Clinical Case Reports

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CASE REPORT

Renal transplant recipient with advanced HIV infection: graft and peripheral cell population analysis

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Funding Information
This work was supported by fund from the Departments of Immunology and Rheumatology and Transplantation.

Received: 29 August 2013; Revised: 1 November 2013; Accepted: 10 October 2013

Clinical Case Reports **2013; 1(2): 79–85**

doi: 10.1002/ccr3.28

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Key Clinical Message

The scenario of a renal transplant recipient who is diagnosed with HIV infection in the late post transplant period is very uncommon. The viral infection effect on immunologic stability, regulatory cells, and allogeneic response during immune quiescence and graft acceptance provides a fertile ground in organ transplantation research and translational immunology.

Keywords

$CCR6^+/CD123^+/IDO^+$ plasmacytoid dendritic cells, Foxp3-expressing T cells, HIV infection, IL-10-producing B cells, mixed lymphocyte reaction, renal transplant recipient, T-cell subsets.

Introduction

The overall experience with organ transplantation in HIV-infected patients is evolving and successful outcomes have been observed when specific criteria are used to select candidates [1–3]. Stock et al. [4] analyzed a cohort of renal transplant recipients with pre-transplant evidence of HIV infection. They reported that patient and graft survival rates were high at 1 and 3 years, with no increases in complications associated with HIV infection. They also observed an unexpectedly high rejection rate, which indicated the need for better immunotherapy [4].

HIV infection is a disease driven by inflammation associated with a pronounced impairment of the T-cell compartment. On the other hand, B cells are mostly considered as positive regulators of the immune response through antibody-dependent response [5]. Nonetheless, reports of interleukin-10 (IL-10)-producing B cells (Bregs) in mice indicate a novel important immunoregulatory role for B cells [6,7]. Bregs ($CD19^+/CD38^{hi}/CD24^{hi}/CD27^+$) suppress the secretion of proinflammatory cytokine tumor necrosis factors-α (TNF-α) in monocytes and $CD4^+$ T cells [8, 9].

Even though studies from Siewe et al. [5] indicate that HIV infection is associated with a decrease in the Breg subset a correlation between Bregs frequency, $CD4^+$, and $CD8^+$ T-cell immune activation and $CD4^+$ T cells counts has also been observed [5]. Some data indicate that Bregs

modulate the differentiation of regulatory T cells (Tregs, $CD4^+/CD25^+/CD127^{low}$) and IL-10 production by Bregs has also shown to lead the accumulation of Tregs. It is likely that during HIV infection, Bregs contribute to a dysregulation of the T-cell compartment by modulating Foxp3-expressing T-cell differentiation [5].

The scenario of a renal transplant recipient (RTR) who is diagnosed with HIV infection in the late post transplant period is very uncommon. The effect of the viral infection on immunologic stability, peripheral blood regulatory cell populations, and allogeneic response has not been clearly established. Previous evidence from our work on RTRs with excellent long-term graft function has shown higher peripheral blood percentages of Tregs, Bregs and regulatory plasmacytoid dendritic cells ($CCR6^+/CD123^+/IDO^+$) [10].

Methodology and Results

We report the case of a 44 year-old male patient who received a living donor renal transplant in 1995; the etiology of the end-stage renal disease was undetermined. The adult donor was his brother, with whom he shares 1-haplotype; the anti-human globulin-complement dependent citotoxicity cross match was negative, the panel reactive antibody result was negative. He spent 1 year in continuous ambulatory peritoneal dialysis and received multiple blood-transfusions prior to transplantation. Serology for cytomegalovirus (CMV) was positive in the donor and the recipient. Serology for HIV was negative in the donor and the recipient. After the transplant procedure, immunosuppressive regimen was based on cyclosporine (Csa) 240 mg/12 h, azathioprine 100 mg/day and prednisone 12.5 mg/day. No induction therapy was used. His baseline serum creatinine (SCr) was 1.1–1.3 mg/dL.

The patient had an uneventful post transplant evolution characterized by a stable renal function and no episodes of acute rejection. In 1999, Csa was withdrawn and his immunosuppressive scheme continued with dual immunosuppression therapy with azathioprine 75 mg/day and prednisone 7.5 mg/day. After Csa withdrawal, graft function improved in 0.9 mg/dL of SCr and an estimated glomerular filtration rate (eGFR) by modified diet in renal disease (MDRD) of 93 mL/min. He referred occasional sexual activity with regular condom use with two heterosexual partners, one of whom he considered to be a high-risk partner.

In September 2012 he presented to the outpatient clinic complaining of 8 kg weight loss in the last 6 months, dysphagia and chronic diarrhea. At this time his immunosuppression therapy consisted of azathioprine 50 mg/day and prednisone 2.5 mg/day. A diagnosis of oral pseudomembranous candidiasis was determined. He was diagnosed with HIV infection by ELISA on two different determinations. He presented with a CD4 and CD8 count of 98 cells/μL and 358 cells/μL, respectively, viral load of 608,322 copies/mL, negative Venereal Disease Research Laboratories, and negative pp65-CMV antigenemia on two determinations. Endoscopic/histologic evaluation revealed evidence consistent with CMV colitis and esophagitis. Treatment was begun with valganciclovir for CMV infection, fluconazole for candidiasis, and prophylaxis with dapsone. He was discharged with close follow-up at the HIV outpatient clinic. At this time, treatment with highly active anti-retroviral therapy (HAART) was postponed due to evidence of recent opportunistic infections.

On November 2012, 1 month later, the patient presented at the emergency department with respiratory distress. He was admitted to the intensive care unit (ICU) requiring respiratory support and norepinephrine. His SCr was 1.08 mg/dL and eGFR 72 mL/min. *Pneumocystis jiroveci* pneumonia was diagnosed. Immunosuppressive therapy was discontinued and the patient received treatment with Piperacilin/Tazobactam 4.5 g i.v./6 h, clarithromycin 500 mg p.o./12 h, trimethoprim/Sulfamethoxazole 160/800 mg (after desensitization), Oseltamivir 75 mg/12 h, unfractioned heparin 500 U/SQ/12 h and prednisone 40 mg/twice a day. Two weeks later, the patient showed an adequate clinical response and was discharged to continue his follow-up as an outpatient with immunosuppressive monotherapy with prednisone 20 mg/day. Renal graft biopsy revealed no evidence of cellular or humoral rejection, and about 20% of interstitial fibrosis and tubular atrophy (Fig. 1). Immunofluorescence and immunoperoxidase stain for C4d were negative.

In December 2012, the patient began treatment with efavirenz 600 mg/day, emtricitabine 200 mg/day, and tenofovir 300 mg/day with adequate tolerance and clinical response. At this time, he continued on immunosuppressive monotherapy with prednisone (20 mg/day). One and a half months later a CD4 and CD8 count at this time revealed 347 cells/μL and 278 cells/μL, respectively, and his viral load was 363 copies/mL. His SCr and eGFR remained stable at 0.7 mg/dL and 92 mL/min, respectively. The patient showed marked improvement and weight gain, with no signs of new episodes of infection or drug side effects.

By March 2013, viral load was undetectable (less than 40 copies/mL) with CD4 and CD8 count of 501 cells/μL and 593 cells/μL, respectively. A second graft biopsy was performed on April 2013, which showed borderline alterations and mild interstitial fibrosis and tubular atrophy (15%), again immunofluorescence panel and immunoperoxidase stain for C4d were negative (Fig. 1). Single-antigen test on three determinations (January, March, and

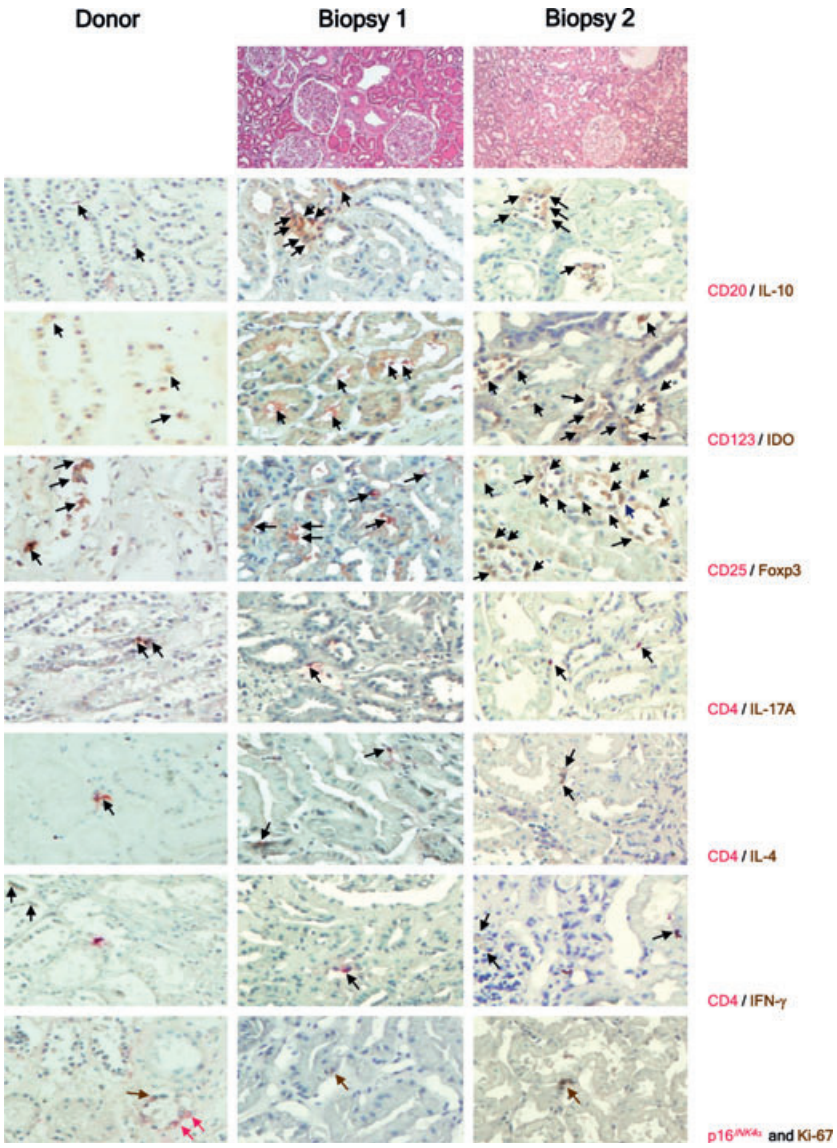

Figure 1. Differences in regulatory cells (IL-10-producing B cells, Foxp3-exppressing regulatory T cells and IDO$^+$ pDCs), immunologic characteristics (Th17, Th2 and Th1), senescence (p16$^{INK4\alpha}$), and proliferation (ki67) in graft biopsies from kidney HIV patient. Renal graft biopsies: Healthy donor (left column), HIV patient biopsy 1 (November 2012, middle column), and biopsy 2 (April 2013, right column). Arrows depict double immunoreactive cells. Magnification: 320×.

May 2013) were negative for donor specific antibodies. The daily dose of prednisone was gradually tapered to a current 10 mg/day and on 14 May 2013, treatment with azathioprine 50 mg/day was restarted. His renal function stability persisted with a SCr of 0.8 to 1.0 mg/dL, without evidence of graft dysfunction or rejection.

Circulating regulatory cell populations analysis by flow cytometry

In a recent publication, our group [10] determined the peripheral blood immunophenotypes of renal transplant recipients (RTRs) with different clinical characteristics. This study divided the groups into patients with excellent long-term graft function and patients with chronic graft dysfunction. In order to characterize the peripheral blood compartment in this patient, we proceeded to follow the same methodology [10]. A group of 10 healthy blood donors, specifically sampled for this study, were included as a control group. A 100-mL peripheral blood sample was drawn on February 2013; the CD4 and CD8 count at this time was 517 cells/μL and 621 cells/μL, respectively. In the analysis of this sample, a predominance of CD19$^+$/CD38^{hi}/CD24^{hi}/CD27$^+$/IL-10-producing Bregs and Tregs was

Table 1. Peripheral blood immunophenotypes of healthy blood donors (n = 10) and the RTR with HIV infection.

	HD (n = 10)	Patient February 2013	Patient March 2013	Patient May 2013
Demographic variables				
Donor age (years), Mean ± SEM	49.4 ± 2.5			
Laboratory variables				
Leukocytes (cells mL^{-1}), Mean ± SEM	$6.4 \times 10^9 \pm 4.1 \times 10^8$	9.8×10^9	4.1×10^9	4.2×10^9
Lymphocytes (%), Mean ± SEM	14.9 ± 0.3	14	24.5	28
CD19-expressing B cells (%)				
$CD19^+$, Mean ± SEM	**12.0 ± 0.9**	**0.7**	**3.8**	**4.12**
IL-10-producing B cells (%)				
$CD19^+/CD38^{hi}$, Mean ± SEM	3.3 ± 0.3	0.51	2.80	2.91
$CD19^+/CD38^{hi}/CD24^{hi}$, Mean ± SEM	2.0 ± 0.3	0.12	0.84	0.90
$CD19^+/CD38^{hi}/CD24^{hi}/IL\text{-}10^+$, Mean ± SEM	0.6 ± 0.05	0.10	0.57	0.44
$CD19^+/CD38^{hi}/IgA^+$, Mean ± SEM	65.3 ± 2.7	60.2	77.1	69.3
$CD19^+/CD38^{hi}/CD23^+/IgD^+$, Mean ± SEM	18.3 ± 3.2	18.6	19.9	39.8
$CD19^+/CD38^{hi}/CD21^+/IgG^+$, Mean ± SEM	52.5 ± 5.6	31.1	61.5	54.4
$CD19^+/CD38^{hi}/CD1d^+/IgM^+$, Mean ± SEM	34.9 ± 4.1	11.8	38.2	13.5
$CD19^+/CD38^{hi}/CD24^{hi}/CD5^+$, Mean ± SEM	33.6 ± 2.5	20.6	37.1	20.8
$CD19^+/CD38^{hi}/CD24^{hi}/CD10^+$, Mean ± SEM	**32.3 ± 2.6**	**15.2**	**19.8**	**19.0**
$CD19^+/CD38^{hi}/CD24^{hi}/CD20^+$, Mean ± SEM	**35.7 ± 2.1**	**18.9**	**21.1**	**20.5**
$CD19^+/CD38^{hi}/CD24^{hi}/CD27^+$, Mean ± SEM	**30.6 ± 3.0**	**74.6**	**49.1**	**44.3**
$CD19^+/CD38^{hi}/CD24^{hi}/CD27^-$, Mean ± SEM	45.3 ± 4.9	22.4	45.1	30.6
$CD19^+/CD38^{hi}/CD24^{hi}/CXCR4^+$, Mean ± SEM	**30.1 ± 1.6**	**10.0**	**26.5**	**12.2**
$CD19^+/CD38^{hi}/CD24^{hi}/CXCR7^+$, Mean ± SEM	**28.0 ± 2.0**	**12.3**	**23.5**	**10.1**
IDO-expressing cells (%)				
$CCR6^+$, Mean ± SEM	16.5 ± 1.6	4.3	12.5	6.7
$CD123^{hi}/CCR6^+$, Mean ± SEM	0.26 ± 0.08	0.13	0.11	0.10
$CD123^{hi}/CCR6^+/IDO^+$, Mean ± SEM	**23.7 ± 1.7**	**8.8**	23.5	35.4
Foxp3-expressing T cells (%)				
$CD4^+$, Mean ± SEM	24.8 ± 1.0	22.9	29.5	20.6
$CD4^+/CD25^{hi}$, Mean ± SEM	0.7 ± 0.1	0.3	0.2	0.1
$CD4^+/CD25^{hi}/Foxp3^+$, Mean ± SEM	**6.3 ± 0.4**	**12.9**	**10.8**	**12.9**
$CD8^+$, Mean ± SEM	14.1 ± 2.1	27.3	20.1	15.1
$CD8^+/CD28^-$, Mean ± SEM	8.0 ± 1.4	17.6	8.5	10.2
$CD8^+/CD28^-/Foxp3^+$, Mean ± SEM	5.9 ± 0.5	5.4	5.2	7.9
Th17 cells (%)				
$CD4^+/CD161^+$, Mean ± SEM	28.3 ± 2.8	22.8	23.2	12.4
$CD4^+/CD161^+/IL\text{-}17A^+$, Mean ± SEM	**0.86 ± 0.08**	**0.37**	0.85	0.76
Th22 cells (%)				
$CD4^+/CD161^-$, Mean ± SEM	0.35 ± 0.06	0.24	0.52	0.1
$CD4^+/CD161^+/IL\text{-}22^+$, Mean ± SEM	7.5 ± 0.4	8.61	10.16	9.5

Bold values represent significant differences in regarding to normal values

observed; in contrast to a low percentage of $CD10^+$/IL-10-, $CD20^+$/IL-10-, $CXCR4^+$/IL-10-, $CXCR7^+$/IL-10-secreting B cells and IDO^+ plasmacytoid dendritic cells (pDCs) (Table 1). A second sample was drawn in March 2013; CD4 and CD8 count at this time was 501 cells/μL and 593 cells/μL, respectively. On this occasion, the predominance of $CD27^+$/IL-10-producing Bregs was not as striking (49.1% vs. 74.6%); this value showed a tendency to become more similar to the percentage observed in the healthy donor group. The percentage of Tregs was only modified slightly (10.8% vs. 12.9%). The frequency of $CXCR4^+$, $CXCR7^+$, and IDO^+ cells was also increased, with a conspicuous tendency on all three cell lines to resemble the amounts observed in the healthy donor group. A third sample was drawn on 1st May 2013 (prior to the reinstatement of azathioprine 50 mg/day); the CD4 and CD8 count at this time was 520 cells/μL and 607 cells/μL, respectively. Regulatory T, B and pDC profile was similar compared with those previously obtained (Table 1).

Tissue regulatory cell population analysis by immunohistochemistry

In order to determine the characteristics of the cellular infiltrate, senescence, proliferation, regulatory cells, and interleukin expression present in the graft compartment,

renal graft biopsies performed 1 month prior to the initiation of HAART therapy (November 2012), and 4 months after the initiation of HAART therapy (April 2013) were stained with specific monoclonal antibodies. A healthy living donor time-zero graft biopsy was included as a control (Fig. 1). Thus, the subpopulation of $CD4^+$/IL-$17A^+$-, $CD4^+$/IL-4^+-, $CD4^+$/IFN-γ^+-expressing T cells, $CD25^+$/$Foxp3^+$ regulatory T cells, $CD123^+$/IDO^+ pDCs and $CD20^+$/IL-10^+-producing B cells was performed with a simultaneous detection using a second-generation EnVision™ G|2 Doublestain System (Dako, Glostrup, Denmark) on renal biopsies [11]. Immunohistochemistry findings showed greater amounts of CD20/IL-10-producing B cells, CD123/IDO pDCs and CD25/Foxp3 T cells in the patient compared to healthy donor tissue. Nonetheless, a noticeable increase in Foxp3-expressing cells was observed in the patient′s last biopsy (Fig. 1).

Mixed lymphocyte reaction

In 1st May 2013, blood samples from the patient, from his 1-haplotype match donor, and from a 6-human leukocyte antigen (HLA) antigen mismatch healthy control were collected. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation against a density gradient (Lymphoprep). One way mixed lymphocyte reactions (MLR) were set up as previously described [12]. A robust proliferative response to the challenge with PBMC's proceeding from a 6-HLA antigen mismatch healthy control was observed, suggesting donor-specific hyporesponse (Fig. 2).

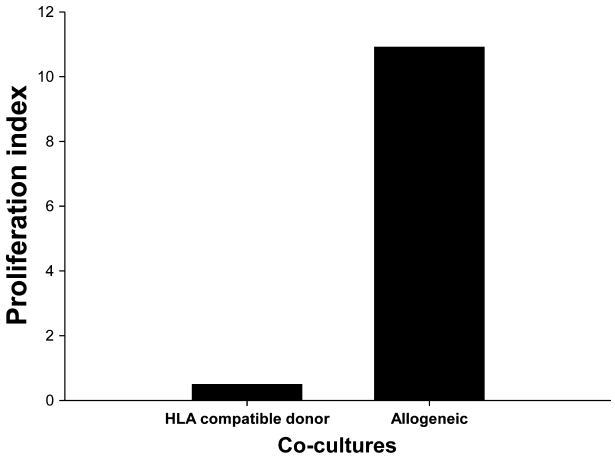

Figure 2. Mixed lymphocyte reaction assay. Cell proliferation was measured according to the CFSE-DA dilution protocol. Different cell generations were gated according to CFSE-DA levels. The gate settings were guided by the proliferation platform provided in the FlowJo software (Tree Star). The different molecular human leukocyte antigen (HLA) typings for the patient, donor, and allogeneic third party were as follows: Patient: [A*24, A*68, B*4002, B*39, CW*0302, CW*07, DRB1*04, DRB1*04, DQB1*0302, DQB1*0302, DRB4*, DRB4*]; Kidney donor: [A*24, A*02, B*4002, B*35, CW*0302, CW*04, DRB1*04, DRB1*04, DQB1*0302, DQB1*0302, DRB4*, DRB4*]; and Third party: [A*02, A*29, B*44, B*44, CW*05, CW*12, DRB1*04, DRB1*07, DQB1*0301, DQB1*02, DRB4*, DRB4*], respectively.

Discussion

The scenario of a RTR with excellent long-term graft function that is diagnosed with HIV infection is very uncommon. Although the restriction toward organ transplantation in an HIV^+ recipient has been overruled, several clinical considerations still remain to be elucidated [1]. In an analysis of functional outcomes in RTR with a pre-transplant diagnosis of HIV performed by Stock et al. [4], despite similar survival rates, they reported a slightly higher 1-year post transplant rejection rate of 12.3% (11.9–12.7) versus 11.6% in the conventional RTR [13]. Furthermore, Malat et al. [14] described a cohort of 92 RTR with HIV in which mixed rejections were seen in 13 HIV^+ RTR (14%) with a median time to antibody mediated rejection (ABMR) of 48 days. Acute cellular rejection occurred in 28% at 1 month and 55% at 12 months [14]. It seems striking that although our patient contracted HIV in the post transplant period, his renal function has remained stable without episodes of rejection.

One could speculate about the tendency to maintain a downregulation of an immune-mediated graft response in a patient that has already developed the required molecular and cellular mechanisms for immune quiescence and graft acceptance prior to the viral infection. It is also likely that during HIV infection, IL-10-secreting Bregs contribute to a dysregulation of the T-cell compartment by regulating Treg differentiation [5], despite or even facing the immune cell reconstitution that follows HAART.

An immunophenotyping of circulating cell subpopulations in the patient was performed. There was a clear predominance of $CD27^+$ Bregs and Tregs; in contrast to a low percentage of other Breg lineages ($CD10^+$, $CD20^+$, $CXCR4^+$, $CXCR7^+$) and regulatory pDCs at an early stage of the cellular reconstitution process. According to our data, the low prevalence of these cells in the peripheral blood suggests a reverse regulation in the periphery and the affected tissue, that is, CXCR7 and CXCR4 are chemokine receptors involved in cell survival and cell migration, respectively [15]. On the other hand, CD10 is a cell membrane metallopeptidase expressed by early B, pro-B, and pre-B lymphocytes and diffuse large B cells. CD10 expression is a well-accepted marker for most transitional T1/T2 B-cell pool, suggesting that these cells are recent emigrants from the bone marrow [9]. CD20 is expressed in mature B cells until plasma cell development

(plasmablasts). It has a central role in the generation of T cell-independent (TI) antibody responses. Although antigen-independent B cells developed normally, in the absence of CD20 expression, antibody formation, particularly after vaccination with TI antigens, is strongly impaired in deficient patients. Consistent with this, TI antipolysaccharide B cell responses is severely impaired in CD20-deficient mice [16]. In this sense, we suggest that the decrease in CD20 expression in the patient could be an immune regulatory mechanism capable to avoid antibody response and to maintain IL-10-producing B cell tolerant phenotype. Further analysis of these cell populations revealed changes in the IL-10-producing Breg cells ($CD27^+$, $CXCR4^+$, $CXCR7^+$) and pDCs, with a phenotype that seemed more similar to the healthy donor group than the one observed in the first determination. Since the peripheral blood analysis was performed after antiviral treatment was started, whether the preponderance of cells with a regulatory profile developed prior to the viral infection as a result of an alloimmune response shifting to a pro-tolerant state during the post transplant course remains elusive. Thus, it is tempting to speculate if the characteristics observed in peripheral blood immune cells analysis during reconstitution is a mere reflection of a previously established balance phenomenon.

As regards the mixed lymphocyte reaction, a hyporesponse from the patient's responder lymphocytes toward his 1-haplotype match donor was observed. However, this quiescent condition did not prevail when these cells were stimulated with a 6-HLA antigen mismatch healthy control (allogeneic). In summary, lymphocytes from this patient were able to proliferate upon foreign antigens, but not in response to his donor HLA antigens.

In the graft compartment, a regulatory cell component seemed to predominate with a marked presence of $CD25^+$/$Foxp3^+$ (Tregs), $CD20^+$/$IL\text{-}10^+$ (Bregs), $CD123^+$/IDO^+ (pDCs), and $CD4^+$/$IL\text{-}4^+$ (Th2) cells in the infiltrate. The stain performed to evaluate $CD4^+$/$INF\text{-}\gamma^+$ (Th1) and $CD4^+$/$IL\text{-}17A^+$ (Th17), showed a practical absence of these cells. The graft biopsy was also evaluated to determine the presence of the cellular senescence marker $p16^{INK4\alpha}$, which was also negative, meanwhile proliferation evaluated by Ki67 expression remained unaltered (Fig. 1). Judging by the graft's cellular infiltrate, speculation about an active regulatory microenvironment that suppresses the Th1 pathway, antigen presentation and subsequent active alloreactivity [10] could be sustained. In a recent publication, we hypothesized a cellular model in which a three-way interaction between Bregs, Tregs, and dendritic cells could explain the phenomenon of graft acceptance by multiple cellular and molecular interactions [10]. The persistence of this tolerogenic cellular infiltrate in the second graft biopsy performed 4 months after the initiation of HAART therapy further supports this hypothesis. Previously, other groups have described the role of regulatory B cells in maintaining immune quiescence in kidney transplant recipients [17–20].

It is important to mention that the effect of HAART therapy on the regulatory component of the immune system has not been adequately characterized. Interestingly, in the cell reconstitution process that was observed in this patient using the cell cytometry analysis, the cellular proportions present in the peripheral blood compartment seemed to have remained constant, with slight changes favoring immunophenotype similarity with the healthy donor group. Further clarifications on whether this phenomenon was due to an active mechanism that existed prior to the viral infection, a consequence of the viral infection itself or a side effect of the HAART therapy warrants further study. Usually, chronic HIV infection is characterized by high T-cell activation, secondary to high turnover, reflected by increased levels of activation cell marker like CD38 and CD25, mostly CD8 cells [21,22]. Likewise, Foxp3 as a marker of Tregs has been associated with HIV infection and as a marker of severity of the infection [23,24]. On the other hand, HAART has been shown to reduce T-cell activation, and studies show a linear correlation between initiation of HAART and a decrease in $CD38^+$/$CD8^+$ T cells (and a lesser decrease in CD4 T cell activation). The results of cytometer analysis in this patient after 6 weeks of therapy, particularly $Foxp3^+$ Tregs still reflect T cell activation, which might be due to an early phase of treatment and could explain the absence of CD8. The role of CD19 cells in HIV infection and changes associated with antiretroviral therapy have not been clearly described [21–23,25].

An intriguing question that necessarily emerges is whether to reinstall the immunosuppressive drug schedule the patient was receiving prior the diagnosis of the HIV infection and its clinical consequences. It is not easy to assume that the immunologic stability observed until now will persist indefinitely [24]. Furthermore, when the low-dose azathioprine the patient was receiving before he developed *Pneumocystis jiroveci* pneumonia was withdrawn, the steroid dose was increased and maintained at 20 mg/day and then progressively tapered to a current dose of 10 mg/day. Therefore, all the findings described at the peripheral blood compartment and at graft level were under the effect of steroids. If we were to hypothesize that the immunologic quiescence the patient displayed before the HIV infection diagnosis took place during the post transplant evolution, then we recognize that this occurred under the effect of azathioprine and steroids.

Undoubtedly, the effect of HIV infection on the molecular and cellular mechanisms that are active during the

processes of immune quiescence and graft acceptance provides for a fertile field in organ transplantation research and translational immunology.

Conflict of Interest

None declared.

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