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# Transplantation of Epcam+Ve Human Hepatic Stem Cells in Liver Cirrhosis Patient and Cellular Immune Response Running Title: Transplantation and Cellular Immune Response

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## Abstract

Liver transplant is considered is the only treatment for liver cirrhosis. Despite the success of organ transplantation, the treatment requires lifelong immunosuppression and major limitation is the availability of donor liver. Hepatic progenitor cell transplantation (HSCT) offers novel but challenging alternatives therapy to liver transplantation for management of liver cirrhosis. In this study, we investigated the cellular immune response by monitoring T-cell, NK-cell and cytokines which play major role in cellular rejection. A total of 5 patients with decompensated liver cirrhosis were enrolled in the study. T-cell (CD3, CD4 and CD8), NK-cells (CD16) was monitored before (Day-1) and after transplantation (Day 1, 7, 15, 30) of human fetal liver-derived EPCAM+Ve cell by flowcytometry. Before and after transplantation, Cytokine-levels (IL2, TNFβ, IFNα, IFNγ and INFβ) were also measured by ELISA. Study has demonstrated that after HSCT patient showed marked clinical recovery and decline in the MELD score and there was no significant increase found in cell mediated response and cytokine levels between pre and post transplantation. Hence this preliminary study demonstrated human fetal liver- derived EPCAM+Ve stem cell transplantation is safety for end stage liver cirrhosis.

**Keywords:** Liver transplantation; Stem cell transplantation; Flowcytometry

## Introduction

Hepatic stem cell transplantation (HCTx) is promising as bridge towards organ transplantation for the treatment of decompensated liver cirrhosis [1]. Fortification of hepatic progenitors from the emergent human liver, with minimum rejection in transplantation remains a major dare for the improvement of therapeutic approaches to liver disorders.

Human fetal liver derived hepatic progenitor's cells recommend an imperative therapy for clinical application [2]. Several investigators have shown that decreased phenotypic expression HLA-II markers in the EpCAM +ve cells isolated from the second trimester fetal liver [2-4]. Rao et al. and Subba et al. described show that EpCAM +ve fetal liver cells express nil levels of HLA-DR marker but they demonstrate the expression of progenitor and liver specific markers suggesting that these EpCAM +ve cells are hepatic progenitors [2,3,5].

Liver cirrhosis is accompanied by cell loss replaced by fibrosis, hence this disease is irreversible in terms of regeneration. Studies demonstrated delivery of cells directly into the liver (autologous, allogeneic) has shown some level clinical improvement [1,6]. Earlier study has demonstrated clinical improvement after cell therapy in the various stages of liver cirrhosis (stage 1-4) [1,7,8].

Various immunological and non-immunological mechanisms are involved in cell survival. The immune system remains the most formidable barrier to transplantation of cells [9]. NK-mediated rejection also has been demonstrated in animal models and allogeneic rejection occurs most frequently due to immune recipient T-cells [10]. The immune system represents effective mechanisms with coordination between both innate and adaptive immune system in rejection of transplanted cells. CD4+ cells play an imperative role in initiating and directing the immune responses whereas CD8+ cells are responsible for cell directed cytotoxicity. Cytokines produced by immune cells, act in autocrine fashion, compelling a significant role in chronic rejection or fibrosis. IFN-γ is particular seems to play a key role in the development of transplant associated vasculopathy. IL2 is a signaling molecule in the immune system which play important role in the development of tregs, the cellular pathway which influences the proliferation. TNF-β and IFN-α help to modulate many immune, inflammatory function and NK cell activation respectively.

Given the importance of the immune status of HCTx in cell therapy, we tried to demonstrate the role of immunoregulatory cells and changes in cytokine levels in peripheral circulation of the recipients to perceive the allogeneic rejection after the therapy.

## Material and Methods

### Patients screening and sample collection

5 male subjects with established cirrhosis and having an average disease length of 5.8 ± 1.0 years were included in the study. Written informed consent from each patient was obtained after briefing them the objectives of the study. The study protocol was approved by Institutional Ethics Committee, Deccan College of Medical Sciences, Hyderabad, India.

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### Hepatocytes isolation and enrichment of EpCAM+ve cells

Hepatocytes isolation and enrichment of EpCAM+ve Cells were done using previously described methods [6].

### Transplantation of EpCAM+ve enriched hepatic progenitor cells

$8.0\times10^6$ fetal hepatic stem cells (EpCAM+ve) were infused through hepatic artery to the liver using femoral artery catheterization [1].

### Sample collection

5 mL venous blood samples (2 mL in K3-EDTA coated vacutainer for cell count and 3 mL in plane tube for serum separation) from each subject on day -1 (before transplantation), day 1, 7, 15, and 30 after transplantation.

### Immunophenotypic analysis

Immunophenotypic analysis was done for CD3 (Per-CP), CD4 (FITC), CD8 (PE) and CD16 (BD Biosciences) antibodies in separate tubes described elsewhere. Data acquisition and analysis were performed using Cell Quest software on a BD Calibur flow cytometer (BD Biosciences).

### Cytokine assay

Serum concentrations of IL2, TNFβ, IFNα, IFNγ and INFβ, were determined by ELISA commercial kits according to the manufacturer's instructions. All tests were performed in duplicate. The ranges of the sensitivity standard curve of the ELISA kits were 0.78-25 pg/mL for IFN-γ, 0.31-10 pg/mL for IL-4, 3.12–100 pg/mL for IL-13, and 1.56–50 pg/mL for IL-10.

### Statistical analysis

Differences in percentages of patients and controls with detectable levels of each cytokine were assessed by Fisher's exact test. A value of less than 0.05 was considered statistically significant, and all tests were two sided. Data analysis was performed with the STATA statistical package.

## Results

### Clinical improvement

After cell transplantation, all the 5 patients demonstrated cumulative decrease in MELD score with increasing time interval. Means of MELD score, serum bilirubin, SGOT/AST (serum glutamic-oxaloacetic transaminase) and alkaline phosphate ($p<0.01$) decreased after 1 months of follow up. In MELD score, statically significant difference was observed between baseline and 1 month follow-up ($p<0.05$). No complication related to procedure was observed during or after cell transplantation. Hepatic angiogram showed no sign of thrombosis in the hepatic artery when analyzed after successive time intervals.

### Immunophenotypic analysis

Immunological markers CD3, CD4, CD8 and NK (Figure 1) cells were analyzed before and after cell transplantation at day-1, day1, day7, day15 and day30. No significant difference was observed in T cell subpopulation before and after allogeneic hepatocytes transplantation (Figure 2).

### Serum cytokine levels

Serum cytokines IL2, TNFβ, IFNα, IFNγ and INFβ were also analyzed with ELISA to find the evoked inflammatory response due

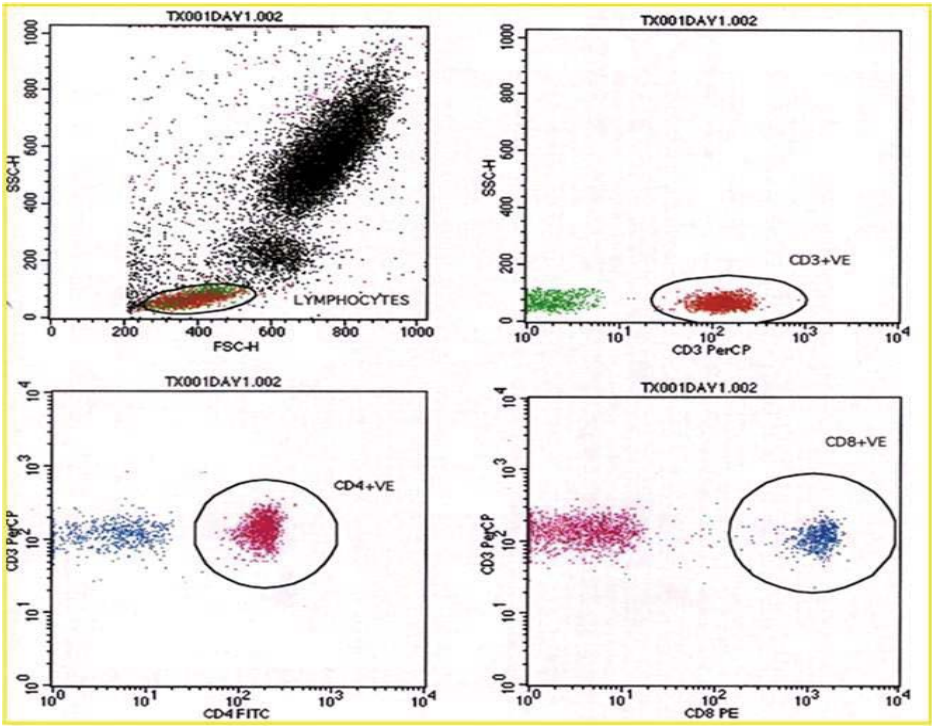


**Figure 1:** Dot plot image showing T-cell subpopulation.

to cell transplantation at day-1, day1, day7, day15 and day30. The level of these inflammatory cytokines did not show any significant difference even after one month. However, slight decrease in IL2 and IFNγ cytokine levels was observed after 7 days of transplantation which again reached to the normal level at day30 (Figure 3). INFβ showed to some extent increased level after cell transplantation but didn't changed significantly in order to induce high level of inflammatory response due to cell therapy (Figure 4).

## Discussion

The site of transplantation of hepatic stem cell plays a major role to avoid the attack of immune cells. Stem cells-based therapeutic approaches have proved their huge impending to change current basic and medical science. However, before applying these cells as regenerative medicines into clinical practice the rejection of allogeneic grafts by the host's immune system is an important concern which requires to be addressed.

Fetal liver derived cells have been revealed to be an affluent source

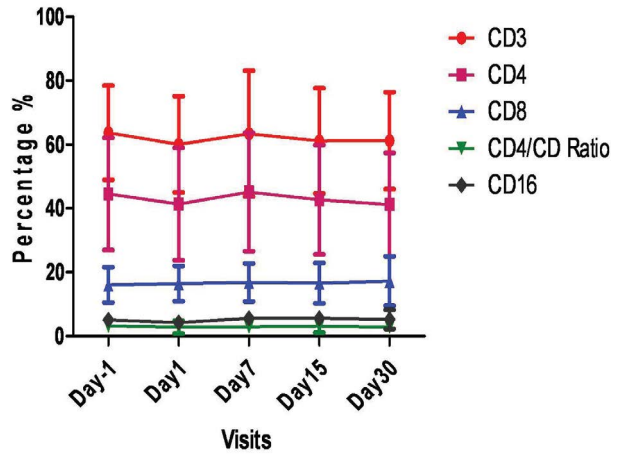


**Figure 2:** Comparative analysis of T-cell subpopulation and Nk-cell before and after transplantation.

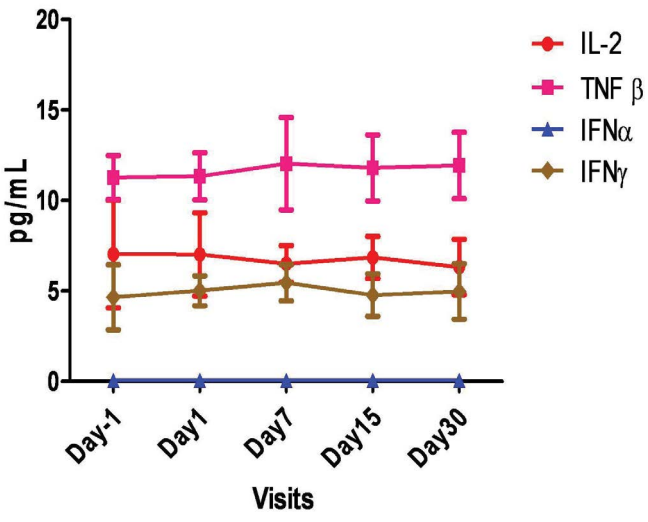


**Figure 3:** Comparative analysis of serum Cytokine levels before and after the transplantation of Hepatic progenitor stem cell.

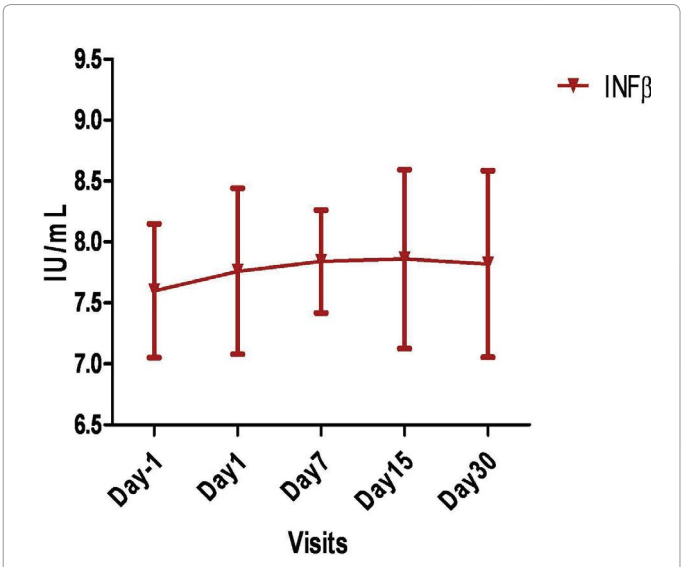


**Figure 4:** Comparative analysis of INFβ levels before and after the transplantation of Hepatic progenitor stem cell.

of hepatic progenitor/stem cells [2,3,5]. Previous study reported that hepatic progenitors with moderate expression of HLA-class I and negative for HLA- class II [2,3,5]. Thus this result supports the use of EpCAM positive fetal liver derived cells for allogenic transplantation because these cells are negative for HLA class II antigen on their surface which otherwise can trigger a rejection reaction in allogeneic transplantation.

Cytokines are endogenous small peptides and they function as critical immunologic mediators in allogeneic transplantation [11]. Allograft rejection is dependent on connections between T lymphocytes, antigen-presenting cells, proinflammatory cytokines (interleukins-1, -2, and -6; tumor necrosis factors; and interferon-gamma) and accessory molecules [12,13]. On the other hand, interleukins-4, -5, -10 and transforming growth factor-beta may repress allograft rejection [14]. Cytokines control mutually aspecific inflammatory and specific immune responses. These aspecific inflammatory changes may not only influence graft function but also influence graft immunogenicity and thus, susceptibility to rejection [15].

Certain aspects of the immune system are meticulous for suppressing inflammation and inhibiting transplant rejection [16]. Essential inhibitory mechanism of the immune system is $T_{reg}$, (or T regulatory cells) [17]. The inflammatory cytokines IL-1β, IL-6, IL-2, IL-21, IL-15, and TNFα, by inhibiting the task of $T_{reg}$ cells and enhancing the activation of cytotoxic T-cells, are accountable for the force of the attack in opposition to the transplanted tissue by the host's immune system [17].

Revelation to elevated levels of the inflammatory cytokines Il-1β, IL-21 or IL-6 facilitate the progenitor cells to expand into aggressive T-cells, while experience to enough levels of a extremely specialized anti-inflammatory cytokine, named transforming growth factor-β (TGFβ), facilitate differentiation into $T_{reg}$ cells [18,19]. Considerably, it has been reported that elevated levels of IL-6 slow down the ability of TGFβ to successfully tempt the differentiation of progenitor cells to $T_{reg}$ cells, leading to an amplify in the number of cytotoxic T-cells [18,19].

The important roles of the inflammatory cytokines IL-2, IL-1β, IL-6, IL-21, IL-15 and TNFα in transplant rejection have been well-

studied by various investigators [20]. By inhibiting the role of $T_{reg}$ cells and promoting the commencement of T-cells, these cytokines are accountable for the passion of molest against the transplanted tissue by the host's immune system [20,21].

Transplant rejection is mainly caused by immunized T-cells, although additional factors may also play a major role. In our study the estimation of T cell subpopulations and NK cells has showed no significant difference in their levels before and after cell transplantation even after 30 days.

Friedman et al., demonstrated that there are significant differences in the cytokine and profiles, and associations with clinical characteristics of Early allograft dysfunction (EAD) and non-EAD recipients, both before and after liver transplantation but in this study the major inflammatory cytokines IL2, TNFβ, IFNα, INFβ and IFNγ did not showed any significant difference in their levels before and after cell transplantation even after 30days. In this perspective the present study has demonstrated decrease in MELD score showing significant clinical improvement after hepatocytes transplantation.

In summary, these data supports previous studies to enhance the cell survival, engraftment, tissue regeneration and control of the inflammatory responses after stem cell transplantation. Furthermore the present study of cytokines suggests stem cell therapy is beneficiary, effective and safe without generating any inflammatory responses and adverse effects.

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### Conflict of Interest

The authors hereby declare no conflict of interest.

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