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ORIGINAL ARTICLE

Peripheral T Lymphocyte Responses in Idiopathic Acquired Aplastic Anemia Patients Treated with Immunosuppressive Ther-

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Abstract:
Background: Idiopathic acquired aplastic anemia is an immune-mediated disorder that manifests as pancytopenia. Raised CD4+ and CD8+ T cell counts and altered ratios play an important role in its pathogenesis. Hematopoietic stem cell transplant is the treatment of choice but not readily available or expensive. In developing countries, the majority of the patients are treated with immunosuppressive drugs.
Objectives: In this study, we aimed to evaluate the effect of immunosuppressive drugs on the absolute CD4+ and CD8+ T cell counts and their ratios.
Methods: In this study, 60 patients and 35 healthy controls were included. CD4+ and CD8+ T cell counts were measured using flow cytometry and Ham's test for Paroxysmal Nocturnal Haemoglobinuria exclusion. Cyclosporine levels of patients receiving the drug were also performed.
Results: A high frequency of acquired aplastic anemia in patients of younger age groups with a slight male preponderance was observed. CD4+ and CD8+ cell counts were lower in younger patients while no significant difference was seen in adult patients. Patients receiving immunosuppressive drugs had a trend toward reduction in raised cell counts particularly in CD4+ T cells. This effect was more pronounced in patients receiving the immunosuppressive drug for more than 6 months.
Conclusion: Immunosuppressive drugs play a key role in restoring immune imbalance by decreasing CD4+, CD8+ T cell counts and their ratios, especially in young patients.

Keywords: Idiopathic acquired aplastic anemia, CD4+ T cells, CD8+ T cells, Immunosuppressive therapy, Cyclosporine, Tacrolimus.
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Introduction

Aplastic anemia (AA) is a clonal hematological disorder, characterized by peripheral blood pancytopenia and bone marrow hypoplasia, in the absence of blasts or abnormal cells and no increase in reticulin fibers (1). AA has an age related presentation with increased incidence in the pediatric and young adult age and then in elderly (2).

AA prevalence has a geographic variation with an increasing incidence worldwide. Approximately, 2-3 cases per million are reported in Europe annually while the incidence is 2-3times higher in Asian countries (3).

Despite having congenital or secondary causes, majority of the cases are idiopathic in nature without any obvious/known causes, likely due to immune mediated pathophysiology(4, 5). Most appropriate explanation is the immune-mediated destruction of CD34+ hematopoietic stem cells (HSCs), resulting in reduced hematopoiesis and hence peripheral cytopenias(6). Bone marrow recovery after immunosuppressive therapy (IST) is also a clue towards its autoimmune nature (6). Altered counts of CD4+ and CD8+ T lymphocytes have previously been shown to play an important role in AA pathogenesis (7). Increased production of myelosuppressive cytokines such as IFNγ, TNFα and certain interleukins by CD4+ and CD8+ T cells in AA could also be responsible for the apoptosis of HSCs(8, 9).

AA is a clonal disorder with a tendency of clonal evolution into other bone marrow disorders such as paroxysmal nocturnal hemoglobinuria (PNH), Myelodysplastic syndrome (MDS), and acute leukemia(5, 10). It has been estimated that nearly half of the AA transforms into PNH, either during treatment or after remission (5).

Hematopoietic stem cells transplant (HSCT) is the treatment of choice, with a response rate of up to 90% with HLA matched sibling donor followed by IST. Standard IST with Anti-Thymocyte Globulin (ATG)/Anti-Lymphocyte Globulin (ALG) alone or in combination with Cyclosporine-A (CsA) has a response rate of 60-70%(11-13).However, these treatment options are either not readily available or very expensive in developing countries including Pakistan. Immunosuppressive drugs particularly CsA is widely used in low-income countries with favorable outcomes. CsA is a calcineurin inhibitor, used for immunosuppression in various indications including AA. Either used alone or in combination with other drugs, CsA is associated with a good response in AA(10). Tacrolimus (Tac), another calcineurin inhibitor, has been tried recently in some parts of the world especially in paediatric AA cases (14). Tac has also shown promising results in AA with lesser toxicity as compared to CsA(15). These immunosuppressive drugs act by lowering the abnormally raised counts and ratios of CD4+ (Helper T cells) and CD8+ (Cytotoxic T cells) hence, restoring immune imbalance. Length of treatment is also an important parameter, not only in the repopulation of hypoplastic bone marrow but also for the prevention of relapse (16). IST for at least six months followed by slow tapering is associated with lower risk of relapse.

In order to understand the immune alteration in AA and the effects of IST on immune imbalance, we measured the absolute CD4+ and CD8+ T cells and CD4/CD8 ratio in IAAA patients receiving IST. Although various immunosuppressive drugs are available and prescribed in IAAA with variable responses, drugs like Tac have a limited experience especially in Pakistan. Empirical evidence is highly suggestive of a good response and least toxicity of Tac in IAAA in patients not responding to CsA alone or in combination.

Methodology

Study design and data collection:

It was a cross-sectional study, conducted from October 2015 to July 2016 on patients with a confirmed diagnosis of IAAA and receiving IST regardless of age, gender and nationality. Patients and healthy controls were enrolled after an informed written consent as per the protocol of Ethical board of Khyber Medical University (KMU), Peshawar, Pakistan.

Study participants

A total of 60 patients with confirmed diagnosis were included after the exclusion of hereditary and secondary AA, concomitant diseases, clonal evolution, incomplete history, non- or poor compliance to treatment and faulty samples. Healthy individuals (n=35) were screened for any major or recurrent illness and anyone with a history of recent significant illness, autoimmune disorder, smoking and allergy were excluded.

In order to better understand the changes in count and ratio of CD4+ and CD8+ T cells of patients receiving IST, we also included newly diagnosed (treatment naïve) and off-treatment (in remission, either complete or partial) cases.

FACS analysis

Absolute cell count was performed on BD FACSCount system (BD Biosciences, USA) using BD reagent kits for CD4+ and CD8+ cells as per the manufacturers guidelines. All patient and control samples were run within 24 hours of collection under specified conditions and the results were recorded for each sample. Controls and calibrations were ensured to be optimum before performance of tests every time.

The published ranges of CD4+ and CD8+ counts and ratio are very wide which could miss any subtle changes within the normal range. To overcome this issue, these published reference ranges (17)were divided into two ranges as; CD4 normal range (400-1500 cells/mm3) into lower normal range (400-1100) and upper normal range (1100-1500), CD8 normal range (200-1100 cells/mm3) into lower normal range (200-800) and upper normal range (800-1100). CD4/CD8 ratio (0.7-3.5) was also divided into lower normal range (0.7-1.5) and upper normal range (1.5-3.5) for the ease of understanding and interpretation.

Cyclosporine levels

Cyclosporine level was done for all the patients receiving CsA alone or in combination with Oxymetholone (CsA + Oxy) using chemiluminescent microparticle immunoassay (CMIA) on the ARCHITECT *i* System, Immunoassay Analyser (Abbott Diagnostics) according to the manufacturers' instructions. Controls and calibrations were run and confirmed before running the patient sample. Only the patients with proper drug compliance and their drug levels within the optimal range were included in this study.

Statistical analysis:

Data were analysed using GraphPad Prism (GraphPad Software, Inc). Frequency, percentages and mean ± SD were calculated as per the spread of data. ANOVA was applied while comparing the mean ± SD of the CD4+, CD8+ cell counts and CD4/CD8 ratio among different treatment categories. A p-value of ≤ 0.05 was taken as significant.

Results:

Demographic details of study participants:

In this study, 58.3% patients were children (age < 18 years) and 41.7% were adults (age ≥ 18 years) with majority of the patients (45%) of 10 to 18 years age followed by 20% in 18 to 30 years age group (**Figure 1A, B**). The control group comprised 62.85% adults and 37.15% children while majority (62.86%) of the individuals were of 10 to 40 years of age (**Figure 1A, B**).

Most of the patients were males (75%) of young age while in the control group, 57.14% were males and 42.86% were females (**Figure 1C**). Majority of the study participants (both patients and controls) were Pakistani nationals and some from Afghanistan (**Figure 1D**).

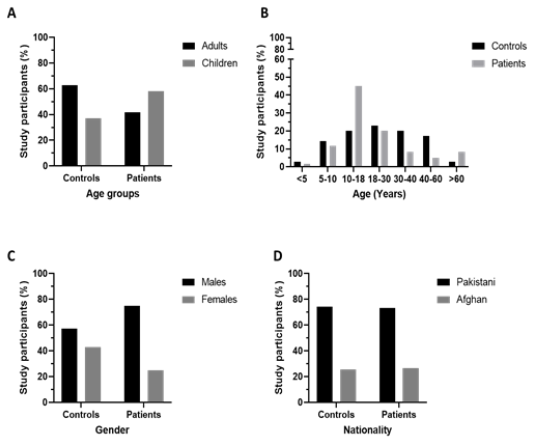

Figure 1: Basic demographic characteristics of the study participant.

Disease severity and treatment categories:

Camitta criteria was used to categorize patients into severity categories of non-severe aplastic anemia (NSAA), severe aplastic anemia (SAA) and very severe aplastic anemia (VSAA)(18). Based on that criterion which accounts for hemoglobin, neutrophil and platelet count, 75% of our patients had VSAA followed by SAA and NSAA **(Figure 2A).**At the time of inclusion into this study, patients were taking different immunosuppressive drugs such as CsA, CsA + Oxy and Tac while some of them were treatment naïve and off-treatment **(Figure 2B)**. As patients were selected randomly therefore, the number of patients in each treatment group was unequal and beyond the control of researchers. Patients were receiving IST for different durations, majority of the CsA and CsA + Oxy users for < 6 months while Tac for ≥ 6 months **(Figure 2C).**

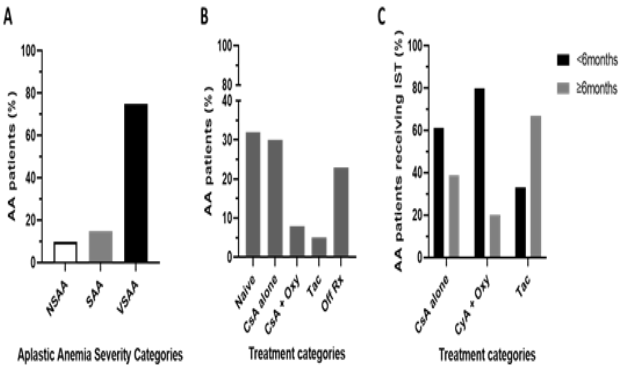

Figure 2: Aplastic anemia severity and treatment categories.

T lymphocyte subsets in study participants:

T lymphocyte subsets (absolute CD4+ and CD8+ T cell counts) and their ratios (CD4/CD8) were assessed in children and adults of patients and controls groups. Most of the children in control and patient groups had their CD4+ cell counts in upper normal and lower normal ranges respectively (**Figure 3A**). No significant difference was seen in CD4+ cell counts of adults in patient and control groups (**Figure 3B**).

In the case of CD8+ cell counts, paediatric patients had their cell counts in lower normal range compared to age matched controls (**Figure 3C**). A wide variation was seen in the CD8+ cell counts in adults in patient and control groups (**Figure 3D**).CD4/CD8 ratios were also calculated from the absolute counts in both control and patient populations. The majority of the participants (100% children and 82.6% adults in control group; 83% children and 80% adults in patient group) had their CD4/CD8 ratios in lower normal range (**Figure 3E, F**).

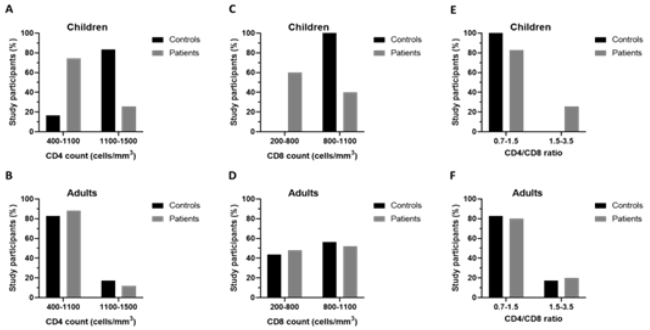

Figure 3: CD4+, CD8+ T cell counts and CD4/CD8 ratios in study participants.

T lymphocyte subsets in response to immunosuppressive drugs and treatment duration

Absolute counts of CD4+ and CD8+ T cells and their ratios were compared among the patient groups in different treatment categories. The mean (± SD) of the CD4+ cell counts of the patients taking CsA alone, CsA + Oxy and Tac were 1012 (± 480.22), 798.0 (± 81.83) and 591.67 (± 147.22) respectively while those of treatment naïve were 1018.79 (± 439.37) and off-treatment were 784.79 (± 208.66) (**Figure 4A**). The mean (± SD) of the CD8+ cell count in CsA receiving patients was 974.06 (± 432.60) while those of CsA + Oxy and Tac were 751.60 (± 241.63) and 901.33 (± 732.18) respectively (**Figure 4B**). CD8+ cell counts were also measured in treatment naïve (790.32 ± 350.66) and off-treatment (776.71 9± 273.43) patients (**Figure 4B**). Similarly, CD4/CD8 ratio was calculated for the patients in each treatment category as shown in **Figure 4C**. CD4/CD8 ratio in treatment naïve patients was 1.40 (± 0.60) while in patients who were compliant to CsA and CsA + Oxy were 1.06 (± 0.38) and 1.11 (± 0.25) respectively. CD4/CD8 ratio in patients taking Tac was 1.19 (± 1.18) and in off-treatment group was 1.09 (± 0.45). Although the data was not statistically significant (one-way ANOVA), the trend in the T cell counts and ratios was suggestive of restoration of raised T cell counts. This was more pronounced in CD4+ T cells.

We also compared the CD4+ and CD8+ T cell counts and ratios in the patients receiving IST for < or ≥ 6 months. Our findings show that the patients receiving IST for ≥ 6 months had their CD4+ cell counts in the lower normal range (**Figure 4D**) while CD8+ counts and CD4/CD8 ratio did not show a significant change even after ≥ 6 months of IST (**Figure 4E, F**).

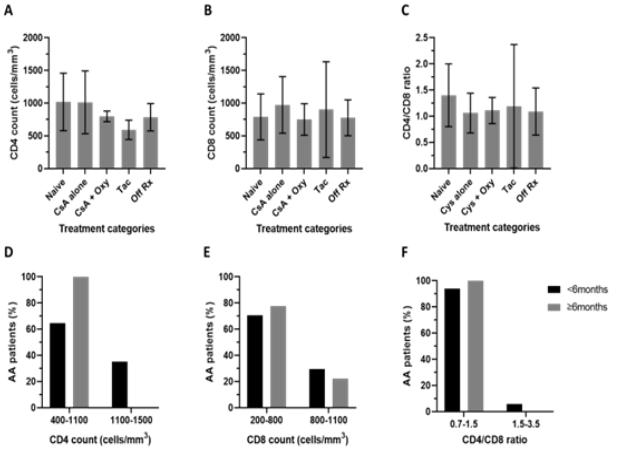

Figure 4: CD4+, CD8+ cell counts and CD4/CD8 ratios in patients receiving immunosuppressive drugs for a different duration.

Discussion

This study was conducted to evaluate the effectiveness of immunosuppressive drugs in decreasing the raised counts of CD4+ and CD8+ T cells in IAAA. Treatment naïve and off-treatment patients were also included for a better comparison of the effects of active IST on CD4+ and CD8+ T cells. None of these patients had undergone HSCT or received ATG/ALG at any stage from the diagnosis to sample collection for this study.

Our findings were consistent with the published literature regarding the bimodal age representation and a slight male preponderance worldwide(17, 19). Patients and controls were randomly selected for inclusion in this study, with majority of them of Pakistani origin and some Afghans.

Due to a high risk of clonal evolution in AA patients, we excluded patients suspected of clonal evolution into PNH, MDS or acute leukemias, however, the possibility of subclinical clonal evolution particularly PNH, could not be completely ruled out. Patients with poor compliance to IST, concurrent illnesses or non-consenting patients were also excluded.

In our study, majority of the children in control group had their CD4+ and CD8+ cell counts in upper normal range which is consistent with other studies, likely due to age-related bone marrow cell production and intercurrent infections(20, 21). Majority of the pediatric patients receiving IST however, had their CD4+ and CD8+ cell counts in lower normal range likely due to a response to the immunosuppressive drugs. However, the adults in both control and patient groups had no significant change in their CD4+ and CD8+ cell counts. Previous studies have demonstrated that adult patients are not good responders to IST compared to children.

In terms of CD4/CD8 ratios, both the children and adults in control and patient groups had their CD4/CD8 ratio in the lower normal range which shows the effectiveness of IST in normalizing the CD4/CD8 ratios.

Treatment naïve patients had raised CD4+ and CD8+ T cell counts which is consistent with the previous studies (22, 23). These raised counts were, however, within the upper normal range of the published reference ranges with none of them above the upper normal range.

HSCT is the treatment of choice in VSAA and SAA followed by IST in patients not eligible for HSCT. Due to non-availability and high cost of ATG/ALG and HSCT, patients are usually started on IST such as CsA as a first -line treatment (24). CsA is the foremost choice for treating IAAA patients in developing countries including Pakistan however, a strict monitoring is required (25, 26). A variable T cell response was seen in patients receiving CsA, likely due to compliance related issues and treatment status, as this group included all patients whether starting or tapering CsA. Patients non-responsive to CsA alone are started on combination therapy with CsA + Oxy in our setup. Patients receiving CsA + Oxy showed a decrease in T lymphocyte counts and improved clinical response, consistent with the published literature (27). To minimize the compliance related impact of CsA on CD4+ and CD8+ cell counts, we excluded the patients with a history of poor or non-compliance and whose blood CsA levels were not within the recommended therapeutic range.

Tac is not widely used as a treatment option in IAAA but in patients where CsA is not favorable treatment option due to ineligibility or intolerance, Tac has emerged as a potent immunosuppressive drug(14, 28, 29). In our study, a small number of patients non-responsive to CsA alone or in combination previously, were started on Tac. Although therapeutic blood level of Tac was never achieved in these patients, an improvement in their platelet counts and overall, well bring was seen. Patients treated with Tac showed a significant decrease in T cell counts, particularly CD4+ T cells. Tac have been shown to acts by decreasing cytokines production responsible for abnormal T cells proliferation especially CD4+ T cells, resulting in normalization of bone marrow function (30). Although treatment with Tac seems to be a better alternative for IAAA, small sample size and small length of treatment are limiting factors and will need further studies for confirmation of these findings.

Patients who were off-treatment and in continued remission were also included to assess the T cell response, if any and the sustainability of that response. Findings of our study suggest that IST is effective in reducing T cell counts which might be sustained over a variable period even after the completion of treat-

ment. Length of IST is also an important parameter for determination of response as well as relapse prevention (31, 32). The relationship between the length of treatment and response of lymphocyte subsets count had not been studied in detail previously. Treatment with CsA for > 6 months and then slow tapering is linked with lower risk of relapse (16). Our study shows that IST for > 6 month is effective in decreasing T cell counts, mainly in CD4+ counts and a small difference was CD8+ counts. CD4/CD8 ratio remained within the lower normal range for majority of patients regardless of treatment duration.

Conclusions

Treatment with CsA alone or in combination with Oxy is effective in reducing raised T cell counts particularly in children. For a better and sustained response, IST for ≥ 6 months followed by slow tapering is beneficial for normalizing the altered T cell biology for an indefinite period.

Ethical approval and consent

The study was approved by the institutional board of studies and informed consent was obtained from each participants included in the study.

Acknowledgment

We thank the study subjects for participating in this study.

Disclosure

The authors report no conflicts of interest.

Author's contributions

GFRwas involved in the execution of the project. MTMK designed, executed the study and wrote the manuscript. SJ and YK helped in organization of data and did the statistical analysis. AH and SK helped in the editing. JA and AST helped in editing. All named authors have read and approved the final version of the manuscript.

Data availability

Available from the corresponding author on reasonable request.

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