

Comparing IRF-4 Gene Expression Between Acute T cell-Mediated Rejection and Stable Renal Transplant Recipients

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Introduction. Renal transplant rejection is one of the clinical challenges, which usually requires administration of immunosuppressive drugs causing serious side effects. Therefore, invention of effective and specific therapeutics is necessary to control undesired immune responses particularly T-cell reactions to allograft. Interferon Regulatory Factor-4 (IRF-4) due to its implication on T cells differentiation and function might be targeted to treat T cell-mediated cellular rejection (TCMR). The aim of this study was to investigate the association between IRF-4 gene expression and acute TCMR, as well as to examine the correlation between IRF-4 gene expression and cellular expression of Programmed cell death-1 (PD-1) and Helios molecules.

Methods. Peripheral blood samples were obtained from 30 patients with biopsy proven acute TCMR and 30 stable recipients. IRF-4 gene expression was quantified using RT-PCR, and cellular expression of PD-1 and Helios were evaluated with flowcytometry.

Results. IRF-4 gene expression was significantly increased in acute TCMR patients compared with stable recipients ($P < .05$). Helios protein expression was slightly decreased in TCMR group but this was not statistically significant. There was a negative correlation between IRF-4 gene expression and PD-1 as well as Helios frequency in the whole studied population.

Conclusion. IRF-4 expression increases in acute TCMR which might also lead to a diminished expression of downstream immunoregulatory molecules such as PD-1 and Helios. Therefore, specific inhibition of IRF-4 may be helpful in managing acute TCMR.

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INTRODUCTION

In recent years renal transplantation has been accepted as an appropriate therapeutic choice for end stage renal diseases (ESRD);¹ however, transplant rejection has still been remaining as the main obstacle in this area. Although immunosuppressive agents could prevent - and once occurred- control graft rejection, these drugs has been demonstrated to impose metabolic disturbance and predispose

the patients to various infectious and neoplastic disorders due to the non-specific suppression of immune system.² For that reason, there are efforts to discover the critical components of immune responses in order to restrain alloimmunity in a more specific manner.³ Among proposed molecules Interferon regulating factors (IRF) family and at top of the list IRF-4, has been introduced as one of the most determining checkpoints in developing

immune responses.⁴

IRF-4 has been shown to be involved in differentiation of naïve CD4+ T cells into T helper 2 (Th2), Th9, Th17, and T follicular helper cells. It cooperates with other transcription factors such as Nuclear factor of activated T-cells (NFAT), basic leucine zipper transcription factor ATF-like (BATF), RAR-related orphan receptor gamma (ROR- γ t), PU.1, SMAD2/3, and B-cell lymphoma 6 (BCL-6) for inducing various T effector cells; however, its role in differentiation and activation of Th1 has not yet been completely understood.^{5,6} IRF-4 also appears to participate in T lymphocytes maintenance, for example in an animal study it was demonstrated that TCD4+ helper cells are significantly prone to apoptosis in *irf-/-* mice when stimulated with *Leishmania major* parasite.⁷

Programmed cell death-1 (PD-1) is a surface receptor involved in T lymphocytes activation-induced cell death. PD-1/PD-L interaction is implicated in peripheral tolerance induction and protects tissues against autoimmune responses.⁸ Helios, a member of IKAROS family zinc finger 2 (IKZF2), is an intra-nuclear transcription factor, which induces natural Treg cells development. Besides, it has been shown that Helios is upregulated in CD4+ CD25+ Treg cells, thus suppression of Helios could diminish the suppressive activity of this subset.⁹ According to the considerable implication of IRF-4 in expansion and function of T lymphocytes, we aimed to evaluate the gene expression of IRF-4 and cellular expression of two molecules negatively regulated by IRF-4 -PD-1 and Helios¹⁰ in stable recipients and acute T cell-mediated rejection (aTCMR) cases. Moreover, as Th1 cells are the main subset responsible for cellular rejection,^{11,12} finding any correlation between IRF-4 expression and TCMR might be suggestive of IRF-4 implication in development and/or activity of Th1 cells.

MATERIALS AND METHODS

Patients

Two groups of adult (18 to 60 years old) renal transplant recipients were enrolled in present study including stable recipients and biopsy proven acute T Cell-mediated rejection (aTCMR) cases. Acute TCMR diagnosis was based on 2018 *Banff Classification of Renal Allograft Pathology* according to the interstitial inflammation (i), tubulitis (t) and

intimal arteritis (v) scores.^{13,14} Samples indicating chronic TCMR, antibody-mediated rejection (ABMR) or mixed TCR/ABMR were excluded from the study. Only 4 patients had donor specific antibody (DSA) tests which all of them were negative. Blood sampling was performed before any therapeutic intervention. Sex- and age-matched stable recipients were selected according to the clinical and laboratory criteria (no creatinine rise in the past three months, no proteinuria, normal physical examination, and -if available- normal biopsy). The patients with any active infectious, autoimmune or allergic disease were excluded. After describing the aims of study, informed consent was obtained from all participants. The laboratory and demographic data of the patients are represented in Table.

RNA Extraction and cDNA Synthesis

RNA was isolated from blood samples using High Pure RNA Isolation kit of Roche Diagnostics (Mannheim, Germany) according to the manufacturer instructions. RNA quality was assessed by NanoDrop1000 spectrophotometer (Thermo Scientific, USA) and samples with A260/A280 ratios: 1.8 to 2.2, and A260/A230 ratios: 2 to 2.2 were presumed as acceptable. RNA reverse transcription to cDNA was performed using Transcriptor First Strand cDNA Synthesis kit of

Table 1. Clinical and Laboratory Data of Studied Population

Group	Stable Recipients	Acute TCMR	P
N	30	30	
Age, y	46.5 ± 13.8	44.4 ± 12.6	> .05
Gender Ratio (M : F)	20 : 10	21 : 9	
Creatinine	1.23 ± 0.33	2.51 ± 1.1	< .001
Urea	30.48 ± 8.4	45 ± 15.6	< .05
GFR	65.95 ± 16.14	28 ± 10.5	< .001
WBC	7040 ± 1745	6972 ± 1940	> .05
TX, y	4.33 ± 2.7	3.87 ± 2.3	> .05
IS Protocol:			
Cyclo/Myco/Pred	9	5	
Tac/Myco/Pred	15	18	
Aza/Cyclo/Pred	5	1	
Tac/Rap/Pred	1	3	
Tac/Aza/Pred	0	2	
Tac/Ever/Pred	0	1	

Abbreviations: TCMR, T cell mediated rejection; GFR, glomerular filtration rate; WBC, white blood cells; TX, transplantation; IS, immunosuppressive; Cyclo, cyclosporine; Tac, tacrolimus; Myco, mycophenolate; Pred, prednisone; Aza, azathioprine; Rap, rapamycin; Ever, everolimus (Data are presented as mean ± SD).

Roche Diagnostics (Mannheim, Germany). cDNA quality was also evaluated by NanoDrop1000 spectrophotometer (Thermo Scientific, USA) and samples with A260/A280 ratios: 1.7 to 2 were stored in -70 °C until use.

Gene Expression Assay

The relative gene expression evaluation was performed using TaqMan Gene Expression Assay. In the real-time polymerase chain reaction (RT-PCR) procedure, each well contained 10 µl Master mix, 1 µL assay mix (interferon regulatory factor 4, reference Hs00180031_m1), 7 µL H2O and 2 µL of diluted sample cDNA of 5 ng/µL, the final reaction volume was 20 µL. The endogenous control was the GAPDH (Hs99999905_m1). The mixture was incubated for 2 min at 50 °C, for 10 min at 95 °C, followed by 45 cycles of 15 sec at 95 °C and 60 sec at 60 °C in Applied Biosystem StepOnePlus Real-Time PCR System (Applied Biosystem, Foster City, CA, USA). The experiments were performed in duplicates.¹⁵ Threshold cycle number was utilized to figure the relative expression between samples. We used the $\Delta\Delta Ct$ (cycle threshold) procedure from which the relative expression = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (\Delta Ct \text{ of TCMR}) - (\Delta Ct \text{ of SR})$.¹⁶

Cell Separation and Flowcytometry

Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-hypaque (inno-train, Germany) gradient. Cells were frozen in FBS with 10% DMSO and stored in liquid nitrogen container tank until the end of the sampling. Thereafter, they were adjusted to a concentration of 1×10^6 cells/mL and stained with mouse PE anti-human CD279 (PD-1)

conjugated Antibody (biolegend United Kingdom). Intranuclear staining was performed for Helios transcription factor using FITC anti-mouse/human Helios Antibody (biolegend USA) according to the manufacturer instruction. Flowcytometry was performed using FACSCalibur (BD FACS Calibur Becton Dickinson, USA); data were analyzed with FlowJo_V10 (FlowJo, USA) software. Unstained cells from each sample were used as control.

Statistical Analysis

Data were presented as mean \pm SD or mean \pm SEM (mentioned in text). One-way ANOVA test was used for comparison between groups. Correlations between scale numeric variables were analyzed by correlation bivariate and linear regression (SPSS 18.0; SPSS Inc., Chicago, USA). *P* values less than .05 were considered as significant.

RESULTS

PD-1 and Helios Expression in Peripheral Blood Mononuclear Cells (PBMC)

The flowcytometric evaluation of PD-1 molecule expression showed no significant difference between patients with aTCMR and stable recipients (6.22 ± 0.89 vs. $6.65 \pm 1.02\%$, mean \pm SEM; *P* > .05) (Figures 1 and 2). Helios expression was slightly lower in patients with aTCMR than in stable recipients but this difference was not statistically significant (0.6 ± 0.09 vs. $0.87 \pm 0.1\%$, mean \pm SEM; *P* > .05) (Figures 1 and 2).

IRF-4 Gene Expression in PBMCs of Acute TCMR and Stable Recipients

The relative expression of IRF-4 gene (to GAPDH

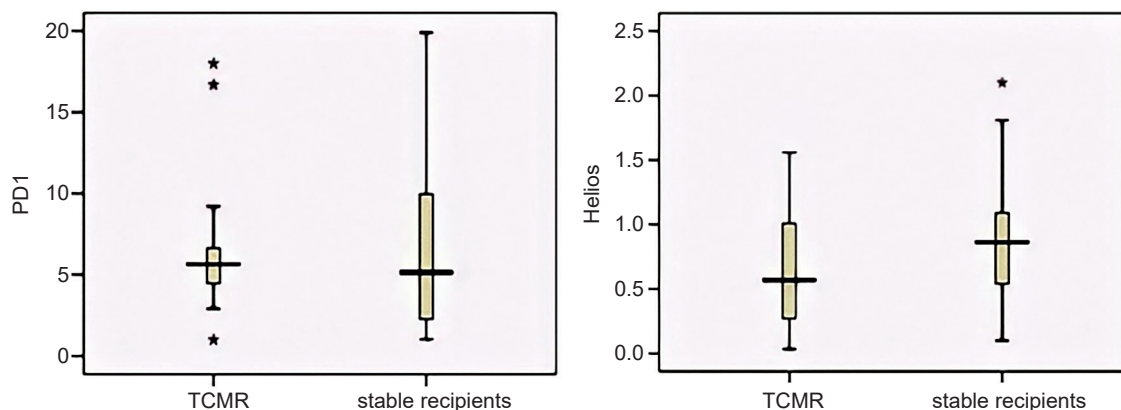


Figure 1. Percentage of PD-1 and Helios Expression in Peripheral Blood Mononuclear Cells of Acute TCMR and Stable Recipients

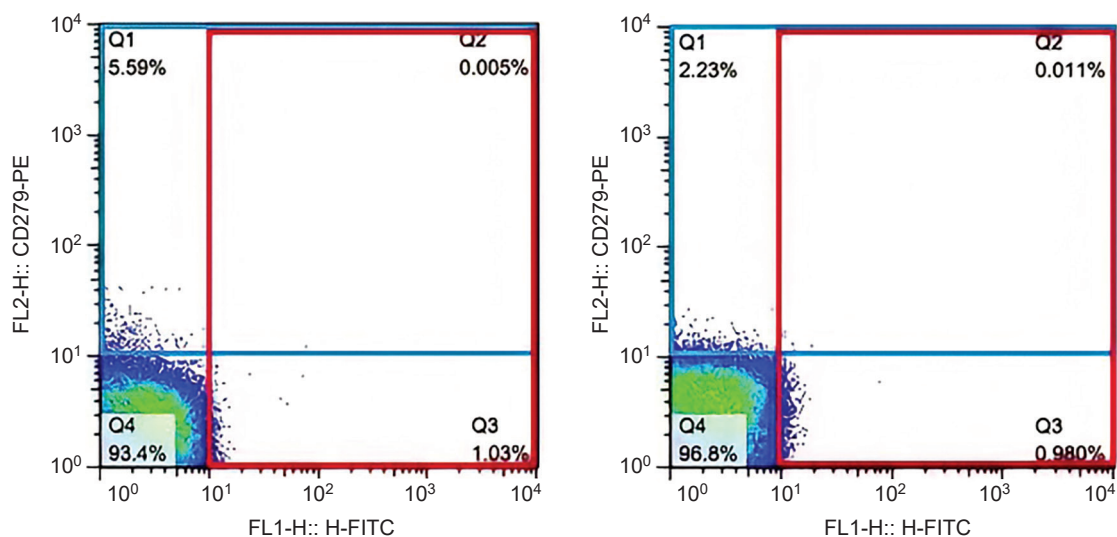


Figure 2. PD-1 (CD279+ Cell [Blue Cadre]) and Helios (H+ Cells [Red Cadre]) Expression Percentage in PBMCs of a Stable Recipient (Left) and an Acute TCMR Patient (Right)

internal control) was significantly increased in patients with acute TCMR comparing to the stable recipients (1.72 ± 1.04 vs. 1.04 ± 0.91 , mean \pm SD; $P < .05$) (Figure 3).

Correlation Between IRF-4 Gene Expression and Molecular Expression of PD-1 and Helios

IRF-4 gene expression showed a significant fair negative correlation with PD-1 frequency on PBMCs with Pearson correlation coefficient of -0.46 ($P < .001$) and R Square of 0.217 . Helios expression was also negatively associated with IRF-4 gene expression in studied population with Pearson correlation coefficient of -0.51 ($P < .001$) and R Square of 0.261 (Figure 4).

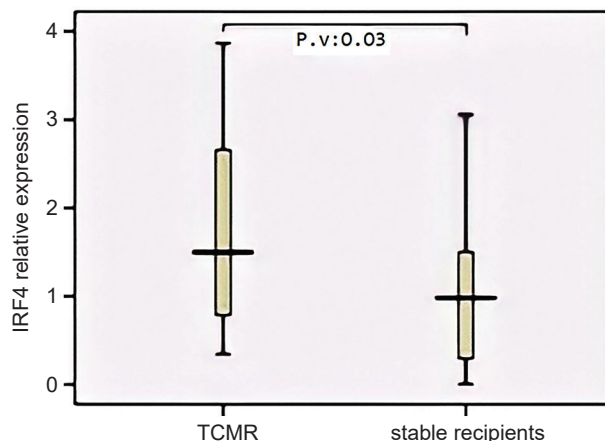


Figure 3. The Relative Expression Level of IRF-4 Gene (IRF4/GAPDH) in Peripheral Blood Mononuclear Cells of Patients with Acute TCMR and Stable Recipients ($P < .05$)

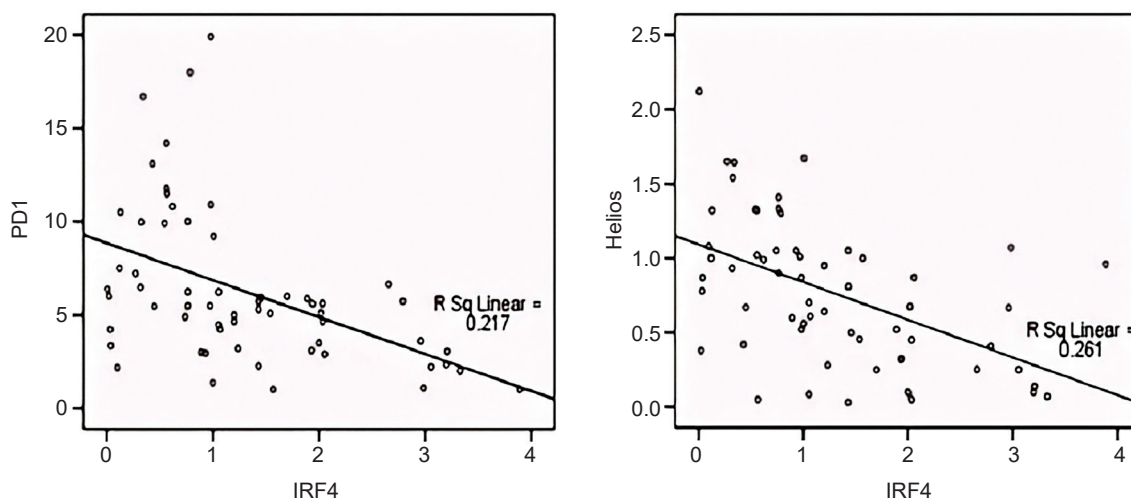


Figure 4. Negative Correlation Between IRF-4 Gene Expression and Molecular Expression of PD-1 and Helios in PBMCs of All Studied Recipients

DISCUSSION

During recent years, Interferon Regulatory Factor-4 has gained interest in various clinical branches; over expression of IRF-4 has yet been demonstrated in rheumatologic diseases such as rheumatoid arthritis and lupus erythematosus.^{17,18} Moreover, cancer studies have shown its involvement in a wide range of hematologic and non-hematologic malignancies; for instance, IRF-4 expression is upregulated in lymphoma, melanoma, multiple myeloma, nonsmall cell lung cancer and many other cancers.^{19,20} In transplantation, the knockout or suppression of IRF-4 gene in animal models has shown beneficial effects on graft survival. It was observed that inhibition of IRF-4 by IRF-4 siRNA resulted in decreased levels of proinflammatory cytokines IL-6, TNF- α and IFN- γ , increased amounts of anti-inflammatory cytokine IL-10 and more M2 macrophage differentiation which, as a result, significantly promoted mice liver transplant and reduced acute rejection scores.²¹ It was also found that Tacrolimus exerts at least a part of its immunosuppressive function in controlling acute liver transplant rejection via downregulation of IRF-4.²² Likewise, Bortezomib -a proteasome inhibitor- increases miR-15b expression that inhibits IRF-4 expression and consequently suppresses T follicular helper CD4⁺ cells' proliferation and differentiation in mice model of renal transplant rejection.²³ IRF-4 is also implicated in acute heart allograft rejection. Wu *et al.* demonstrated that IRF-4 inhibition led to the PD-1 and Helios genes over expression and improved mice heart graft survival. Heart transplant from IRF-4-deficient mice showed significantly better outcomes comparing to the wild type. Furthermore, this study exhibited that CD4⁺ T lymphocytes are the main cells suppressed due to the IRF-4 deletion. T cells dysfunction (consequent to the IRF-4 suppression) was irreversible after 30 days, the result which introduces IRF-4 as an appealing molecule to manipulate for preventing allograft rejection.¹⁰

Our study on human renal transplantation showed comparable findings. IRF-4 gene expression was significantly elevated in acute TCMR patients. Helios frequency in peripheral blood mononuclear cells was slightly decreased in rejecting cases compared to the stable recipients; however, PD-1 expression was almost similar between two groups. In addition, statistical analysis revealed

a fair negative correlation between IRF-4 gene expression and PD-1 as well as Helios frequency in PBMCs of all studied population which confirms the previous findings about negative regulation of these molecules by IRF-4(10). These and above-mentioned results suggest a detrimental role for IRF-4 in transplantation and propose it as a suitable factor to be targeted in immunosuppression particularly for treating acute TCMR. On the other hand, one animal study showed that during chronic infection, IRF-4 along with Basic Leucine Zipper ATF-Like Transcription Factor (BATF) and nuclear factor-activated T cells c1 (NFATc1) could induce exhausted TCD8⁺ phenotype and inhibited memory T cell formation probably through downregulation of transcription factor T cell factor 1 (TCF1);²⁴ suggesting a positive role of IRF-4 in transplantation. Furthermore, the critical role of IRF-4 in development, proliferation and activation of T and B lymphocytes, dendritic cells and macrophages should be taken in account while complete lack of IRF-4 has been shown to impair immune responses in mice knockout models.²⁵

In addition to the abovementioned study about Tacrolimus, Tang *et al.* in a more recent experiment on animal model of liver transplantation demonstrated that Tacrolimus suppressed the function of IL-21-producing T follicular helper via impeding the expression of the BATF/JUN/IRF4 complex, thus prevented acute graft rejection.²⁶ Since Tacrolimus has been widely used for protecting the allografts from immune attack,²⁷ it might be helpful to identify its detailed mechanisms of action in order to improve efficiency. We could not find any statistical correlation between Tacrolimus administration and IRF-4 expression (Data not shown) maybe due to the unequal number of patients receiving Tacrolimus⁴⁰ and cyclosporine.²⁰

Although present study has focused on implication of IRF-4 in TCMR, antibody mediated rejection (AMR) could also be affected by expression level of this transcription factor. It has yet been shown that IRF-4 along with the other member of IRF family -IRF-8- are profoundly involved in B lymphocytes differentiation and maturation such as pre-B cell induction, B cell tolerance, marginal zone B cell initiation, B lymphocytes proliferation in response to the external stimulations, germinal center reaction and finally plasma cell generation.^{25,28} Therefore, blocking IRF-4 activity might be beneficial for

preventing from AMR particularly for presensitized transplant recipients. In present study, we have focused on T-cell mediated rejection to explore the role of IRF-4 in Th1 cells alloreactivity; however, it is suggested to conduct a similar study in pure AMR cases in order to reveal the implication of IRF-4 in antibody mediated rejection.

According to the negative consequences of IRF-4 activation for transplantation outcome (as well as certain rheumatologic diseases and malignancies), partial inhibition of this factor might be recommended for managing certain clinical situations. For this purpose, there have been a few studies conducted, for instance, Yang *et al.* found that IRF-4 inhibition using IRF4-siRNA decreased experimental autoimmune encephalomyelitis (EAE) scores, diminished Th1 and Th17 cells number in brain tissue and increased the infiltration of Treg cells. IRF4-siRNA could also restrain Th1 and Th17 cell development in vivo and in vitro. In addition, in Dendritic/T cells co-culture, IRF4-siRNA-treated DCs induced significantly less IFN- γ and IL-17 secretion from T cells. It was also observed that adoptive transfer of CD11c+ DCs from IRF4-siRNA-treated mice could ameliorate EAE because these DCs exhibited reduced expression of IL-6, and TNF- α cytokines while increased IL-10 expression.²⁹ According to the recent trends in interfering with genetic pathways for prevention and treatment of rejection,³⁰ one study showed that selective targeting of IRF-4 by synthetic microRNA-125b-5p could control multiple myeloma (MM) both in vitro and in vivo;³¹ finally, there is evidence of downregulation of IRF-4 levels in MM cell line and bone marrow samples following Lenalidomide administration, a drug which is used for treating hematological malignancies in combination with chemotherapy.³² Although these findings suggest IRF-4 inhibition for protecting the allograft from rejection, further studies are required to find optimal methods to inhibit this factor which do not interfere with the normal development and function of immune cells.

CONCLUSION

Taken together, IRF-4 appears to play a detrimental role in transplantation as its expression increases in acute T cell mediated rejection and its expression level shows a negative correlation with PD-1 and Helios immunoregulatory factors'

frequency. Therefore, IRF-4 inhibition might be suggested as a therapeutic option for selective immunosuppression post-transplantation.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ETHICAL APPROVAL

All the techniques carried out in present study involving human participants were in accordance with the standards of the institutional research committee and with the Helsinki declaration and its later amendments or comparable ethical standards. It has ethical approval of Tehran university of medical sciences (code: IR.TUMS.CHMC.REC.1970.040).

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