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Cryptic B Cell Response to Renal Transplantation

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Transplantation reliably evokes allo-specific B cell and T cell responses in mice. Yet, human recipients of kidney transplants with normal function usually exhibit little or no antibody specific for the transplant donor during the early weeks and months after transplantation. Indeed, the absence of antidonor antibodies is taken to reflect effective immunosuppressive therapy and to predict a favorable outcome. Whether the absence of donor-specific antibodies reflects absence of a B cell response to the donor, tolerance to the donor or immunity masked by binding of donor-specific antibodies to the graft is not known. To distinguish between these possibilities, we devised a novel ELISPOT, using cultured donor, recipient and third-party fibroblasts as targets. We enumerated donor-specific antibody-secreting cells in the blood of nine renal allograft recipients with normal kidney function before and after transplantation. Although none of the nine subjects had detectable donor-specific antibodies before or after transplantation, all exhibited increases in the frequency of donor-specific antibody-secreting cells eight weeks after transplantation. The responses were directed against the donor HLA-class I antigens. The increase in frequency of donor-specific antibody-secreting cells after renal transplantation indicates that B cells respond specifically to the transplant donor more often than previously thought.

Key words: accommodation, rejection, renal transplant, tolerance

Abbreviations: AP, alkaline phosphatase; ASC, antibody-secreting cells; DMEM, Dulbecco's modified Eagle's medium; DSA, donor-specific antibodies; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunosorbent spot assay; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HLA, human leukocyte antigen; IRB, institutional review board; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PRA, panel-reactive antibodies.

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Introduction

Allogeneic transplantation reliably evokes humoral immune responses against histocompatibility antigens in animals. In the seminal instance in 1938, Gorer (1) reported that 21 of 22 naïve black mice engrafted with allogeneic sarcoma cells had detectable allo-specific antibodies. A similar if less intense response was observed after skin allografting (2). So sure was Gorer of this antibody response, he concluded its absence must reflect a limitation in the method used (3).

The antibodies produced in response to allogeneic transplantation recognize products of the major histocompatibility locus and formation of those antibodies is said to be ''the invariable consequence of single or repeated antigen stimulation by transplantation of the skin or other normal tissues (4). Consistent with this concept, Auchincloss et al. (5) detected cytotoxic antibodies in each of 14 C57BL/6 mice engrafted with skin from BALB/c mice. Klein et al. (6) detected cytotoxic allo-specific antibodies in 12 of 14 mice transplanted with congenic fetal hearts. Not only do allo-specific antibodies mark allo-immunity, they also have been used to map H-2 (6–9), and as key reagents for recognizing histocompatibility antigens (10,11).

However, while allo-specific antibodies provide a sensitive index of allo-immunity in mice, these antibodies are usually detected in only scant quantities or not at all during the early months after clinical transplantation and are far from universal thereafter. Although kidney transplant recipients with late graft dysfunction often have donor specific antibodies in their blood (12), recipients with no evidence of graft dysfunction during the first year usually do not. Screening sera by microcytotoxicity, Martin et al. (13) detected newly produced anti-donor HLA antibodies in only 23 (9%) of 266 renal transplant recipients. Using this method, Halloran et al. (14) detected anti-donor HLA class I antibodies in the blood of 13 (20%) of 64 renal transplant recipients and all subjects with detectable anti-donor antibodies had episodes of rejection.

Using a sensitive flow cytometry method, Scornik et al. (15) detected anti-donor IgG in 19 (40%) of 48 renal transplant recipients who had rejection but in only 2 (9%) of 22 who did not. Using a similar method, Christiaans et al. (16) detected antibodies against donor lymphocytes in the blood of 17 (12%) of 143 renal transplant recipients. Using a sensitive ELISA, Varnavidou-Nicolaidou et al. (17) detected anti-donor HLA antibodies in 45 (17%) of 264 renal transplant recipients and Cardarelli et al. (18) in only 10 (4%) of 251 renal transplantation recipients. Using an ELISA to test subjects thought to be at high risk of rejection, Zhang et al. (19) detected anti-donor HLA antibodies in only 11 (22%) of 49 renal transplant recipients. Ho et al. (20) detected anti-HLA antibodies in the blood of 221 (23%) of 950 cardiac allograft recipients during the first year after transplantation; however, antibodies specific for the donor were for the most part limited to the 23 subjects who experienced antibody-mediated rejection. Li et al. (21) detected antibodies against donor HLA in only 5 (6%) of 87 recipients of living-related kidney transplants. Using single antigen beads, Smith et al. (22) detected antibodies against donor HLA in the blood of 57 (25%) of 224 cardiac transplant recipients. These and other reports clearly show that human organ transplant recipients more often than not have little or no donor-specific antibody. Detection of anti-donor antibodies even in the absence of T cell or B cell complement-dependent cytotoxicity cross-matches is associated with increased incidence of acute and/or chronic antibody-mediated rejection (23). On the other hand, induction therapies consisting of antibody and B cell depletion treatments clearly decrease incidence of antibody mediated rejection (24) suggesting that preventing anti-donor antibodies from forming may be necessary to reduce transplant rejection. However, the appearance antidonor antibodies could instead be a consequence of kidney dysfunction, as a failing kidney may no longer absorb the antibodies which accumulate in the blood.

Absence of donor-specific antibodies in the blood of renal transplant recipients might be explained in several ways. Absence of donor-specific antibodies might reflect the effective immunosuppression of the clinical transplant recipients preventing T cell-dependent B cell responses, which presumably would otherwise reliably generate these antibodies in humans as they do in mice. Absence of donor-specific antibodies might reflect B cell tolerance, as described in mice by Nemazee and Burki (25), and by Goodnow et al. (26) for proteins in mice and by Fan et al. (27) in human recipients of ABO incompatible heart transplants. Absence of donor-specific antibodies might also give a false impression of absence of a B cell response if the antibodies produced in the response were absorbed to the graft. Consistent with the latter possibility, others (4,28–32) and we (33,34) have shown that large amounts of donor-specific antibody can be absorbed by organ transplants and cleared partially or fully from the blood.

Distinguishing B cell responses characteristic of tolerance, immunosuppression or immunity is relatively simple in some settings. If the stimulating antigen is known, homogeneous and available in large amounts, the antigen can be used as a target for determining the number of antibody-secreting cells by ELISPOT (35,36). In the conventional ELISPOT, the number of antigen-specific antibody-secreting cells remains small and stable if the response is absent, suppressed or circumscribed by anergy. The number of antigen-specific antibody-secreting cells decreases markedly if deletional tolerance occurs (27) and increases markedly if immunity occurs (37). Evaluating humoral immunity to HLA by this approach is not easily accomplished, however, because the immunizing antigens are highly polymorphic and not available in sufficient abundance to allow screening of B cells for the broad range of specificities represented in the HLA system; although testing using one or a limited number of specificities has been described (38,39). And, even if the antigens were to be available, the representation of those antigens as isolated proteins could differ profoundly from the representation of the antigens on a cell surface, leading to over- or underestimation of responses.

To overcome the limitations of detecting and measuring humoral immunity to organ transplants, we devised a way to enumerate antibody-secreting cells in the blood that recognize donor-specific antigens, as expressed on the surface of donor cells. We show here that the number of cells secreting donor-specific antibodies directed against HLA class I antigen(s) increases in kidney transplant recipients who have no evidence of rejection and we consider the implications for the prevalence of allo-immunity and the biology of the graft.

Materials and Methods

Human subjects

Nine adults (eight males and one female; ages 34–60) who were to be first-time recipients of living-donor renal transplants at the University of Michigan were entered into this study. Eight recipients received kidneys from living donors (two from related donors, six from unrelated donors) and one from a deceased donor. Involvement of the subjects was approved by the Institutional Review Board (IRB) of the University of Michigan and informed consent was obtained. The recipients had no history of immunological disease or treatment with immune-modulating agents and had no antibodies against the donor HLA detectable by Luminex single antigen assay (One Lambda, CA) measured at the University of Michigan histocompatibility laboratory. Following renal transplantation, the subjects received maintenance immunosuppression at the time posttransplant samples were collected. Five subjects were treated with cyclosporine, mycofenolate mofetil and prednisone in a tapering dose, and four were treated with tacrolimus, mycophenolate mofetil, alafacept and prednisone in a tapering dose. Thymoglobulin was administered before transplantation to four recipients of unrelated living donor kidneys. No subject experienced rejection during the course of this study. Sera from adults with or without antibodies directed against a broad range of HLA, as determined by the level of ''panel reactive antibody'' (PRA) served as positive or negative controls for ELISA.

Collection and storage of blood and tissue samples

Heparinized peripheral blood was obtained immediately prior to transplantation and 6–8 weeks afterwards. Blood was layered on Ficoll–Hypaque (GE

Healthcare, Piscataway, NJ) and the peripheral blood mononuclear cells were isolated, frozen and stored in liquid nitrogen. Plasma fractions were stored at −80°C for later use.

At the time of renal transplantation, a 10 × 10 mm^2 segment of perinephric soft tissue was removed from the donor kidney and a 10 × 2 mm^2 segment of Scarpa's fascia from the wound edge of the recipient. These samples were diced with scissors into 1 × 1 mm^2 fragments and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 2.5 mM L-glutamine, with penicillin and streptomycin.

Stimulation of peripheral blood mononuclear cells

Aliquots of peripheral blood mononuclear cells were thawed and cultured in RPMI supplemented with 10% FBS, 2.5 mM L-glutamine, with penicillin and streptomycin. The cells were stimulated with 5 mM CpG oligonucleotide (ODN 2006, Sigma-Aldrich, St. Louis, MO), 2 ng/mL IL-15 (BD Pharmingen, San Jose, CA), 10 ng/mL CD40L (Insight Genomics, Falls Church, VA) and with 50 ng/mL IL2 (Insight Genomics) in complete RPMI medium with 10% FBS, as modified from methods previously reported (40,41) for 5 days, with fresh medium provided at day 2.5. The number of B cells in each sample of PBMC was determined by FACS using FITC-labeled monoclonal anti-CD79 (Southern Biotech, Birmingham, AL). PBMCs had between 9% and 11% B cells. Transplantation did not change the fraction of B cells in PBMCs.

Measuring the frequency of donor-specific antibody-secreting cells

The frequency of antibody-secreting cells was determined using a novel cellular ELISPOT in which fibroblasts, rather than purified antigen, served as the target. To establish the target layer, aliquots of frozen fibroblasts were thawed and cultured in DMEM with 20% FBS, penicillin and streptomycin in 96-well plates to approximately 90% confluency. To prevent ingestion of bound antibody, fibroblasts were lightly fixed by incubation with 4% paraformaldehyde, pH 7.4 for 15 min at 25°C and washed three times with phosphate-buffered saline (PBS), pH 7.4, containing 1% FBS. To enumerate donor-specific antibody secreting cells, 2.5 × 10^5 stimulated PBMCs were re-suspended in fresh medium containing 5 mM CpG oligonucleotide (ODN 2006, Sigma-Aldrich), 10 ng/mL IL-15 (BD Pharmingen), 10 ng/mL CD40L (Insight Genomics) and 50 ng/mL IL2 (Insight Genomics) in complete RPMI medium with 10% FBS, added to each well in 96-well plates containing the fixed, confluent donor, recipient or third party fibroblasts. The plates were maintained at 37°C and 5% CO_2 for 24 h, and then washed three times with PBS containing 1% FBS. Secreted antibody bound to fibroblasts was detected using goat anti-human IgM- or anti-human IgG-antibodies conjugated with alkaline phosphatase (AP).

To enumerate total IgM- or IgG-secreting cells a conventional ELISPOT was performed. Each well of Immuno Spot M200 filter plates (Millipore, Billerica, MA) was moistened with 30 μL/well of 35% ethanol in water for 30 s, washed three times with sterile PBS and coated with 100 μL of 5 μg/mL of unconjugated goat anti-human IgG- or goat anti-human IgM-antibodies (Southern Biotech) in PBS. The wells were washed with sterile PBS, blocked with 10% FBS in PBS at room temperature for 1 h, and then stored at room temperature with PBS for up to 2 h before use. To each well 1 × 10^5 stimulated PBMCs resuspended in fresh medium containing 10% FBS were added. The plates were maintained at 37°C and 5% CO_2 for 24 h, and then washed three times with PBS containing 1% FBS. Secreted antibody bound to the fibroblasts was detected using goat anti-human IgM or anti-human IgG, conjugated to horseradish peroxidase (HRP) (Southern Biotech). Bound antibody was developed according to the manufacturers' instructions. The number of positive counts was recorded by ImmunoSpot S5 UV analyzer (CTL Analyzers, LLC) and with software version ImmunoSpot 5.0.9, set to count the center 70% of each well. Counts were corrected for the surface fraction assayed. The number of specific spots was determined by subtracting the number of spots measured in plates incubated with developing antibody without PBMCs (background). Assays were performed in triplicate.

HLA class I blockade was performed by adding 100 μg/mL mouse monoclonal antibodies that recognize HLA-A, B and C (W6/32) (Santa Cruz Biotechnology, Santa Cruz, CA) to monolayers of fibroblasts, for 60 min at 4°C, prior to incubation with PBMCs and developed as described above.

HLA typing

HLA typing was performed at the University Michigan histocompatibility laboratory for both donor and recipient by reverse sequence specific oligonucleotide, according to standard procedures.

HLA specific antibody detection in blood

The presence of anti-HLA antibodies in blood was determined at the University Michigan histocompatibility laboratory by Luminex Single Antigen Assay (One Lambda). The laboratory reports median fluorescence intensities (MFIs). MFIs above 700 were considered positive.

Assay for donor-specific antibodies

Levels of antibodies in the blood specific for donor or third-party cells was determined by ELISA as described previously (42), using cultured fibroblasts as targets. Monolayers of confluent fibroblasts were prepared as above and fixed with paraformaldehyde. Samples of heparinized plasma were heat inactivated at 55°C for 30 min, diluted in PBS containing 1% FBS, applied to wells and incubated for 60 min at 4°C. The wells were washed with PBS containing 1% FBS and then incubated with goat anti-human IgM and goat anti-human IgG antibodies conjugated to AP (Santa Cruz Biotechnology) and developed according to the manufacturers' instructions. Absorbance was read at 405 nm on an ELISA plate reader Biotek Synergy 2 equipped with Gen5 v.1.04.4 software (Biotek Instruments, Inc, Winooski, VT). Absorbance in wells incubated only with secondary antibody and in wells with autologous fibroblasts were subtracted from readings obtained with donor fibroblast wells. The assays were performed in triplicate.

Statistical analysis

Statistical analysis was performed using Prism 6 software (Graph pad, La Jolla, CA). The results of paired samples (before and after transplantation) were compared using paired t-test analysis. Comparison of averages between groups was performed using unpaired two-tailed t-tests.

Results

To evaluate the B cell response to transplantation, we devised an approach to enumerating donor-specific antibody-secreting cells. When an antigen recognized by B cells is known and available in quantity, antibody-secreting cells can be assayed by a conventional ELISPOT. Perry et al. (38) and Heidt et al. (39) used this approach enumerate antibody secreting cells specific for common HLA class I antigens. Fan et al. (27) used the approach to enumerate antibody secreting cells specific for blood-group antigens. However, in many circumstances the histocompatibility antigens recognized by B cells are incompletely known or defined or may not be available in quantity on a surface that models the surface of a cell. Accordingly, we adapted an approach

originally used by Larson et al. (43) to measure the B cell response to epithelial tumor antigens, but using donor fibroblasts instead of tumor cells as targets. Using fibroblasts rather than B cells of the donor allowed us to focus on HLA-class I encoded antigens and to avoid the possibility that polyreactive B cells of the donor, potentially comprising half of the B cells isolated from the blood, might recognize and stimulate B cells of the recipient.

At the time of organ harvesting and transplantation, fibroblasts from nine living donors and recipients were explanted, expanded in culture and cryopreserved. A sample of these cultured cells is shown in Figure 1. To avoid as much as possible testing preexisting or acquired responses to HLA class I, we investigated only those subjects with no donor-specific antibodies (DSA), detected by Luminex Single Antigen Assay (One Lambda) using a secondary antibody against IgG, before and after transplantation. One subject had pretransplant reactivity against one HLA-class I haplotype and another subject had reactivity against two HLA-class I antigens not present in the donor, after transplantation. No recipient developed antidonor antibodies after transplantation during the time of the study. To determine whether fibroblasts cultivated from tissues of kidney donors and recipients express HLA on cell surfaces and hence could serve as cellular targets for the ELISPOT, we tested the cultured cells for binding of murine monoclonal antibodies specific for a nonpolymorphic domain of HLA-A, B and C. Figure 2A and B show that fibroblasts explanted from transplant donors and recipients expressed appreciable levels of HLA-A, B and C and did not express HLA-class II.

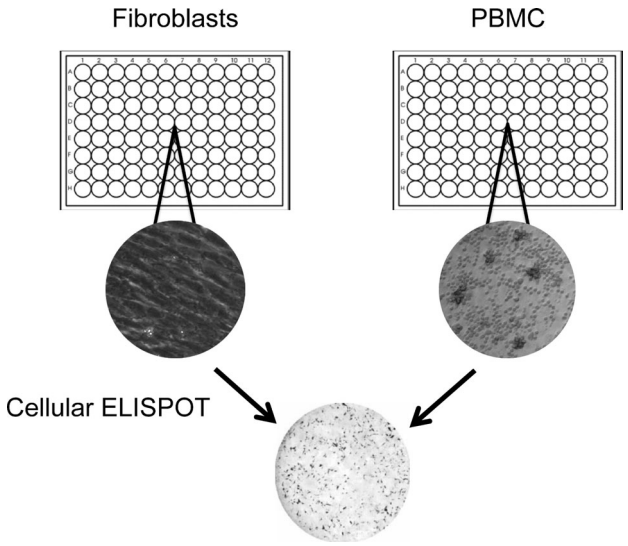

Figure 1: Cellular ELISPOT diagram . Samples of perinephric fascia from donors and Scarpa's fascia from recipients of renal transplants were explanted and used to establish primary cultures of fibroblasts. The fibroblasts generally reached confluence at day 30. PBMCs collected at the time of transplantation and 6–8 weeks later were cultured for 5 days prior to overlaying onto the fixed fibroblast layer and incubated for 24 h. Antibodies bound to the fibroblasts were detected with goat anti-human IgM or IgG, AP conjugated and developed as with a regular ELISPOT. A typical cellular ELISPOT well is shown.

We next asked whether the recipients of renal transplants with normal graft function and lacking evidence of donor specific antibodies, detected by with Luminex Single Antigen beads, and no evidence of rejection have nonetheless antibodies specific for a donor cell surface antigen, perhaps an HLA not represented by the beads, that might be present in their transplants. To address this question we used the cultured fibroblasts as targets for ELISA. Figures 2CC and 3A and B show that the nine renal transplantation recipients studied had little or no antibody specific for fibroblasts of the transplant donor before or after transplantation. This result is consistent with the observations of many cited above (13–22) and others not cited in this paper, that most recipients of functioning kidney transplants have little or no detectable donor-specific antibodies in their blood during the early months after transplantation. In contrast, sera obtained from subjects sensitized to a broad range of HLA antigens in the population, as indicated by a high level of PRA, reacted strongly in the fibroblast ELISA (Figure 2C). The absence of allo-reactive antibody in the circulation of recipients could reflect failure to respond to antigen owing to immunosuppression, immunological ignorance, anergy or tolerance, on the one hand, or a response masked by absorption of allo-specific antibody to the graft, on the other.

Detecting such cryptic B cell responses can be challenging because normal cells can, over a period of hours, absorb enough antibody specific for plasma membrane antigens to constitute 1% of total cellular protein (44) and the activation of complement that may ensue promotes uptake and degradation (45). Thus, the absorption of antibodies and activation of complement in an organ transplant can leave scant amounts of cell-specific antibody for detection by ELISA or immunopathology (46). Still, any B cell response should be ascertainable by enumerating antibody-secreting cells in the blood. Thus, to determine whether the absence of allo-reactive antibodies in the circulation of renal transplant recipients reflects failure to respond to the antigen or a response belied by binding of antibodies to the graft, we measured the number of circulating allo-specific antibody-secreting B cells before, and two months after, transplantation by ELISPOT assay using lightly fixed monolayers of cultured cells as a target. Because some fraction of B cells in normal individuals might recognize auto- as well as donor-antigens (47,48), we subtracted the number of B cells responding to recipient cells from the number responding to donor cells in our determination of the donor-specific response. This approach undoubtedly underestimates the B cell response to the transplant, but it does lend greater confidence to the responses we report.

Figure 4 shows the average number of anti-specific antibody-secreting cells measured by ELISPOT using donor

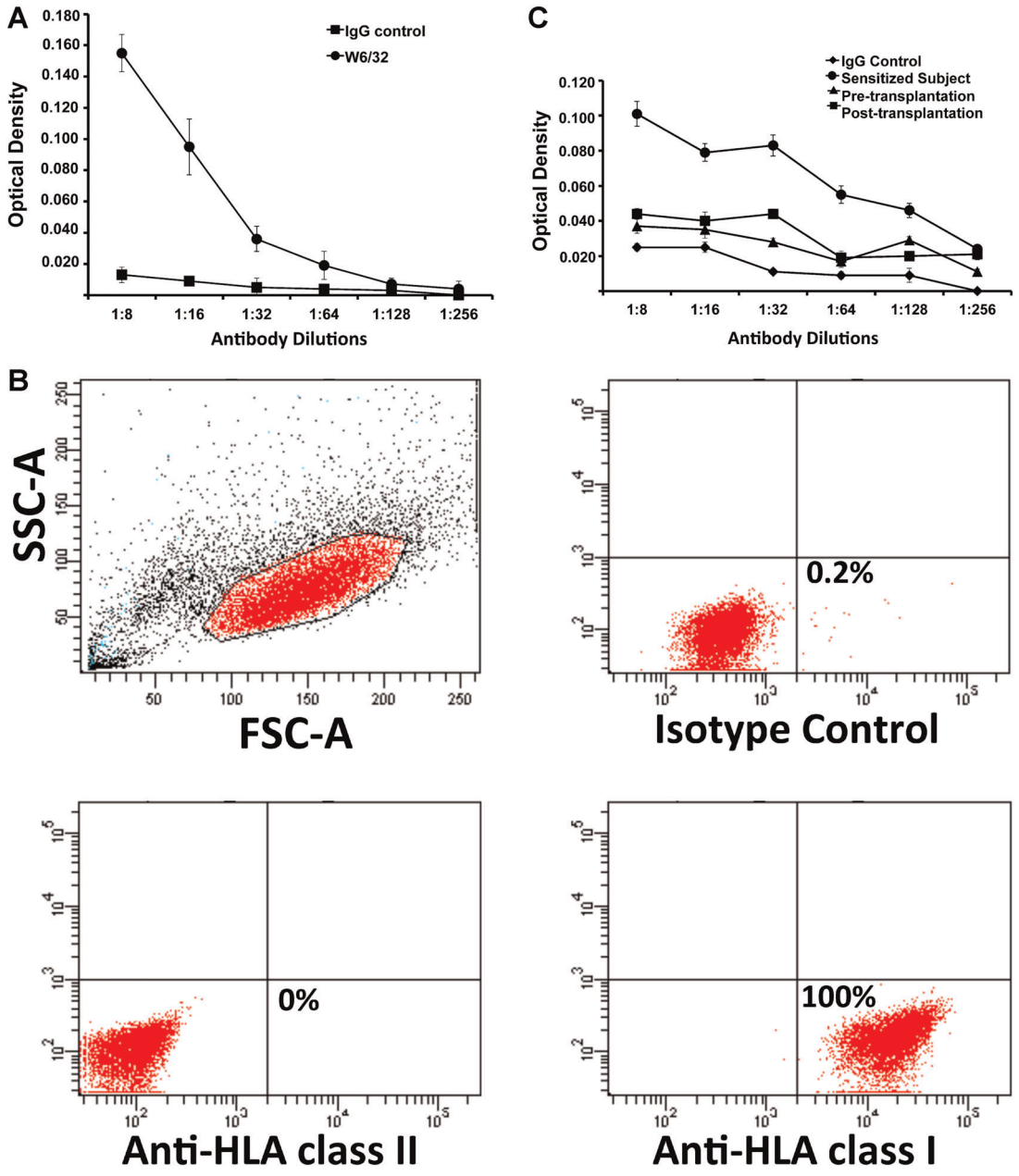

Figure 2: Use of cultured human fibroblasts as a target to assay for donor-specific antibodies. (A) Expression of HLA-class I on cultured human fibroblasts. The figure shows that cultured fibroblasts express HLA-class I detected by binding of a murine monoclonal antibody specific for a nonpolymorphic domain of HLA-A, B and C (W6/32) and measured by ELISA. Standard errors of triplicate measurements are indicated. (B) Flow cytometry analysis of HLA-class I and class II expression by cultured fibroblasts. Shown is the light scatter plot with the lymphocyte gate depicted in red. Dot plots show staining with antibody isotype control, anti-HLA class I and anti-HLA class II antibodies. The fractions of positively stained cells are noted. (C) Use of cultured fibroblasts for assay of donor-specific antibodies in the serum of renal transplant recipients. Cultured donor fibroblasts were incubated sequentially with serial dilutions of serum from a highly sensitized subject and with serum obtained before and two months after renal transplantation and with AP labeled anti-human IgG. Donor-specific IgG was measured by detecting the optical density at 405 nm (Y-axis). The assay detects binding of IgG from a highly sensitized subject but minimal or no binding of IgG from the renal transplant recipient or pooled IgG used a control. Standard errors of triplicate measurements are indicated.

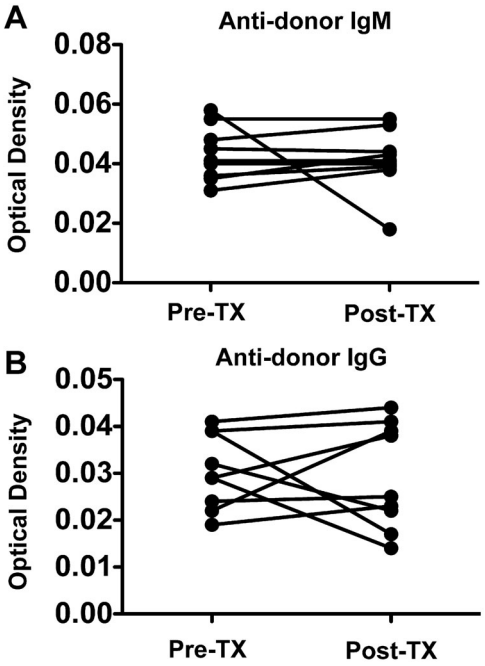

Figure 3: Donor-specific IgM and IgG in renal transplant recipients . Serum was obtained from nine subjects before and two months after renal transplantation. The serum diluted 1:16 was assayed for binding of IgM (A) or IgG (B) to donor fibroblasts and expressed as absorbance at 405 nm (Y-axis). The results show that renal transplant recipients had no detectable donor-specific antibodies two months after transplantation. The average ODs and standard deviations for each population were 0.04 $\pm$ 0.003, IgM pre-TX; 0.04 $\pm$ 0.004, IgM post-TX; 0.03 $\pm$ 0.003, IgG pre-TX; 0.03 $\pm$ 0.004, IgG post-TX.

fibroblasts as targets for nine renal transplant recipients with normal graft function. At the time of transplantation, these subjects had a discernable fraction of B cells (average of 86 antibody-secreting cells per 5×10^4 B cells (Figure 4A–C) specific for the transplant donor, as indicated by the production of IgM antibodies that defined the sites where the B cells bound to allogeneic fibroblasts. Also, as expected, a smaller but still discernable fraction of B cells recognized autologous fibroblasts (Figure 4A). Considering the difference between donor-reactive and auto-reactive B cells to represent the donor-specific response, Figure 4B and C shows that donor-specific IgM-secreting cells increased from an average of 86 to an average of 144 antibody-secreting cells per 5×10^4 B cells, in the nine renal transplant recipients during the 8 weeks after transplantation. The increase in the number of donor-specific IgM-secreting B cells suggests that, following transplantation, some fraction of the B cells committed to recognizing the donor expanded in all subjects studied. In contrast, in a typical recipient the total number of IgM-secreting cells in blood, enumerated using conventional ELISPOT, decreases slightly after transplantation (from an average of 1417 antibody-secreting cells per 5×10^4 B cells before to an average of 1003 antibody-secreting cells after transplantation) (Figure 4F).

As might be expected of recipients with normal graft function and no prior episodes of rejection, the number of allo-specific IgG-secreting cells was much lower than the number of IgM-secreting cells and the mean changed minimally, from 16 per 5×10^4 B cells to 28 per 5×10^4 B cells, after transplantation (Figure 4D). In contrast, the total number of IgG-secreting cells decreased after transplantation (from an average of 411.7 per 5×10^4 B cells pretransplantation, to an average of 265.7 per 5×10^4 B cells, posttransplantation) (Figure 4F). However, in five subjects, the number of IgG secreting cells increased markedly (from 0 to 29 per 5×10^4 B cells, from 0 to 19 per 5×10^4 B cells, from 19 to 41 per 5×10^4 B cells, from 16 to 42 and from 9 to 32 per 5×10^4 B cells, Figure 4E and marked in red in Figure 4D). The subjects showing the greatest incremental increase in the number of IgG donor-specific B cells were well at the time of this analysis. Figure 4D also shows that the number of donor-specific IgG-secreting cells decreased significantly in two recipients (from 29 to 14 per 5×10^4 B cells in one, and from 18 to 8 per 5×10^4 B cells in another, Figure 4E and marked in green in Figure 4D). Whether these changes reflect sampling, clonal deletion or anergy remains to be established in longer follow-up studies.

To ensure that the ''response'' was truly donor-specific, we determined the number of antibody-secreting cells specific for third-party fibroblasts. Figure 5A and B shows that the number of IgM- or IgG-secreting cells specific for third-party fibroblasts did not change after transplantation. Thus, B cell responses after transplantation, as determined here, are donor-specific, and the relative absence of B cell responses to third-party cells did not reflect idiosyncratic suppression of antibody secretion by cultured B cells, since samples from recipients bearing grafts from which fibroblast targets were derived exhibited strong responses.

Since B cell responses were observed in all subjects and were donor-specific and since fibroblasts do not express HLA class II (Figure 2B), the most likely targets were HLA class I antigens. To test this concept, monolayers of fibroblasts were blocked with a saturating concentration of a murine monoclonal antibody that recognizes HLA-A, B and C and then used the blocked cells as targets for ELISPOT as described above. HLA class I blockade dramatically decreased the reactivity of pretransplant (from 102.9 IgM ASC per 5×10^4 B cell, to 19.8 IgM ASC per 5×10^4 B cells, on average) and posttransplant (from 193.8 IgM ASC per 5×10^4 B cells to 32.8 IgM ASC per 5×10^4 B cells, on average) (Figure 5C). HLA class I blockade also decreased the reactivity of pretransplant donor-specific IgG-secreting cells (from 12.5 IgG ASC per 5×10^4 B cells to 4.5 IgG ASC per 5×10^4 B cells, on average) and posttransplant (from 19.3 IgM ASC per 5×10^4 B cells to 5 IgG ASC per 5×10^4 B cells, on

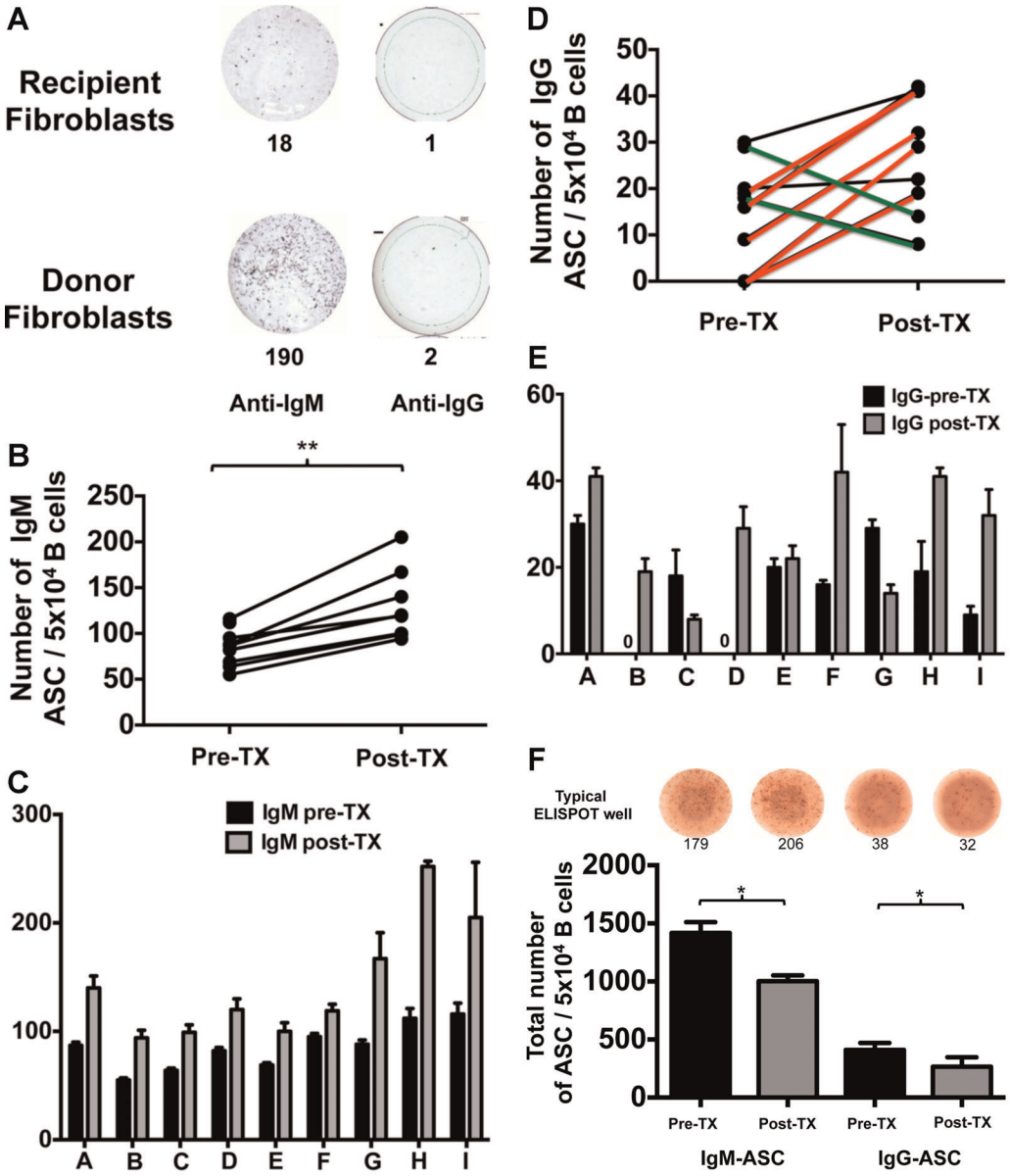

Figure 4: Donor-specific antibody-secreting cells in renal transplant recipients . The number and specificity of donor-specific antibody-secreting cells were determined using a novel ELISPOT in which cultured fibroblasts were used as targets and following incubation with 5×10^4 stimulated B cells in unfractionated PBMC for 24 h. Antibody bound to the fibroblasts was detected by ELISPOT using murine monoclonal anti-human-IgM or -IgG. (A) Typical cellular ELISPOT wells. Cellular ELISPOT wells revealing anti-donor or anti-recipient (autologous) IgM or IgG antibodies. Numbers indicate the ELISPOT readings. (B) Frequency of B cells producing donor-specific IgM detected by cellular ELISPOT before and 2 months after renal transplantation. The number of antibody-secreting cells producing donor-specific IgM increased significantly after renal transplantation ($p = 0.0001$, paired t-test). (C) Frequency of B cells producing donor-specific IgM detected by cellular ELISPOT before and 2 months after renal transplantation in individual recipients. Averages of ASC before and after transplantation were compared by paired t-test ($p = 0.0001$). (D) Frequency of B cells producing donor-specific IgG detected by cellular ELISPOT before and 2 months after renal transplantation. The number of antibody-secreting cells producing donor-specific IgG increased (indicated in red) in four and decreased in two (indicated in green) of the nine subjects after renal transplantation. Pre- and posttransplant values did not significantly differ by paired t-test analysis ($p = 0.0574$). (E) Frequency of B cells producing donor-specific IgG detected by cellular ELISPOT before and 2 months after renal transplantation in three typical patients. Individual experimental values and standard errors are shown. Averages of ASC before and after transplantation were compared by paired t-test ($p = 0.0574$). (F) Frequency of total IgM and IgG producing cells before and 2 months after transplantation for a typical recipient. Recipients had 1417 IgM and 411.7 IgG secreting B cells per 5×10^4 B cells pretransplantation, and 1003 IgM and 265.7 IgG secreting B cells per 5×10^4 B cells posttransplantation, respectively. The number of IgM or IgG secreting B cells before and after transplantation did significantly differ by t-test analysis (for IgM $p = 0.0256$, for IgG $p = 0.0337$).

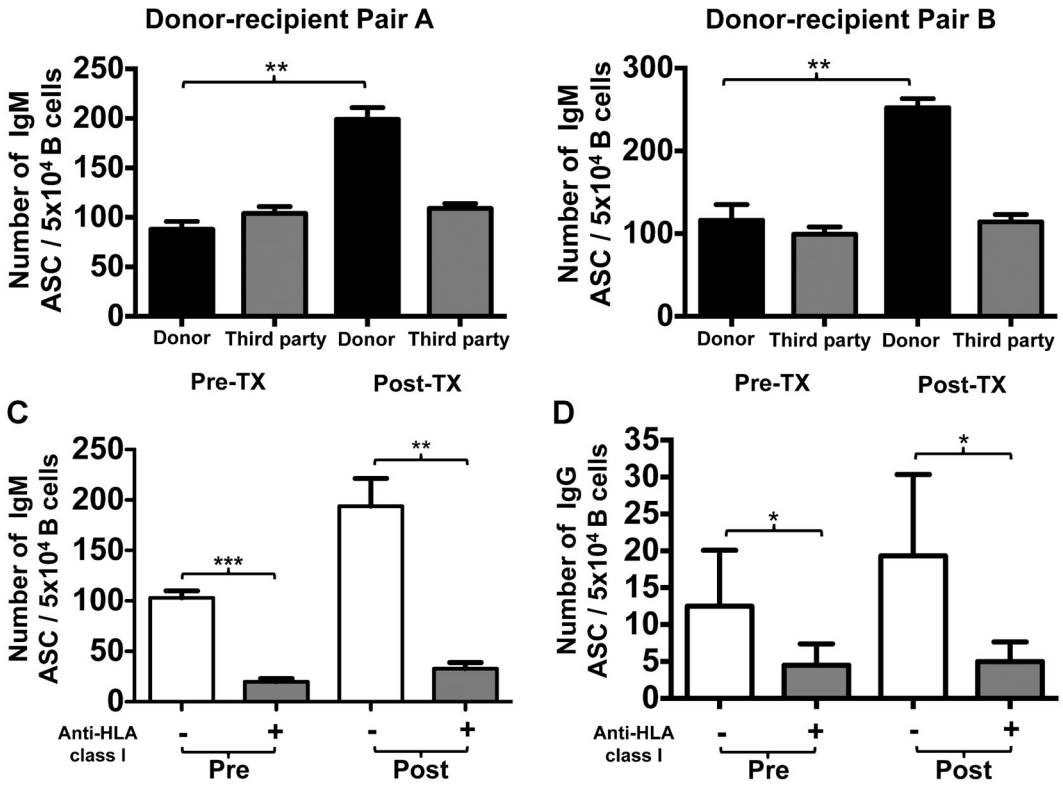

Figure 5: (A) and (B) Specificity of donor-specific B cells for donor and not for third party fibroblasts . Shown are results from cellular ELISPOT of B cells obtained from two recipients with donor or third party fibroblasts. The number of antibody-secreting cells producing donor-specific IgM increased significantly (pair A, $p = 0.0015$; pair B, $p = 0.0035$, unpaired, t-test) after renal transplantation while the number the number of antibody-secreting cells producing third party-specific IgM did not (pair A–B, $p = 0.6$; pair B–A $p = 0.3$). (C) and (D) Specificity of donor-specific B cells for HLA class I. To determine the extent to which donor-specific B cells recognize HLA class I versus other potential targets on fibroblast cell surfaces, cultured donor fibroblasts were blocked with murine anti-HLA class I antibodies and then the ELISPOT was performed. C and D depict the number of anti-donor-specific IgM (C) or IgG (D)-secreting cells before and 2 months after transplantation with (+) or without (−) MHC class I blockade and shows that donor-specific antibody-secreting cells predominantly recognize HLA class I. HLA blockade was specific since incubating with an isotype control did not decrease anti-donor reactivity. Averages and standard errors of samples obtained from four distinct recipients were assayed in triplicate. Comparison of the means was by paired t-test (IgM pretransplantation $p < 0.0001$, IgM posttransplantation, $p = 0.001$, IgG pretransplantation, $p = 0.0482$; IgG posttransplantation, $p = 0.0468$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

average) (Figure 5D). HLA blockade was specific since incubating with an isotype control did not decrease anti-donor reactivity.

Discussion

Here we report advances in concept and technique regarding the immunological response to clinical kidney transplantation. The advances stem from our application of a novel method to probe antibody secreting cells and our finding that the number of donor-specific antibody-secreting cells in the blood of transplant recipients increases during the two months following transplantation. This finding establishes that B cells in humans, as in experimental animals, do respond reliably to allogeneic transplantation and sets the stage for understanding the fate and impact of those B cells in disease and health of grafts.

Although B cells of mice usually respond to allogeneic transplantation (6), it is not intuitive that B cells of humans would do likewise. Clinical transplant recipients are treated aggressively with immunosuppressive agents that inhibit T cell activation, especially during the early weeks after transplantation, and the B cell response to MHC class I antigens is T cell dependent. Thus, one might anticipate an attenuation of the B cell response in parallel with attenuation of the T cell response (49,50). Also consistent with the possibility that immunosuppressive therapy might impair B cell responses to allo-antigens is the weak primary response to vaccines (51–54) and the absence of detectable donor-specific antibodies during the first several months after successful transplantation (22,30,55,56). We also detected no donor-specific antibodies in the subjects we investigated. Of course, the trauma of surgery might activate and potentially expand populations of B cells nonspecifically; however, the expanded population of B

cells recognize donor HLA class I but not autologous or third party HLA class I and hence the expansion is donor-specific. The number of B cells secreting IgG specific for the donor MHC class I exceeded by 3.5-fold the number obtained by others (39). This difference probably reflects the fact that our assay detected antibodies directed against multiple MHC class I expressed by donor fibroblasts.

Use of cultured cells rather than purified antigens as a target for ELISPOT has some advantages and limitations worthy of comment. The B cells identified by this assay and hence the antibodies they produce recognize antigens as expressed in native conformation and in the context of the native constituents of the donor cell membrane. At the same time, the method excludes from detection B cells that recognize only denatured, degraded, or artificially clustered antigens and B cells producing antibodies the binding of which would be sterically blocked because of steric hindrance or blocking by the membrane associated glycocalyx. Accordingly, we have previously found that some antibodies capable of binding to antigens that can be isolated from cell membrane do not actually bind to those antigens on intact membranes (57,58). Another potential disadvantage of the approach we took is that B cells potentially directed against MHC class II are excluded. Human endothelium commonly expresses MHC class II proteins (59), and hence the fraction of responding cells we describe may underestimate the total fraction of antibody-secreting cells responding. However, accurate detection of cells secreting antibody specific for HLA class II would be confounded by the properties of the most readily available HLA-class II targets (B cells and phagocytes). Up to one-half of circulating B cells are polyreactive, bearing surface Ig that recognizes Ig and B cells and many phagocytes bear Fc receptors.

From our findings we can conclude that the recipients of clinical allografts do not exhibit tolerance or ''suppressed'' immunity to their grafts. However, it is possible that antibody secretion *per se*, as opposed to B cell stimulation, is hindered in some way by immunosuppressive agents. Whether the antibody-secreting cells we detected represent activated B cells secreting antibodies that bind to the graft is consistent with our findings, but not proved. Others (4,28–32) and we (33,34) have shown that a normal organ or a normally functioning organ graft can absorb large amounts of antidonor antibodies, leaving little or none remaining in the blood. Some antibodies bound to a graft might induce accommodation, a condition in which a graft or other tissue exhibits acquired resistance to antibody-mediated injury (60,61), and this condition might explain ongoing absorption of antidonor antibodies (59,61). If the increase in the number of donor-specific B cells does reflect accommodation, then accommodation might well be a common outcome of allo-transplantation during at least the first several months and the classical way of detecting accommodation, that is the simultaneous presence of antibody in the blood and normal graft function (62,63), might significantly underestimate the prevalence. In this case, our original definition of accommodation needs to be revised to encompass some organ transplant recipients who have no detectable antibody against the donor.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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