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ORIGINAL ARTICLE

Alloreactive natural killer cells promote haploidentical hematopoietic stem cell transplantation by expansion of recipient-derived CD4$^+$CD25$^+$ regulatory T cells

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alloreactive natural killer cells, dendritic cells, haploidentical hematopoietic stem cell transplantation, regulatory T cells, tolerance.

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Summary

Alloreactive NK cells (Allo-NKs) have been shown to exert advantageous effects on the outcomes of haploidentical hematopoietic stem cell transplantation (Haplo-HSCT) for cancer treatment. However, the mechanisms of action of Allo-NKs remain unclear. We established a novel Haplo-HSCT conditioning regimen composed of Allo-NKs and a low dose of immunosuppressive drugs (Allo-NKs + Chemo) to investigate alternative mechanisms besides direct cytotoxicity. The inhibitory effects of different cell subsets on the donor–recipient mixed lymphocyte reactions (MLRs) were evaluated after Haplo-HSCT. The quantities and functions of CD4$^+$CD25$^+$ regulatory T cells (Tregs) and dendritic cells (DCs) in the spleen and the thymus were examined. Our results showed that the Allo-NKs + Chemo regimen induced systemic tolerance, and that CD4$^+$CD25$^+$ Tregs played a significant role in inducing and maintaining systemic tolerance after Haplo-HSCT. Alloreactive NK cells promoted the expansion of recipient-derived CD4$^+$CD25$^+$CD127$^-$ Tregs in the thymus and the spleen which could be amplified *in vitro* by the immature donor-derived DC subset isolated from the thymus of Allo-NKs + Chemo-treated mice. Our findings suggested that Allo-NKs are capable of inducing systemic tolerance after Haplo-HSCT by assembling donor-derived immature DCs to expand recipient-derived Treg cells in the thymus.

Introduction

Allogeneic hematopoietic stem cell transplantation (Allo-HSCT) has been applied as a therapeutic approach for the treatment of solid tumors which are insensitive or resistant to the traditional chemotherapy in the animal models and clinical trials of renal carcinoma, breast cancer, and ovarian cancer [1–3]. However, clinical application of Allo-HSCT is limited by the availability of major histocompatibility complex (MHC)-matched donors and the Allo-HSCT-induced severe complications, such as exhaustion of bone marrow, lethal graft-versus-host disease (GVHD) and infection. Haploidentical HSCT (Haplo-HSCT) has been applied as an alternative approach for patients who have no HLA-identical related donors [4]. However, these patients still have high risks of morbidity and mortality because of myeloablative chemotherapy conditioning and severe GVHD.

Recent studies have shown that high doses of chemotherapy conditioning and complete deletion of the host hematopoietic system are not essential for the anti-tumor effects of Allo-HSCT [5,6]. Our previous study demonstrated that the inherited donor chimerism in hosts, named fetal–maternal microchimerism, could benefit the outcome of activated haploidentical peripheral blood stem cells (Haplo-PBSCs) treatment in multiple advanced-stage

cancer patients by relieving the symptoms and prolonging the survival time [7,8]. Therefore, modified Allo-HSCT procedures with milder immunosuppressive conditioning regimens which are capable of inhibiting donor–recipient response and inducing acquired donor chimerism in hosts will be more feasible and beneficial in advanced-stage cancer patients who need but cannot tolerate the conventional Allo-HSCT treatment.

Contemporary studies have shown that an adaptive cell subset, the alloreactive NK cells (Allo-NKs) in conditioning regimens may benefit donor engraftment in human and rodent [9,10]. NK cells play significant roles in regulating innate immune response through directly recognizing and killing mutated and virus-infected cells and have been recently reported to participate in induction of allograft tolerance [11]. The activation state of NK cells is regulated by receptors that transduce inhibitory or activating signals [12]. NK cells can be activated by priming of the cell-surface receptors, including immunoreceptor tyrosine-based activation motif-bearing NK receptor complexes, NKG2D receptor complexes, and CD244 [13,14]. Alternatively, they may also be activated by preventing ligation of the inhibitory receptors, known as the killer immunoglobulin-like receptors (KIRs), and the MHC-I molecules on target cells which cause phosphorylation of the intracytoplasmic immunoreceptor tyrosine-based inhibitory motif in KIRs [15,16]. In Haplo-HSCT transplant models, the lack of specific ligands on recipient cells to bind to KIRs on the NK cells from donor grafts results in activation of donor-derived NK cells, known as alloreactive NK cells (Allo-NKs) [17,18].

Both clinical trials and animal studies have demonstrated that Allo-NKs in nonmyeloablative conditioning regimens promote donor engraftment, decrease the risk of lethal GVHD, and inhibit tumor relapse [19–21]. We recently reported that when compared with chemotherapy (Chemo) alone, Allo-NKs combined with low doses of immunosuppressive drugs fludarabine and cyclophosphamide (Allo-NKs + Chemo) induce and maintain donor chimerism *in vivo*, and dramatically inhibit growth of Lewis lung cancer (LLC) or relapsed tumors after Haplo-HSCT in a mouse model [22,23]. Although direct lysis of recipient-derived hematopoietic stem cells, dendritic cells (DCs), lymphocytes and tumor cells has been proposed to explain the beneficial effects of Allo-NKs in Haplo-HSCT [9], the precise mechanisms of action of the Allo-NKs remain unclear. The purpose of this study was to determine whether alternative mechanisms are involved in Allo-NKs' beneficial effects on Haplo-HSCT. Our findings suggested that Allo-NKs pretreatment can amplify recipient-derived regulatory T cells (Tregs) by assembling donor-derived immature DCs in the thymus, and induce systemic tolerance, which benefits Haplo-HSCT.

Materials and methods

Mice and Haplo-HSCT

All animal experiments were performed according to protocols approved by the Experimental Animal Committee at Tianjin Medical University, Tianjin, China. Six to eight-week-old female C57Bl/6 (H-2D$^{b/b}$, recipient) and BALB/C × C57Bl/6 F1 hybrid (H-2D$^{b/d}$, donor) mice were obtained from Vital River Laboratory Animals Corporation (Beijing, China). All mice were maintained in pathogen-free conditions at the Experimental Animal Center of Tianjin Medical University.

A MHC-I-mismatched Haplo-HSCT model was applied for bone marrow transplantation. Bone marrow cells were isolated from donor mice (BALB/C × C57Bl/6 F1 hybrid) by flushing of femora and tibiae. Cells were suspended in RPMI-1640 medium at the concentration of 5×10^7/ml. For bone marrow transplantation, bone marrow cells (1×10^7 cells in 200 μl medium/mouse) were injected into recipient mice (C57Bl/6) through the tail vein.

C57Bl/6 mice (recipients) were randomly divided into two groups. Group 1 (Chemo regimen, 15 mice): Mice were preconditioned with continual peritoneal injection of fludarabine (100 mg/kg body weight/day) for 3 days followed by fludarabine and cyclophosphamide (150 mg/kg body weight/day) for another 2 days before Haplo-HSCT. Group 2 (Allo-NKs + Chemo regimen, 15 mice): After the same chemotherapy as given to Group 1, mice were injected with 2×10^6 Allo-NKs intravenously along with Haplo-HSCT. After purification of NK cells using the MACS magnetic isolation kit, the purity of NK cells in the Allo-NKs infusion was 74.63 ± 5.49% as defined by NK1.1$^+$ cells. We observed that 22.24 ± 2.95% of donor-derived NK cells were Allo-NKs, which expressed high levels of Ly49A, a characteristic molecule of Allo-NKs exclusively targeting to the H-2D^{d-} C57Bl/6-derived cells (data not shown). Ten normal C57Bl/6 mice without Haplo-HSCT were used as controls.

Cell isolation

The donor-derived Allo-NKs were isolated by negative selection from the spleen of BALB/C × C57Bl/6 F1 hybrid mice using the MACS magnetic mouse NK cell isolation kit II (Miltenyi Biotec, Bergisch-Gladbach, Germany). The recipient-derived CD3$^+$ T cells, CD4$^+$ T cells, CD8$^+$ T cells, and CD4$^+$CD25$^+$ Treg cells were isolated by positive selection from the spleen of the C57Bl/6 mice using the MACS magnetic mouse pan-T cell isolation kit, CD4$^+$ T cell isolation kit, CD8$^+$ T cell isolation kit, and CD4$^+$CD25$^+$ Treg cell isolation kit II, respectively (Miltenyi Biotec). The recipient-derived CD11c$^+$ cells were

isolated from the thymus of the C57Bl/6 mice using the MACS magnetic mouse $CD11c^+$ cell isolation kit (Miltenyi Biotec). The purity and viability of isolated cells were determined by flow cytometry and Trypan blue staining, which indicated a purity of >80% and a viability of >95%.

Mixed lymphocyte reaction (MLR)

Splenic effectors (2×10^5 cells/well in a 96-well culture plate) of the Allo-NKs + Chemo-treated mice, the Chemo-treated mice and C57Bl/6 controls were prepared at indicated time points after Haplo-HSCT, and were stimulated by irradiated (60 Gy) F1 spleen cells at ratios of 1:5, 1:10 and 1:20 for 96 h at 37 °C in a humidified atmosphere with 5% CO_2. Cell proliferation was determined by bromodeoxyuridine (BrdU) labeling method using a BrdU Cell Proliferation Assay kit (Calbiochem/Merck, San Diego, CA, USA) according to the manufacturer's instructions. Proliferation of the effectors was determined by comparing the amounts of incorporated BrdU in experimental samples to control samples. The proliferation of cells is reflected by Stimulation Index (SI), which was calculated using the following formula: SI = [(experimental counts)/(responder control counts + stimulator control counts)]. The $CD4^+$ T cells, $CD8^+$ T cells, $CD4^+CD25^+$ cells, and $CD4^+CD25^-$ cells were isolated from the recipients of different groups on day 23 after Haplo-HSCT. The different cell subsets were added into a conventional unidirectional haploidentical MLR system consisting of normal C57Bl/6 and irradiated F1 spleen cells at the concentration of 2×10^4 cells/well, and co-cultured for 3 days to evaluate their capacity to attenuate the donor–recipient MLRs. The culture supernatants were collected for the detection of donor-stimulated IFN-γ secretion by enzyme-linked immunosorbent assay (ELISA). All experiments were performed at least three times.

In vitro induction of Tregs

Splenic $CD3^+$ T cells (2×10^6/well) isolated from normal C57Bl/6 mice were co-cultured with the thymic $CD11c^+$ DCs (1×10^5/well) isolated from the Allo-NKs + Chemo-treated mice or the Chemo-treated mice on day 14 after Haplo-HSCT. After co-culturing in 24-well plates for 5 days at 37 °C in a humidified atmosphere with 5% CO_2, T cells were isolated and labeled with anti-mouse fluorescein isothiocyanate (FITC)-conjugated CD25, PE-conjugated CD127, and PE-Cy5-conjugated CD4 antibodies (BD Pharmingen, San Diego, CA, USA) for phenotype analysis. The induced T cells were added into a conventional unidirectional haploidentical MLR system as described above, and co-cultured for 3 days for determining whether the induced T cells inhibited the donor–recipient MLRs as the isolated recipient-derived T cells did. The culture supernatants were collected for detecting donor-stimulated IFN-γ secretion. All experiments were performed at least three times.

Flow cytometry analysis

The graft chimerism in the recipient's bone marrow and spleen were detected using FITC-labeled anti-mouse $H\text{-}2D^d$ mAb (BD Pharmingen). The proportions and absolute counts of Tregs in the recipient spleen, thymus and stimulated $CD3^+$ T cells were detected using anti-mouse FITC-conjugated CD25, PE-conjugated CD127, and PE-Cy5-conjugated CD4 antibodies and TrueCount tubes (BD Pharmingen). The proportion and maturity of DCs in the recipient thymus and spleen were detected using FITC-conjugated anti-mouse CD11c, PE-conjugated anti-mouse CD40 and CD106 mAbs (BD Pharmingen). The appropriate isotype controls, such as FITC-, PE-labeled anti-mouse IgG1, and IgG2b,κ were used (BD Pharmingen). Cells were incubated with antibodies for 30 min on ice in the dark. Then, the cells were washed twice with PBS containing 0.2% BSA and fixed using 1% paraformaldehyde. After lysis of red blood cells with FACS lysis solution (BD Pharmingen), the cells were analysed using a FACSAria flow cytometry (Becton Dickinson, Mountain View, CA, USA). At least 50 000 events were acquired for each analysis. All samples were measured at least three times.

Quantitative real-time RT-PCR (qRT-PCR)

Total cellular RNA was extracted from the isolated $CD4^+CD25^+$ T cell subset and $CD4^+CD25^-$ T cell subset from the spleens and thymuses of the Allo-NKs + Chemo-treated mice and the Chemo-treated mice using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The RNA samples were reverse-transcribed to cDNA using murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) and the mRNA expressing levels of forkedhead box P3 transcription factor Foxp3 and cytokines, including IL-2, IL-4, IL-10, TGF-β and IFN-γ were quantified by qRT-PCR using the SYBR®Premix Ex Taq™ system (Takara Bio, Tokyo, Japan) following the manufacturer's instruction. The relative amounts of indicated genes were normalized by β-actin and calculated using the formula: $2^{-\Delta Ct}$ ($\Delta Ct = Ct_{target\ gene} - Ct_{\beta\text{-}actin}$). All experiments were repeated at least four times. The primers for detecting Foxp3, IL-2, IL-4, IL-10, TGF-α, IFN-γ and β-actin are listed in Table 1.

Table 1. Primers for real-time quantitative RT-PCR.

Gene name	Primer sequences	Product size (bp)
IL-2	Upstream: 5′-GGAGCAGCTGTTGATGGACCTAC-3′ Downstream: 5′-AATCCAGAACATGCCGCAGAG-3′	173
IL-4	Upstream: 5′-TCTCGAATGTACCAGGAGCCATATC-3′ Downstream: 5′-AGCACCTTGGAAGCCCTACAGA-3′	183
IL-10	Upstream: 5′-GACAACATACTGCTAACCGACTCCT-3′ Downstream: 5′-GCCTGGGGCATCACTTCTAC-3′	110
TGF-β	Upstream: 5′-GTGTGGAGCAACATGTGGAACTCTA-3′ Downstream: 5′-TTGGTTCAGCCACTGCCGTA-3′	143
IFN-γ	Upstream: 5′-CGGCACAGTCATTGAAAGCCTA-3′ Downstream: 5′-GTTGCTGATGGCCTGATTGTC-3′	199
Foxp3	Upstream: 5′-AGTTCCTTCCCAGAGTTCTT-3′ Downstream: 5′-GGCGAACATGCGAGTAAACC-3′	162
β-actin	Upstream: 5′-CCTGGGCATGGAGTCCTGTG-3′ Downstream: 5′-AGGGGCCGGACTCGTCATAC-3′	131

Western blot

The $CD4^+CD25^+$ cells isolated from the spleen and thymus of Allo-NKs + Chemo- and Chemo-treated mice on day 23 and the induced $CD3^+$ T cells stimulated by thymic DCs of both groups for 5 days were washed using PBS and lysed in cell lysis buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethanesulfonyl fluoride, 1 mM NaF and 1 μg/ml of aprotinin and leupeptin, pepstatin) on ice. After centrifugation, soluble cellular protein concentration was determined using Micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). For Western blot analysis, proteins were separated on SDS–PAGE and transferred to polyvinylidene fluoride membranes. The membrane was incubated with rabbit polyclonal anti-Foxp3 antibody (Poly6238; BioLegend, San Diego, CA, USA) overnight at 4 °C. Then the membrane was incubated with horseradish peroxidase (HRP)-conjugated mouse secondary antibodies (Zhongshanjinqiao, Beijing, China) for 1 h at room temperature. Bound HRP was detected by using SuperSignal West Pico chemiluminescent Substrate (Pierce Biotechnology).

Enzyme-linked immunosorbent assay

The ELISA analysis for detecting IFN-γ secretion was performed according to the manufacturer's protocol (Genzyme, Cambridge, MA, USA), and the concentration of IFN-γ in each sample was calculated according to the standard curve.

Statistical analysis

All values are presented as the mean ± standard deviation (SD). The statistical significance of the differences between mean values was determined using an SPSS 13.0 software package (SPSS Inc., Chicago, IL, USA). The quantitative data were analysed using one-way ANOVA and LSD method. The level of statistical significance was set at $P < 0.05$.

Results

Allo-NKs induced systemic tolerance and contributed to the maintenance of donor chimerism after Haplo-HSCT

The donor–recipient MLRs and donor chimerism were examined *in vitro* every week after Haplo-HSCT. Both Allo-NKs + Chemo and Chemo treatments attenuated the donor–recipient MLRs in the first month after Haplo-HSCT. However, the proliferation of recipient lymphocytes was only fully inhibited in mice treated with Allo-NKs + Chemo regimen on day 28 after Haplo-HSCT, with a much higher inhibitory rate (35.87 ± 2.38%) than that in the Chemo-treated group (20.26 ± 4.02%) ($P < 0.05$, Fig. 1a). Consistently, the chimeric rates in the Allo-NKs + Chemo-treated group on day 14 and day 23 were 21.80 ± 2.03% and 27.30 ± 6.01% in the bone marrow (BM), 17.00 ± 1.56% and 48.20 ± 2.40% in the spleen respectively. The chimeric rates maintained at higher levels of 28.70 ± 5.90% in the BM and 46.40 ± 5.00% in the spleen on day 90 after Haplo-HSCT. However, the chimeric rates in the Chemo-treated mice on day 14 and day 23 were 8.60 ± 0.82% and 9.20 ± 1.61% in the BM, 11.40 ± 3.89% and 9.45 ±1.48% in the spleen respectively. The chimeric rates declined to the undetectable levels (<2%) on day 90, which indicated a transient chimerism in the Chemo-treated mice ($P < 0.05$).

In order to determine whether the beneficial effect of Allo-NKs on Haplo-HSCT was caused by the direct cytotoxicity against recipient cells, the numbers of karyocytes in the BMs and spleens of both groups were counted. No significant differences were identified between the two groups during the first month after Haplo-HSCT. This result suggested that additional mechanisms besides direct cytolysis are involved in Allo-NKs-induced systemic tolerance, which contributes to the maintenance of donor chimerism.

The $CD4^+CD25^+$ T cells in Allo-NKs + Chemo-treated mice played a pivotal role in inducing systemic tolerance

In order to define which cell subset plays the key role in inducing systemic tolerance after Haplo-HSCT, we isolated the $CD4^+$ and $CD8^+$ T cells subsets from the spleens of both recipient groups on day 23 after Haplo-HSCT, and added them into the conventional haploidentical MLR system composed of normal C57Bl/6 cells and inactivated F1 cells. When added at a ratio of 1:20, the $CD4^+$

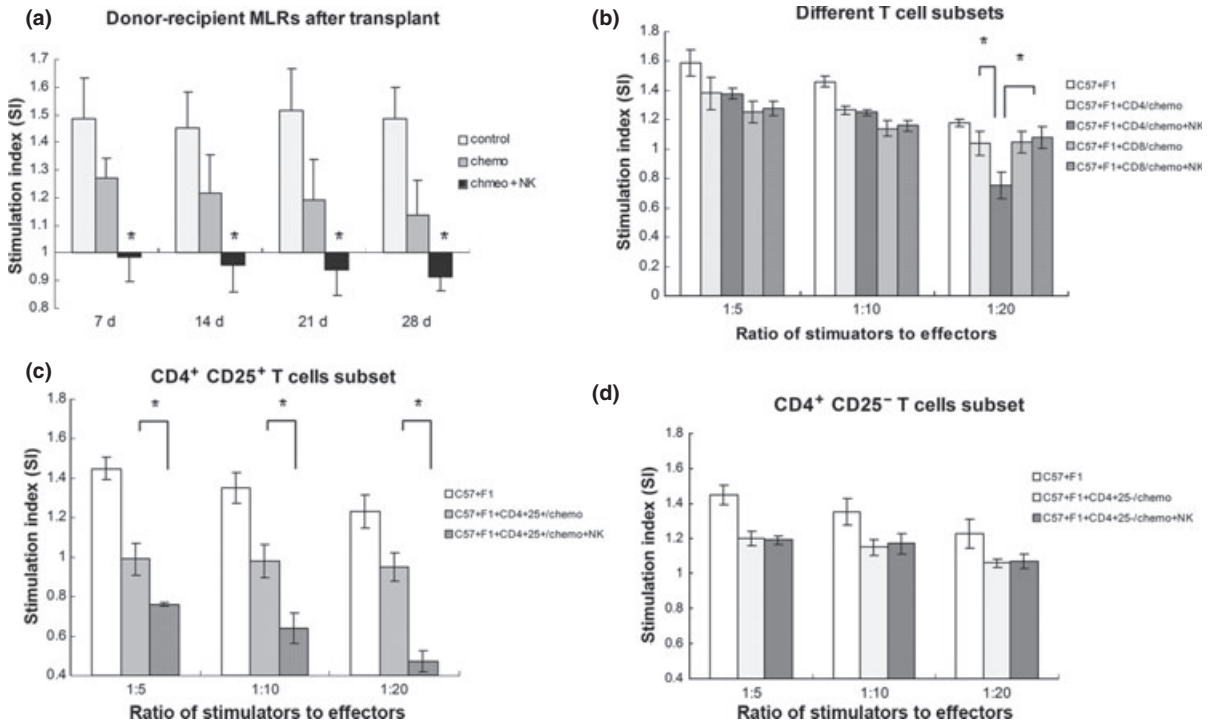


Figure 1 Alloreactive NK cells (Allo-NKs) + Chemo regimen induced systemic tolerance in which the $CD4^+CD25^+$ T cells played a dominant role in suppressing host-versus-graft rejection. (a) The donor–recipient mixed lymphocyte reactions (MLRs) were measured by BrdU incorporation, in which the recipient splenocytes from both Allo-NKs + Chemo and Chemo-treated mice were stimulated by irradiated donor cells at indicated time points after haploidentical hematopoietic stem cell transplantation (Haplo-HSCT). The proliferation of recipient lymphocytes was fully inhibited (*) in the Allo-NKs + Chemo-treated mice when compared with that in the Chemo regimen treated group. (b) $CD4^+$ T cells or $CD8^+$ T cells were isolated from the splenocytes of Allo-NKs + Chemo- and Chemo-treated mice on day 23 after Haplo-HSCT using magnetic isolation kits, and added into the conventional haploidentical MLR system to evaluate their immunosuppressive effects. The $CD4^+$ T cells of Allo-NKs + Chemo-treated mice significantly inhibited the donor–recipient MLRs when compared with the $CD8^+$ T cells of the same mice and the $CD4^+$ T cells of Chemo-treated mice. (c, d) The $CD4^+CD25^+$ (c) and $CD4^+CD25^-$ (d) T cells were isolated from both groups, and their suppressive effects on donor–recipient MLRs were assessed as described in Methods. The $CD4^+CD25^+$ T cells of the Allo-NKs + Chemo-treated mice, rather than the $CD4^+CD25^-$ T cells, showed significantly stronger suppressive effects on MLRs than the counterparts from Chemo-treated mice. The means and error bars depicted in the graph were collected from five mice of each group.

T cells of the Allo-NKs + Chemo-treated mice inhibited the donor–recipient MLRs by 36.20 ± 7.40%, which was significantly higher than the $CD8^+$ T cells from the same mice (8.17 ± 6.38%) and the $CD4^+$ T cells of the Chemo-treated group, 11.66 ± 6.95% ($P < 0.05$, Fig. 1b). To further identify what subset of $CD4^+$ T cells that were responsible for the inhibitory effects, we isolated the $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from both groups. The $CD4^+CD25^+$ T cells of the Allo-NKs + Chemo-treated mice, rather than the $CD4^+CD25^-$ T cells, showed potent suppressive effects on MLRs. These cells inhibited the donor–recipient MLRs by 58.0 ± 3.68%, 51.85 ± 5.92% and 54.47 ± 4.47% at all ratios ranged from 1:5 to 1:20, which is significantly stronger than the counterparts from Chemo-treated mice (27–31%) ($P < 0.05$, Fig. 1c, d). These findings suggest that the $CD4^+CD25^+$ T cells in the spleens of the Allo-NKs + Chemo-treated mice are responsible for the induction and maintenance of systemic tolerance.

$CD4^+CD25^+CD127^-$ Tregs expressing higher levels of Foxp3 and IL-10, but lower level of IFN-γ, increased in the spleen and thymus of the Allo-NKs + Chemo-treated mice

As express lower level or lack of expression of CD127 has been reported in the population of functional Tregs in the $CD4^+CD25^+$ T cells in human and rodent, we analysed the $CD4^+CD25^+CD127^-$ cell population in the spleen and thymus at indicated time points after Haplo-HSCT. On day 14, <5% of the total $CD4^+$ T cells were $CD4^+CD25^+CD127^-$ in the spleens of Allo-NKs + Chemo- or Chemo-treated mice (Fig. 2a, Q4). However, on day 23, a newly emerging cluster of

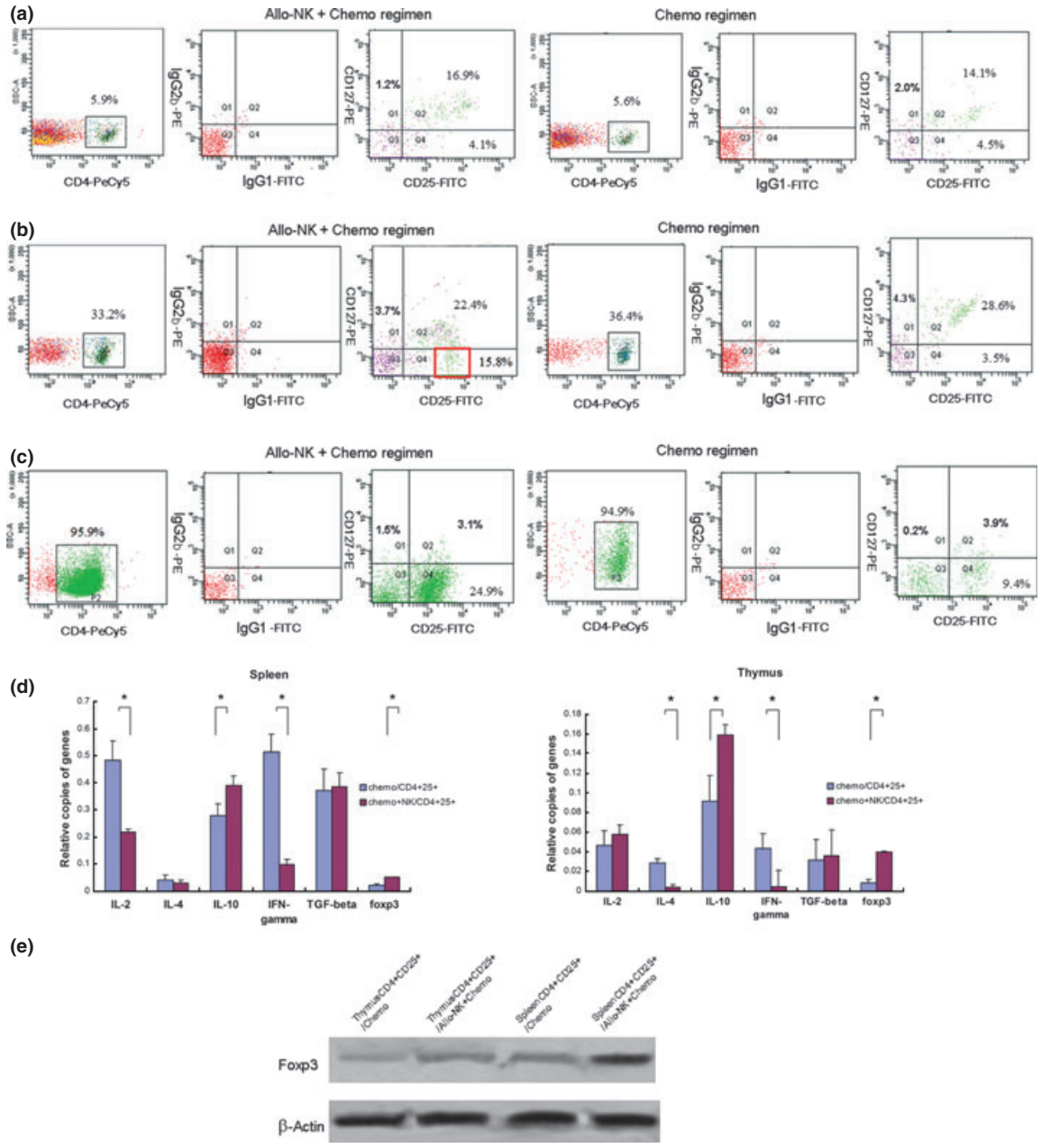


Figure 2 A newly amplified Tregs subset expressing higher level of Foxp3 and IL-10, lower level of IFN-γ was identified in the spleen and thymus of alloreactive NK cells (Allo-NKs) + Chemo-treated mice on day 23 after haploidentical hematopoietic stem cell transplantation (Haplo-HSCT). (a, b) The proportions of $CD4^+CD25^+CD127^-$ Tregs in recipient spleens of either Allo-NKs + Chemo- or Chemo-treated mice were detected on day 14 (a) and day 23 (b) after Haplo-HSCT by flow cytometry. Cells in Q4 quadrant represent $CD4^+CD25^+CD127^-$ T cells, a predominant Treg phenotype. The newly emerging cluster of $CD4^+CD25^+CD127^-$ cells with higher CD25 expression (red box) was detected exclusively on day 23 in the spleens of Allo-NKs + Chemo-treated mice. (c) Accordingly, the proportion of $CD4^+CD25^+$ $CD127^-$ Tregs in recipient thymuses of Allo-NKs + Chemo-treated mice was significantly higher than that in the thymuses of Chemo-treated mice. (d) Real-time RT-PCR was performed to compare the mRNA expression levels of Foxp3, IL-2, IFN-γ, IL-4, IL-10, and TGF-β in $CD4^+CD25^+$ T cells isolated from the spleen and the thymus of both groups on day 23 after Haplo-HSCT. Significantly higher mRNA levels of IL-10 and Foxp3 in the splenic and thymic $CD4^+CD25^+$ T cells of the Allo-NKs + Chemo-treated mice was determined comparing with the Chemo-treated group. In contrast, the mRNA levels of IFN-γ, IL-2 and IL-4 decreased simultaneously. (e) Furthermore, Foxp3 expression at protein level in $CD4^+CD25^+$ T cells was detected using Western blot method. The Foxp3 protein in the splenic and thymic $CD4^+CD25^+$ T cells of Allo-NKs + Chemo-treated mice was significantly higher than the counterpart of Chemo-treated. The flow cytometry dot-plot and Western blot was based on the data of one representative experiment. The means and error bars depicted in the graph were collected from five mice of each group ($*P < 0.05$).

$CD4^+CD25^+CD127^-$ cells with high level of CD25 expression (median fluorescence intensity ranged from 10^3 to 10^4) were identified exclusively in the spleens of Allo-NKs + Chemo-treated mice, and the proportion increased to 17.45 ± 2.20% of total $CD4^+$ T cells (Fig. 2b, Q4). In contrast, the proportion of this subset was significantly lower in the spleens of Chemo-treated mice, which was only 5.45 ± 1.8% of total $CD4^+$ T cells ($P < 0.05$). Simultaneously, the proportion of $CD4^+CD25^+CD127^+$ cells, which were regarded as competent effector T cells in the spleens of Allo-NKs + Chemo-treated mice was lower comparing with that of Chemo-treated ones (Fig. 2a, Q2). We calculated the absolute numbers of Tregs in the spleen of both groups on day 23 after Haplo-HSCT, and determined the Tregs to be 400 ± 45.3 cells/μl in Allo-NKs + Chemo-treated group and 99 ± 32.6 cells/μl in Chemo-treated group ($P < 0.05$). The proportions of $CD4^+CD25^+CD127^-$ Tregs in recipient thymus of both groups were detected on day 23 as well. Accordingly, 24.21 ± 2.73% of $CD4^+$ T cells in the thymuses of Allo-NKs + Chemo-treated mice were $CD4^+CD25^+CD127^-$ Tregs, which was significantly higher than 8.75 ± 1.63% in the thymuses of Chemo-treated mice, with an abundance of 1150 ± 120.7 cells/μl and 398 ± 41.7cells/μl respectively ($P < 0.05$, Fig. 2c).

To confirm that the newly emerged $CD4^+CD25^+$ $CD127^-$ T cells were functional Tregs, we analysed the cytokine profile and Foxp3 expression level in the $CD4^+CD25^+$ T cells isolated from the spleens and the thymuses of both groups on day 23 after Haplo-HSCT. We found significantly higher mRNA levels of IL-10 and Foxp3 in the splenic $CD4^+CD25^+$ T cells of the Allo-NKs + Chemo-treated mice, which were 1.4- and 2.5-fold higher, respectively, in comparison to the Chemo-treated group. In contrast, the mRNA levels of IL-2 and IFN-γ in Allo-NKs + Chemo-treated group were 2.2- and 5.3-fold lower respectively, than the Chemo-treated group ($P < 0.05$, Fig. 2d). Consistent with the results from the spleens, the mRNA levels of IL-10 and Foxp3 in the thymic $CD4^+CD25^+$ T cells of the Allo-NKs + Chemo-treated mice increased by 1.7- and 4.8-fold compared to the Chemo-treated ones, whereas the levels of IFN-γ and IL-4 decreased by 9.8- and 6.9-fold respectively ($P < 0.05$, Fig. 2d). These results were consistent with the unique cytokine profiles observed in the Tregs. Furthermore, Foxp3 expression at protein level in $CD4^+CD25^+$ T cells of Allo-NKs + Chemo- and Chemo-treated mice was detected using Western blot method. The Foxp3 protein in the splenic and thymic $CD4^+CD25^+$ T cells of Allo-NKs + Chemo-treated mice was higher than that of Chemo-treated mice (Fig. 2e). Thus, our data indicated that Allo-NKs pretreatment promoted amplification of $CD4^+CD25^+$ Tregs, rather than activating competent Th cells in the recipients, contributing to the inhibitory effects on host-versus-graft (HVG) rejection.

Amplification of Tregs was correlated with assembly of immature donor-derived DCs in the thymus of the Allo-NKs + Chemo-treated mice

In order to define the mechanisms by which Tregs were induced in the recipients, we detected composition and maturity status of the $CD11c^+$DC populations in the

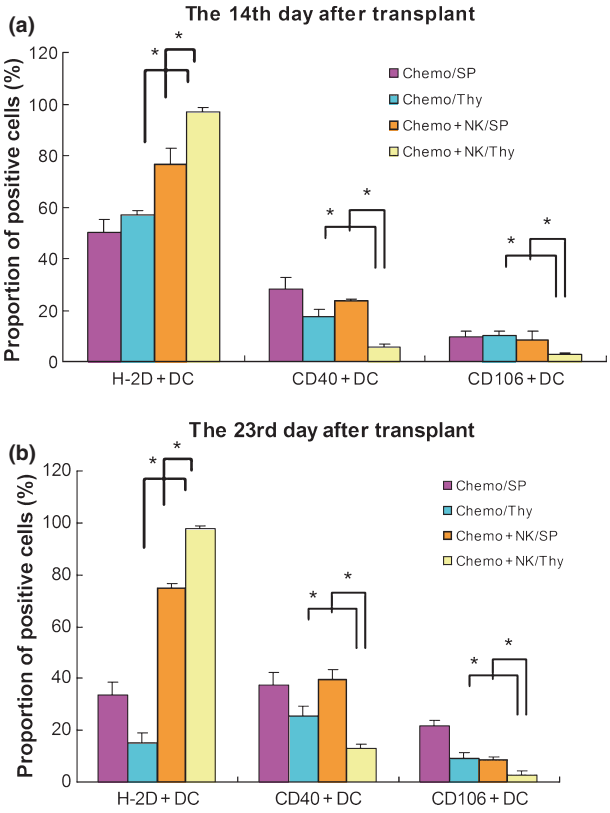
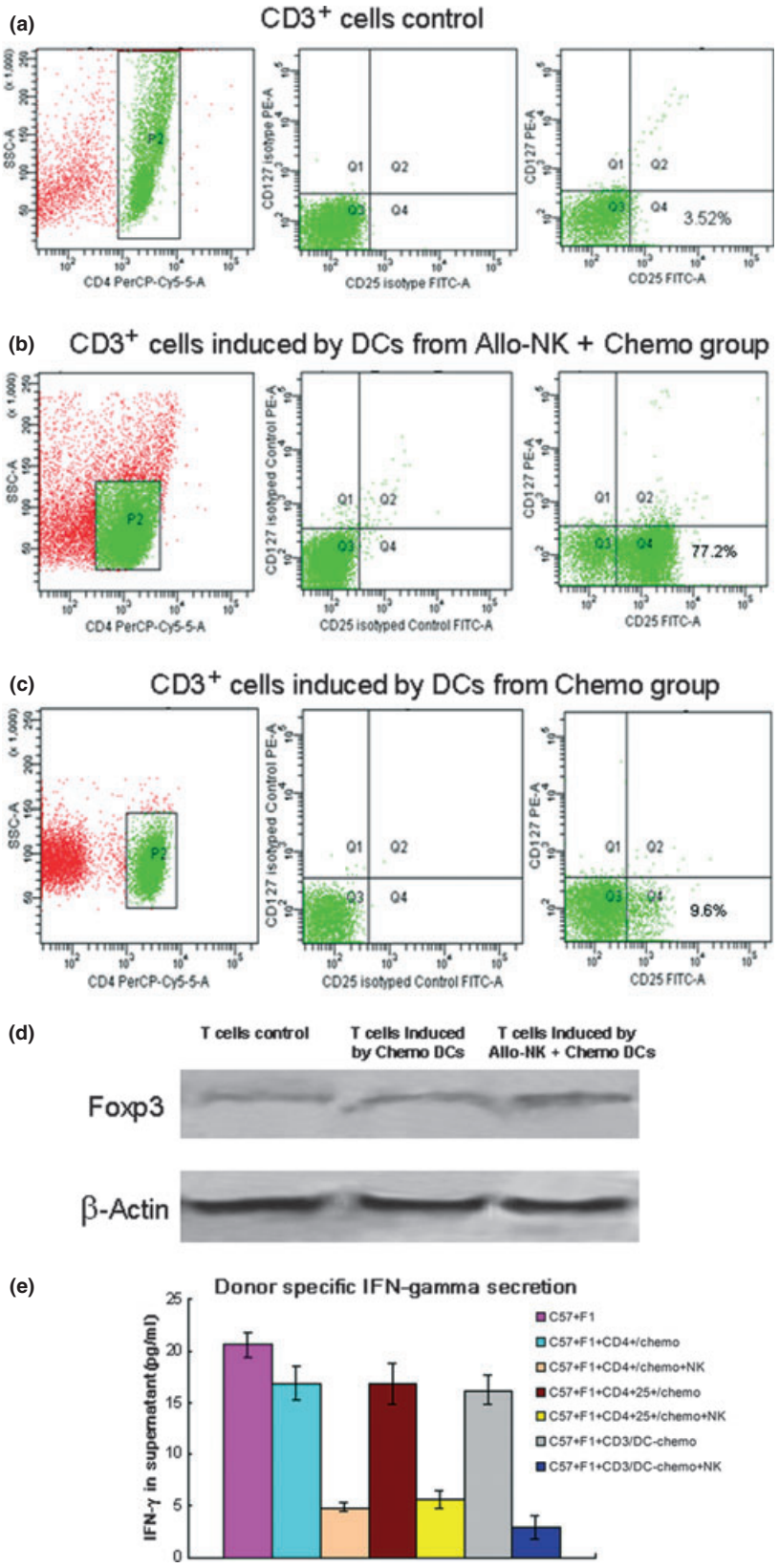


Figure 3 Immature donor-derived $CD11c^+$DCs assembled in the thymus of the alloreactive NK cells (Allo-NKs) + Chemo-treated mice (a, b) DCs were isolated from the thymus and spleen of either Allo-NKs + Chemo regimen treated mice or Chemo regimen treated mice on day 14 (a) and day 23 (b) after haploidentical hematopoietic stem cell transplantation (Haplo-HSCT). The phenotypes of DCs were analysed using anti-mouse fluorescein isothiocyanate-conjugated CD11c, PE-conjugated $H\text{-}2D^d$, PE-conjugated CD40, and PE-conjugated CD106 antibodies by flow cytometry. The proportion of donor-derived $CD11c^+$DCs ($H\text{-}2D^{d+}CD11c^+$ cells) in the thymus of Allo-NKs + Chemo-treated mice was higher than that in the spleen of the same mice and in the thymus of Chemo-treated ones either on day 14 or day 23. In addition, the donor-derived DCs in the thymus of Allo-NKs + Chemo regimen-treated mice displayed immature phenotypes with down-regulated expression of CD40 and CD106 for at least 1 week. The means and error bars depicted in the graph were collected from five mice of each group.



spleens and the thymus of Allo-NKs + Chemo- or Chemo-treated mice on day 14 and 23 after Haplo-HSCT. All $CD11c^+$ DCs in the thymus of Allo-NKs + Chemo-treated mice were donor-derived ($H\text{-}2D^{d+}CD11c^+$ cells), whose percentage in total $CD11c^+$DCs was 96.87 ± 1.89% on day 14 and 97.87 ± 1.13% on day 23. The proportion of donor-derived $CD11c^+$DCs in the thymus of the Allo-NKs + Chemo-treated mice was higher than that in the spleen of the same mice (76.53 ± 6.03% on day 14 and 74.83 ± 1.81% on day 23), and than that in the thymus of the Chemo-treated mice (57.13 ± 1.57% on day 14 and 14.97 ± 4.16% on day 23) ($P < 0.05$). Furthermore, the donor-derived DCs in the thymus of Allo-NKs + Chemo-treated mice displayed immature phenotypes and were lacking in expression of CD40 and CD106 on surface when compared with those either in the spleen of the same mice and in the thymus of Chemo-treated mice, in which the proportions of $CD40^+$DCs and $CD106^+$DCs were 5.87 ± 0.92% and 2.77 ± 0.66% on day 14, 13.33 ± 1.20% and 2.93 ± 1.35% on day 23 respectively ($P < 0.05$, Fig. 3a, b). The immaturity of donor-derived DCs was maintained for at least 1 week until Tregs amplification was observed in the thymus and spleen of Allo-NKs + Chemo-treated mice on day 23 after Haplo-HSCT. Thus, these results suggest that pretreatment with Allo-NKs may promote Treg amplification by assembling more immature donor-derived DCs, which can induce inhibitory Tregs, in the thymus.

The immature donor-derived DCs isolated from the thymus of the Allo-NKs + Chemo-treated mice induced amplification of functional $CD4^+CD25^+$ Tregs *in vitro*

We further tested the hypothesis that the immature donor-derived DCs can induce recipient's $CD4^+$ T cells to develop $CD4^+CD25^+$ Tregs. A co-culture system was established using $CD3^+$ T cells isolated from the spleens of normal C57Bl/6 mice and $CD11c^+$ DCs isolated from the thymus of Allo-NKs + Chemo or Chemo-treated mice on day 14 after Haplo-HSCT. The proportion of $CD4^+CD25^+CD127^-$ Tregs in $CD4^+$T cells increased from 5.41 ± 2.04% to 70.23 ± 8.66% after co-culturing with the DCs from Allo-NKs + Chemo-treated mice, much higher than that after the co-culture with DCs from Chemo-treated mice (11.74 ± 3.95%) ($P < 0.05$, Fig. 4a–c). The numbers of Tregs induced by the thymus DCs were 1053 ± 105.2 cells/μl for Allo-NKs + Chemo-treated group, and 147 ± 40.3 cells/μl for Chemo-treated group ($P < 0.05$). Furthermore, Foxp3 expression at protein level in the $CD3^+$ T cells induced by immature thymic DCs of Allo-NKs + Chemo-treated mice was slightly higher when compared with that in the counterpart induced by the thymic DCs of Chemo-treated mice and control T cells (Fig. 4d).

In order to evaluate the inhibitory effects of amplified Tregs, the induced $CD3^+$ T cells were added into a conventional haploidentical MLR system and donor-stimulated IFN-γ secretion was detected. The $CD3^+$ T cells induced by the DCs from Allo-NKs + Chemo-treated mice showed significant inhibitory effects, which were as potent as the $CD4^+$ T subset and the $CD4^+CD25^+$ Tregs subset isolated from the spleens of Allo-NKs + Chemo-treated mice on day 23 after Haplo-HSCT, with an inhibition of 84.11 ± 6.06% on IFN-γ secretion by C57Bl/6 splenocytes in the donor–recipient MLR system. In contrast, only 21.05 ± 10.54% of donor-stimulated IFN-γ secretion was inhibited by the $CD3^+$ T cells induced by the DCs from Chemo-treated mice ($P < 0.05$, Fig. 4e). These results suggested that the donor-derived immature DCs in the thymus of Allo-NKs + Chemo-treated mice were able to amplify functional $CD4^+CD25^+$ Tregs *in vitro*.

Discussion

Adoptive cell therapy for hematologic or solid malignancy has been increasingly applied in recent years, contributing to a rapid growth in gene engineering and molecular immunology. Syngeneic adoptive cell therapies are

Figure 4 The donor-derived immature DCs induced amplification of functional $CD4^+CD25^+CD127^-$ Tregs *in vitro*. (a–c) Normal C57Bl/6 $CD3^+$ T cells were co-cultured with the $CD11c^+$ DCs isolated from the thymus of alloreactive NK cells (Allo-NKs) + Chemo- or Chemo-treated mice on day 14 after Haplo-HSCT. The proportions of $CD4^+CD25^+CD127^-$ Tregs were detected by flow cytometry. (a) The $CD3^+$ T cells control. (b, c) The proportion of $CD4^+CD25^+CD127^-$ Tregs in the T cells stimulated by the DCs from Allo-NKs + Chemo (b) or Chemo (c) treated mice after 5 days of co-culture. (d) Foxp3 expression at protein level in the $CD3^+$ T cells induced by immature thymic DCs of Allo-NKs + Chemo-treated mice was slight higher when compared with that in the counterpart induced by the thymic DCs of Chemo-treated mice and control T cells. (e) The $CD3^+$T cells induced by the thymic DCs of both groups were added into a conventional haploidentical mixed lymphocyte reaction (MLR) system and donor-stimulated IFN-γ secretion in the supernatants was measured by enzyme-linked immunosorbent assay. The $CD3^+$ T cells induced by the DCs of Allo-NKs + Chemo-treated mice showed significant inhibitory effects comparing with the $CD3^+$ T cells induced by the DCs of Chemo-treated ones, which were as potent as the $CD4^+$ T subset and the $CD4^+CD25^+$ Tregs subset isolated from the spleens of Allo-NKs + Chemo-treated mice on day 23 after Haplo-HSCT. The flow cytometry dot-plot and Western blot were based on the data of one representative experiment. The means and error bars depicted in the graph were collected from five mice of each group.

regarded as an efficient treatment strategy in either post-operative or advanced cancer patients [24,25]. However, tumor-induced anergy may attenuate the immune responses to tumor cells and compromise the anti-tumor effects during the treatment of advanced solid malignancies. We and other researchers have demonstrated that fetal-maternal microchimerism can potentiate the anti-tumor effects of activated haplo-PBSCs both *in vivo* and *in vitro* [8,26], suggesting that haplo-PBSCs might be a feasible alternative source for adoptive cell therapy to treat metastatic and drug-resistant cancer patients after generating optimal donor chimerism in recipients. In our recent studies, we reported that Allo-NKs combined with a low dose of chemotherapy (Allo-NKs + Chemo) induced sustainable donor chimerism *in vivo* and protected recipients from severe GVHD and transplant-related death, furthermore inhibited growth of LLC tumors after Haplo-HSCT [22,23]. The Allo-NKs-mediated direct lysis of recipient cells, including hematopoietic stem cells, DCs and lymphocytes, has been proposed to explain the sustained inhibition of HVG rejection after transient cell transfusion [9]. However, the precise mechanisms underlying this effect are still unclear. Therefore, in this study we investigated in-depth the alternative mechanisms of Allo-NKs-dependent induction of systemic tolerance after Haplo-HSCT.

We found that Allo-NKs in the conditioning regimen promoted and maintained donor chimerism in both BM and spleen, which was consistent with persistent full inhibition of donor–recipient MLRs. Furthermore, no significant decrease of the number of karyocytes in the BM and spleen was observed during the first month after Haplo-HSCT in Allo-NKs + Chemo-treated mice. Thus, induction of systemic tolerance via Allo-NKs-mediated cytolysis is unlikely to be a major mechanism, and certain regulatory cell subsets in Allo-NKs + Chemo-treated mice may play an essential role in down-regulating HVG rejection.

Tregs are a subset of thymus-derived $CD4^+$ T cells that constitutively express CD25 and Foxp3 genes that play crucial roles in tumor immunity, susceptibility to autoimmune disease, and transplantation tolerance [27–30]. Recent reports indicated that lower expression of CD127 is a consistent and specific phenotype marker of $CD4^+CD25^+$ Tregs both in human and rodent [31,32]. It was reported that Tregs are essential in inhibiting alloreactive responses, controlling HVG rejection, attenuating GVHD and promoting hematopoietic engraftment after total body irradiation-based conditioning regimens in humans and rodents [33–35].

In this study, we found that a new $CD4^+CD25^+CD127^-$ T cell subset developed in the thymus and spleen of Allo-NKs + Chemo-treated mice on day 23 after Haplo-HSCT, and that these cells displayed potent inhibitory effects on the conventional haploidentical MLR *in vitro*. This is consistent with other reports that a proportional expansion of Tregs appears in recipients after mismatched Allo-HSCT in favor of establishing HVG tolerance and donor hematopoietic engraftment [36]. It has been reported that both donor and recipient derived Tregs are capable of inducing selective tolerance *in vivo* against mismatched donor antigens in sublethally conditioned mice models, although the immune response to the third-party antigens and polyclonal stimulation were reserved [34,36–38]. However, our observations, in which most of $CD4^+CD25^+$ T cells were recipient-derived and the proportion of donor-derived cells was <20% (data not shown), were somewhat different from this conclusion. Our results were consistent with other reports which propose that the recipient-derived Tregs population is the most important trigger for the induction of HVG tolerance, whereas both recipient- and donor-derived Tregs take part in the maintenance of the tolerance status [8,37,39].

It is well-known that after Allo-HSCT, the naïve recipient-derived T cells can be educated and converted to Tregs by several factors, such as the regulatory cytokines GM-CSF [40] TGF-β and IL-2 [41], immature myeloid DCs [42] and conditioned plasmacytoid DCs [43]. It was reported that an Allo-NKs-based conditioning regimen has the capacity of protecting the architectural organization and cellular composition of the thymic stromal compartment from GVHD and maintaining regular thymopoiesis in a nonmyeloablative Allo-HSCT model [44]. Therefore, we traced the infused Allo-NKs *in vivo*, and found that Allo-NKs were mainly arrested in the thymus after infusion and lasted for 1 week before the immature donor-derived $CD11c^+$ DCs appeared (data not shown). Markey *et al.* demonstrated that donor conventional DCs are the major population in presenting alloantigens after BM transplantation, and maintainable immaturity of $CD11c^{hi}$ DCs promote induction of peripheral tolerance after Allo-HSCT by impairing differentiation of Th1 cells and increasing Treg cells in recipients [45]. Consistent with their report, we observed that the donor-derived immature DCs persisted for at least 1 week until a newly amplified $CD4^+CD25^+CD127^-$ Tregs subset was detected in the thymus and spleen. The functional test revealed that the immature $CD11c^+$ DCs isolated from the thymus of Allo-NKs + Chemo-treated mice were capable of inducing the amplification of functional $CD4^+CD25^+CD127^-$ Tregs from C57Bl/6 $CD3^+$T cells, leading to a significant inhibition of IFN-γ secretion in a conventional haploidentical MLR. Overall, our findings suggested that Allo-NKs pretreatment promote immune tolerance to haploidentical donor antigens by assembling donor-derived immature DCs and amplifying recipient-derived Tregs in the thymus.

Authorship

JY performed research and wrote the paper. XR wrote the paper. FY and HL analysed data. SC and XA contributed important reagents. YC and HS performed research. NZ collected data. XH designed research.

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