Comparison of the Th1, IFN-γ Secreting Cells and FoxP3 Expression between Patients with Stable Graft Function and Acute Rejection Post Kidney Transplantation

Banafsheh Nazari1, Aliakbar Amirzargar1,2, Behrouz Nikbin1,2, Mohsen Nafar3, Pedram Ahmadvour3, Behzad Einollahi4, Mahboob Lesan Pezeshki5, Seyyed Mohammad Reza Khatami5, Bita Ansari5, Hassan Nikuinejad6, Fatemeh Mohamadi1, Mahdi Mahmoudi2, Samaneh Soltani2, and Mohammad Hossein Nicknam1,2

1 Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
2 Molecular Immunology Research Center, Tehran University of Medical Sciences, Tehran, Iran
3 Department of Nephrology, Shahid Labbafinejad Medical Center, Shahid Beheshti Medical University, Tehran, Iran
4 Department of Nephrology, Baqiyatallah University of Medical Sciences, Tehran, Iran
5 Department of Nephrology, Imam Khomeini Hospital, Tehran University of Medical Sciences, Tehran, Iran

Received: 29 June 2012; Received in revised form: 25 September 2012; Accepted: 28 October 2012

ABSTRACT

There are limited clinical investigations identifying the percentage of T helper 1 (Th1) and T regulatory (Treg) cells in stable as well as rejected kidney allografts, a concept which needs to be more studied. The aim of our study was to compare the percentage of CD4+ IFN-γ+ cells, the number of IFN-γ secreting cells and the amount of FoxP3 expression in patients with or without stable graft function, to determine the roles of these immunological factors in stable and rejected renal allografts.

In this prospective study, 3 months after transplantation 30 patients who received renal transplants from unrelated living donors were enrolled and divided into two groups, 20 patients with stable graft function and 10 patients with biopsy proven acute rejection.

The percentage of Th1 CD4+ IFN-γ+ cells was determined on PBMC by flow cytometry and the number of IFN-γ secreting cells by ELISPOT method. Furthermore, FoxP3 expression of PBMCs was measured by Real Time PCR method. The results of these assessments in both groups were statistically analyzed by SPSS 14.0. Our results showed that the percentage of Th1 CD4+ IFN-γ+ cells and the number of IFN-γ secreting cells were significantly higher in the patients with acute rejection in comparison to the stable graft function group (p<0.001). In addition, the level of FoxP3 gene expression was higher in the group with stable graft compared to the acute rejection group.

The higher percentage of CD4+ IFN-γ+Th1 subset and number of IFN-γ secreting cells and also the lower expression of Foxp3 could prone the patients to acute rejection episode post transplantation. By these preliminary data, it is suggested that monitoring of Th1 cells post transplantation, as an immunologic marker could predict the possibility of rejection episodes.

Keywords: Graft Rejection, Interferon-gamma, Kidney Transplantation, Th1 Cells

Corresponding Authors: Mohammad Hossein Nicknam, MD, PhD; and Aliakbar Amirzargar, PhD; Molecular Immunology Research Center and Immunogenetic Laboratory, Department of Immunology, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran. Tel: (+98 21) 8895 3009, Fax: (+9821) 6641 9536, E-mail: nicknam_m@yahoo.com
IFN-γ and FoxP3 Expression in Kidney Transplantation

INTRODUCTION

End Stage Renal Disease (ESRD) lowers quality of life of the patients and dialysis as a therapeutic method increases the risk of a variety of diseases such as Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infection. Renal transplantation can change the patient’s life to be more effective. Although the immunosuppressive drugs can increase the chance of the graft acceptance, allograft rejection happens as a result of post transplant immune reactions. It has been shown that 3 months and 1 year survivals of transplanted kidneys from living donors are 99.5% and 98.5%, respectively.¹

Reduction of the immunosuppressive drugs to the maintenance dose with the least side effects and a graft with an optimum function are the goals of transplantation medicine. In this regard, it is important to know the exact cellular and immunological mechanisms of the allograft rejection process and to find a way inducing tolerance and reducing the specific immune responses to transplant antigens.

Undoubtedly, CD4+ IFN-γ+ Th1 cells are very crucial cells in triggering immune responses. Different studies suggest that these cells can cause transplant rejection.² It has been previously shown that the IFN-γ knockout mouse models are rejection tolerant. This finding provided a key role of IFN-γ T cells to initiate the rejection process, however further studies revealed conflicting findings. In the absence of IFN-γ, the allografts in heart and kidney transplant animal models not only showed stability but also showed an accelerated rejection associated with increased parenchymal necrosis.³⁴ In addition, it has been reported that patients with clinical signs of acute rejection show higher serum levels of IFN-γ and IL-2 than those with a functional effective allograft in pre and post-transplant periods.⁵⁶

In T cell-mediated rejection, IFN-γ is a prominent cytokine in the rejection process.⁷⁸ Both CD4+ and CD8+ Treg cells express FoxP3 protein, but CD4+ Treg cells induce more effective immunological reactions in regulation of immune responses⁹ and are the main Treg-cell population which express the gene of FoxP3 in thymus and peripheral tissues.¹⁰ It has been shown that the induction of FoxP3 gene expression in naïve T cells leads to their differentiation toward regulatory T cells. This gene encodes forkhead box P3 transcription factors and its dysfunction can cause autoimmune diseases that affect multiple organs in humans and mice, a concept which shows the importance of this gene.¹¹ The exact paths of Treg function is not well known yet. However, different mechanisms such as production of inhibitory molecules and cytokines, competition with other T cells for IL-2 production, and interaction with the APCs have been mentioned.¹² According to a variety of data in Th1 and Treg roles in the pathogenesis of kidney transplantation rejection, we evaluated the changes of peripheral blood Th1 and Treg cells in patients receiving an unrelated living kidney transplant.

PATIENTS AND METHODS

This double center study from 2010 to 2011 was performed under an Ethics Committee-approved protocol at Tehran University of Medical Sciences. After filling out informed written consent in the study, all patients receiving graft from unrelated living donor, including 20 men, and 10 women with mean age of 48.8 years old were enrolled in this study. Pre-existing conditions in most of the studied patients as shown in table 1 were blood pressure, nephrolithiasis and diabetes (Table 1). Patients receiving cadaveric or second graft were excluded. Acute rejection defined in terms of increase patients’ serum creatinine, which was proved by biopsy. Stable graft function characterized by showing balanced serum creatinine and no experience of delayed graft function. In this study, 10 ml of the patients’ blood specimens were collected in EDTA containing tubes and used for flow cytometry analysis, ELISPOT assay, and Real time PCR. All samples were collected three months after the transplantation. The patients in both groups underwent the same protocol of adjusted immunosuppressive therapy (Cyclosporine, Prednisolone and Mycophenolate mofetil). All the patients in acute rejection group were biopsy-proven. The obtained data from each group was compared and statistically analyzed by spss version 14 software.

PBMC stimulation and flow cytometry analysis PBMCs were isolated from blood samples. The PBMCs were cultured alongside PMA (Phorbol myristate acetate) /Ionomycin (Calcium Ionophore) and BD GolgiStop™ for 5 hours. Afterward the cells were fixed by cold BD Cytofix™ Fixation Buffer. BD Perm/Wash™ buffer permeabilized the fixed PBMCs.
Table 1. Demographic data and clinical characteristics of the studied patients.

<table>
<thead>
<tr>
<th>Topics</th>
<th>Stable Graft</th>
<th>Acute Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Female/ Male</td>
<td>7/13</td>
<td>5/5</td>
</tr>
<tr>
<td>Mean age</td>
<td>38.8±14.54</td>
<td>36.8±8.45</td>
</tr>
<tr>
<td>Disease type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood Pressure</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>Diabetes</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Polycystic Kidney disease</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Nephrolithiasis</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>IgA Nephropathy</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Nephrotic Syndrome</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Kidney stone</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Then cells were stained by fluorescent monoclonal antibodies, specific for human CD4/IFN-γ and analyzed by BD FACS calibur flow cytometer and WinMDI 12.9.

By using lymphocytic gate, CD4+ T cells were gated and then the percentage of CD4+IFN-γ+ cells were measured and compared between the two groups. Independent-sample T test was used for comparison of the differences between two groups.

**Elispot**

ELISPOT test was divided into two steps; aseptic and non-aseptic. Aseptic Procedure was performed in laminar flow hood. Capture antibody diluted in sterile ELISPOT Coating buffer (ratio 1 to 250) with a volume of 100µl was added to each well of the plate and was incubated overnight at 4°C, then washed two times with sterile ELISPOT Coating Buffer, each well was filled by RPMI-1640 and incubated at 37°C for one hour and then 100µl of 10^5 transplant recipient’s cell suspension was added. Six wells were allocated to each sample. The first two wells were considered as positive controls and contained 4 µl mitogen (PHA), the second two wells were used as negative controls, and the next two wells were added by donor and recipient cells (ratio 1:5, respectively). The plates were incubated for 48 hours at 37°C with 5% CO2. In Non-aseptic procedure, wells were emptied and washed 3 times. Diluted biotinylated detection antibody (11µl) was added to each well and decanted after 2 hours of incubation. Each well was washed 4 times by ELISPOT Wash Buffer and after adding diluted Avidin-HRP reagent, the plate was washed 5 times: 3 times with ELISPOT Wash Buffer and 2 times with PBS. Then it was incubated with 100µl/well freshly prepared AEC Substrate Solution for one hour. The reactions were stopped by washing each well 3 times with 200 µl distilled water. The spots representing the IFN-γ secreting cells were counted by dissecting microscope.

**Real Time PCR**

Total RNA was extracted from PBMC using High Pure RNA Isolation Kit, Cat No:11828665001, Roche Applied Science. cDNA synthesized from the extracted RNA using Transcriptor First Strand cDNA Synthesis Kit, Cat No: 04897030001, Roche Applied Science. The amount of FoxP3 gene expression was measured through Taqman primer probe Comparative CT method using ABI 7300 Real-Time PCR system. Briefly, this method is based on comparison the expression of a gene with an other gene and shows relative amount of target genes in patients’ samples. Acute Rejection group was determined as test group and Stable graft group was considered as control group. β-Actin house keeping gene was used as endogenous control. 2^\Delta\Delta CT showed relative fold changes of gene expression.

**RESULTS**

The results of this study showed that the percentages of Th1 CD4+IFN-γ+ cells of peripheral blood of rejected graft patients were significantly higher than those in the stable graft patients (8.66±1.9 vs. 17.7±2.8, p=0.00) (Figure 1).
IFN-γ and FoxP3 Expression in Kidney Transplantation

Figure 1. CD4+ IFN-γ+ cell percentages in flow cytometry analysis. Percentage of Th1 cells were significantly higher in Acute Rejection group compared to stable kidney graft group.

Figure 2. Result of ELISPOT assay showed higher spots in acute rejection group compared to stable graft group. Each spot represents amount of IFN-γ secretion by cells in a patient’s blood sample.

Figure 3. Analysis of comparative Foxp3 gene expression showed that in Stable graft group FoxP3 gene expression was higher than Rejection group.

The data obtained from ELISPOT assay also indicated considerable increase in the number of spots among the acute rejection patients (230.6±13.7 vs 54.4±11.9, p=0.00) suggesting a highly significant increase in the IFN-γ+ secreting population (Figure 2).

FoxP3 gene expression was determined by the comparison of FoxP3 ∆CT changes in the sample group (patients with acute rejection) with the control group (patients with stable graft). The level of FoxP3 gene expression in Stable graft group was 1.63 times higher than the acute rejection group p.value (Figure 3).

DISCUSSION

Allograft acute rejection is a cause of death in kidney transplant recipients and can lead to the development and progression of chronic rejection.6, 12, 13 T cells as major mediators of immune responses to the grafts can cause acute rejection through different cytokines.14 Several studies have shown that acute rejection is mediated by Th1 subset of T cells through IFN-γ dependent delayed-type hypersensitivity responses and cytotoxic T-cell stimulation.15 IFN-γ can provide and maintain inflammatory environment through induction of Th1 cells as well as Nitric oxide...
(NO) and indoleamine 2, 3 dioxygenase (IDO) where it can cause suppressive and anti-inflammatory effects through induction of Treg cells.

Therefore, it is postulated that IFN-γ has contradictory effects on immune responses. The important role of T cells in transplant immunology motivated us to design this study to evaluate effect of Th1 and Treg cells on acute rejection procedure. Our results were similar to the findings of D’Elios et al. They studied acute rejected grafts and concluded that the inflammatory condition caused by Th1 cells was due to allo-immune responses.

A. Loverre et al studied on 72 renal transplanted patients and showed that the number of Th1 cells decreased in the circulation of patients undergoing delayed graft function (DGF) 24 hours after transplantation and the rate of T-bet+ cells raised in the allograft of these individuals compared with those without DGF. They concluded that such early reduction of Th1 cells in the circulation of the patients with DGF should be due to migration of Th1 cells to the inflammatory environment of the graft. Therefore, it seems that the results of their study were not in accordance with ours, but it is noteworthy that in our study, levels of Th1 cells were evaluated 3 months post transplantation. This longer time could cause raising of the Th1 cells in the graft as well as in the circulation. The higher number of Th1 cells in our study could reveal that these cells make a pivotal role in the alloimmunization and finally in acute rejection episodes.

In contrast with the inflammatory role of Th1 cells, Treg cells can cause anti-inflammatory effects and prolong graft survival by their immune suppressing function. Also allogeneic transplantation mouse models have shown that the induction and maintenance of transplantation tolerance is mainly mediated by Treg cells. Some studies have pointed out to the fact that Tregs cells can reduce graft injury by inhibiting alloimmune responses. Demirkiran and his colleagues showed that the percentage of Treg cells reduced in patients with acute rejection episodes during the first year after transplantation compared to non-rejecting group. This reduction was statistically significant.

Wang and his colleagues in 2011 indicated that the percentage of FoxP3+CD4+ cells in the peripheral blood of the patients undergoing acute rejection increased when compared to the patients with functioning transplants. They explained that this unexpected finding was because of T-cell activation, since it was reported that FoxP3 could also be considered as a T-cell activation marker. They also reported an increased FoxP3 gene expression in the grafts undergoing acute rejection to stable graft function. In contradiction with these findings, our study indicated that levels of FoxP3 gene expression were higher in patients with functional renal transplants compared to patients with acute rejection. Due to Treg suppressive and regulatory effects of Treg in the graft inflammatory environment. Wang and his colleagues reported that Foxp3+ Treg-cell removal from mouse models of kidney transplantation could cause kidney rejection. They showed a relationship between Foxp3+ cells and tolerance induction.

Taken together, our findings indicated the importance of Th1 cells in acute rejection mechanism and significance of Treg cells in induction of graft stability. Also this study showed the importance of IFN-γ and its ability to alter the outcome of transplantation. The importance of other cytokines such as IL-2, IL-6 and IL-15 on acute rejection development should be studied. It is suggested to consider Th1/ Treg cell ratio changes in peripheral blood of patients with allograft kidney transplantation as a predictor factor for graft survival.

ACKNOWLEDGEMENTS

The authors are thankful to the Tehran University of Medical Sciences grants commission for funding this project (grant no. 9959).

We are also grateful to the staff of the transplantation ward at Shahid Labbafinejad Medical Center, Shahid Beheshti Medical University and Baqiyatallah University of Medical Sciences for their kind collaboration in taking the blood samples and collecting clinical data.

Declaration of conflicts of interest: The authors have no conflict of interest.

REFERENCES

3. Miura M, El-Sawy T, Fairchild RL. Neutrophils mediate


