Original Paper

PU.1-Silenced Dendritic Cells Induce Mixed Chimerism and Alleviate Intestinal Transplant Rejection in Rats via a Th1 to Th2 Shift

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Abstract
Background/Aims: Intestinal transplantation is an effective treatment for end-stage bowel failure; however, graft rejection and the toxicity associated with non-specific immunosuppression are major limitations of this procedure. Studies have shown that mixed chimerism can produce post-transplantation immune tolerance. Here, we demonstrate that in rat intestinal transplantation, PU.1-silenced dendritic cells (DCs) plus bone marrow (BM) cell transfusion results in mixed chimerism, and we investigate the mechanisms responsible for the effects of mixed chimerism rejection. ***Methods:*** In a model of intestinal transplantation, male Brown Norway rats were the donors, and female Lewis rats were the recipients that were randomly divided into 4 groups: control, BM, BM-imDCs and BM-PU.1. The dynamic changes in graft morphology, rejection scoring and serum concentrations of Th1/Th2-related cytokines were investigated on postoperative days 0, 7, 14, 21, and 30. ***Results:*** The BM-PU.1 group had better graft health, milder pathologic injuries, and lower rejection grades compared with the other groups. The rates of mixed chimerism were significantly highest in the BM-PU.1 group and correlated with decreases in serum IL-2 and increases in serum IL-10. ***Conclusion:*** Transfusion of PU.1-silenced DCs and BM cells induces stable mixed chimerism and has the potential to reduce pathologic injuries via a pro-Th2 shift in the Th1/Th2 balance.

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Introduction

Intestinal transplantation has become an effective treatment for end-stage intestinal diseases and patients who fail on total parenteral nutrition (TPN) [1, 2]. Over the last decade,

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the curative effects of intestinal transplantation have improved significantly due to surgical advances, effective immunosuppressive regimes, and a decrease in lethal infections. Graft rejection rates for isolated intestinal transplants have decreased from 80% to 30–40%, and 1-year patient and graft survival rates have increased to 90% and 80%, respectively, at some experienced transplant centers [3-5]. Although complex immunosuppressive regimes are still needed to attenuate rejection episodes and lengthen survival times, their long-term use can result in sepsis and infections. Today, developing a more effective immunomodulatory strategy that does not incorporate long-term immunosuppression is the key goal, and such a strategy could even be described as the 'Holy Grail' [6].

The establishment of mixed chimeras, which are characterized by the co-existence of donor and host hematopoietic cells, has been successfully demonstrated in pre-clinical and clinical trials [7]. The recipient receives special cells from the donor and establishes a cell population that mixes donor cells with recipient cells [8]. In recipients of transplanted allografts, the use of donor-derived bone marrow cells induces tolerance and helps to achieve long survival times. However, the routine use of mixed chimeras has been hampered by the need to condition the recipient to accept the donor bone marrow cells [9-11]. Often, myeloablation and depletion of immune cells is necessary to condition the recipient for mismatched bone marrow transplants, which presents risks due to toxic side-effects. Graft-versus-host disease (GVHD) is also a significant problem when attempting to establish mixed chimeras after myeloablation [6]. To resolve these problems, we silenced the key regulatory factors involved in DC maturation, which may lead to a stunted capacity to prime the immune response and improved chimera stability. The transcription factor PU.1 is predominantly expressed in DCs and is essential for DC differentiation. Recent studies have provided evidence that the PU.1 gene plays a critical role in DC maturation [12] by serving not only as a transcriptional activator but also as a repressor. For instance, in bone marrow-derived DCs, PU.1 up-regulates the expression of DC-characteristic genes, such as class II transactivator (CIITA), CD80, CD86, and IL-12 p40 [13, 14], while suppressing the expression of Th2 cytokines, including IL-13 and IL-5 [15]. PU.1 regulates its target genes through epigenetic modifications, including histone deacetylation and DNA methylation [16, 17]. Our previous study using an intestinal allograft model showed that compared with immature DCs, PU.1-silenced DCs had better and more stable effects in reducing the inflammatory response [18], and therefore, we aimed to establish stable mixed chimerism in a rat model of heterotopic intestinal transplantation (HIT) using simultaneous transfusion of PU.1-silenced DCs and donor-derived bone marrow cells without irradiation or other cytoreductive conditioning. We observed the formation of mixed chimerism, which was accompanied by beneficial effects on histopathological structural integrity, grades of rejection, recipient survival rates, and the ratio of serum levels of IL-2 and IL-10. The objectives of this study are to present a new conditioning approach and improve understanding of the mechanism by which chimerism attenuates injury in transplantation and prolongs graft survival.

Materials and Methods

Animals and Experimental Design

Inbred male Brown Norway (BN) rats and female Lewis rats (250 - 280 g) were obtained from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). Rats were provided with ad libitum access to standard chow and tap water and housed on a 12-h light/dark cycle in a specific pathogen-free animal facility. All animals received humane care, and the study protocols complied with Nanjing University's guidelines and animal research laws. All experimental procedures were approved by our Institutional Animal Care and Use Committee.

Donor dendritic cell isolation and transfusion

Bone marrow cells of BN rats were used for DC generation according to the methods described by Lutz [19] and our previous study [20]. Briefly, BN rats were sacrificed, and femurs and tibias were obtained

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by dissection. Bone marrow cells were flushed from the bones using phosphate-buffered saline (PBS). The resulting cell suspensions were centrifuged and treated with red cell lysis buffer to remove red blood cells. A portion of the harvested cells were diluted in 0.85% saline solution at a final density of 4×10^6/ml for use as BM cells. Some of the cells were cultured in six-well plates (density, 4×10^6/ml) in RPMI1640 with 5 ng/ml of recombinant rat granulocyte macrophage colony-stimulating factor (GM-CSF) and 5 ng/ml of interleukin (IL)-4 (Peprotech, NJ, United States). Nonadherent granulocytes were removed after 48 h of culture. The cytokine-enriched medium was refreshed every other day. On day 7, non-adherent and loosely adherent cells were harvested and identified as immature DCs. Trypan blue exclusion testing showed more than 95% cell viability.

PU.1 silencing

1) Small interfering RNAs (siRNAs) targeting the PU.1 gene were synthesized by Jima Corporation (Shanghai, China). The sequences of the PU.1-specific siRNAs were as follows: sense, 5'-AGC GAU CAC UAU UGG GAU UTT-3'; antisense, 5'-AAU CCC AAU AGU GAU CGC UTT-3'. The sequences of the negative control siRNAs were as follows: sense, 5'-UUC UCC GAA CGU GUC ACG UTT-3'; anti sense, 5'- ACG UGA CAC GUU CGG AGA ATT-3'. 2) Lipofectamine 2000 (Invitrogen, United States) was used according to the manufacturer's instructions to transiently transfect the siRNAs into imDCs for 24 h. 3) The transfected cells were re-plated and cultured in the presence of 10 μg/ml LPS (Sigma Aldrich, United States) for an additional 48 h, and the harvested cells were designated as PU.1-silenced DCs and negative control-silenced DCs. 4) Real-Time PCR and WB were used to monitor the transfection efficiency of the PU.1 siRNAs.

Flow cytometric and mixed lymphocyte reaction (MLR) analysis of cultured cells

Surface molecule expression and phenotypic analysis of the PU.1-silenced DCs was determined by fluorescence-activated cell sorting using a Calibur flow cytometer equipped with Cell Quest (Becton Dickinson, New Jersey, United States). The following antibodies were purchased from eBioscience Corporation (CA, United States): phycoerythrin (PE)-coupled anti-CD86, PE-coupled anti-CD80 and fluorescein isothiocyanate-coupled anti-MHCα The restricted marker for rat DCs, OC-62 Alexa Fluor, was purchased from BioLegend (CA, United States). For the MLR analysis, T cells (2×10^5) purified from rat splenocytes were used as responder cells and were plated with DCs from the different experimental groups at varying ratios. We used Nylon Fiber Column T (Wako, Japan) according to the manufacturer's instructions to separate T-cells. Cells were cultured for 3 d and pulsed with 1 μCi of 3H-thymidine (PerkinElmer, Woodbridge, United States) for the final 18 h. The cells were subsequently harvested onto glass fiber filters, and the incorporated radioactivity was quantified using a liquid scintillation counter.

Experimental groups

The allogeneic segmental heterotopic intestinal transplantation (male BN rats to female Lewis rats, HIT) was performed under pentobarbital anesthesia in a sterile field using the cuff technique. A 15-20-cm segment of ileum was removed from a donor and transplanted into the recipient. After vascular anastomosis, a stoma was generated in the abdominal wall of the distal intestine to observe pathological changes. The study included 4 groups: the control group, the pure HIT administration group (n = 6); the BM group, HIT with transfusion of donor-derived bone marrow cells (2×10^7 cells, day 0) via the portal vein (PV) (n = 6); the BM-imDCs group, HIT with transfusion of imDCs (2×10^7 cells, day -2) plus donor-derived bone marrow cells (2×10^7 cells, day 0) (n = 6); the BM-PU.1 group, HIT with transfusion of PU.1-silenced DCs (2×10^7 cells, on day -2) plus donor-derived bone marrow cells (2×10^7 cells, day 0) (n = 6). The donor marrow cells helped to induce mixed chimerism (Fig. 1).

Histology

Graft tissues were immersion-fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (HE). Specimens from the stomas were obtained at 0, 7, 14, 21 and 30 days after HIT, and rejection grading was performed to estimate the degree of rejection [21].

Real-time PCR analysis

Real-time PCR for the sex-determining region of the Y chromosome (SRY) was performed using samples of recipient blood, liver, spleen and intestine obtained on day 30. After genomic DNA extraction, the

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Fig. 1. Flow diagram.
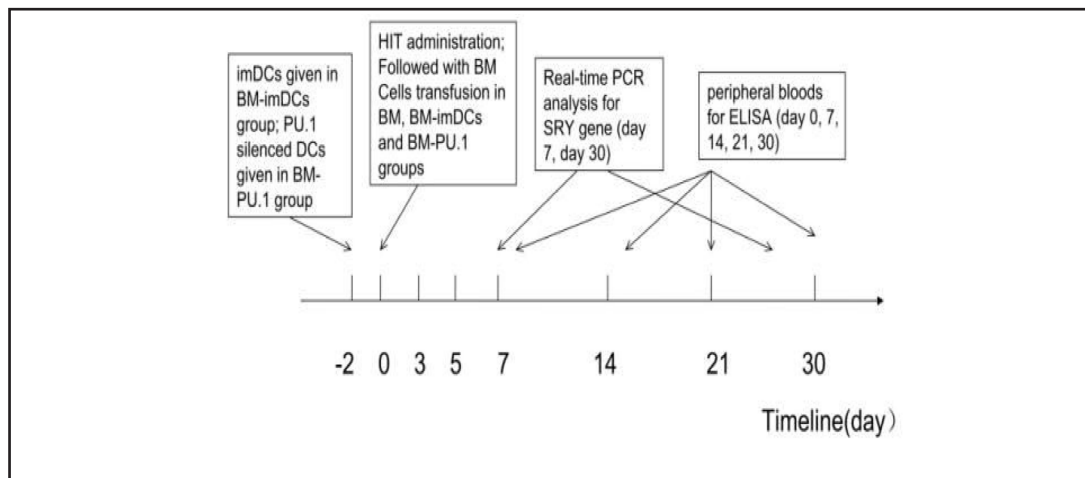
PCR mixture was prepared using SYBR Premix Ex TaqTM (TaKaRa, Dalian, China). The primers used for PCR were 5'-AAG TCA AGC GCC CCA TGA-3' (sense) and 5'-TGA GCC AAC TTG TGC CTC TCT-3' (antisense) [22]. Each 25-μL sample was denatured at 95°C for 40 15-s cycles, and then subjected to annealing and extension (60°C, 1 min, ABI 7900HT Fast RealTime PCR System, Applied Biosystems Inc., USA). Genomic DNA from male BN rats was mixed at proportions of 100%, 20%, 4%, 0.8% and 0.16% with genomic DNA from female Lewis rats. The mixed DNA samples were used to establish standard curves [23].

Enzyme-labeled immunosorbent assay (ELISA)

Peripheral blood was collected via the tail vein on days 0, 7, 14, 21 and 30 for ELISA. Serum levels of Th1-related cytokines (IL-2) and Th2-related cytokines (IL-10) were assayed in duplicate using a commercial rat IL-2 or IL-10 ELISA Kit (R&D Systems, Minneapolis, United States) according to the manufacturer's instructions.
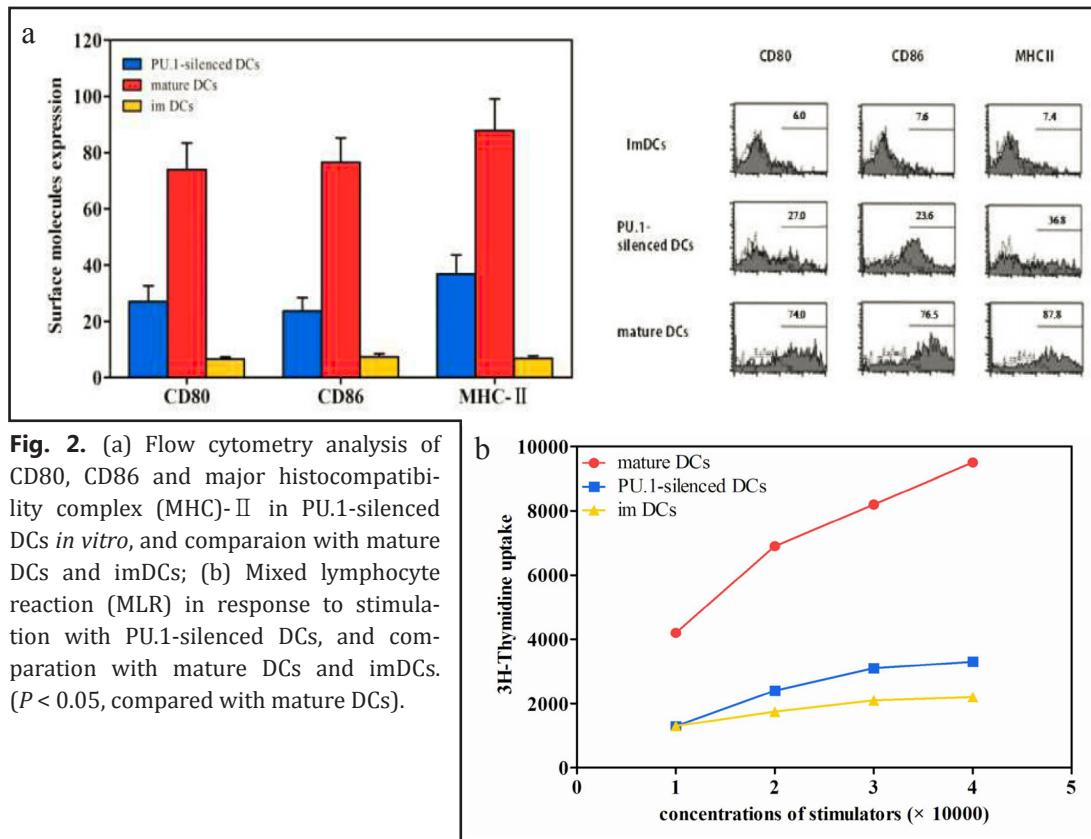
Statistical Analysis

All results are expressed as the mean ± SD. Statistical analyses were performed using one-way analysis of variance (ANOVA) with a post hoc test to ascertain the p-values of differences between groups. All analyses were performed using SPSS 13.0 (SPSS, Chicago, III). $P < 0.05$ was considered significant.

Results

In vivo detection of the immunophenotype and tolerogenic functions of PU.1-silenced DCs

PU.1-silenced DCs were obtained from bone marrow cells cultured in low-dose GM-CSF and IL-4 for 7 days. Similar to the phenotypic characteristics of imDCs (6.0% ± 1.2%, 7.6% ± 1.8% and 7.4% ± 1.3%), PU.1-silenced DCs displayed low expression levels of CD80, CD86 and MHCα(27.0% ± 5.6%, 23.6% ± 4.8% and 36.8% ± 6.8%, respectively) compared with mature DCs (74.0% ± 9.4%, 76.5% ± 8.7% and 87.8% ± 11.3%, respectively) (Fig. 2a). In an MLR, PU.1-silenced DCs inhibited the proliferation of naive Lewis splenocytes as effectively as immature DCs (Fig. 2b). In contrast, when pre-treated with mature DCs, a significantly higher response was observed ($P < 0.05$).

SRY gene real-time PCR assays were used to observe the establishment of mixed chimerism on day 30 (Fig. 3). The relative SRY gene expression in the BM-PU.1 group was higher than that in the BM group (3.03 ± 0.16% vs 1.15 ± 0.07% in the blood, $P < 0.05$; 0.86 ± 0.05% vs 0.21 ± 0.02% in the liver, $P < 0.05$; 4.08 ± 0.12% vs 1.23 ± 0.05% in the spleen, $P < 0.05$; and 2.01 ± 0.27% vs 0.71 ± 0.09% in the intestine, $P < 0.05$, respectively). There were no significant differences in relative SRY gene expression between the BM group and the BM-imDCs group.

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Fig. 2. (a) Flow cytometry analysis of CD80, CD86 and major histocompatibility complex (MHC)-Ⅱ in PU.1-silenced DCs *in vitro*, and comparaion with mature DCs and imDCs; (b) Mixed lymphocyte reaction (MLR) in response to stimulation with PU.1-silenced DCs, and comparation with mature DCs and imDCs. ($P < 0.05$, compared with mature DCs).

Fig. 3. Real-time PCR analysis for sex-determining region of Y chromosome (SRY) in tissues (blood, liver, spleen and intestine) of female Lewis recipient on day 30. Data were expressed and compared in different groups as the Mean ± SD (*p < 0.05, compared with control group; # p < 0.05, compared with BM group; n = 6).
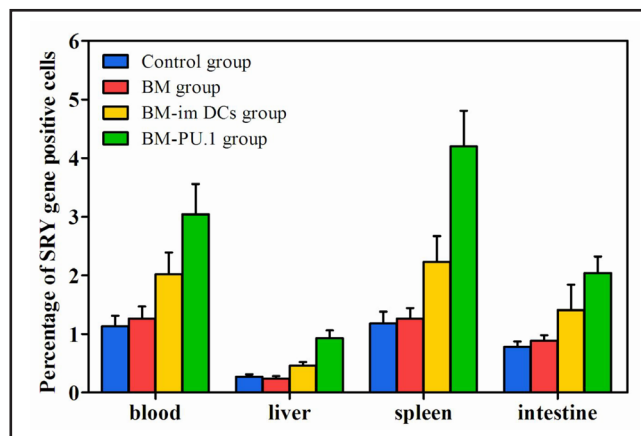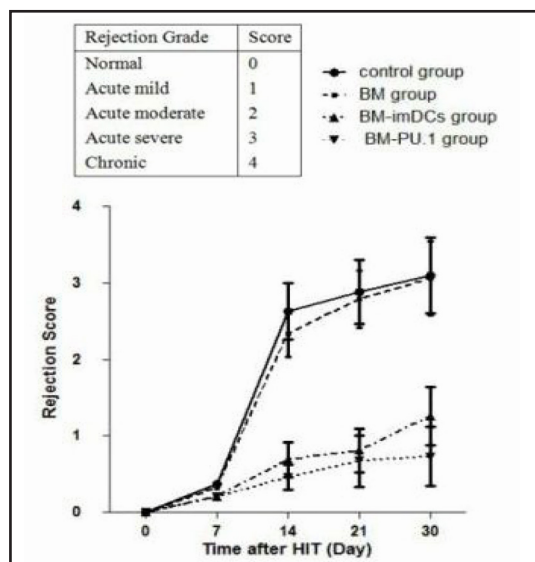

Histopathological examination of grafts

Histopathological examination of the grafts revealed different degrees of lymphocyte infiltration and villous edema at different time points. Evaluation of the histological grade of injuries demonstrated significantly lower scores in the BM-PU.1 group than in the BM group at days 14, 21 and 30 (0.5 ± 0.2 vs 2.7 ± 0.3, $P < 0.05$; 0.7 ± 0.3 vs 2.8 ± 0.3, $P < 0.05$; 0.8 ± 0.3 vs 3.1 ± 0.3, $P < 0.05$, respectively) (Fig. 4).

Changes in cytokine expression

ELISA was used to determine the levels of serum IL-2 and IL-10 after intestinal transplantation. The levels of IL-2 increased significantly and peaked on day 14 in the control, BM-imDCs and BM-PU.1 groups. However, in the BM group, IL-2 levels exhibited a decreasing trend after day 7. All of the curves returned to almost the same level at day 30. The extent of the increase in IL-2 was reduced in the BM-imDCs and BM-PU.1 groups compared with

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Fig. 4. Histological changes in intestinal grafts after HIT in Lewis rats. The evaluation of the histological grade of grafts was based on the score system (Criterion: normal = 0; mild, moderate and severe rejection = 1, 2, and 3, respectively; chronic rejection = 4). Data were expressed as the Mean ± SD.
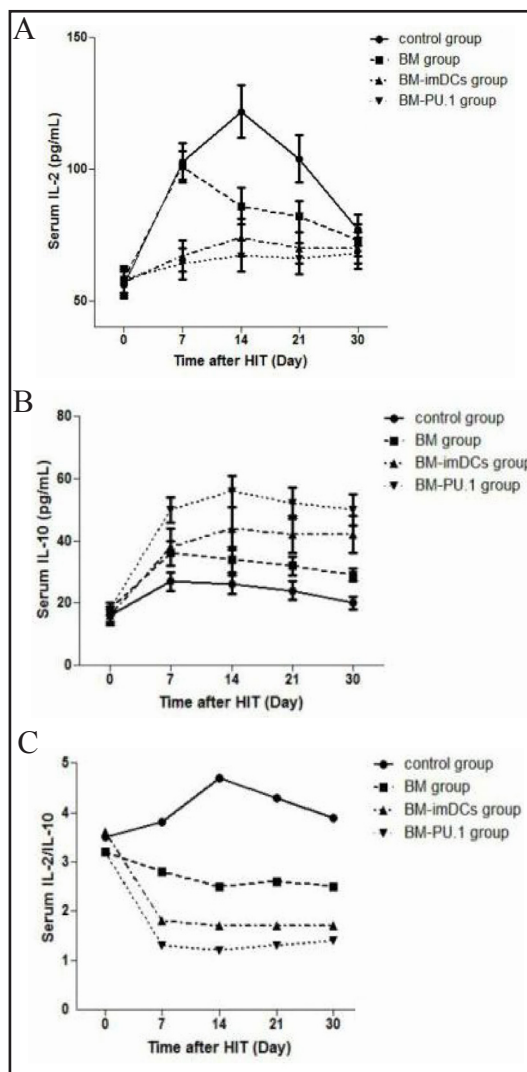
Fig. 5. Trends of serum IL-2, IL-10 and IL-2/IL-10 ratio after HIT by ELISA. Data were expressed as the Mean ± SD.

the BM group ($P < 0.05$). The IL-2 level in the BM-PU.1 group was slightly lower than that in the BM-imDCs group on days 21 and 30 after HIT ($63.87 ± 3.20$ pg/ml vs $81.62 ± 3.47$ pg/ml, $P < 0.05$; $67.22 ± 2.20$ pg/ml vs $72.06 ± 3.40$ pg/ml, BM-PU.1 vs BM on day 21 and 30, respectively) (Fig. 5a). Conversely, the IL-10 levels increased after HIT in all groups (Fig. 5b). The IL-10 levels in the control group and BM group peaked on day 7 and then gradually decreased. However, in the BM-imDCs and BM-PU.1 groups, peak levels were observed at day 14 and remained high until day 30. The duration and amplitude of the increase in IL-10 was larger in the BM-PU.1 group than in the other groups ($53.07 ± 0.94$ pg/ml vs $34.05 ± 0.88$ pg/ml, $P < 0.05$; $50.25 ± 0.85$ pg/ml vs $32.53 ± 0.93$ pg/ml, $P < 0.05$. BM-PU.1 vs BM on day 21 and 30, respectively). The level of IL-10 in the BM-imDCs group was lower on days 21 and 30 than that in the BM-PU.1 group.

To determine the Th1/Th2 balance, the IL-2/IL-10 ratios were calculated using the data presented in Fig. 5a and Fig. 5b (Fig. 5c). The IL-2/IL-10 ratio in the control group increased and peaked 14 days after HIT. The IL-2/IL-10 ratio decreased sharply in the BM-PU.1 and BM-imDCs groups on day 7 and then remained at a relatively stable level. The IL-2/IL-10 ratio decreased slightly in the BM group and remained at a higher level after day 14. Fourteen

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days after HIT, the IL2/IL-10 ratios in the control, BM, BM-imDCs and BM-PU.1 groups were 4.7 ± 0.2, 2.6 ± 0.3, 1.8 ± 0.2 and 1.3 ± 0.1, respectively ($P < 0.05$).

Discussion

Intestinal transplantation is almost the only life-saving procedure in patients with end-stage intestinal disease who fails on TPN and without other available medical or surgical treatment options. However, it is associated with increased morbidity and mortality, which acute and chronic rejection remains a major cause of both early graft loss and unsatisfied long term recipient survival. In recent years, effective immunosuppressants are provided to induce general immunosuppression during the peri-operative period, but they also direct toxicity and some serious side effects yet. How to inducing donor specific immune tolerance and getting a satisfactory graft condition remains a challenge for all transplantation.

Mixed chimerism has been shown to be an attractive method for inducing tolerance in major organ transplantation without the need for lifelong immunosuppression [24, 25]. However, the establishment of long-term and stable chimerism without myeloablative treatments remains a major challenge. In previous studies investigating intestinal transplantation in mice, imDCs were shown to be an alternative to myeloablative conditioning. They established long-term (> 100 days) bone marrow chimeras in mice [6] and increased the induction of CD4+ CD25+ FoxP3+ regulatory T cells, an indicator and long-term marker of tolerance [26].

We designed an innovative strategy of inducing mixed chimerism after intestinal transplantation in rats. Donor-derived bone marrow cells were transfused through the PV to induce mixed chimerism, while PU.1-silenced DCs were administered 2 days before surgery to facilitate the process. Our results showed that chimerism was successfully established; it was stable and without notable side effects. Even on day 30, bone marrow cells from the donors were observed in the blood, liver and spleen of the recipients and in the small intestine of the recipient adjacent to the grafted donor small intestine. We believe that this strategy has the potential to be an effective method for inducing immune tolerance and reducing toxicity due to myeloablative or cytoreductive recipient conditioning [27]. Histopathological analysis revealed that the BM-PU.1 treatment markedly attenuated mucous atrophy and submucous fibroplasias, especially after day 14. Recipients that did not exhibit long-term stable chimeras and recipients with no chimeras rapidly rejected the allografts, demonstrating the importance of chimerism in the establishment of tolerance.

The Th1/Th2 balance plays an important role in transplantation immunology, and the representative cytokines IL-2 and IL-10 play fundamental roles in regulating this balance. Differentiated Th1 cells excrete IL-2 and IFN-γ, which promote the maturation of cytotoxic T lymphocytes and correlate highly with the severity of transplant rejection and failure. Th2 cells secrete IL-10 and IL-4, which are anti-inflammatory cytokines. IL-10 can down-regulate the synthesis of a broad range of inflammatory cytokines and inhibit allogeneic T cell proliferation by blocking the synthesis of IL-12 in DCs. The production of high levels of endogenous IL-10 has been associated with the function of regulatory T cells and the induction of transplantation tolerance to allogeneic antigens [28]. IL-4 has previously been shown to play a critical role in the establishment of mixed chimerism in irradiation-treated mice. Skewing the immune response towards a Th2 cytokine profile may be one mechanism for inducing immune tolerance. In this study, when severe acute rejection was observed on day 7, high IL-2, low IL-10 and a high IL-2/IL-10 ratio were observed in the control group. This ratio was significantly converted in the BM-PU.1 group, and the values were maintained at a lower level from day 7 to day 30. These results indicate that when used in HIT, PU.1-silenced DCs followed by bone marrow transfusion can induce T-cell anergy, produce stable mixed chimerism, and lead to Th2 polarization, converting the IL-2/IL-10 ratio. These findings are consistent with previous reports showing that induction of allogeneic mixed

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chimerism using imDCs and bone marrow transplantation leads to prolonged tolerance in cardiac and skin transplantation [6].

To our knowledge, it is the first report of the establishment of long-lasting mixed chimeras in rats through pre-conditioning with PU.1-silenced DCs and donor derived bone marrow transfusion. This conditioning regime induces more stable mixed chimerism, promote a Th2 shift in Th1/Th2 balance and facilitate the induction of immune tolerance. It has a potential to allow the long-term survival of grafts without additional need for immunosuppression or myeloablative therapies. Based on these findings, our study may suggest to being among potential clinical solutions to prevent rejection and achieve immunological tolerance prior to organ transplantation. However, further studies are still planned to investigate whether immunosuppression can halt allograft rejection after chimera loss.

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Disclosure Statement

The authors do not have any conflict of interest, personal or organizational, financial or familial, real or apparent, in participating in this procurement.

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