, IL2R, interferonγ, and IL6R in the supernatants of B-cell cultures 40 h after activation. Before and after activation, only low levels of IL4, transforming growth factorβ, IL6R, IL2R, and interferonγ were detectable in supernatants from all groups of patients. After 40 h of activation and independently of the stimuli, no difference was observed for IL10, IL6, and tumor necrosis factorα levels for the different groups of patients (Figure 6). Altogether, no absence of response or cytokine polarization was observed in the conditions of stimulation used in DF patients compared with HV and other recipients.

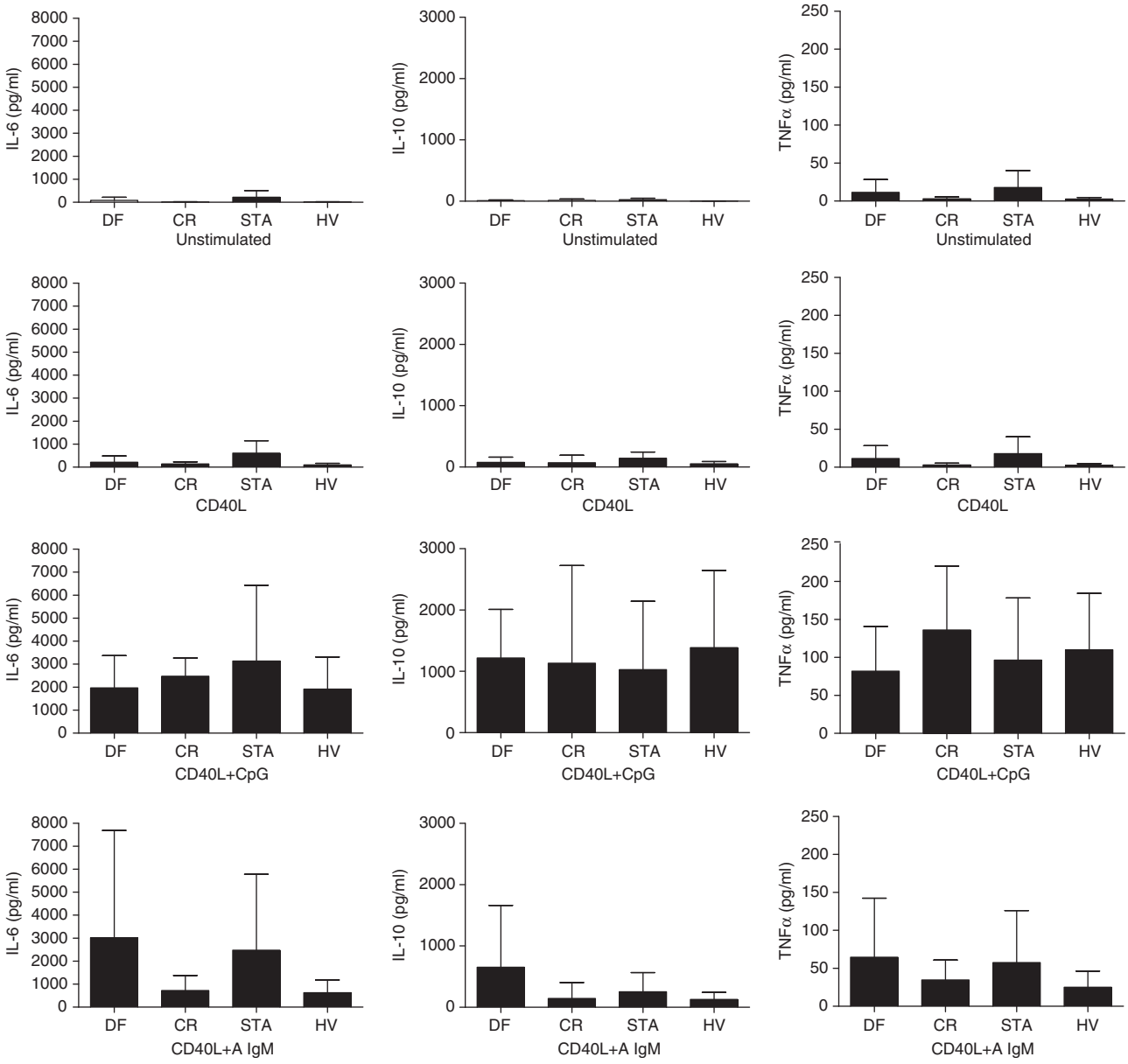


**Figure 6 | B cells from three drug-free long-term graft function (DF) patients, five patients with stable graft function, five healthy volunteers and five patients with chronic rejection were purified from peripheral blood mononuclear cells by negative selection.** B cells were either unstimulated or stimulated with CD40L alone, CD40L and CpG or CD40L and anti-human immunoglobulin M (IgM). Supernatants were collected at different time points (0, 16, 24, and 40 h after stimulation). Aliquots of the supernatant were analyzed for different cytokines (data are presented for interleukin (IL)-6, IL-10, and tumor necrosis factor (TNF)α), by Luminex xMAP technology. Results were shown after 40 h of activation.

### Development of allo- and autoantibodies in DF patients

We studied anti-donor antibodies by applying the Luminex Single antigen technique to sera from DF patients (Labscreen Single Antigen; One lambda, Canoga Park, CA, USA). Out of the 12 DF patients (mismatched cadaveric graft), 10 displayed no anti-human leukocyte antigen antibodies and 2 displayed anti-donor antibodies (DQ7 and A33/DQ8). The MFI were 21,800 for DQ7, and 9200 and 193,00 for A33 and DQ8, respectively (Table 2, Supplementary Table SD). It has been suggested that patients submitted to a tolerance induction protocol develop a substantial autoimmune response.[30] We investigated whether DF patients also exhibited an autoimmune component using protein microarrays. Although each individuals exhibit high numbers of potential autoantibodies (mean = 3921, s.e.m. = 320, at a threshold of 500 relative fluorescence units (RFU)), the number of total autoantibodies measured for the four groups of individuals was not statistically different, whatever the threshold used (Supplementary Figure SH). Similarly, when the autoantibody signal for HV was subtracted to analyze only autoantibodies due to transplantation, the number of autoantibodies in each group remained not significantly

**Table 2 | Summary of clinical data: patients with drug-free long-term graft function (DF), kidney recipients with stable graft function under standard immunosuppression (STA), patients under standard immunosuppression with deteriorating kidney graft function (CR) and healthy volunteers (HV)**

| Groups | Age (years) | Gender (female/all) | Time between graft and analysis (months) | Creatinemia (μmol/l) | Proteinuria (g/24 h) | Donor (living vs deceased) | Number of HLA mismatches | Time between immuno-suppression withdrawal and analysis (years) | Donor specific Abs |
|---|---|---|---|---|---|---|---|---|---|
| *DF* | | | | | | | | | |
| Mean | 52 | 3/12 | 177 | 100 | 0.12 | 1/12 | 3 | 8 | 2/12 |
| s.d. | 18 | | 97 | 36 | 0.20 | | 2 | 8 | |
| Min | 27 | 3 | 59 | 35 | 0.00 | 1 | 0 | 3 | 2 |
| Max | 82 | 9 | 368 | 142 | 0.59 | 11 | 4 | 27 | 10 |
| *CR* | | | | | | | | | |
| Mean | 54 | 17/31 | 102 | 256 | 3 | 1/31 | 3 | | |
| s.d. | 14 | | 69 | 99 | 3 | | 2 | | |
| Min | 27 | 14 | 20 | 84 | 0 | 1 | 0 | | |
| Max | 78 | 17 | 304 | 492 | 12 | 30 | 6 | | |
| *STA* | | | | | | | | | |
| Mean | 49 | 12/34 | 123 | 121 | 0 | 3/34 | 3 | | |
| s.d. | 14 | | 67 | 34 | 0 | | 1 | | |
| Min | 24 | 12 | 29 | 68 | 0 | 3 | 0 | | |
| Max | 76 | 22 | 269 | 215 | 1 | 30 | 6 | | |
| *HV* | | | | | | | | | |
| Mean | 44 | 13/29 | | | | | | | |
| s.d. | 11 | | | | | | | | |
| Min | 25 | 13 | | | | | | | |
| Max | 66 | 16 | | | | | | | |

Abbreviations: Abs, antibodies; HLA, human leukocyte antigen.

different (Supplementary Figure SI). Because the spectrum of specificity of these autoantibodies could vary in each group and individual, M-statistics were performed using the Protoarrays manufacturer's software to identify differentially expressed antoantibodies in DF patients. Among the 98 targets with a *P*-value inferior to 0.01, 51 targets (48 unique proteins) exhibited a higher prevalence in the DF group and 47 targets (44 unique proteins) in other groups (Supplementary Tables SA and SB). For both lists, no unique pathway or biological process was highlighted, whereas ontologies such as phosphorylation (GO: 0016310) and signal transduction (GO:0007165) were enriched for targets with lower prevalence in the DF group (false discovery rate $<0.01$) using Gominer software. Furthermore, among the 92 proteins, 36 are known to be expressed by the kidney.[31] Focusing on particular proteins, autoantibodies against proteins known to be expressed in B cells were identified: CHEK1, PIM2, LCK, ZAP70, IKBKB, PDGFRA, PSMA4, and LPXN (Supplementary Figure SJ).

## DISCUSSION

We previously reported that DF patients are characterized by a high number of peripheral B cells.[7] This observation was confirmed by two international networks (M. Hernandez-Fuentes and KA Newell, Am Transplant Congress, Boston, 2009) in kidney recipients, whereas no increase was observed in drug-free patients after a liver transplantation.[32] This increased number of B cells was supported by an enriched B-cell transcriptional profile not only compared with patients with chronic rejection, as confirmed by others (M. Hernandez-Fuentes and KA Newell, Am Transplant Congress, Boston, 2009) but also compared with stable patients under immunosuppression.

These observations led us to perform a detailed analysis of the B-cell compartment in DF patients, compared with stable patients under immunosuppression. Ever since the first cases of operational tolerance in the clinic were described, the problem of adequate comparators has remained unsolved. This paradox is due to the clinical situation of these patients that display stable graft function but no longer receive immunosuppression, a state that, until now, was only achievable in rodents. HV share with DF patients the absence of immunosuppression but have not received a transplant. Patients with chronic rejection have a transplant but are under immunosuppression and likely display a contrasted inflammatory response. Stable patients who share graft function stability with DF patients are probably the best controls but are under immunosuppression. Finally, it may be useful to compare the profile of DF patients with chronic rejection because no biopsy was available for DF patients in whom a 'minimal' form of subclinical chronic rejection cannot be excluded.

We report that DF patients are characterized by a significant increase in absolute value (not in frequency) of memory B cells that express costimulatory/migratory molecules, compatible with an inhibitory/regulatory B-cell profile.

These changes were significant not only compared with stable patients under immunosuppression but also patients with histologically proven signs of chronic rejection, a cohort of patients where enhanced B-cell activity was expected. These observations led us to hypothesize that B cells could contribute to the maintenance of long-term graft function.

The imbalanced and decreased CD32a/CD32b ratio in DF patients suggests that some mechanisms of regulation by the low-affinity type IIb immunoglobulin G (IgG) Fc-binding (CD32b)[33–37] overcome activation mediated by CD32a.

Interestingly, DF patients also exhibited an upregulation of the peripheral BAFF-R/BAFF ratio that is crucial for the maturation of B cells and for maintaining peripheral B-cell numbers.[28,29,38,39] In support of these data, we report that genes related to B-cell cycle were also overexpressed in DF patients. Finally, TACI/BAFF ratio was also increased in these patients suggesting that B cells remain under the control of TACI, a negative regulator of mature B cells[40] and that these observations do not reflect a general deregulation of B-cell factors. We previously reported that BANK1 was one of the key leader genes upregulated in DF patients.[23] BANK1 is an adaptor protein highly expressed in peripheral B cells[41] that is a modulator of hyperactive B-cell responses by inhibiting AKT activation upon CD40 signaling.[24] DF patients, both at the peripheral blood mononuclear cells and B-cell level, displayed a higher level of BANK1 transcripts than HV suggesting that, again, this profile is not linked to the absence of treatment. DF patients also displayed B cells expressing the CD1d and CD5 molecules. Recently, a $CD1d^{h}$ $CD5^{+}$ B-cell subset producing IL10 has been identified as a unique subset of potent regulatory B cells in mouse[42] and in human.[43] CD1d is also important for the generation of an optimal Th2 T-cell response[44] that promotes a favorable context for tolerance.[45] Nevertheless, B cells from DF patients only produced low levels of IL10 after 40 h activation and no difference was observed between the groups of patients and HV in terms of IL4, IL6R, IL2R, tumor necrosis factorα, transforming growth factorβ, and interferonγ production. These data show that B cells from DF patients are not hyporesponsive to polyclonal activation and display a similar pattern of activation as the other patients after short *in vitro* activation.

Finally, we looked at the development of allo- and auto-antibodies in DF patients. To date, 11 of these 12 patients still display stable graft function and are immune-competent decades after transplantation.[46] Two displayed antibodies reacting with donor major histocompatibility complex (DQ7 and A33/DQ8). One patient still has a stable function 6 years after antibody appearance. The second rejected his graft 4 years after interruption of immunosuppression (12 years after transplantation) and 2 years after appearance of antibodies. A biopsy for the latter patient revealed lesions of transplant glomerulopathy without C4d staining. Finally, we investigated whether DF patients also exhibited an autoimmune component as previously described in induced 'tolerant' patients.[30] Consistent with previous knowledge, we found that all individuals, even HV, exhibit antibodies directed against a wide spectrum of antigens, such as internal, cell surface, circulating proteins, carbohydrates, nucleic acids, lipids, and haptens.[47] We also found that DF patients were not characterized by a different level of circulating autoantibodies but could have different specific autoantibodies. Indeed, while Li *et al.*[31] demonstrated an enrichment of kidney-specific autoantibodies produced after renal transplantation, there was the same proportion of produced autoantibodies between the different groups of recipients. Interestingly, we identified 98 autoantibody targets with significantly higher or lower prevalence in DF patients compared with the others. However, no clear pathway was identified arguing that autoantibody production could not belong to a mainstream biological process. Focusing on particular proteins, autoantibodies against proteins expressed in B cells were identified such as CHEK1, PIM2, LCK, ZAP70, IKBKB, PDGFRA, and LPXN. Thus, CHEK1 autoantibody exhibited a higher prevalence in the DF group and its gene was found to be overexpressed in our previous study.[16] Furthermore, Porcheray *et al.*[30] found that PSMA4 autoantibody was highly expressed in one tolerant patient and we also found that PSMA4 autoantibody was highly expressed in one DF patient and expressed less in all other patients. Finally, we found that Leupaxin, a member of the paxillin superfamily, was one of the autoantibodies largely increased in DF patients. This molecule, which is preferentially expressed in hematopoietic cells including B cells, has been shown to suppress the secretion of IL2, likely by the inhibition of JNK, p38 MAPK, and AKT signaling. This molecule thus has an inhibitory role in BCR signaling and B-cell function.[48]

Overall, our study points to several characteristics in B cells from DF patients. Further studies are required to explore if B cells are indeed involved in long-term graft acceptance decades after withdrawal of immunosuppression.

## MATERIALS AND METHODS

### Patients

The study was approved by the University Hospital Ethical Committee and the Committee for the Protection of Patients from Biological Risks. All 106 age-matched kidney transplant patients included for study gave informed consent (Table 2 and Supplementary Tables SC and SD).

*(i) Patients with DF (n = 12)*: patients with a stable kidney graft function (creatinemia $<150$ μmol/l and proteinuria $<1$g/24 h) in the absence of immunosuppression for at least 1 year (range: 3–21 years).[16] Treatment was stopped due to non-compliance ($n=9$), post-transplant lymphoproliferative disorder ($n=2$), or calcineurin inhibitor toxicity ($n=1$). (ii) *Kidney recipients with stable graft function under standard immunosuppression* (STA; $n=34$): had a creatinemia $<150$ μmol/l and proteinuria $<1$ g/24 h for at least 3 years. (iii) *Patients under standard immunosuppression with deteriorating kidney graft function* (CR; $n=31$): with a creatinemia $>150$ μmol/l and/or proteinuria $>1$ g/24 h, classified according to the Banff classification criteria.[49,50] Five out of the 31 patients had a transplant glomerulopathy, 18/31 were suspicious for humoral rejection (C4d or circulating anti-human leukocyte antigen

antibodies), 8/31 had an active humoral component (C4d and circulating anti-donor antibodies). (iv) HV ($n=29$): age-matched healthy volunteers (mean age: $44 \pm 11$ years; range 25–66; sex 16M/13F) had a normal blood formula and no infectious or other concomitant pathology for at least 6 months before the study.

### Biological samples

Venous blood samples were collected in EDTA vacutainers. Peripheral blood mononuclear cells were separated on a Ficoll layer (Eurobio, Les Ulis, France) and frozen in TRIzol reagent (Invitrogen, Cergy Pontoise, France).

### RNA cDNA and real-time quantitative PCR

RNA was extracted using the TRIzol method (Invitrogen), according to manufacturer's instructions. Genomic DNA was removed by DNase treatment (Roche, Indianapolis, IN, USA). RNA quality and quantity was determined using an Agilent 2100 BioAnalyzer (Palo Alto, CA, USA). RNA was reverse transcribed using polydT oligonucleotide and Maloney leukemia virus reverse transcription (Invitrogen). Real-time quantitative PCRwas performed using commercially available primer and probe sets HPRT: Hs99999909_m1, CD32a: Hs00234969_m1, CD32b: Hs00269610_m1, BANK1: Hs00215678_m1, BAFF-R: Hs00606874_g1, BAFF: Hs00198106_m1, APRIL: Hs00742713_s1, TACI: Hs00963364_m1, BCMA: Hs03045080_m1, CD19: Hs00174333_m1 from Applied Biosystems (Foster City, CA, USA). HPRT and CD19 were used as endogenous controls to normalize RNA amounts. Relative expression between a sample and a reference was calculated according to the $2^{-\Delta\Delta Ct}$ method.[51]

### Flow cytometry

The mature Bm cell sub-populations were studied according to their expression of CD38, IgD, and CD27.[52,53] Flow cytometry was performed on a BD LSRII analyzer with FlowJo software (TriStar Inc., Ashland, OR, USA) using monoclonal antibodies: CD19 (IgG1/J4.119), CD38 (IgG1/HIT2), IgD (IgG2a/IA6), CD80 (IgG1/L307.4), CD86 (IgG1/IT2.2), CD27 (IgG1/M-T271), CD5 (IgG1/UCHT2), CD40 (IgG1/5C3), CD62 L (IgG1/Dreg56), IgM (IgG1/G20-127), CD21 (IgG1/IV B98), CD138 (IgG1/BP100), CD1d (IgG1/M-T101) (BD PharMingen, San Diego, CA, USA), CD32 (IgG2a/2E1) and CD20 (IgG1/B9E9) (Immunotech, Luminy, France).

### Microarray data analysis

Raw gene-expression files[16] are available at the Stanford Microarray Database http://smd.stanford.edu/cgi-bin//publication/viewPublication.pl?pub_no=654. Gene set enrichment analysis (http://www.broad.mit.edu/gsea/) was run on 4942 genes by using version 2.5 of the C2 gene set database and the 1252 gene sets comprising more than 10 and less than 500 genes were used. Default parameters and 10,000 random sample label permutations were used. Gene sets with a false discovery rate inferior to 25% and a nominal *P*-value inferior to 5% were selected. Gominer software[22] was performed to investigate biological processes and functions and to group the genes into biologically coherent categories.

### Detection of alloantibodies

Human leukocyte antigen antibodies were detected by a multiplex screening test (LAT-M; One lambda, Canoga Park, CA, USA). Donor-specific antibodies were detected by Luminex Single antigen (Labscreen Single Antigen).

### Autoantibody immunoprofiling

Serum immunoprofiling was performed on ProtoArray human protein microarrays v4.0 by the ProtoArray Service team (Invitrogen). Each microarray contained approximately 8000 recombinant proteins spotted in duplicate. One ProtoArray was used per sample and a negative control assay (buffer with no serum) was run in parallel. Microarray slides were prepared according to the manufacturer's protocol. Arrays were scanned using an Axon GenePix 4000B fluorescent microarray scanner, and data extraction was performed using GenePix Pro 6.0 software (Molecular Devices, Sunnyvale, CA, USA). Microarray quantile normalization, background subtraction, and average calculation of duplicates were performed using Invitrogen's proprietary Protoarray Prospector Software v5.2.1 and then the signal from the negative control array was subtracted from each slide. To compare the level of total autoantibody in each group, we compared the number of targets at multiple thresholds of RFU, according to the fact that the filtering procedure could induce a bias[31] (Supplementary Figure SH). To analyze autoantibodies due to transplantation, the mean signal of healthy volunteers was subtracted for each patient array (delta intensity signal). Potential autoantibodies were selected if the delta intensity signal was superior to given thresholds fixed at 0, 50, (ref. 30) 100, 500, (ref. 54) 1000 or 5000 RFU (Supplementary Figure SI). To identify autoantibodies specific to DF patients, a statistical analysis was performed using Protoarray Prospector Software and M-statistics according to Babel *et al.*[55] To select reliable autoantibodies and following the manufacturer recommendations, additional thresholds were imposed requiring that signals be at least 500 RFU and a minimum signal difference of 200 RFU had to be observed between samples from DF patients and samples from the other groups.

### B-cell activation

B cells were purified by negative selection using magnetic beads (Dynabeads; Invitrogen, Paisley, UK) in accordance with manufacturer's instructions. Purification was typically 97% $CD19^+$. B cells were stimulated with CD40L (Alexis biochemical; Enzo Life Sciences Inc., Farmingdale, NY, USA), CD40L, and CpG ODN 2006 (Hycult biotechnology; Uden, The Netherlands) or CD40L and anti-human IgM (Jackson ImmunoResearch Lab, West Grove, PA, USA). B cells were incubated with conjugated antibodies (all from BD Pharmingen, San Diego, CA, USA): anti-CD19, anti-CD80, and anti-CD25. Supernatants were collected at 0, 16, 24, and 40 h following stimulation and analyzed for cytokines (IL-4, IL-6, IL-10, tumor necrosis factor$\alpha$, interferon$\gamma$, IL-2R$\alpha$, IL-6R, and transforming growth factor$\beta$1) by Luminex xMAP technology (MILLIPLEX MAP; Millipore, Billerica, MA, USA), in accordance with manufacturer's protocol.

### Statistical analysis

The non-parametric Kruskal–Wallis test with Dunn's post-test was used for comparison of more than two groups using GraphPad Prism software v.4. Differences were defined as statistically significant when $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***). Correlations were analyzed by linear regression and mean values were compared using the Mann–Whitney test or Wilcoxon's test for paired series. Categorical variables were compared by the $\chi^2$-test with the Yates correction when at least one of the calculated figures was $<5$.

### DISCLOSURE

All the authors declared no competing interests.

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**SUPPLEMENTARY MATERIAL**

**Table SA.** 51 targets of autoantibodies with higher prevalence in the DF group compared to the other groups identified with M-statistics with *P*-values inferior to 0.01.

**Table SB.** 47 targets of autoantibodies with lower prevalence in the DF group compared with the other groups identified with M-statistics with *P*-values inferior to 0.01.

**Table SC.** Description of the patient groups included for study: 31 patients under standard immunosuppression with deteriorating kidney graft function (CR) and 34 kidney recipients with stable graft function under standard immunosuppression (STA).

**Table SD.** Description of the patient groups included for study: 12 patients with drug-free long-term graft function (DF) and 29 healthy volunteers (HV).

**Figure SA–SG.** Heat maps of the seven sets of genes related to B cell pathways of 61,100, 160, 22, 15, 60, and 38 genes identified with gene set enrichment analysis in the blood of patients with drug-free long-term graft function compared with patients with stable function under immunosuppression.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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