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ORIGINAL ARTICLE

Kidney transplant recipients possess less depressing plasma than healthy controls in KwaZulu-Natal (South Africa) – a paradoxical observation

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ABSTRACT

The reduced activation of lymphocytes of patients on immunosuppressive drugs is well documented. Human plasma has been reported previously to play a role in lymphocyte proliferation. Several factors, including alpha globulin and lipoproteins, have been proposed as modulators of lymphocyte proliferation.

Aim: To measure the ATP response of peripheral blood mononuclear cells (PBMCs) from kidney transplant patients and healthy controls following phytohaemagglutinin (PHA) stimulation and to compare the effect of plasma of transplant recipients and of healthy controls on lymphocyte activation.

Methods: Peripheral blood mononuclear cells from the blood of healthy controls and kidney transplant patients on regimens based respectively on cyclosporine, sirolimus and tacrolimus, were separated by density gradient centrifugation. Cells were counted and incubated overnight with and without PHA. The luciferin–luciferase enzyme reaction, which induces bioluminescence, and the Turner Biosystem luminometer were used to measure intracellular ATP levels in relative light units, which were converted to ng/mL using an ATP standard curve. A chi-squared test using the Instat 3 program (Graphpad®) was used to compare results.

Results: PHA stimulation of PBMC from healthy individuals produced a 47% increase in ATP production. This increase was reduced to 31% when transplant patient plasma was added ($P < 0.05$). However, when plasma from healthy controls was added instead, paradoxically, the ATP production decreased further to 14%. A similar difference between patient and control plasma was recorded using PBMCs from transplant patients. The reduction in ATP production was the greatest in PBMCs from transplant patients on the tacrolimus-based regimen ($P = 0.0388$). Receiver operator characteristic (ROC) for ATP level revealed an area under the curve of 0.986. The cut-off value of ATP level between kidney transplant and control using the Youden index was 595 ng/mL, with a sensitivity of 93.3% and specificity of 99.9%

Conclusion: Plasma isolated from patients on immunosuppressive drugs suppressed the response of lymphocytes to PHA stimulation. Paradoxically, plasma from healthy controls suppressed T cell activation even more severely. If confirmed in a more extensive study, this observation may be used to influence the choice of replacement fluid in the practice of plasma exchange in transplantation.

Keywords: plasma; peripheral blood mononuclear cells; T cell activation; kidney transplantation.

INTRODUCTION

Despite improvements in immunosuppressant protocols, drug-related complications are still a significant cause of premature death of patients with a functioning transplanted organ [1]. Some complications associated with immunosuppressive drugs include rejection, infection, malignancies, cardiovascular disease, stroke, and diabetes [1-4]. The lack of a tool to closely monitor the effect of these drugs on the immune system has contributed

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to patients being over- or under-immunosuppressed, increasing morbidity and mortality [2,5].

An assay that involves adenosine triphosphate (ATP) and bioluminescence has been proposed as a non-invasive, sensitive and straightforward tool to monitor the immune response of transplant patients [6-10]. It measures the early events in the activation/proliferation of lymphocytes [6]. This assay is now much improved compared with the methods commonly used to measure the proliferation of lymphocytes, which generally require large quantities of blood, are technically demanding and require radioactive isotopes [11]. ATP is present in all metabolically active cells; cell injury and death rapidly reduce cytoplasmic ATP. Therefore, ATP may be used to assess immune cells' functional integrity following immunosuppressive therapy. Bioluminescence is achieved due to the enzyme luciferase, which is based on the gene from the firefly, Photuris pennsylvanica. Luciferase is used to generate a stable glow-type luminescent signal.

In the study reported here we evaluated the ATP bioluminescence assay as a potential diagnostic tool to assess the effect of immune-suppressant medications on the immune response in a cross section of the transplant patient population attending Inkosi Albert Luthuli Central Hospital (IALCH) renal clinic, in Durban, South Africa. Instead of using isolated T cells, as reported by Kowalski et al., we used peripheral blood mononuclear cells (PBMCs), which are closer to the in vivo situation and technically much simpler [3,6]. Using the percentage increase removes the variability concern between individuals as each patient's phytohaemagglutinin (PHA)-stimulated PBMCs, with the unstimulated PBMCs as its control. Ultimately, our study explored ATP as a determinant of viability and thus the level of immune suppression of lymphocytes. PHA, being essentially a T cell antigen in a PBMC preparation, the results of our method should be comparable with those of Kowalski et al. [3]. We have previously reported the use of PBMC stimulation and the ATP bioluminescence method in two studies on the effects of extracts of Sutherlandia frutescens, a medicinal plant used as an immune booster [12,13]. The reduction of lymphocyte proliferation by human plasma has been reported before [14].

Our study also compared the effect of plasma from transplant recipients and healthy controls on lymphocyte activation.

MATERIALS AND METHODS

This is a case–control study where cases were kidney transplant patients on various immunosuppressive drugs and controls were healthy individuals (not on any immunosuppressive drug). Ethical approval for the study was granted by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Ethics # H172/05).

Participants

Thirty-eight kidney transplant patients, including eight cadaver kidney transplant recipients attending Inkosi Albert Luthuli Central Hospital, were recruited following informed consent. Among the 30 living donor kidney transplant recipients, 4 were spouses of the donors, and 26 were either parents, children, or siblings. Twenty-two kidney transplant patients were on a cyclosporin (Neoral®)-based immunosuppressive regimen, whereas 10 were on a sirolimus (Rapamune®)-based regimen and six on a tacrolimus (Prograf®)-based regimen. All patients received low-dose prednisone and either azathioprine or mycophenolate mofetil. Fifteen healthy individuals were also recruited as controls (Table 1).

Table 1. Demographics and clinical characteristics of participants.

Group	n	Age (yr) (range)	Race	Gender	Age of transplant (yr)	Drug levels (ng/mL)	Creatinine levels (μmol/L)
Cyclosporine	21	$\bar{x}$: 53.5 (39–68)	Asians: 14 Africans: 2 Whites: 4 Coloured: 2	M:10 F:12	<1: 0 1–10: 11 >10: 11	C2; $\bar{x}$ = 884; SEM = 46.83 (506–1390)	$\bar{x}$ = 728; SEM = 8.2 (85–261)
Sirolimus	10	$\bar{x}$: 41.5 (19–64)	Asians: 6 Africans:4	M: 5 F: 5	<1: 0 1–10: 8 >10: 2	$\bar{x}$ = 8.67; SEM = 1.16 (3.2–15.10)	$\bar{x}$ = 159.6; SEM = 31.7 (192–333)
Tacrolimus	6	$\bar{x}$ = 26.5 (15–38)	Asians: 4 Africans 1 White: 1	M: 4 F: 2	<1: 1 1–10: 5	$\bar{x}$ = 8.32; SEM = 0.98 (6.10–12.70)	$\bar{x}$ = 125.2; SEM = 14.6 (74–160)
Controls	15	$\bar{x}$ = 41 (18–64)	Asians: 10 –	M: 5 F: 5 –			
P value		0.0003					0.3562

Abbreviations: $\bar{x}$, mean; SEM, standard error of the mean; χ^2, chi-squared test.

Methods

Cells isolation and culture

Peripheral blood mononuclear cells were separated from blood obtained from transplant patients attending the IALCH renal clinic and control blood samples, using density gradient centrifugation. Cells were counted with a haemacytometer to include 50,000 cells per well.

Cells in culture medium (RPMI with fetal bovine serum albumin and antibiotics penicillin and streptomycin) were plated in triplicate on white opaque, 96-well microtitre plates. They were then incubated with the stimulant phytohaemagglutinin (PHA) at 37°C in a CO_2 incubator for 15–18 hours. Cells were plated in the presence of the stimulant PHA and concurrently in the absence of PHA to evaluate ATP synthesis in response to stimulation and basal ATP activity. The plasma effect was further assessed by adding plasma from either healthy individuals or transplant patients on various immunosuppressive treatment regimes. In the culture experiments without plasma, all 22 participants on cyclosporine were tested; however, in the experiments using plasma, only 17 were tested, due to a technical error.

ATP bioluminescent assay

The Promega CellTiter-Glo® (Madison, WI, USA) Luminescent Cell Viability Assay was used [15,16]. It lyses the cell to release intracellular ATP and signals the presence of ATP on the basis of the luciferin/luciferase enzyme reaction. The light produced by this reaction was measured with a Turner Biosystem luminometer (Sunnyvale, CA, USA), and the readings were recorded in relative light units (RLU).

ATP standard curve

The assay plate was assembled to include ATP standard concentrations of 10,000 ng/mL, 1,000 ng/mL, 100 ng/mL, 10 ng/mL and 1 ng/mL. The light measured by a luminometer is proportional to the concentration of ATP produced (Figure 1). Subsequently, the assay was performed using cultured cells from patients and controls. For ease of reference, we name the assays described above as ATP–PBMC assays.

Stimulation normalised ratio (SNR)

To consider the variation of T cell response to PHA stimulation, we propose standardising the response using a ratio called the stimulation normalised ratio (SNR). It is calculated by dividing the ATP produced by the PBMCs of a particular sample *x* (PBMC*x*), when exposed to PHA, minus the ATP generated when not exposed to PHA by the corresponding difference using PBMC from control cells (PBMC*c*), as represented by the formula below.

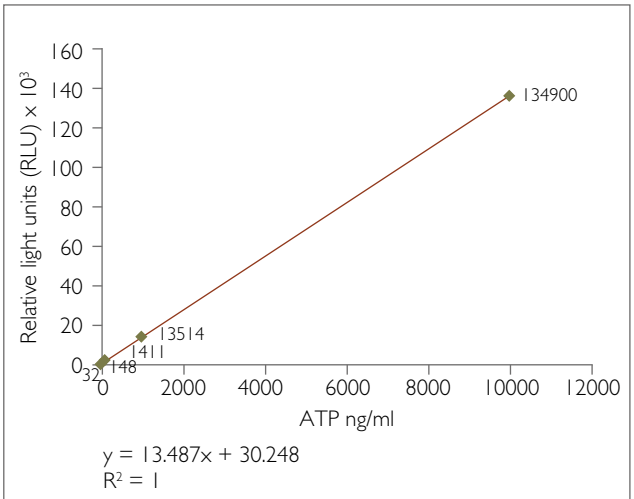

Figure 1. The relationship between relative light units (RLU) and ATP concentration (ng/ml).

$$SNR = \frac{(PBMCx + PHA) - PBMCx}{(PBMCc + PHA) - PBMCc}$$

Hence, the SNR for the positive control = 1 as $x = c$. When recorded in our experiments, the SNR results were as expressed in Table 1. The SNR values for PBMCs from patients treated with cyclosporine, sirolimus and tacrolimus were 0.24, 0.25 and 0.29, respectively.

Statistical analysis

Descriptive statistical methods providing means and standard errors of the mean were used to analyse the data. According to the normality test, groups were further compared using parametric or non-parametric tests. The Instat 3 program from Graphpad® (San Diego, CA, USA) was used. The test for association between relevant variables was performed by logistic regression analysis using SPSS version 27 (IBM, New York, USA). The receiver operator characteristic (ROC) was derived to determine cut-off values. Groups were compared using the chi-squared test.

RESULTS

As an internal control, all experiments included a known concentration of ATP to determine the ATP standard curve from which the ATP concentration for each patient

and control sample was calculated. The relationship between relative light units and cell number was further established by measuring the RLU corresponding to a known number of cells (PBMCs).

Cultures of PBMCs from transplant patients and controls with phytohaemagglutinin

ATP concentrations for each patient and control were recorded and analysed. No difference in ATP was found in unstimulated PBMCs from healthy controls or from any of the three groups of transplant patients ($P > 0.05$; χ^2) (Table 1). This result means that regardless of immunosuppressive treatment, unstimulated PBMCs of transplant patients and controls were equally viable after 24 h, 48 h or 72 h of incubation. However, PHA stimulation of PBMCs from control patients produced more ATP than PBMCs from any transplant patient on any of the three immunosuppressive regimens ($P < 0.05$; χ^2). No significant difference in PHA-induced ATP production was recorded between patients grouped according to their immunosuppressive regime (Table 1, Figure 2). PHA stimulation of PBMCs from healthy individuals led to a 47% increase in ATP production.

Association of transplant status with demographic variables and ATP increase

To evaluate the association between kidney transplant status with demographics and ATP increase, the receiver operator characteristic of the ATP–PBMC assay was derived. Results are shown in Figure 3; the area under the curve (AUC) was 0.986. The cut-off value between kidney transplants and controls using the Youden index (the closest point to 1) was 595 ng/mL, with a sensitivity of 93.3% and specificity of 99.9% [17].

We further applied logistic regression analysis to assess the association of transplant status with demographic variables and ATP increase. Univariate logistic regression revealed that kidney transplantation was associated with age and ATP increase with OR (CI) of 1.137 (1.010–1.279) and 0.991 (0.983–0.998), respectively (Table 2). No association was seen with either gender or race. However, a bivariate logistic regression analysis of transplant status between age and ATP increase revealed that the association of ATP increase persisted, whereas age was no longer associated with kidney transplant status (Table 3). Hence ATP increase was independently associated with kidney transplant status.

Cultures of PBMCs from transplant patients and controls with phytohaemagglutinin and plasma from transplant patients

In cultures containing PBMCs with PHA as well as plasma from patients, ATP production was reduced compared to PBMCs with PHA only (Table 4). This reduced ATP production was observed for all PBMC cultures (from both controls and patients). PBMCs from healthy controls nevertheless produced more ATP than those from patients. No statistically significant differences were observed in PBMCs from patients on cyclosporine-, sirolimus- or tacrolimus-based regimens.

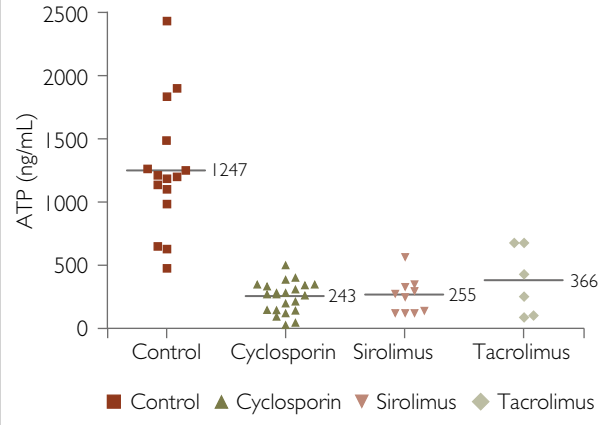

Figure 2. The average ATP produced following PHA stimulation of lymphocytes in the control group as well as in the kidney transplant recipient groups on cyclosporin, sirolimus and tacrolimus, respectively. ATP level was significantly higher in controls compared to transplant patients ($p < 0.0005$; χ^2). The differences of immune response between patients on cyclosporine, sirolimus and tacrolimus regimen were not statistically significant ($p = 0.244$; χ^2).

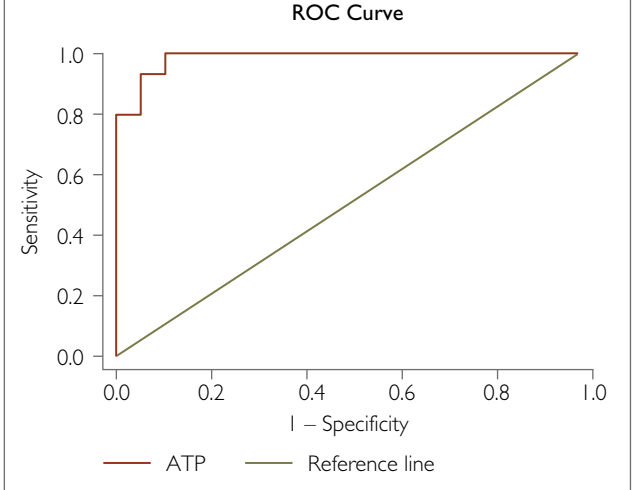

Figure 3. Receiver operator characteristic (ROC) curve for ATP level, showing the area under the curve (AUC) of 0.986. The cut-off value of ATP level between kidney transplant and control using the Youden index (the closest point to 1) is 595 ng/mL, with a sensitivity of 93.3% and specificity of 99.9%.

Table 2. Univariate logistic regression of transplant status and selected variables.

Variables	Odds ratio	95% confidence interval	P
Age	1.137	1.010–1.279	0.034
Gender	1.000	0.248–4.028	1.000
Race	1.580	0.433–5.763	0.488
ATP increase	0.991	0.983–0. 998	0.017

Table 3. Bivariate logistic regression of transplant status and selected variables.

Variables	Odds ratio	95% confidence interval	P
Age	1.085	0.88–1.339	0.444
ATP increase	0.992	0.985–1.000	0.038

Table 4. PHA stimulation of PBMCs according to origin of plasma.

	Unstimulated PBMC only (ATP ng/mL) (basal ATP activity)	Stimulated PBMC+PHA (ATP ng/mL)	SNR	Stimulated PBMC+PHA+ patient plasma (ATP ng/mL)	SNR	Stimulated PBMC+PHA+ control plasma (ATP ng/mL)	SNR	P
Control n = 10	2988 ± 216	42351 ± 236	1	3762 ± 196	0.62	3313 ± 200	0.26	<0.0059
Cyclosporin n = 17	2406 ± 175	264 9± 178	0.22	2510 ± 207	0.08	1915 ± 150	–0.39	<0.003
Sirolimus n = 10	2250 ± 242	2505 ± 255	0.20	2336 ± 229	0.04	1834 ± 151	–0.33	<0.0361
Tacrolimus n = 6	2325 ± 470	2691 ± 501	0.29	2149 ± 100	–0.14	1252 ± 64	–0.86	<0.0173
p^*	0.6891	<0.0005		0.0055		<0.0001		
p^{**}	0.5228	0.8737		0.3891		0.0388		

Abbreviations: SNR (stimulation normalised ratio) = (ATP increase of case/ATP increase of control); p^*, comparison of all column data including control data; p^{**}, comparison of column data excluding control data; $p < 0.05$ considered statistically significant.

Cultures of PBMCs from transplant patients and healthy controls with phytohaemagglutinin and plasma from healthy controls

However, when plasma from healthy controls replaced plasma from patients in the above experiments, there was a further reduction in ATP production. The decrease was greatest in PBMCs from patients on a tacrolimus-based regimen, with SNR of –0.86 compared to –0.39 and –0.33 for those on cyclosporine and sirolimus, respectively. This difference was statistically significant ($P = 0.0388$; χ^2) (Table 4).

DISCUSSION

The assessment of the immune response has been successfully conducted using an assay approved by the US Food and Drug Administration, known as ImmuKnow™, manufactured by Cylex Inc. (Columbia, MD, USA) [5]. Briefly, it involves an overnight incubation of whole blood with PHA, after which CD4 cells are selected using paramagnetic particles coated with a monoclonal antibody to the CD4 epitope, followed by lysis of the cell and measurement of ATP [6,18]. We propose a more straightforward and less expensive method using PBMCs instead of isolated CD4 cells. This method is easier to set up, much less costly, and much closer to the in vivo situation. It is, therefore, more relevant to real life. The implication of the lower cost of this method means that it is more easily used than the Cylex method when resources are limited [6].

The following aspects of the assay protocol we evaluated differed from the ImmuKnow™ immune cell function assay. Studies by Barten et al. and Copeland and Yatscoff revealed that using whole blood, as in the ImmuKnow™ assay, when stimulating the cells with PHA, allows the lymphocytes to be maintained in the presence of the immunosuppressive drugs, which are partitioned between the membranes of the red cells [19,20]. A prolonged incubation time of 5–7 days without red blood cells results in lymphocytes recovering from suppression and the recall responses and alloreactivity. Because the assay under investigation requires only overnight incubation of PBMCs with PHA in the absence of red blood cells, any concern of the lymphocytes reverting to their original strength is negligible [6]. Transplant recipients are on a variety of immunosuppressant drugs, which inhibit a range of immune cells, not just CD4 T cells. Although cyclosporin and tacrolimus specifically target T lymphocytes, sirolimus, on the other hand, has a non-specific antiproliferative effect. It targets the cell cycle, which includes all dividing cells and

azathioprine and mycophenolate mofetil, preventing the expansion of T cells and B cells [5]. Hence, we resolved to use PBMCs rather than CD4 cells as it is an advantage not to limit the study by isolating CD4 T cells from their natural environment.

An ATP standard was included in each microtitre plate to generate a standard curve, from which the intracellular ATP levels of control and patient test samples could be directly calculated after each assay run. The relative light units measured for control and patient samples were converted to ATP (recorded as ng/mL) based on equivalent relative light unit values in the ATP standard curve. As depicted in Figure 1, the known ATP is plotted on the x-axis, and the amount of light produced is measured in RLU on the y-axis. The ATP standard ranged from 1 ng/mL to 10,000 ng/mL. The linearity of the curve was assessed using the correlation coefficient (r^2).

Our findings correlated with previous studies, which showed that the measure of intracellular ATP is directly proportional to the number of living cells in the sample [21,22]. The relationship between RLU, a measure of the intracellular ATP detected by the luminescent signal, and the number of cells in culture was linear ($r^2 = 0.99$). Converting the RLU values to the corresponding ATP (ng/mL) values using the ATP standard curve confirmed that this assay exhibited a linear correlation between ATP produced and the increasing concentration of cells, that is, from 2,500 to 200,000 cells per well. This finding highlights the sensitivity of the ATP bioluminescence assay.

The results demonstrate also that in vitro PHA stimulation of PBMCs from kidney transplant recipients on cyclosporine-, tacrolimus- and sirolimus-based regimens produces 2 to 3 times less ATP than in normal controls. Based on the percentage increase and the actual ATP production, this assessment method seems to distinguish between patients on immunosuppressive drugs and healthy individuals. However, there were no statistically significant differences in the PBMC response among the three immunosuppressive regimens. Kowalski et al. found a similar result when comparing patients on cyclosporine versus tacrolimus [6]. Although these results need to be confirmed in a larger population, the side effect profile must carry greater weight when choosing a regimen for a given patient. The role or degree of the immunosuppression appears not to discriminate among the three regimens. Also, other immunosuppressive agents and their combination in the triple therapy – that is, steroid and azathioprine or mycophenolate mofetil – may play a significant role that is not adequately acknowledged. The combination of immunosuppressive agents may not necessarily be additive, and indeed it may be synergistic or antagonistic.

Currently, the adjustment of immunotherapy in transplant patients relies essentially on the blood levels produced by individual drugs used to monitor the treatment. Besides transplant patients, our assay can be used to assess any condition with an altered immune response. For example, in autoimmune disorders such as lupus, patients are subjected to powerful immunosuppressive drugs without proper monitoring of cellular immune function. This method could help manage patients to avoid over- or under-immunosuppression. In evaluating HIV-infected individuals, this method may assist in unmasking ineffective CD4 cells instead of waiting and relying on CD4 cell counts only. In vitro assessment of the immune response has great potential. Further investigation will assist in defining and expanding its use.

PHA stimulation of PBMCs from healthy individuals produced a 47% increase in ATP production. The ATP increase was reduced to 14% when normal plasma was added ($P < 0.05$). However, when healthy control plasma was replaced by plasma from patients, the ATP increase improved to 31%. A similar difference between patient and control plasma was recorded using PBMCs from transplant patients (Table 1). It appears that plasma isolated from patients on immunosuppressant drugs reduced PHA-induced stimulation of lymphocytes.

Intuitively, as transplant patients receive various immunosuppressive drugs which are regularly measured in their blood, their plasma should be expected to limit T cell proliferation more strongly than plasma from healthy donors. Indeed and counter-intuitively, plasma from healthy controls not on immunosuppressive medication produces even more significant inhibition of T lymphocyte activation. Therefore, it can be hypothesised that the plasma from healthy individuals contains factors that suppress the response of lymphocytes to PHA stimulation. The reduction of lymphocyte proliferation by human plasma has been reported previously [14]. Alwahaibi et al. have recorded that serum levels of interleukin (IL)-2, a T cell growth factor, were significantly higher in kidney transplant recipients than in healthy controls [23]. The high IL-2 levels may explain why T cell proliferation is greater in the presence of serum or plasma from transplant recipients than from healthy controls. However, it does not explain the reduction in the T cell response to PHA after the addition of plasma from either kidney transplant recipients or healthy controls. Furthermore, a report of IL-2 therapy restoring regulatory T cell dysfunction induced by calcineurin may underscore the complexity of mechanisms at play in the paradoxical observation of T cell activation described here [24].

Factors reported to regulate human lymphocyte proliferation include alpha globulin and lipoproteins [25,26]. Immunoregulation by low-density lipoproteins were shown to inhibit mitogen-stimulated human lymphocyte proliferation after initial activation. Reduced activation of lymphocytes of patients on immunosuppressive drugs is well documented [3,5,8,27,28]. The role of plasma exchange in managing diseases such as vasculitis and Goodpasture disease is well established and has regained interest recently [29-31].

Further analysis of plasma from healthy individuals and transplant patients may help identify factors that may account for the inhibitory effect on lymphocytes. A more extensive study should provide more understanding of the above observation. Some protocols use albumin as a replacement fluid [30,31]. The suppressing role of the plasma on T cell activation in this study should be confirmed in a larger one. An in vivo plasma exchange trial that compares the type of replacement fluid is warranted.

A further clinical implication of the lack of significant differences in T cell responses to PHA, from the patients on the three regimens, may be the role played by antimetabolites such as MMF or azathioprine. In view of the nephrotoxicity of calcineurin inhibitors, a careful further reduction of their dosages, under the umbrella of antimetabolite cover and the ATP–PBMC monitoring test, needs to be considered. Of note, several kidney transplant recipients, from the pre-cyclosporine era, lived with a functional graft for decades, under an azathioprine and prednisone regimen.

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Conflicts of Interest

Alain Assounga was a member of the Astellas Advisory Board on anaemia.

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