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Immunosuppression Boost With Mycophenolate Mofetil for Mixed Chimerism in Thalassemia Transplants

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A B S T R A C T

Declining mixed chimerism (MC) portending impending graft failure is an undesirable outcome. However, for hemoglobinopathies in a stable state of MC, residual host cells persist without rejection in 30% to 40% of patients after hematopoietic stem cell transplantation (HSCT). Early detection and level of MC have been attributed to be significant in predicting the outcome of MC. Common clinical approach on MC is removal of immunosuppression. We retrospectively evaluated MC in transfusion dependent thalassemia patients who underwent HSCT in our institution between September 2013 and January 2022 to determine the outcome of MC on the basis of our approach of immunosuppression boost in comparison to conventional approach of immunosuppression tapering. Among 90 patients, 22 (24.4 %) had MC at some time point after transplantation with a median follow-up of 496 (67-1492) days. Immunosuppression withdrawal was done in 12 (54.5%) patients, whereas immunosuppression boost was given in 8 (36.3%) patients. In the immunosuppression withdrawal group, 2 (16.6%) patients evolved to complete chimerism, 5(41.6%) patients had persistent MC (PMC), whereas 5 (41.6%) patients had secondary rejection. All these rejections were at median of 186 (89-251) days after transplantation. In the immunosuppression boost group, all patients (n = 8) had PMC with no secondary rejection until median follow-up of 255(97-812) days after transplantation. We acknowledge that we need more experience with our unconventional approach of immunosuppression boost to obtain statistical significance in comparison to the conventional approach of tapering of immunosuppression.

Allogeneic hematopoietic stem cell transplantation (HSCT) still remains the most feasible and affordable cure for transfusion-dependent thalassemia (TDT), although the world of gene editing is evolving at a very fast pace [1]. However, graft rejection is an undesirable outcome that usually follows declining mixed chimerism (MC) and has been reported in 5% to 30% of patients [2–4]. In a stable state of MC, residual host cells persist without graft rejection in 30% to 40% patients after HSCT for hemoglobinopathies [5]. Many of these patients exhibit coexistence of donor and host cells providing transfusion independence, which is the primary aim of transplantation [5]. Possible factors that lead to MC are conditioning regimen, stem cell dose, immune status of recipient, and post-HSCT immunosuppression [6,7]. The early detection and level of MC have been attributed to be significant in predicting the outcome of MC [2]. Various measures have been taken to reduce MC and, hence, subsequent graft rejection by either intensifying myeloablative conditioning or by modulating immunosuppression [8,9]. The commonest clinical approach on MC is removal of immunosuppression and giving donor lymphocyte infusion (DLI) [10]. Giving DLI, in case of dropping chimerism in malignant disorders where graft-versus-leukemia effect is desirable and graft-versus-host disease (GVHD) is acceptable, is a common practice. The fact that it can lead to significant GVHD, both acute and chronic, is a major concern when a clinician needs to think about DLI as a modality for the salvage of donor chimerism in nonmalignant disorders [10]. It is also essential to acquire deep understanding about evolution of MC, mainly to intervene in time to prevent rejection. We retrospectively evaluated the course of MC in TDT patients who underwent HSCT between September 2013 and January 2022 to determine the outcome of MC on the basis of our unconventional approach of immunosuppression boost in comparison to the conventional approach of immunosuppression tapering.

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Table 1
Comparison between CC and MC Groups (N = 90)

	MC Group	CC Group	*P* Value
Number of patients	22 (24.4%)	68 (75.5%)	
Mixed chimerism according to conditioning			
Flu/Bu/Cy/ATG (n = 46)	15 (32.6%)	31 (67.3%)	
Thio/Treo/Flu (n = 44)	7 (15.9%)	37 (84.0%)	
Age at HSCT (yr)	6.5 (1.3-13)	8 (1-15)	.114
Gender			.507
Male	15 (68.1%)	41 (60.2%)	
Female	7 (31.8%)	27 (39.7%)	
Pesaro risk			.309
Class 1	5 (22.7%)	15 (22%)	
Class 2	14 (63.6%)	33 (48.5%)	
Class 3	3 (13.6%)	20 (29.4%)	
Stem cell source			.137
BMSC	15 (68.1%)	34 (50%)	
PBSC	7 (31.8%)	34 (50%)	
ABO mismatch			
Matched	17 (77.2%)	40 (58.8%)	
Major mismatch	4 (18.1%)	24 (35.2%)	.785
Minor mismatch	1 (4.5%)	4 (5.8%)	
Donor/recipient gender			
Male/male or female/female	9 (40.9%)	23 (33.8%)	.834
Female/male	6 (27.2%)	19 (27.9%)	
Male/female	7 (31.8%)	26 (38.2%)	
Median stem cell dose			
CD34+ cells (10×10^6/kg)	3.89 (0.3-9.1)	4.33 (0.2-8.01)	.402

MATERIALS AND METHODS

Patient Population

Ninety TDT patients who underwent HSCT from an HLA-identical sibling in our institution were evaluated retrospectively for chimerism studies. Patient characteristics are summarized in Table 1. Risk classification was as per classifications by Lucarelli et al. [11] and Mathews et al. [12]. Study was approved by our Institutional Review Board. All data were collected from systematically maintained hospital records.

Conditioning Regimen and GVHD Prophylaxis

Patients who underwent transplantation between September 2013 and September 2018 received treosulfan/thiotepa/fludarabine (Treo/Thio/Flu), whereas patients who underwent transplantation between October 2018 and January 2022 received fludarabine/busulfan/cyclophosphamide/antithymocyte globulin (Flu/Bu/Cy/ATG) regimen. We experienced significant number of transplant-related mortality with Treo/Thio/Flu regimen; hence, our team decided to change our regimen to Flu/Bu/Cy/ATG and since then we have no transplant-related mortality until now.

All patients in Treo/Thio/Flu group received intravenous thiotepa 8 mg/kg on day −6, treosulfan 14 g/m^2/d on day −5 to day −3, and fludarabine 40 mg/m^2/d on day −5 to day −2. Patients in the Flu/Bu/Cy/ATG group received intravenous fludarabine 30 mg/m^2 on day −18 to −13, ATG 4.5 mg/kg over 3 days (day −12 to day −10), oral busulfan 14 mg/kg for boys and 12 mg/kg for girls on day −9 to day −6, and cyclophosphamide 50 mg/kg/d for girls and 40 mg/kg/d for boys on day −5 to day −2. GVHD prophylaxis included oral cyclosporine at a loading dose of 10 mg/kg/dose twice a day, and thereafter a maintenance dose according to the levels and intravenous methotrexate 10 mg/m^2 on days 1 and 7 mg/m^2 on days 3 and 6.

Stem Cell Source and Engraftment

Source of stem cells was peripheral blood in Treo/Thio/Flu and bone marrow in Flu/Bu/Cy/ATG conditioning group. Neutrophil recovery was defined as the first of 3 consecutive days with an absolute neutrophil count $>0.5 \times 10^9$/L and a platelet count $>20 \times 10^9$/L unsupported for 7 days.

Chimerism Study

Chimerism was assessed at day 28, 60, 90, 6 months, and 1 year after HSCT for those with complete chimerism (CC) or more frequently and beyond 1 year in MC. Chimerism analysis was done on DNA extracted from whole blood. In general, for those with significant drop in blood counts, monitoring was done once in 1 to 4 weeks, and intervention steps were initiated as mentioned below. In case of sex-mismatched transplants, chimerism was tested with fluorescein in situ hybridization for XY chromosomes. In case of same gender, variable number tandem repeat was used for chimerism testing.

Definitions of Chimerism Status

MC was defined as donor cells <95% at any point post-transplant. MC was categorized in 3 levels according to percentage of donor cells (level 1, 90%-95%; level 2, 75%-89%; and level 3, 5%-74%) [13]. Sorted MC was not performed on these patients as it is not available in our center. Transient MC is defined as MC that subsequently reverts to CC or rejection, whereas those who continued to have MC with stable graft function were considered to have persistent MC (PMC). Graft failure was defined as either the absence of hematopoietic reconstitution of donor origin on day 28 after transplantation (primary graft rejection) or as confirmed loss of donor cells (<5%) after transient engraftment of donor cells, leading to transfusion dependence (secondary graft rejection) [13].

Therapeutic Interventions

We used the conventional approach of tapering of immunosuppression and an unconventional approach of increasing immunosuppression by adding mycophenolate mofetil (MMF) and by keeping a higher therapeutic level of cyclosporine (200-250 ng/dl). These immunosuppressive drugs were continued for at least 1 year after transplantation, and, if persistent MC was achieved with stable hemoglobin levels, tapering was done, first with MMF followed by cyclosporine. DLI was given only if the patient had no GVHD.

Statistical Methods

Descriptive statistical methods were used to evaluate all variables. The association between quantitative variables and chimerism group was determined using the Mann-Whitney test. Differences in proportions were assessed using chi-square or Fisher's exact test. Logistic regression was used to evaluate the predictability of risk factors. For all tests, a two-sided *P* value ≤.05 was considered statistically significant. SPSS version 28.0 software was used for the analysis.

RESULTS

Frequency of MC

Among 90 patients included in this analysis, 22 (24.4 %) had MC at some time point post-transplant with median follow-up of 496 (67-1492) days. (Figure 1) At day +28, 9(10%) patients had MC, whereas 81(90%) remained in CC. Subsequently, 2 (2.5%) among 81 patients with CC on day +28 developed MC between day +28 and +60, and 9 (11.3 %) out of the remaining 79 patients with CC developed MC between day+60 and +90. Two (2.8%) patients developed MC between day+90 and 6 months and none developed MC between 6 months and 1year and after 1-year post HSCT. Comparison of patients with CC or MC showed no significant difference between the baseline characteristics. (Table 1)

Outcome based on Time of Onset of MC

Among 9 patients who had MC by day 28, 3 (33.3%) patients had graft rejection, whereas 1 patient (13.9%) evolved to CC and 5 (55.5%) patients had PMC (Figure 1). Of the 2 patients who developed MC between day 28 and 60, neither had graft rejection, and neither evolved to CC. In the day 60 to 90 group (n = 9), 2 (22.2%) patients had graft rejection, whereas 2 (22.2%) evolved to CC, and 5 (55.5%) patients remained in PMC. Among the 2 patients who developed MC between day 90 and 6 months, all remained in PMC. However, on statistical analysis, rejection was not associated with the day of onset of MC ($P = .625$).

Outcome based on Level of MC

Five (22.7 %) patients had level 1 MC, 15(68.1%) patients had level 2, and 2(9.0.%) patients had level 3 MC (Figure 2). With interventions, 1 patient (out of 5 [20%]) with level 1 MC evolved to CC, and 4 (80%) continued with PMC. None of these patients had graft rejection. Among 15 patients with level 2 MC, 9 (60%) remained in PMC, 2 (13.3%) evolved to CC, and 4 (26.6%) had graft rejection. Rejection occurred in 1 (out of 2 [50%]) patient with level 3 MC, whereas 1 (50%) patient had PMC. However, risk of rejection of graft was not found to be

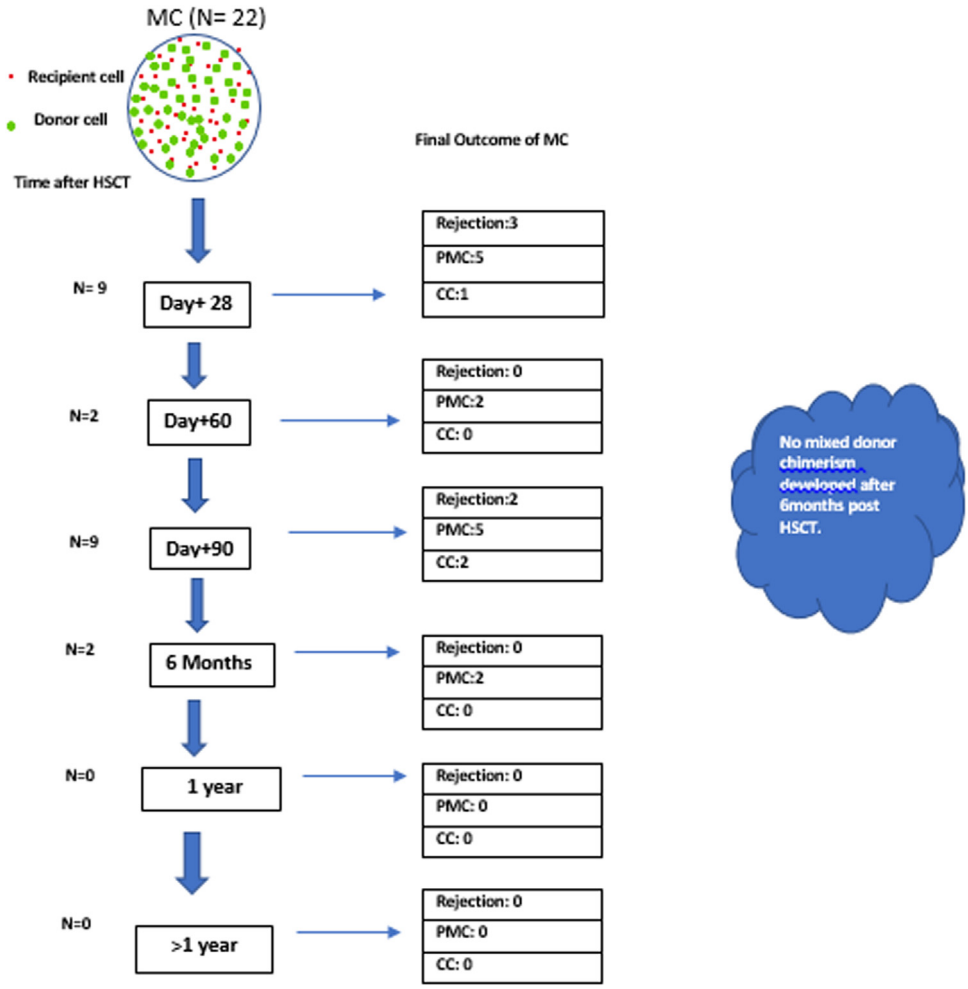

Figure 1. Outcome on the basis of time of onset of MC. Outcome according to the onset of MC has been depicted here. There was no MC development after 6 months after transplantation (HSCT). Graft rejection was not found to be related to time of onset of MC ($P = .625$).

significantly higher with increasing levels of MC ($P = .982$) (Figure 2)

Among patients who had MC by day 28 (n = 9), only 1 (11.1%) had level 1 MC, 7 (77.7%) had level 2 MC, and 1 (11.1%) had level 3 MC. In patients who developed MC between day 28 and day 60 (n = 2), 1 (50%) had level 1 MC, 1 (50%) had level 2 MC, and none had level 3 MC. When patients developed MC between day 60 and day 90 (n = 9), 3 (33.3%) had level 1 MC, 3 (33.3%) had level 2 MC, and 2 (22.2%) had level 3 MC. Among the patients who had MC between day 90 and day 180 (n = 2), all patients had level 2 MC, and none had either level 1 or level 3 MC. None of the patients developed MC beyond 6 months after HSCT.

Therapeutic Interventions and Outcome of MC

Immunosuppression withdrawal was done in 12 (54.5%) patients, whereas immunosuppression boost was done in 8 (36.3%) patients. Only one received DLI, and immunosuppression was kept same for that patient. One patient did not require intervention as he was with level 1 MC and normal hemoglobulin. Among patients where immunosuppression withdrawal was attempted, 2 (16.6%) evolved to CC, and 5 (41.6%) had PMC, whereas 5 (41.6%) had secondary rejection. In the group who received immunosuppression boost, all (n = 8) patients had PMC with no secondary rejection until last follow-up (Figure 3). The median duration of persistence of MC in this group was 255 (97-812) days whereas all patients (who rejected) in immunosuppression withdrawal group had rejection at median of 186 (89-251) days. Out of all augmented patients, 5 are still on MMF and cyclosporine. Three patients who are off MMF are still on cyclosporine with persistent MC. The levels of MC are tested regularly every 4 to 8 weeks depending on the case scenario. We certainly are not planning to continue immunosuppression indefinitely, even though we have not experienced any side effects with the long-term use of MMF.

Evolution of MC

With a median follow-up of 496 (126-1492) days, among the 22 patients with MC, 3 (13.6%) patients evolved to CC, 5 (22.7%) progressed to rejection, and 14 (63.6%) remained in PMC. All patients with rejection did so within the first year after HSCT. It was also observed that patients (n = 3) who evolved to CC achieved so within first year after HSCT. This suggests that although high numbers of residual host cells (RHC) early after HSCT have high risk of rejection, at 1 year after HSCT there is a state of tolerance between donor and recipient cells resulting in PMC (Figure 3). All the patients with PMC (n = 14) in our cohort are transfusion independent with median hemoglobin of 9.05 (7.2-13) gm/dL and chimerism ranging from 50% to 94%. Four (28.5%) patients have level 3 PMC (50%-73%).

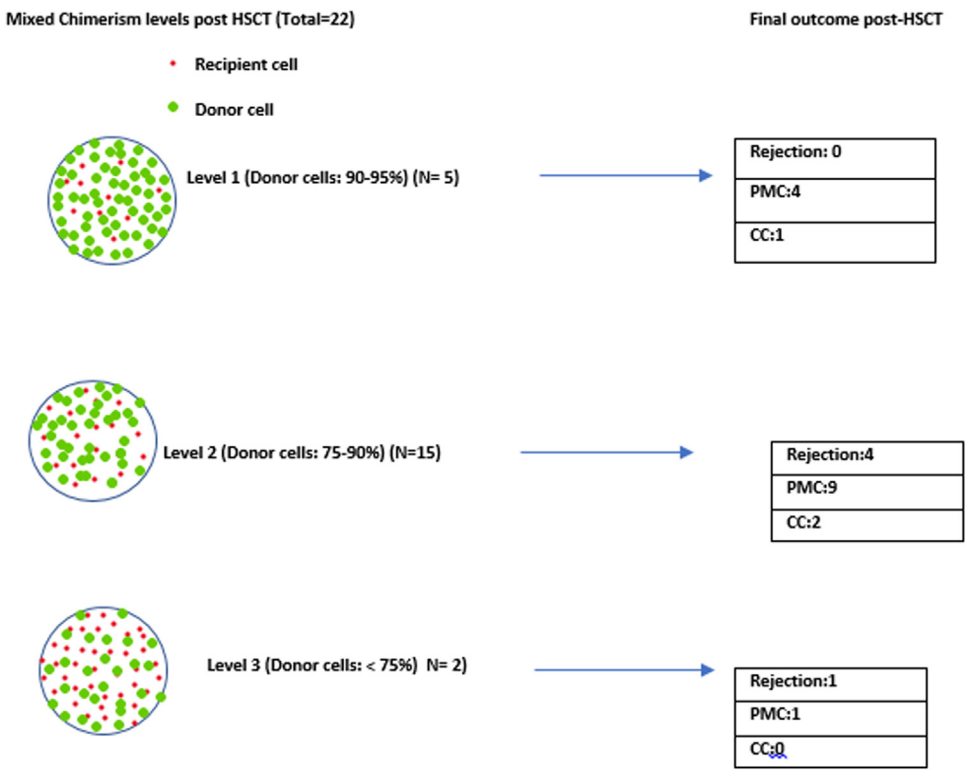

Figure 2. Outcome of MC on the basis of initial levels of MC. Outcome according to level of MC (at onset) has been depicted here. Graft rejection was not associated with levels of MC ($P = .982$).

Risk Factors for MC

We attempted to evaluate the predictability of various factors for MC. Recipient's age, sex, risk classification, CD34+ dose, donor sex, graft source, ABO mismatch between donor and recipient were evaluated as the potential risk factors. However, none of them were statistically significant.

DISCUSSION

Transfusion independence can be achieved with stable MC in TDT after transplantation. It has been determined that therapeutic chimeric level as low as 19% to 24% is adequate for the successful correction of thalassemia major phenotype [14]. The study by Andreani et al. [15] shows that most of the erythrocytes present in long-term transplanted patients of hemoglobinopathies, even in a state of MC, were of donor origin. However, risk of rejection is the main concern with MC. Therefore the evolution of MC after its first detection has been focused mainly on intervening in time to save the graft.

In our study, chimerism evaluation at the first month after HSCT demonstrated 90% full donor chimerism. Of the 10% with MC at first evaluation at day 28, most (88.8%) remained in PMC at the second evaluation 2 months after HSCT. At the first year follow-up, 45.4% patients had stable MC, whereas another 18.1% patients are yet to reach 1 year who otherwise have normal hemoglobin. In a study by Fouzia et al. [10], 34.8% of patients had MC in the first year after HSCT [10]. These findings correlate with the literature in terms of the early and later incidence of MC after HSCT for TDT. Early MC has been reported to be between 30% to 35% by several other studies [2,16]. In contrast, there are few reports of late-onset MC [17]. In our study, of patients who had complete chimerism on day 28, 13/90 (14.4%) developed MC later (within 6 months of HSCT). We have not observed development of MC beyond 6 months after HSCT. Rejection can occur even with the late occurrence of MC [6,4]. Although all rejections in our study were observed before 1 year of transplantation, all patients with PMC were transfusion independent with a median hemoglobin of 9.05 (7.2-13) gm/dL, which is lower than that reported in the study by Fouzia et al. [10] (11.5 [9-13.6] gm/dL).

We had 5 (22.7%) patients with level 1, 15 (68.1%) patients with level 2, and 2 (9.0%) with level 3. Fouzia et al. [10] had reported MC at level 1 in 43.5%, level 2 in 30.4%, and level 3 in 26.1% cases [10]. Most of our rejections occurred in level 2 MC (26.6%) and level 3 MC (50%). Because our study had fewer patients with level 3 MC, it is difficult to corroborate our results to the above-mentioned Indian study, which had reported that graft rejection was higher with level 3 MC [10]. The evolution to CC/PMC appears to be higher (77.27% versus 65%) and rejection rate lower (22.7 % vs 35%) in our cohort in comparison to study by Andreani et al. [17] among all levels of MC. It suggests that the presence of higher number of RHC with level 3 MC at day +28 post HSCT should be the indicator for early interventions.

In addition to the severity of MC, timing of MC has also been considered as a predictor of the long-term outcome of MC in the literature [17]. In our study, all patients who experienced secondary rejection had onset of MC at less than 120 days after HSCT. Although the sample size was very small, it was also observed that not only the first chimerism at day 28, but also the later chimerism (in our study first 3 chimerism studies [days 28, 60, and 90]) might be more representative of the outcome. As elicited in the above-mentioned studies, the probability of rejection is related to both the number of donor cells, as well as the time of onset; although contrary to that, we did not find a significant association of rejection with either level of MC or time of onset. The possible explanation for this could be the small number of cases and a reduction in

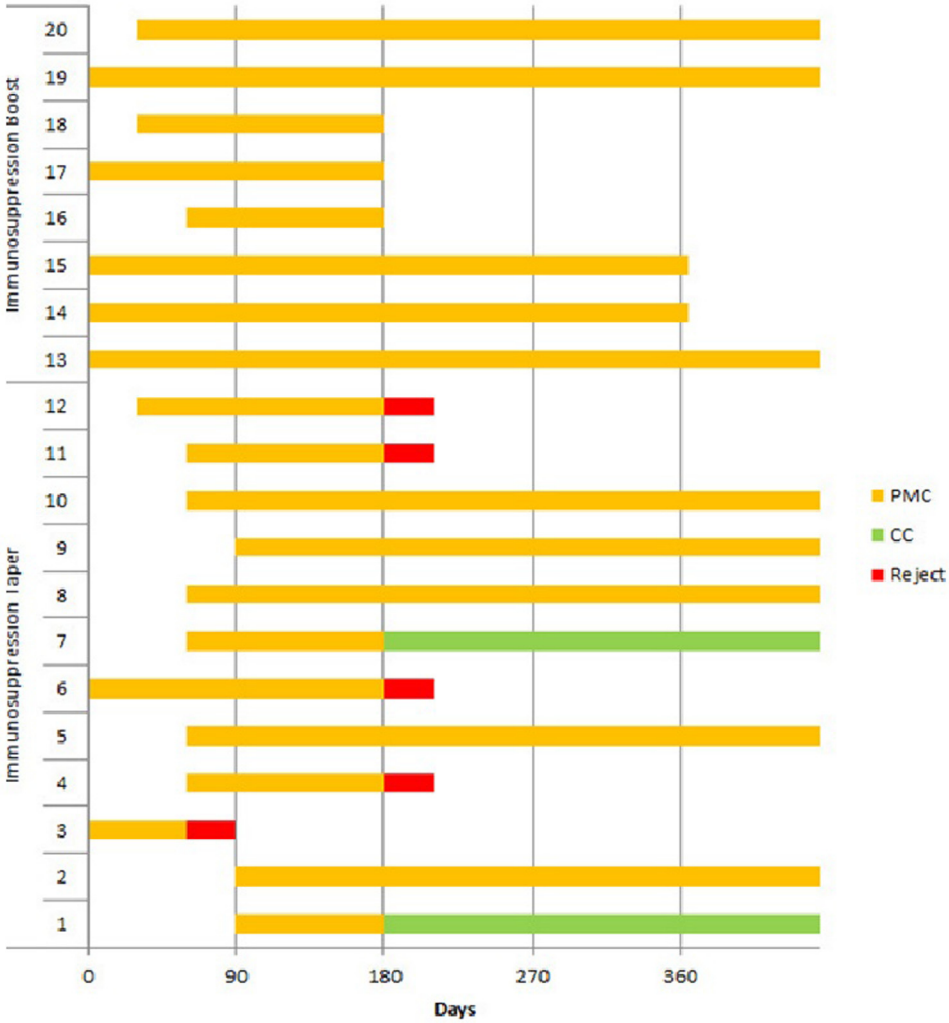

Figure 3. Swimmer plot depicting the course of MC in immunosuppression boost and immunosuppression tapering group. X-axis denotes number of days after transplantation, and Y-axis denotes the number of patients in each group.

secondary rejection (to none) after the modification in our approach for MC management.

The issue of intervention for MC has been controversial. When and how to intervene in MC remain critical questions to be answered. Various study groups have used the principle of withdrawal of immunosuppression [18,19]. The emergence of hematopoiesis in a recipient could be related to inadequate myelosuppression and immune suppression by the conditioning regimen, but the exact mechanism is still a riddle. With intensification of conditioning, Anurathapan et al. [20] show a lesser incidence of MC in TDT transplants. This principle of increasing immunosuppression suggested to us to change our approach for management of MC in thalassemia transplantations. We started using a different approach of immunomodulation (i.e., increasing immunosuppression by adding MMF to cyclosporine). We observed marked difference in rejection rate between 2 approaches because we achieved no rejection in cases of immune boost, and there were 22.7% rejections when immunosuppression withdrawal was attempted in all levels of MC. However, we could not prove its statistically significance (P = .40) This may be due to the small number of patients who received immunosuppression boost. The additional concern in tapering immunosuppression was that the reversal to CC was at the expense of acute GVHD (32.6%) and chronic GVHD (17.4%) in other reports [10]. However, we did not encounter GVHD after withdrawal of immunosuppression in our cohort.

We also attempted to evaluate differences in 2 groups (MC versus CC) in baseline demographics and other factors related to patient, donor, graft characteristics, and other transplantation variables that could predispose to MC. However, we could not find any significant variable that was associated with MC in our study cohort.

We are aware of shortcomings of our study in that we need more experience by treating a significant number of patients with MC with our unconventional approach of immunosuppression boosting to obtain statistical significance in comparison to a conventional approach. Hence, longer duration of follow-up is essential to determine its effectiveness to hold up its expectation of no secondary rejection even after years. Also, long-term side effects of MMF need to be monitored. In support, a recent report by Kharfan-Dabaja et al. [21] highlights the complexities associated with MC in nonmalignant disorders, and it still remains an area of future research.

CONCLUSION

Because early and level 3 MC after transplantation are significant risk factors for graft rejection, every attempt should be

made to prevent rejection. On attempting withdrawal of immunosuppression, we encountered secondary graft rejection in 5 (22.7%) of 22 patients with MC. On the contrary, an immunosuppression boost via MMF along with ongoing cyclosporine resulted in a complete absence of secondary graft loss. We acknowledge that our finding needs to be validated in future studies with large number of patients.

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