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Original Article

Determination of Pre-Transplant Mean Counts of CD 28 Negative T Lymphocytes in Early Acute Rejectors and Non - Rejectors of Renal Allograft

Muhammad Aftab Hassan, Hamid Nawaz Tipu, Muhammad Omair Riaz, Sara Waqar Khan, Mustajab Alam

Department of Immunology, Armed Forces Institute of Pathology, Rawalpindi/National University of Medical Sciences (NUMS) Pakistan

ABSTRACT

Objective: To determine pre-transplant mean counts of CD 28 negative T lymphocytes in early acute rejectors and non rejectors of renal allograft.

Study Design: Prospective longitudinal study.

Place and Duration: Department of Immunology, Armed Forces Institute of Pathology Rawalpindi, Pakistan from Nov 2023 to July 2024.

Methodology: The study population included 45, ABO compatible, Human leukocyte antigens matched patients undergoing living-donor kidney transplantation. Expression of CD 28 on T lymphocytes was detected by flowcytometry. After 3 months of transplantation those who had normal renal function test were included in the non-rejectors group and those who had biopsy proven rejection were included in the rejectors group. Independent samples t-tests were used to compare mean cell counts between rejectors and non-rejectors, with a *p*-value <0.05 considered statistically significant.

Results: Mean counts (cells/ ul) and percentages of CD 28 positive and negative T cells were determined. Within three months of transplant, 13.3% (6 patients) of the participants developed early acute rejection, and the mean age of rejectors was significantly lower (27.50±10.07 years) than those of non-rejectors (38.7±10.27 years; *p*=0.017). The percentage of CD 3+ CD 28^{-}T lymphocytes was significantly lower in rejectors (21.1±2.8%) compared to non-rejectors (25.2±8.2%), with a statistically significant difference (*p*=0.02).

Conclusion: In this study, renal transplant recipients with high numbers of 28 negative T cells had lower incidence of early acute rejection. This knowledge may be used to guide further studies to evaluate the role of CD 28 as predictive marker in renal transplant.

Keywords: Graft rejection, Kidney transplantation, Renal replacement therapy, Transplantation immunology.

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INTRODUCTION

Chronic kidney disease (CKD) is one of the major public health problems globally with estimated prevalence of 13.4%.[1] According to previous studies CKD prevalence in Pakistan was reported to be 12.5% - 29.9%.[2] In CKD patients, kidney transplantation is considered the preferred treatment as successful transplantation leads to reduced mortality and morbidity.

Currently renal allograft is monitored through a complex process involving physical examination, immunological investigations and histopathological findings on renal biopsy using Banff Classification.[3] This extensive workup and risk associated with renal biopsy are not only cumbersome for patients but also contribute to financial burden. Therefore, new biomarkers (e.g donor-derived cell free DNA, micro RNA) have been the focus of ongoing medical research to overcome these limitations and to improve allograft monitoring.[4,5] One such marker is expression of CD 28 on T lymphocytes. CD 28 negative (CD 28-) T lymphocytes can serve as a potential risk biomarker in renal allograft recipients as high counts of CD 28- cells are associated with a lower risk of transplant rejection.[6] At birth, virtually all human T cells express CD 28 which is a co-stimulatory molecule and plays a role in T cell activation. In young adults, up to 20-30% of their CD 8 T lymphocytes lose CD 28 expression.[7] In ESRD pro-inflammatory uremic environment leads to premature immune senescence and dysfunctional T-cell immunity.[8,9]

Therefore, it is reasonable to hypothesize that pre-transplant levels of CD 28- cells may be different among the early acute rejectors and non-rejectors of renal allograft. This study was done to ascertain the levels of CD 28- T lymphocytes as a non-invasive marker to predict post transplantation outcomes in allograft recipients allowing close surveillance of patients at risk. Moreover, after validation this non-

Correspondence: Dr Muhammad Aftab Hassan, Department of Immunology, AFIP Rawalpindi Pakistan

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invasive technique can be employed for future testing as diagnostic and clinical aid in patient selection and treatment modification.

METHODOLOGY

This prospective longitudinal study was carried out after institutional review board approval and after obtaining written informed consent from patients (IRB no: READ-IRB/23/3534). The sample size for this study was determined using the WHO sample size calculator by keeping a population mean of 6.4 for CD 28- T cells was used, the estimated sample size was calculated to be 45.[10] This study utilized a non-probability consecutive sampling technique to select live related renal transplant candidates.

Inclusion Criteria: Renal transplant recipients of either gender with age ranging from 18 to 60 years, who were ABO-compatible & Human leukocyte antigen (HLA) matched at A, B & DR loci with donor and with negative CDC or flow cytometric crossmatch were included.

Exclusion Criteria: Patients who had previously received immunosuppression induction therapy, undergoing re-transplantation, HIV-positive or HLA mismatched to donor were excluded from the study.

Data collection involved gathering detailed demographic, clinical, and immunological information, including confounding factors such as CMV serostatus and diabetes mellitus. Blood specimens of all the study participants were collected in EDTA tubes, 48 hours prior to the renal transplant. The primary investigation involved flow cytometric analysis of CD28 expression on T lymphocytes using fluorochrome-labeled monoclonal antibodies (BD Biosciences), staining protocol was followed as per manufacturers recommendations. An isotype control (mouse IgG1/IgG2) was used to differentiate between CD28+ and CD28- cells on peripheral blood lymphocytes. A total of 50,000 cells were acquired on FACS Canto II instrument, with the target population gated on an FSC/ SSC dot plot. Data analysis was performed using BD FACS DIVA software. Absolute counts and percentages of different T cell subsets were recorded for all the participants. Patients after three months post-transplantation were divided into rejectors and non-rejectors based on graft outcomes. Biopsy-proven rejection based on Banff criteria was used to identify rejectors of renal allograft while those who had normal graft function and normal renal function test were included in non-rejectors group. Early acute rejection was defined as the development of biopsy-proven acute allograft rejection within 3 months after kidney transplantation, based on the Banff criteria.[11] Main components of the study methodology are presented in Figure-1.

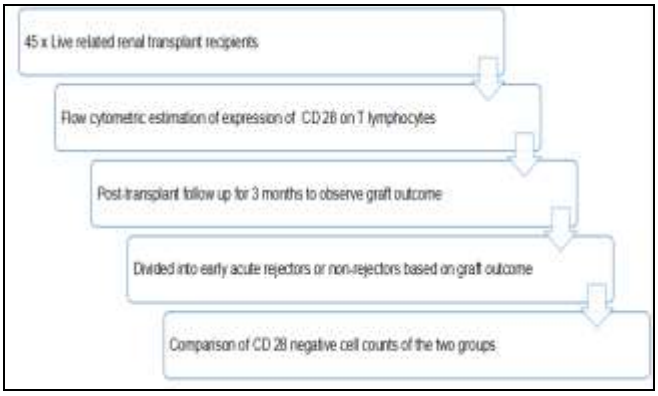

Figure-1: Study methodology and Follow-up[11]

Data analysis was conducted using the Statistical Package for the Social Sciences (SPSS), version 27.0. Categorical variables, such as the gender of recipients and donors, were summarized using frequencies and percentages. Continuous variables, including age and $CD28^-$ T cell counts (cells/µL), were expressed as means with standard deviations or as medians with interquartile ranges, as appropriate based on data distribution. The Shapiro-Wilk test was employed to assess the normality of continuous variables. Continuous variables following the assumption of normality ($p>0.05$), were presented as Mean±SD and those not following the assumption of normality ($p<0.05$), were expressed by Median (IQR). Categorical variables, such as recipient and donor gender, were expressed as frequencies and percentages. Independent samples t-test or Welch's t-tests were used to compare mean values between rejectors and non-rejectors, and p-value <0.05 was considered statistically significant. For variables exhibiting non-normal distribution Mann–Whitney U test was applied and p-value of less than 0.05 was considered statistically significant . The Chi-Square test or Fishers exact test was used to compare categorical variables, including recipient and donor gender, hypertension and diabetes mellitus; a p-value of less than 0.05 was considered significant.

RESULTS

In this study 45 patients undergoing renal transplant were selected as per inclusion criteria. Pre-transplant counts of CD 28+ and CD 28- T lymphocytes were measured in all the participants by

flowcytometry. Among the participants 37(82.2%) were males and 8(17.8%) were females. Patient demographics and clinicopathological details are given in Table-I.

The absolute counts of CD4+ CD 28⁻T lymphocytes were also lower in rejectors compared to non-rejectors (83.3±41.4 vs. 113.5±54.2), though this difference was not statistically significant (p=0.11).

Table-I: Demographic and Clinicopathological Characteristics of Study Participants(n=45)

Parameter(s)		Post-transplant Status		Total	*p*-value
		Rejectors	Non-Rejectors		
Recipient's Age Mean ± SD (years)		27.5±10.07	38.7±10.27	37.2±10.8	0.017*
Donor's Age Median (IQR) (years)		23.5(19.5 - 27.5)	35(25 - 41)	35(24 - 39)	0.066^
Recipient's Gender	Male	4(8.8%)	33(73.3%)	37(82.2%)	0.286#
	Female	2(4.4%)	6(13.3%)	8(17.7%)	
Hypertension	yes	5(11.1%)	30(66.6%)	35(77.7 %)	0.725#
	no	1(2.2%)	9(20 %)	10(22.3%)	
Diabetes Mellitus	yes	1(2.2%)	4(8.8%)	5(11.1%)	0.529#
	no	5(11.1%)	35(77.7%)	40(88.8%)	
Donor's Gender	Male	0(0 %)	11(24.5%)	11(24.5%)	0.311#
	Female	6(13.3%)	28(62.2%)	34(75.5%)	

** Independent Sample t-test*
^ Mann Whitney U test
 # Chi-Square test/ Fishers exact test

Table-II: T Cell Subset Analysis of Rejectors and Non-Rejectors of Renal Allograft (n=45)

Parameter	Absolute Counts (cells/ ul)		*p*-value	Percentages (%)		*p*-value
	Rejectors (n = 6)	Non-rejectors (n=39)		Rejectors (n = 6)	Non-rejectors (n=39)	
	*Mean±SD / †Median (IQR)	*Mean±SD / †Median (IQR)		*Mean±SD / †Median (IQR)	*Mean±SD / †Median (IQR)	
Lymphocytes	†1640.5 (1548.8 – 1946.4)	†2021.6 (1544.3 – 2548.0)	0.308a	†27.9 ± 9.13	†25.8 ± 6.1	0.448b
CD3+ T cells	†1117.4 (1002.7 – 1204.4)	†1374.4 (1119.1 – 1750.9)	0.176a	†65.6 ± 8.5	†68.2 ± 8.9	0.530b
CD 3+ CD 28+ T cells	†715.0 (594.7 – 866.1)	†790.7 (610.4 – 1039.4)	0.442a	†43.9 ± 11.9	†40.5 ± 6.8	0.513c
CD 3+ CD 28- T cells	*376.9 ± 172.2	*525.8 ± 197.7	0.089b	*21.1 ± 2.8	*25.2 ± 8.2	0.029c
CD 4+ CD 28+ T cells	†496.4 (339.4 – 596.7)	†544.9 (449.7 – 762.6)	0.166a	†28.2 ± 8.9	†29.5 ± 5.7	0.750c
CD 4+ CD 28- T cells	†77.7 (66.1 - 111.1)	†99.0 (72.3 – 144.8)	0.211a	†4.1 (3.9 – 4.4)	†5.0 (3.4 – 6.8)	0.404a
CD 8+ CD 28+ T cells	†269.2 (188.3 – 330.2)	†220.2 (166.9 – 291.5)	0.546a	†16.7 (13.3 – 19.7)	†11.9 (9.8 – 12.7)	0.027a
CD 8+ CD 28- T cells	*293.6 ± 144.2	*412.3 ± 169.8	0.112b	*16.5 (14.3 – 18.4)	*17.6 (16.0 – 23.2)	0.300a

a Independent sample Mann-Whitney U test
b Independent Sample t-test
c Welch's t-test
**Mean ± SD*
† Median (IQR)

Among these 45 participants 6(13.3 %) developed early acute rejection (within 3 months of transplantation) while 39(86.7 %) had normal renal function. Out of 6 patients in the rejectors group, 4(66.6 %) were males and 2(33.3 %) females. The mean age of those experiencing rejection was significantly lower (27.5±10.07 years) compared to non-rejectors (38.7±10.27 years, p=0.017). When comparing CD3+ CD28- T lymphocyte counts, rejectors had lower absolute counts (376.9±172.2 vs. 525.8±197.7), but the difference was not statistically significant (p=0.08). However, the percentage of CD3+ CD28- T lymphocytes was significantly lower in rejectors than in non-rejectors (21.1±2.8% vs. 25.2±8.2%, p=0.02). The effect size, calculated using Cohen's d, was 0.671, indicating a moderate to large difference between the two groups.

Similarly, CD8+ CD28⁻T lymphocyte counts were higher in non-rejectors than in rejectors (412.3±169.8 vs. 293.6±144.2), but this difference was also not statistically significant (p=0.112).

These findings align with previous research on immunological aging, suggesting that early acute rejection (EAR) is more common in younger individuals with lower CD3+ CD28⁻T cell counts. This supports the hypothesis that CD3+ CD28- T cells increase with age, and their loss may reduce the risk of EAR. In this study it was observed that rejection was more pronounced in younger patients with lower CD4+ CD28- T cells as compared to non-rejectors (p=0.017). T cell subset analysis of the patients is presented in Table-II while comparison of T cell subsets of rejectors and non rejectors is presented in Figure-2.

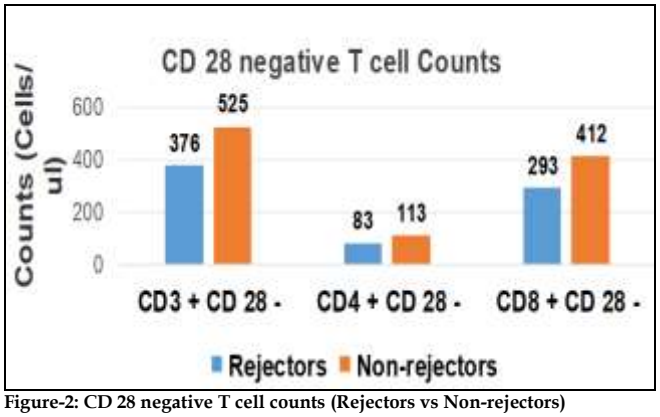

Figure-2: CD 28 negative T cell counts (Rejectors vs Non-rejectors)

DISCUSSION

In this study the determination of pre-transplant mean counts of CD 28- T lymphocytes in early acute rejectors and non - rejectors of renal allograft was done. The results of this study show that mean cell count of CD 28- T cells were lower in rejectors as compared to non rejectors (p=0.02). These findings are consistent with the previously published study by Dedeoglu B *et al*., according to which higher number as well as percentage of CD 4+ 28- T cells, is associated with a lower risk for EAR.[6] In another study published in 2017 by Cortes-Cerisuelo M *et al*., similar findings were reported, in which patients possessing higher frequency of CD 28+ T cells were more likely to experience acute rejection.[12] It is pertinent to mention that in previous studies, it was hypothesized that increased immunological aging at the time of renal transplantation carries a lower risk for allograft rejection.[10,13] A study published by Betges MGH *et al*., in 2020, reported that levels of CD 28- CD 8+ T cells lower in rejectors as compared to non rejectors, 32.5±2.7 & 41.6±1.3, respectively (p-value = 0.003).[10]

It is important to highlight that loss of CD 28 on the cell surface of T cells is one of the features of T-cell aging. In previous studies it was reported that in young individuals CD 28- T cells are present in a very low frequency.[7,14] CD 28- T cells, which are highly differentiated and exhibit an exhausted phenotype, may outcompete alloreactive T cells for resources within the immune system. In the context of kidney transplantation, these senescent CD 28- T cells may have diminished alloreactive potential, contributing to a lower risk of early rejection. In a study published in 2015, Maly *et al*., reported that CD 28- T cells, especially the CD 4+ subset, often exhibit a restricted T-cell receptor repertoire compared to CD 28+ T cells.[14] This restricted repertoire may result in a reduced ability of CD 28- T cells to recognize and respond to alloantigens, contributing to a lower risk of early rejection. In a study published in 2023, Betjes et al. reported that these cells show an exhausted phenotype.[15] Furthermore, in different studies limited immunological activity of these CD 28- T cells is well established. It is important to highlight that role of CD 28 is being investigated in different organ transplants. In liver transplant recipients, higher frequencies of CD 4+ CD 28+ T cells were found in allograft rejecting patients.[16] According to a Chinese study by Lei Geng *et al*., after liver transplantation, a higher frequency of $CD8^{+}CD28^{-}$ T-suppressor cells seems to be essential for maintaining stable graft function (p-value; <0.01). These cells are also significant in decreasing the need for immunosuppressive treatment due to their immunoregulatory properties.[17]

The findings of this study indicated that while the percentage of CD 28- T lymphocytes varied between early acute rejectors and non-rejectors of renal allografts, this difference was statistically significant (p=0.029). It is noteworthy that the observed trend of lower CD4+ CD 28- T cells in rejectors, along with their younger age compared to non-rejectors, aligns with previous studies. This pattern is consistent with the understanding that CD 28- T cells increase with age as part of immunological aging.[18] Consequently, younger patients, who have lower levels of CD 4+ CD 28- T cells, experienced more pronounced rejection episodes than older non-rejectors (p=0.041). A study by Kusztal *et al*., reported similar findings, indicating that the intensity of rejection is notably weaker in older kidney allograft recipients. These observations may be linked to age-related immunological changes, particularly the decreased expression of CD 28 and the enhanced expression of CTLA-4 following stimulation.[19] Similarly a study by Betjes *et al*., highlights the potential for reducing maintenance immunosuppression in elderly kidney transplant recipients due to immunological aging and donor-specific hypo responsiveness, which lowers T-cell-mediated rejection.[15]

Considering these findings, it is justifiable to hypothesize that premature T-cell aging and the risk for EAR after kidney transplant are associated with each other. However, further multicenter studies, with larger patient populations are needed to investigate these findings in more heterogeneous populations and to define cut-off values to be utilized in routine lab and clinical practice. In future such a prognostic

marker could help for optimal risk assessment and tailoring personalized immunosuppressive regimen. As previously the presence of CD 28- T cells has shown some potential as a predictive marker for transplant outcomes in kidney transplantation, but further research is needed to establish its clinical utility.

The strength of the current study is the number of recipients forming a relatively homogenous group, all living related renal transplant cases, and receiving the same immune suppressive drugs. In addition, this is an ongoing study and follow-up of patients is planned at 6 months, 1 year and then on yearly basis for long term monitoring.

It's important to mention that the association between CD 28- T cells and transplant outcomes may vary across different patient populations, immunosuppressive regimens, and types of organ transplantation. The existing studies have primarily focused on living-donor kidney transplant recipients receiving specific immunosuppressive drugs. Therefore, the generalizability of these findings to other transplant populations remains to be determined. Furthermore, additional areas require exploration, such as the impact of CD 28- T cells on long-term graft survival and chronic rejection, beyond their association with early acute rejection. Also the role of different immunosuppressive strategies, as well as the need for standardized protocols for assessing CD 28- T cells, remain critical for advancing their clinical application.[15]

To summarize, while the presence of CD 28- T cells, shows promise as a predictive marker for transplant outcomes, there are several aspects that require further investigation. Long-term allograft outcomes, immunosuppressive strategies, standardization of assessment, and combination with other biomarkers are important considerations for advancing the understanding and clinical utility of CD 28- T cells in predicting transplant outcomes.

LIMITATIONS OF STUDY

This study has several limitations. It included only recipients with HLA-matched donors, limiting the generalizability to non-HLA matched transplant recipients. Furthermore, deceased donor kidney transplant is very limited in Pakistan therefore those patients were not included.

CONCLUSION

In conclusion, renal transplant recipients with high numbers of 28- T cells, had better graft survival at 3 months. This is the first study from Pakistan, in which role of CD 28 was determined in living related renal transplant recipients. This knowledge may be used to guide further studies to evaluate the role of CD 28 as predictive marker of renal allograft survival.

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Authors Contribution

Following authors have made substantial contributions to the manuscript as under:

MAH & HNT: Conception, study design, drafting the manuscript, approval of the final version to be published.

MOR & SWK: Data acquisition, data analysis, data interpretation, critical review, approval of the final version to be published.

MA: Conception, data acquisition, drafting the manuscript, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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