Mechanisms of thymic repair and immune reconstruction of in vitro-induced precursor T cells as a modified haplo-identical HSCT regimen

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Research Article

Keywords: precursor T, hematopoietic stem cell transplantation, thymic repair, immune reconstitution, RANK/RANKL

Posted Date: March 22nd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1456346/v1

Abstract

Purpose Diseases relapse and severe infection caused by delayed immune reconstitution are still challenges in the haplo-identical hematopoietic stem cell transplantation (haplo-HSCT). Non-myeloablative transplant regimens also only alleviated these complications and did not solve the above problems. Among them, the thymus damage caused by preconditioning including irradiation and chemotherapy and graft-versus-host disease (GVHD) is the primary issue. Accelerating the recovery of thymus-derived T cells after transplantation is a key to solving above problem.

Methods Therefore, we established a cell culture system using Notch ligand Delta4 and IL-7 that could induce and amplify hematopoietic stem cells differentiation and proliferation into precursor T (preT) cells in vitro. Non-myeloablative HSCT protocol was modified by using induced preT and G-CSF mobilized donor splenic mononuclear cells (MNC) co-infusion. Thymic GVHD, thymic repair, thymus-derived T cell development were compared in thymus-irradiated mice treated with MNC+preT and only MNC by polychromatic immunofluorescence tracking and T cell receptor Vβ detection.

Results The data showed that pre T cells highly expressed CC9 and RANKL markers restored thymic architecture, weakened GVHD effects, enhanced immuno-tolerance and accelerated immune reconstitution by activating the RANK/RANKL pathway.

Conclusion The modified non-myeloablative regimen containing preT cells will be more conducive to the widespread use of haplo-HSCT in clinical work.

Introduction

Haplo-identical hematopoietic stem cell transplantation (haplo-HSCT) is a reliable and effective treatment for hematological malignancies (1-5). However, the T cell reconstitution after transplantation is slow due to thymus injury caused by irradiation and chemotherapy, natural thymus aging, thymus destruction by allogenic donor lymphocytes, which are the main reasons for serious infection, acute and chronic GVHD, diseases relapse and other serious complications affecting the wide application of haplo-HSCT (6). Non-myeloablative transplant regimens also only alleviated these complications and did not completely solve the above problems. It is well known that early T cells are derived from donor mature T cell expansion after transplantation. The expression of a single T cell antibody and the lack of donor and recipient bidirectional immune tolerance is the main cause of severe infection and severe GVHD. Donor T cells or naive cells derived through thymus development have better potential for polyclonal T cell antibody expression and better donor and recipient immune tolerance can well solve the above problems. Unfortunately, thymic-derived T cells due to thymic damage after transplantation usually need to recover slowly after more than one and half years after transplantation. Therefore, how to reduce thymus injury, accelerate thymocytes differentiation and maturation, and induce the immune tolerance during thymus reconstruction has always been the research focus of transplant experts. It was reported that precursor T cells with low immunogenicity restore thymus structure by entering the recipient's thymus and enhancing

T-cell regeneration in an immune-deficient mouse model (7-9). However, it has not been applied in mice that possess normal immunity.

Thus, we attempted to infuse granulocyte stimulating factor (GCSF)-mobilized donor mouse spleen monocytes and Notch signal activated donor precursor T cells to thymus-irradiated haplo-identical mice. We observed the pathway and time points of precursor T cell thymus homing and T-cell regeneration. We also analyzed the effects of precursor T cells on restoring the structure of impaired thymus and weakening the thymus GVHD, aiming to find evidence and new stem cell therapy for the application of precursor T cells to enhance immune recovery after haplo-HSCT.

Methods

Generation of precursor T cells ex vivo in a feeder-free culture system

Six-well culture plates were incubated with 1 μg/ml DLL4-Fc (Sino Biological, China) and 5 μg/ml RetroNectin (Takara, Japan) for 24 h at 4°C, washed twice with cold PBS and incubated further for 1 h with 2% HSA diluted in PBS. Mouse lineage negative hematopoietic progenitor cells were seeded at a density of 1×105/well and cultured in αMEM medium (Gibco, USA) containing 20% FBS (Gibco, USA), 30 ng/ml mouse SCF, 5 ng/ml mouse Flt3L, and 5 ng/ml mouse IL-7 (Sino Biological, China). Culture system was refreshed via half-medium change, twice a week and maintained for about ten days.

Mice and Non-myeloablative haplo-HSCT model

Six to eight-week-old male C57BL/6-Gt(ROSA)26Sortm1(CAG-tdTomato)/Vst mice (C57BL/6-tomato) were purchased from the Jackson Laboratory (USA). Six to eight-week-old male C57BL/6N mice and female CB6F1 mice (the hybrid offspring of C57BL/6N and Balb/c) were purchased from Vital River Laboratory (China). Donor C57BL/6N mice were treated with GCSF, q12h for 5 days, and then sacrificed and splenic cells were collected. A stable haploid mouse transplantation model with reduced preconditioning was already established in our lab and 6Gy thymus irradiation was used as recipient mice preconditioning in this experiment. Four hours before transplantation, the upper and lower thymus of recipient CB6F1 mice were blocked using a lead plate, so that only the thymus received 6Gy Co60 irradiation at a rate of 105cGy/min. Each recipient CB6F1 mice received 6×107 GCSF mobilized donor splenic cells after irradiation could lead to stable donor chimerism in bone marrow.

Cells dose and groups setting

There were 24 mice in the MNC + preT group. Each received 6×107 GCSF mobilized donor splenic mononuclear cells (MNC) and 1×107 precursor T cells(preT) through the caudal vein. A total of 18 mice were assigned to the MNC group, and each received only 6×107 GCSF mobilized donor MNC. Eighteen mice in the control group received an equal volume of PBS.

Detection of precursor T cell chimerism in the thymus of recipient mice

We used flow cytometry to distinguish between wild type donor cells and recipient thymocytes. PE positive precursor T cells originated from C57BL/6-tomato mice were used. No reduction in the fluorescence signal was observed owing to the stable tomato gene.

Tracking precursor T cell in the thymus via immunofluorescence

The thymus of recipient mice in the MNC + preT group that were harvested 3 days, and 1, 2, and 3 weeks after transplantation were fixed in 4% paraformaldehyde (Solarbio, China), cut into 5 μm slices, stained with anti-Cytokeratin 8 (red; thymus cortical), anti-Cytokeratin 5 (green; thymus medullary), and anti-Tdtomato (purple; precursor T cells or mature CD3 cells originated from precursor T cells), and then observed under a confocal microscope. The results are representative of three independent experiments.

Detection of precursor T cell differentiation in the recipient thymus

The thymus of recipient mice in the MNC + preT group that were harvested 3 days, and 1, 2, 3, 4, and 8 weeks after transplantation were ground into single-cell suspensions using a 70 μm filter (Falcon, USA). A lysis buffer (Kainuobio, China) was used to lyse erythrocytes. Then, the thymocytes were stained with anti-mouse-CD4-APC-CyTM7 and anti-mouse-CD8-BV421 (Biolegend, USA) to determine the stage of T-cell differentiation. The PE was used as ideal biomarkers to distinguish thymocytes that originated from precursor T cells among all thymocytes.

Evaluation of thymus structure restoration

For the pathological evaluation, all mice were sacrificed 8 weeks after transplantation. Harvested thymus was fixed in 4% paraformaldehyde (Solarbio, China), cut into 5 μm slices, and stained with hemotoxylin and eosin (HE) and immunofluorescent dye (same as detecting precursor T cell location using immunofluorescence). The harvested thymus was also stained with RANK antibody (R&D, USA) to detect the RANK expression within thymus after transplantation.

Thymus regeneration factor keratin growth factor (KGF) detection was performed using 500 μl of peripheral blood collected in anticoagulant centrifuge tubes 8 weeks after transplantation and these were placed at room temperature for 1 h. The serum was separated via centrifugation at 1000×g for 10 min at room temperature. KGF levels were detected using a mouse KGF ELISA kit, according to the manufacturer's instructions (Elabscience, China).

Evaluation of immune recovery

Central immune recovery

Thymus samples were harvested after 1, 3, and 8 weeks and made into a single-cell suspension as described above and stained with anti-mouse-CD4-APC-CyTM7, anti-mouse-CD8-BV421 (Biolegend, USA), and anti-mouse-TCR Vβ 2–17 (BD, USA). The cells were then examined using a flow cytometer. Mice

serum was collected as described above and detected using mouse IL-7 Elisa Kit, according to the manufacturer's instructions (R□D, USA).

Peripheral immune recovery

Exactly 50 μl of peripheral blood was collected through the caudal vein every week after transplantation, and erythrocytes were lysed with lysis buffer (BD, USA). Then, the peripheral blood cells were stained with anti-mouse-CD4-APC-CyTM7, anti-mouse-CD8-BV421, and anti-mouse-CD3-FITC (Biolegend, USA) and examined using flow cytometry.

Detection of precursor T cell function in attenuating GVH effects within the thymus

Exactly 50 μl of peripheral blood from each mouse was collected and tested to count white blood cells (WBCs) every week after transplantation. The mice thymus were harvested 8 weeks after transplantation were ground into single-cell suspensions as described above and stained with anti-mouse-CD4-APC-CyTM7, anti-mouse-CD8-BV421, anti-mouse-FasL-PE, and anti-mouse-IFN-γ-PE (Biolegend, USA). The fluorescence signal was detected using flow cytometer.

Statistics

The data and error bars were presented as the mean ± standard error of the mean. All statistical analyses were performed using the Prism (GraphPad Software, San Diego, USA) and SPSS software (IBM, USA). To determine statistical significance, a two-tailed unpaired Student's t-test was used. Significance was determined as $P < 0.05$.

Results

Precursor T cells were generated in a feeder-free culture system

Hematopoietic progenitor cells from the BM of male C57BL/6-tomato mice were stimulated with DL4-Fc that was used to precoat the cell culture plate and cultured for about ten days. Large numbers of precursor T cells, including double negative (DN) 2 stage cells (CD4-CD8-CD44 + CD25+) and DN3 stage cells (CD4-CD8-CD44-CD25+) were harvested (Fig. 1a-b). And the highly expression of CCR9 and RANKL was found in precursor T cells (Fig. 1c).

Stable donor chimerism was achieved in recipient mice after cell infusion

Complete donor chimerism in recipient mice peripheral blood, bone marrow and thymus was achieved within 4 weeks after transplantation (Fig. 1d-f). To eight weeks after transplantation, different degrees of GVHD symptoms including wrinkled hair, rash and weight loss were observed in MNC group and MNC + preT group.

Tracking precursor T cells migration in recipient mouse thymus

The highly expressed CCR9 and RANKL in precursor T cells contributed to thymus homing and crosslinking with RANK, which was expressed in thymic epithelial cells (TEC). The thymocytes originating from infused precursor T cells could be detected from 3 days to 8 weeks in the recipient mice thymus. Three days after cell infusion, precursor T cell chimerism was still low (3.1 ± 2.4%) (Fig. 1g). Then, the chimerism increased to more than 60% just two weeks later and slowly decreased to less than 1% at + 8 week. Thus, a temporary wave of T cell development was observed (10)

Then the migration of precursor T cells in recipient mice thymus were tracked. A few precursor T cells (purple) entered the thymus cortex from 3 days to 1 week after infusion, while they were still absent in the thymus medulla at this time (Fig. 1h). After 2 weeks, more precursor T cells were found in the thymus cortex and a certain amount of precursor T cells appeared on the thymus medulla. Therefore, allogeneic precursor T cells could migrate from the cortex to the medulla inside the recipient thymus, just the same as normal lympho-progenitor thymic migration and T-cell differentiation.

The phenotype of precursor T cells was then measured (Fig. 1i). They were still at the DN stage 3 days after infusion (93.8 ± 2.9%). After one week, less than 10% of preT cells had differentiated into DP thymocytes and two weeks later, a rather high percentage of preT cells had differentiated into the DP stage (70.6 ± 10.6%). The single positive (SP) thymocytes, including CD4 + and CD8 + cells, were less than 20% at this time. From week 4 to 8, the percentage of CD4 + and CD8 + SP cells were gradually increased to 36.4 ± 10.6% and 7.9 ± 4.4%. While the percentage of DP cells were decreased to 52.2 ± 14.4%. Combining phenotype change and migration pathway, we presumed that allogeneic precursor T cells could enter irradiated recipient mice thymus, start T-cell generation from the cortex to the medulla, and finally exit the thymus after differentiation to naive T cells.

Impaired thymus structure was restored after preT cell infusion

Through HE pathological observation, the irradiated thymus of mice in the control group was recovered 8 weeks after transplantation, with an intact basic structure, and a normal number of lymphocytes and thymus corpuscles (Fig. 2a).

Unsurprisingly, the thymus of mice in the MNC group were severely impaired and disorganized. The number of lymphocytes and thymus corpuscles were largely decreased. Instead, more blood vessels and fibrous tissue emerged. After precursor T cell infusion, although the thymus structure was not as good as the control group, it was much better organized than the MNC group. Immunofluorescence pathology is a valuable method for studying the areas of the thymus cortex and medulla. The thymus of the control and MNC + preT groups had a cortex, medulla, and a defined corticomedullary boundary, while the thymus of the MNC group exhibited a rather poor boundary between the cortex and medulla and the medulla was even absent in some samples (Fig. 2b).

Eight weeks after precursor T cell infusion, higher RANK expression of thymic epithelial cells (TEC) was found in MNC + preT group, comparing to that in MNC group. No significantly difference could be found between MNC + preT group and control group. (Fig. 2c)

The KGF level in the MNC + preT group was significantly higher than that in the MNC group (14.1 ± 2.8 vs 8.4 ± 5.1, P = 0.0243) (Fig. 2d). It proved the thymus of mice in the MNC + preT group actively initiated restoration after precursor T cell infusion.

Immune reconstitution was accelerated upon precursor T cell infusion

The total number of thymocytes and the T-cell development level represented by the proportion of cells at DP stage are vital indexes of central immune reconstruction. One week after transplantation, the number of thymocytes were significantly decreased in both the MNC + preT and MNC groups compared to the control group (1.8 ± 1.2×106 and 2.7 ± 1.9×106 vs 47.1 ± 1.9×106, P□0.01). And the percentage of DP cells in MNC + preT group and MNC group were 14.1 ± 1.7% and 15.5 ± 4.5%, respectively, which were significantly lower than 71.5 ± 0.3% in Control group (P□0.01). Proving that the thymus was severely impaired and T-cell regeneration was severely delayed after donor splenic cell infusion (Fig. 3a-b). At 3 weeks after transplantation, the number of thymocytes and percentage of DP cells in MNC + preT group were higher than that in MNC group (11.5 ± 6.9×106 vs 2.3 ± 0.8×106, P = 0.08;75.5 ± 0.6% vs 60.4 ± 4.9%, P□0.01). To 8 weeks after cell infusion, the indices of the MNC + preT group were significantly higher than those of the MNC group (37.9 ± 24.0×106 vs 12.3 ± 15.8×106, P = 0.008; 56.2 ± 10.4% vs 28.4 ± 24.6%, P = 0.005). This indicates that central immune reconstruction was significantly accelerated with precursor T cells' help at the cellular level.

IL-7 is a key cytokine in the development of T-cell generation in the thymus, and its secretion level is closely related to the maturation process of T cells. Serum IL-7 levels in the MNC + preT and control groups were significantly higher than those in the MNC group (55.1 ± 31.6 vs 14.5 ± 14.3, P = 0.0167), suggesting that infusion of preT cells promoted T-cell regeneration in the thymus (Fig. 3c).

TCR Vβ expressions of several DN and DP thymocytes in the MNC + preT group were higher than those in the MNC group, and the expression of Vβ9 was significantly different among them (0.93 ± 0.05 vs 0.37 ± 0.30, P = 0.015) (Fig. 3d). This suggested that precursor T cells enriched the diversity of the TCR repertoire. However, there was no significant difference in TCR expression between the two groups for SP CD4 + and SP CD8 + T cells. Then, these two transplant groups were compared with the control group. TCR expression in the former two groups was higher than those of Vβ9 and Vβ12. No significant differences were found in other loci.

The status of peripheral immune reconstitution was closely related to central immune reconstitution due to the continued export of naive T cells from the thymus. The peripheral blood CD4:CD8 ratio, which reflects immune homeostasis, was normal in the control group, and no inverted lymphocyte subsets were found (Fig. 3e). At 4 weeks and 8 weeks after transplantation, the CD4:CD8 ratio of mice in the MNC + preT group was significantly higher than that in the MNC group (1.2 ± 07 vs 0.6 ± 0.5, P = 0.018; 2.3 ± 1.2 vs 1.6 ± 0.6, P = 0.013). The percentage of peripheral blood CD3 cells 8 weeks after was also significantly higher in the former group (15.1 ± 6.7 vs 7.0 ± 5.1, P = 0.04) (Fig. 3f). It demonstrated that the precursor T cells accelerated T-cell regeneration of peripheral blood and reconstitution of peripheral immune cells.

Immune tolerance induced by infusing preT cells attenuated donor GVHD or GVH effects in thymus

We assumed that a more severe immune response occurred in this group. As shown in Fig. 3e, the peripheral blood CD4:CD8 ratio was lower in the MNC group compared with MNC + preT group. As decreased CD4:CD8 ratio is closely related to GVHD, and the GVH effect mediated by CD8 + T cells is the main reason for recipient tissue damage (11), we measured the FasL and IFN-γ expression in thymocytes, which was positively correlated with the GVH effect (12). Percentages of FasL-positive CD4 + and CD8 + cells in the MNC + preT group were significantly lower than those in the MNC groups (0.36 ± 0.19 vs 1.84 ± 1.45, P = 0.03; 0.58 ± 0.17 vs 4.65 ± 2.46, P = 0.003) (Fig. 4a-b). The percentage of IFN-γ-positive CD8 + cells was also lower than that in the MNC group (0.92 ± 0.66 vs 2.42 ± 1.38, P = 0.02) (Fig. 4b). As a result, GVH effects within the recipient thymus were attenuated after precursor T cell infusion. Owing to this, the thymus structure and function soon recovered to normal immune status.

Discussion

Thymus is the vital central immune organ. During the process of hematopoietic stem cell transplantation, serious damage to thymus gland is caused by high-dose irradiation and chemotherapy in preconditioning or GVHD by the large number of donor mature lymphocytes homing to recipient thymus (7–10, 13–17). The recovery of thymic tissue, structure and function, and the recovery of the number and function of donor T cells through thymic selection and development are key factors in haplo-HSCT success. Therefore, researchers tried to add allogeneic precursor T cells that have thymus homing ability during transplantation to rapidly replenish the "seed" for T-cell differentiation within the thymus (8, 10, 18). However, they were mostly applied to immunodeficient mice, thus these models do not mimic the functions and effects of preT cells on wild type mice and also not mimic the clinical haplo-HSCT model (9, 15, 16). Here, we used wild type mice with normal immunity and MHC barrier as recipients and GCSF mobilized splenic cells that contained a large number of donor mature lymphocytes as a transplant to mimic the immune response that occurred in haplo-HSCT. It has not been studied how allogeneic precursor T cells enter the recipient thymus and whether it is different from the normal thymus homing process. Thymus homing and T-cell development effects of allogeneic precursor T cells were first detected in this study. The CCR9 and RANKL highly expressed allogeneic precursor T cells first entered the low-dose-irradiated thymus cortex and differentiated for 2–3 weeks, then migrated to the thymus medulla, and finally exported after completing the complete T-cell development process. Recipient mice thymus achieved high donor chimerism (≥95%) at 2 weeks after transplantation. Interestingly, the precursor T cell chimerism reached its peak now (≥60%) and could be accounted for more than half of the total donor cell chimerism. Although two weeks ago, the precursor T cell infusion dose was only 1/6 of that of spleen cells.

Next, we observed a significant increase in number of thymocytes and the percentage of DP thymocytes in MNC + preT group compared to MNC group. Indicating that precursor T cells not only homed and proliferated fast in the recipient thymus, but also promoted the donor stem cells' thymus homing and T cell reconstitution. The serum IL-7 level of MNC + preT group at 8 weeks after transplantation was also

higher than MNC group. TCR Vβ expressions of several DN and DP thymocytes in the MNC + preT group were higher than those in the MNC group, and the expression of Vβ9 was significantly different among them. It demonstrated that the diversity of the TCR repertoire was enriched with precursor T cells' infusion (19). However, there was no clear clinical significance of Vβ9 difference. The upregulation of TCR Vβ9 was positively correlated with GVT effect, while the upregulation of other sites such as β4, β11, β23 was positively correlated with the occurrence of GVHD (20). In the meantime, the increased percentage of CD3 cells and CD4:CD8 ratio in the peripheral blood demonstrated the regeneration of peripheral immune cells after precursor T cells' infusion.

Above results were closely related to the repaid recovery of damaged thymus (21, 22). In our study, the thymus in the MNC + preT group had an intact structure and a clear boundary between the cortex and medulla. The highly disorganized and medulla-lacking architecture was observed in the thymus of the MNC group. Obviously, damaged recipient thymus was restored effectively with precursor T cells infusion. The biomarkers of precursor T cells generated in vitro were detected and the highly expressed of RANKL was found. RANKL could accelerate T cell proliferation by contributing to thymus homing and crosslinking with RANK, which was expressed in thymic epithelial cells (TEC) (23). Eight weeks after precursor T cell infusion, higher RANK expression of thymic epithelial cells (TEC) was found in MNC + preT group, comparing to that in MNC group in our experiment .Then the fast proliferation of DP and SP thymocytes could in turn induce the reconstruction of thymic medullary cells (24, 25). Therefore, the restored thymus structure may be due to the enhanced RANKL-RANK cross-talk between lymphocytes and TECs (8).

Finally, we found attenuated GVHD effects of donor lymphocytes within the recipient thymus by infusing precursor T cells (26). The donor mature lymphocytes could enter the recipient thymus soon after transplantation. As mentioned above, the thymic structure in the MNC + preT and MNC groups was severely impaired just one week after transplantation. The total number of thymocytes and percentage of DP thymocytes were significantly decreased. Strong GVHD effects were observed in our study. If there was no intervention, the thymic architecture would be severely damaged and the medulla would be absent as mentioned above 8 weeks after transplantation. The thymus medulla is important for negative selection in the T-cell development (27), and a lack of negative selection due to missing medulla could largely delay the reconstitution of immuno-tolerance. In our experiment, higher FasL and IFN-γ expression of CD4 and CD8 T cells were detected in MNC group compared to MNC + preT group. Precursor T cells infusion through RANK/RANKL pathway activation not only promotes thymic epithelial cell development, thereby increasing the functional recovery of thymic double-negative selection, while the rank RANK/RANKL pathway can also promote the proliferation of thymic Treg cells.(28) So the combination of these effects induces immune tolerance in donor and recipient while attenating the GVHD response.

Conclusion

We used wild type mice with normal immunity as the recipient in a haplo-HSCT mouse model with ex vivo generated precursor T cells infusion to mimic clinical haplo-HSCT. The thymus homing and T-cell

regeneration of allogenic precursor T cells were observed. In vitro-induced allogeneic precursor T cells highly expressed CC9 and RANKL surface markers, which promoted thymic homing, development, maturation, and polyclonal TCR expression of pro-allogeneic T cells by activating the RANK/RANKL pathway, while also acted as immune tolerance and reduced GHVD effects. Our results provide a potential therapeutic option to optimize the haplo-HSCT scheme. It further opened a new field of T cell therapy for artificial induction of allogeneic precursor T cells in vitro to repair the irradiation and chemotherapy damaged thymus and to compensate for the immune function recovery of patients with immune deficiency caused by multiple reasons.

Declarations

Funding

This work was supported by National Natural Science Foundation of China NO. 81670110

Conflict-of-interests disclosures: The authors declare no competing financial interests.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Hui-Sheng Ai, Kai-Xun Hu designed and analyzed the experiments and wrote the manuscript. Yang-yang Lei; Yi Wang; Kai-Xun Hu, Mei Guo, Chang-Lin Yu, Jian-Hui Qiao; Bo Cai; Xin-rui Chen; Shan Jiang performed and analyzed the experiments and prepared the figures. All authors have read and approved the final manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate: Not applicable

Consent to participate

All authors have reviewed and approved the manuscript for submission.

Consent for publication

All authors have reviewed and approved the manuscript for submission.

Acknowledgements: Not applicable

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Figures

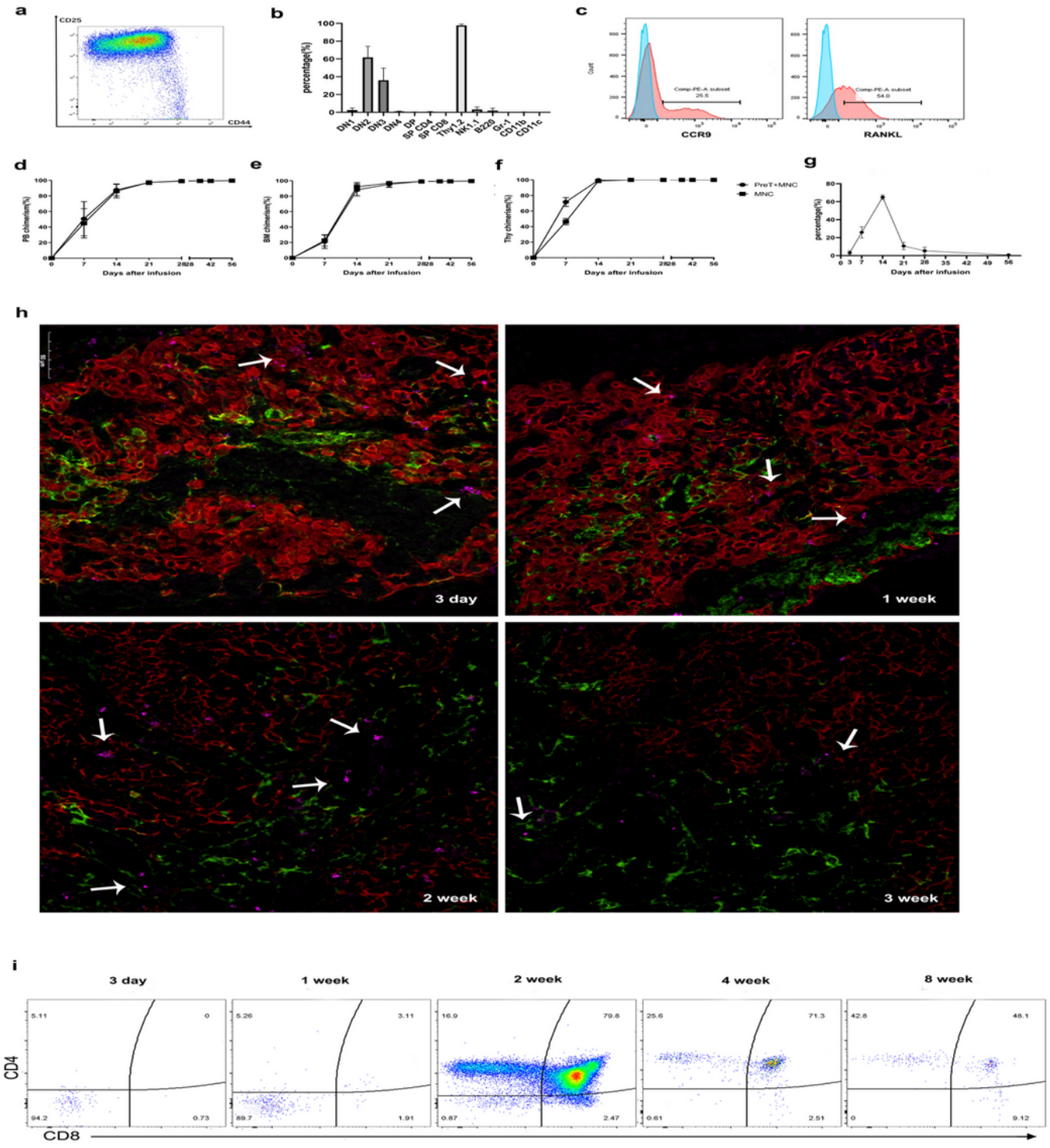

Figure 1

Allogenetic preT cells generated ex vivo could homing recipient thymus and start T-cell development process. (a-b) Cellular phenotype of precursor T cells after ten-day culture. (c) CCR9 and RANKL were highly expressed in surface of precursor T cells. (d-f) Donor chimerism was stable in peripheral blood,

bone marrow and thymus of recipient mice (donor cells were only H-2Kb positive while recipient cells were both H-2Kb and H-2Kd positive). (g) Thymocytes that originated from precursor T cells were PE positive while the thymocytes originated from wild-type donor mice and recipient mice were PE negative. Then the percentage of precursor T cell originated cells' chimerism was analyzed and counted by flow cytometry. (h) Mice thymus tissue were stained with anti-Cytokeratin 8 (red; thymus cortical), anti-Cytokeratin 5 (green; thymus medullary) and anti-Tdtomato (purple; precursor T cell originated cells). The migration and location of precursor T cell originated cells were observed under an immunofluorescence microscope (White arrows indicated clusters of precursor T cell originated cells). (i) The precursor T cells started T-cell regeneration process from DN stage to SP stage after thymus homing.

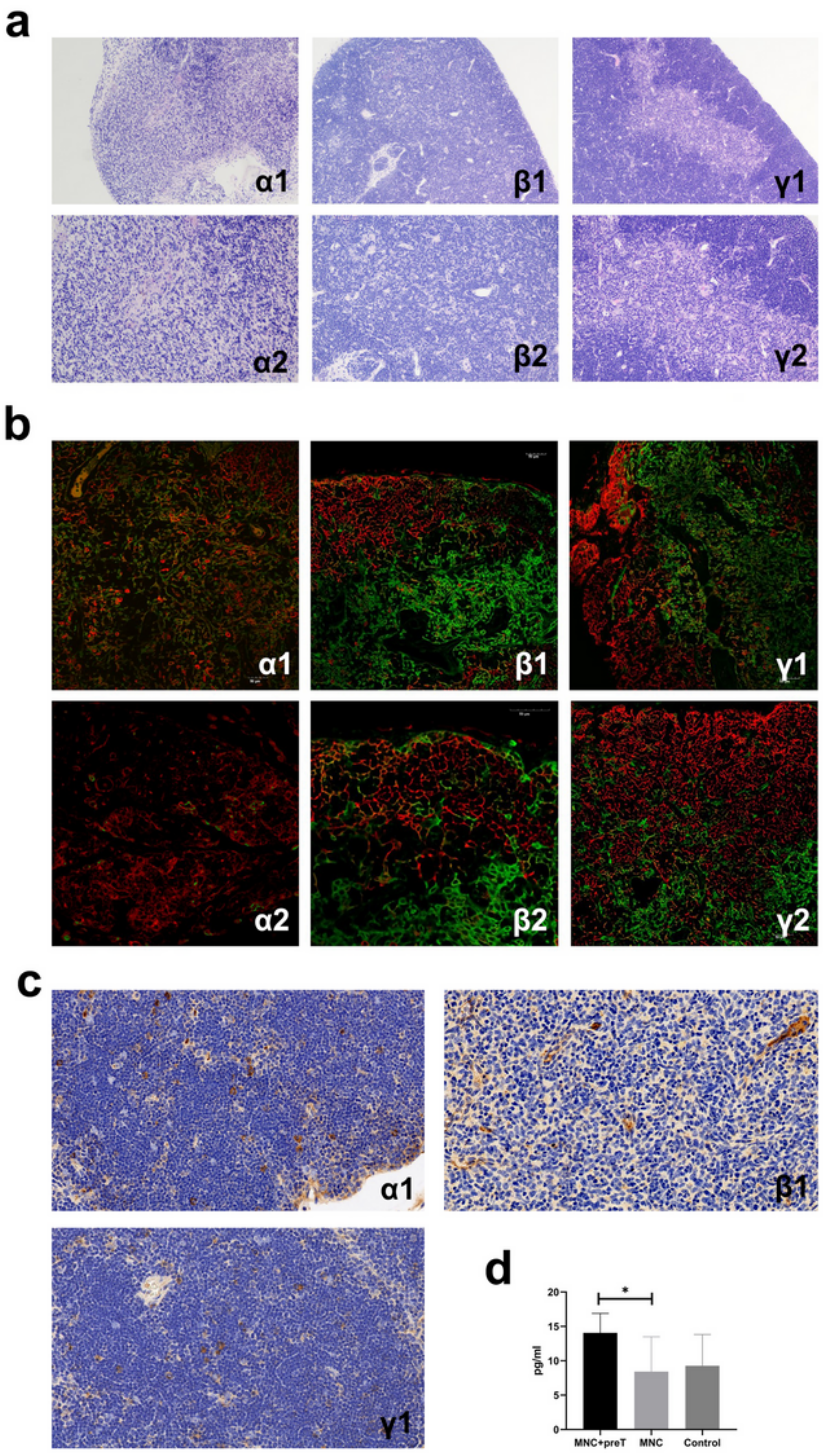

Figure 2

Structure of recipient mice thymus was severely impaired after infusion of donor splenic cells and restored by co-infusion of preT cells. Pathological observation and Serum KGF level measuring were conducted at 8 weeks after transplantation. (a) Thymus tissue was stained with HE dye and observed under a microscope (α: MNC group, β: MNC+preT group, γ: Control group). (b) The distribution of cortex (red) and medulla (green) in thymus with similar immunofluorescence staining as Figure 1h (no anti-

Tdtomato tag here). (c) Thymus tissue was stained with RANK antibody and observed under a microscope (RANK expression was located in brown areas surrounding the thymic lymphocytes). (d) Serum KGF level was measured according to the protocol of instructions.

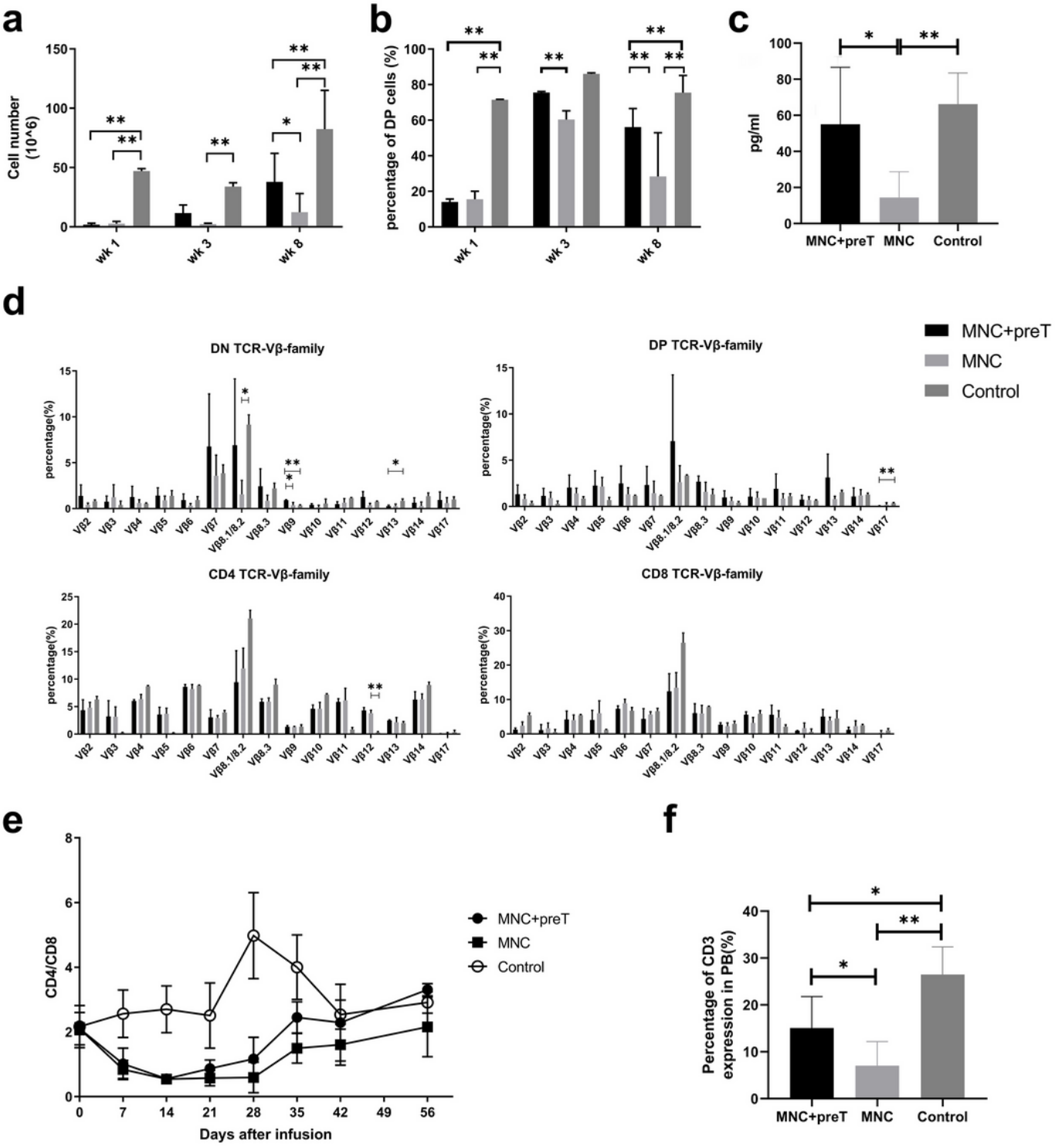

Figure 3

The central and peripheral immune reconstitution was accelerated via preT cell infusion. (a-b) The number of thymocytes and percentage of DP thymocytes in three groups at 1, 3 and 8 weeks after transplantation. (c) Detected serum IL-7 level of mice in three groups at 8 weeks. (d) TCRVβ expressions of DN, DP, SP CD4+ and SP CD8+ thymocytes at 8 weeks. (e-f) The fluctuating peripheral blood CD4:CD8 ratio and percentage of peripheral blood CD3 at 8 weeks after transplantation.

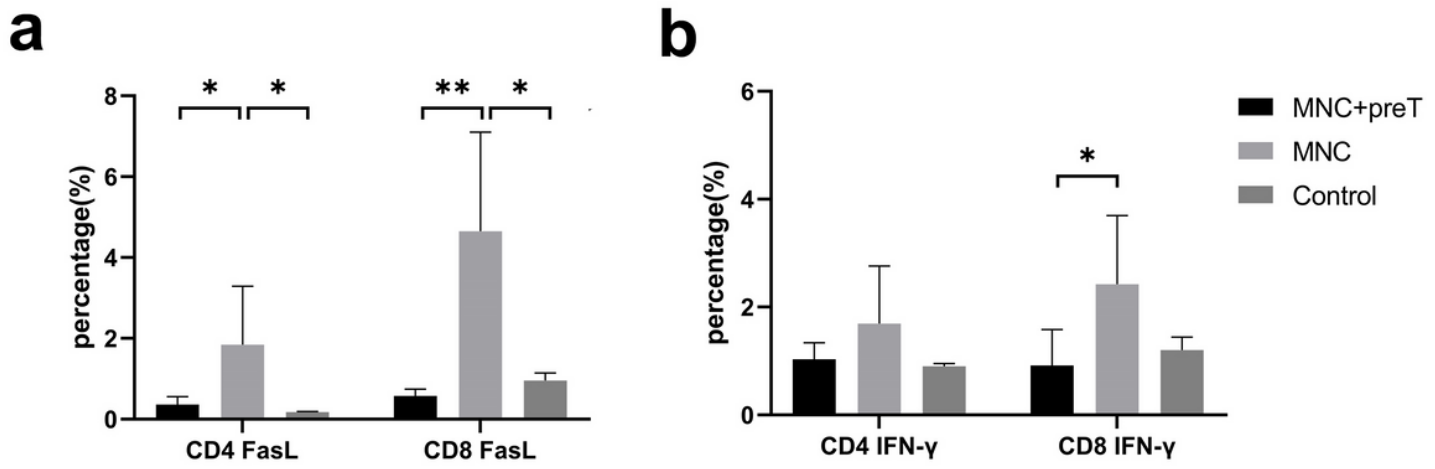

Figure 4

(a-b) The thymocytes FasL and IFN-γ expression in each group at 8 weeks after transplantation.