Low-dose post-transplant cyclophosphamide can mitigate GVHD and enhance the G-CSF/ATG induced GVHD protective activity and improve haploidentical transplant outcomes

Yu Wang, Ying-Jun Chang, Lu Chen, Lan-Ping Xu, Zhi-Lei Bian, Xiao-Hui Zhang, Chen-Hua Yan, Kai-Yan Liu, and Xiao-Jun Huang

© 2017 Taylor & Francis Group, LLC

ABSTRACT

Use of high-dose, post-transplant cyclophosphamide (PTCy) results in low rates of graft-versus-host-disease (GVHD) and favorable immune reconstitution, although with higher rates of relapse and somewhat high rates of graft failure. We hypothesized that permissible dose reduction of PTCy might be feasible. The current study attempts to establish a murine model and focus on regulatory T cells (Tregs) to clarify the immunological mechanisms for GVHD prevention by low-dose PTCy. In addition, a prospective, clinical cohort study in haploidentical, T-cell replete transplantation is initiated to support the rational. We found that acute GVHD could be alleviated by low-dose PTCy and could be further mitigated after the combined use of low-dose PTCy and antithymocyte globulin (ATG) in mice. Flow-cytometric analyses in mice showed that low-dose PTCy could increase the number of Tregs and the effect on Tregs is significantly prominent with the combined use of low-dose PTCy and ATG. In the clinical cohort study, the cumulative incidence of grades II-IV acute GVHD in combined treatment cohort with low-dose PTCy and ATG/cyclophosphamide colony-stimulating factor (G-CSF) (17%; 95% CI, 5–29%) was significantly lower than that in matched-pair cohort (33%; 95% CI, 25–41%; P = 0.04) and that in historical cohort (56%; 95% CI, 42–70%; P < 0.001). In-vivo immune reconstitution analysis showed that low-dose PTCy could facilitate suppressive Tregs reconstitution. In conclusion, low-dose PTCy is sufficient for GVHD abrogation under lymphopenic situation and can enhance the protective effect of ATG/G-CSF on GVHD. Intensiﬁed conditioning followed by low-dose PTCy might be a feasible option for patients undergoing haploidentical transplantation.

Introduction

Over the past 2 decades, new approaches to induce immune tolerance after haploidentical hematopoietic cell transplants (haplo-HCT) have effectively controlled the intense alloreactivity, resulting in markedly improved outcomes. Successful establishment of T-cell-replete (TCR) protocols including T-cell modulation with antithymocyte globulin (ATG)/granulocyte colony-stimulating factor (G-CSF) and post-transplant cyclophosphamide (PTCy) provides alternative treatment options for patients without matched donors. Each strategy has its own advantages and disadvantages. ATG/G-CSF-represented regimen produces essentially universal engraftment with limited relapse and favorable survival, albeit with relatively high rates of graft-vs.-host disease (GVHD), when compared with HLA-identical transplantations. While use of high-dose PTCy results in low rates of graft-vs.-host disease (GVHD) and favorable immune reconstitution, although with higher rates of relapse and somewhat high rates of graft failure. Thus, novel strategies are needed to refine each approach and improve the outcomes of patients treated in each paradigm.

The mechanisms underlying the crossing of HLA barriers of high-dose PTCy included in-vivo selectively destroy rapidly proliferating alloreactive T cells, regulatory T cells (Tregs) persistence and expansion owing to increased expression of aldehyde dehydrogenase (ALDH), and the long-lasting intrathymic clonal deletion of anti-host T cells. The selective action of high-dose PTCy allows memory T cells of the recipient to escape Cy and increase the risk of graft failure, regardless of the conditioning intensity in recent European experience; also, in the preclinical and clinical studies that examined engraftment, PTCy dose of 50 mg was inadequate while high dose (150–200 mg/kg) of PTCy is most effective at preventing graft rejection in mouse model and the engraftment-promoting effects of pre- and post-transplantation Cy are additive. However, the therapeutic anti-leukemia immune response might be alleviated owing to an excessive cytotoxicity with high-dose Cy administration, which may contribute to the relative high relapse after high-dose Cy protocol.

Recently, in a mouse model, the effectiveness of an intermediate dose of 66 mg/kg PTCy, rather than 200 mg/kg, was
demonstrated to ameliorate GVHD by depleting donor allo-reactive T cells and rapidly recovering donor Tregs under lymphopenic conditions while sparing other populations to provide immunity against tumor antigens. Notably, a more vigorous depletion of cells was observed at a higher concentration of PTCy, which might lead to excessive Cy-mediated deletion or inactivation of tumor specific T cells. Meanwhile, in G-CSF/ATG protocol, rapid reconstitution of Tregs has been demonstrated after non-inherited maternal-antigen (NIMA)-mismatched donor HCT compared with non-inherited paternal-antigen (NIPA)-mismatched donor transplant, which translated into a lower incidence of acute GVHD after NIMA-exposed donor HCT. Based on the above findings, we postulate that lower-dose of PTCy could be sufficient for tolerance induction to warrant alleviating GVHD without compromising graft-vs.-leukemia (GVL) effect.

Given that excellent engraftment achieved with ATG and G-CSF (reported to be 99–100%), it is inferred that graft failure is not a particular concern under this circumstance when compared with administering high-dose PTCy alone. Instead, further reducing GVHD merits examination, especially after HCT from maternal donor or collateral relatives with high risk of GVHD occurrence. Furthermore, Tregs are involved in the immune-modulatory effects of both PT/Cy and ATG/G-CSF, whereas the relevant mechanisms are different in some aspects. Therefore, it is conceivable that ATG/G-CSF conditioning followed by low-dose PTCy could reduce GVHD without influencing GVL. Thus, to elucidate the hypothesis, the current study attempts to establish a murine model and focus on Treg cells to clarify the immunological mechanisms of low-dose PTCy and the potentially synergistic GVHD-prevention effect by its combined use with ATG/G-CSF. Furthermore, a prospective, clinical cohort study is initiated to support the rational. Results of clinical outcomes and immune reconstitution are reported. This novel procedure in TCR haplo-HCT with intensified conditioning followed by low-dose PTCy might be feasible options to promote protection against GVHD and improve haploidentical transplant outcomes.

Results

Acute GVHD could be alleviated by low-dose PTCy and could be further mitigated after the combined use of low-dose PTCy and ATG in mice

We first established a GVHD mouse model that could cause a high level of acute GVHD (aGVHD) without any precaution. Flow cytometric analyses of H2-Kb showed that all recipient mice exhibited a donor genotype on day 7, which meant a successful engraftment. According to the loss of weight and disease score, 1 × 10^7 bone marrow (BM) cells and 4 × 10^7 spleen cells were used as the final composition of mixture.

Next, we investigate if aGVHD could be alleviated by low-dose PTCy. As shown in Fig. 1, mice given low-dose PTCy alone (group D), which were injected 10mg/kg PTCy, demonstrated a significantly lower level of aGVHD according to disease score (Fig. 1B), as compared with GVHD-positive, untreated group (group B, p = 0.007). In addition, at day 14 post allo-HCT, we collected specimens of intestine, liver, lung and spleen. Histopathological analysis and the appearance of the mice at day 24 post allo-HCT showed that group D exhibited a lower degree of aGVHD (Fig. 1C, D). From the above findings, we concluded that low-dose PTCy could alleviate acute GVHD in mouse model.

To determine whether ATG followed by low-dose PTCy could further alleviate aGVHD, we detected the appropriate dose of ATG in the established aGVHD model which was partially effective but insufficient to prevent aGVHD. In mice model, the serum of mouse thymocyte antibody was used as ATG and 0.05 ml per mouse per day was used in the following experiments. We then investigate if aGVHD could be further alleviated after the combined use of low-dose PTCy and ATG. As expected, mice in combined-treatment group (group E), which were injected ATG followed by low-dose PTCy, demonstrated a significantly lower level of aGVHD according to weight loss (p = 0.02 and p = 0.01, respectively; Fig. 1A) and disease score (p = 0.04 and p = 0.02, respectively; Fig. 1B), as compared with mice given ATG alone (group C) or low-dose PTCy alone (group D). In addition, histopathological analysis and the appearance of the mice post allo-HCT showed that combined treatment group (group E) exhibited a lower degree of aGVHD (Fig. 1C, D). From the above findings, we concluded that low-dose PTCy could enhance the protective effect of ATG on GVHD in mouse model.

The number of Treg cells could be increased by low-dose PTCy and the effect on Tregs is significantly prominent with the combined use of low-dose PTCy and ATG in mice

To further detect how low-dose PTCy effected on aGVHD, we monitored the dynamics of CD4+ T cells, CD8+ T cells and Treg cells post allo-HCT (Fig. 2). In this experiment, Treg cells were recognized as CD4+ CD25+ Foxp3+ T cells.

As shown in Fig. 2, the number of T cells in the combined treatment group (group E) was not only comparable with that in GVHD-positive, untreated group (group B) but also with that in ATG-alone (group C) or low-dose PTCy-alone (group D) group, which suggested that neither low-dose PTCy alone nor the combined use of low-dose PTCy and ATG affected the number of CD4+, CD8+ T cells and undoubtedly, the total number of T cells.

On the other hand, Treg cells were influenced by treatment (Fig. 2). Firstly, the number of Treg cells in low-dose PTCy-alone group (group D) had a marked trend to be significantly higher than that in GVHD-negative group (group A, p = 0.08) and tended to be higher than that in GVHD-positive, untreated group (group B, p = 0.13), which indicated the expansion of Treg by low-dose PTCy. Secondly, the combined-treatment group (group E) had significantly higher number of Treg cells than that in GVHD-negative group (group A, p = 0.01) and GVHD-positive, untreated group (group B, p = 0.02), which revealed that low-dose PTCy and ATG had synergistic effect on Treg cells. Altogether, we could conclude that the ATG
followed by low-dose PTCy could promote the recovery of Treg cells without affecting CD4+ or CD8+ T cells.

**Study population**

All of the patients in the study cohort completed the prescribed combination of ATG and G-CSF along with low-dose PTCy. The cohorts were balanced with respect to patient and donor characteristics (Table 1).

**Low-dose PTCy significantly reduced acute GVHD**

As shown in Fig. 3A–B, the 100-day cumulative incidence of grades II-IV aGVHD in study cohort A with low-dose PTCy was significantly lower than that in cohort B (contemporary matched-pair control without low-dose PTCy, P = 0.04) but the rate was similar between the 2 cohorts for grades III-IV aGVHD (P = 0.15); whereas, the incidences of both grades II-IV and grades III-IV aGVHD in cohort A were significantly lower than that in cohort C (historical control without low-dose PTCy, both P < 0.001). The median interval for developing aGVHD grades II-IV in cohort A (31 days, range, 23–60 days) was similar to that in cohort B (25 days, range, 9–65 days; P = 0.55), but was significantly later than that in cohort C (16 days, range, 8–54 days; P = 0.001). A multivariate analysis showed that the use of low-dose PTCy remained to be a significant factor influencing acute GVHD (Table 2).

**Low-dose PTCy reduced moderate-to-severe chronic GVHD**

The 1-year cumulative overall incidences of chronic GVHD were comparable among the 3 cohorts (Fig. 3C). However, the rate of moderate-to-severe chronic GVHD in cohort A was comparable to the rate in cohort B (P = 0.36) but had a marked trend to be significantly lower than that in cohort C (P = 0.06, Fig. 3D). A multivariate analysis showed that the use of low-dose PTCy significantly reduced the incidence of moderate-to-severe chronic GVHD, compared with cohort C (Table 2).

**Low-dose PTCy could facilitate suppressive Treg cells reconstitution without affecting CD4+ or CD8+ T cells reconstitution**

Surprisingly, at the end of the 1st, 2nd and 3rd months after HCT, the numbers of both true Treg fractions (Fr I and II, as indicated below in the part of “immune reconstitution” of “Method” section), but not that of Fr III or total CD4+CD25+Foxp3+ T cells, were increased in patients after low-dose PTCy treatment (cohort A) when compared with the parallel cohort (cohort B, Table 3). Levels of CD3+, CD4+, and
CD8$^+$ T-cells were comparable between the 2 cohorts at the end of the 1st, 2nd and 3rd months after HCT (Table 3).

**Haematopoietic recovery, infection and transplant outcomes**

All subjects in the study cohort exhibited haematopoietic recovery after transplantation. One patient in each control cohort died of infection at day 40 and day 8 after HCT without myeloid recovery, respectively. The median time to myeloid recovery was one day shorter in cohort B (13 days, range, 10–20 days) than in cohort A (14 days, range, 12–21 days) and was similar between cohort A and C (13 days, range, 10–20 days). The platelet recovery at day 100 in cohort A (100%) was comparable to that in cohort B (85%; 95% confidence interval (CI), 79–91%; $P = 0.98$) and cohort C (87%; 95% CI, 77–97%; $P = 0.97$).

The cumulative incidences of cytomegalovirus (CMV) reactivation at day 100 in Cohort A (75%; 95% CI, 62–88%) was comparable to that in Cohort B (85%; 95% CI, 79–91%; $P = 0.10$) and that in Cohort C (83%; 95% CI, 73–93%; $P = 0.13$); The cumulative incidences of Epstein-Barr virus (EBV) reactivation at day 100 in Cohort A (15%; 95% CI, 4–26%) was comparable to that in Cohort B (29%; 95% CI, 21–37%; $P = 0.07$) and that in Cohort C (26%; 95% CI, 13–39%; $P = 0.18$). Late-
onset severe pneumonia\textsuperscript{31} (LOS, >3 months after HCT) occurrence were also comparable among the 3 cohorts (2% in cohort A vs. 4% in cohort B vs. 2% in cohort C, $P = 0.76$).

Table 2. Multivariate analyses of outcomes.

| Outcome                                         | Hazard Ratio (95% CI) | P value |
|-------------------------------------------------|-----------------------|---------|
| Non-relapse mortality                           |                       |         |
| Cohorts                                         |                       |         |
| Cohort A                                        | 1.0                   | <0.001* |
| Cohort B                                        | 2.14 (0.96–4.80)      | 0.06    |
| Cohort C                                        | 5.08 (2.20–11.75)     | <0.001  |
| Other significant factors                       |                       |         |
| Mononuclear cell count                          | 0.75 (0.60–0.94)      | 0.01    |
| CD34\^+ cell count                              | 0.69 (0.49–0.98)      | 0.04    |
| CD4/CD8 ratio                                   | 1.53 (1.07–2.19)      | 0.02    |
| Acute GVHD $\geq$ grade-2                      |                       |         |
| Cohorts                                         |                       |         |
| Cohort A                                        | 1.0                   |         |
| Cohort B                                        | 2.14 (0.96–4.80)      | 0.06    |
| Cohort C                                        | 5.08 (2.20–11.75)     | <0.001  |
| Chronic GVHD moderate to severe                 |                       |         |
| Cohorts                                         |                       |         |
| Cohort A                                        | 1.0                   | 0.02*   |
| Cohort B                                        | 1.42 (0.70–2.86)      | 0.32    |
| Cohort C                                        | 2.56 (1.21–5.41)      | 0.01    |
| Survival                                        |                       |         |
| Cohorts                                         |                       |         |
| Cohort A                                        | 1.0                   | 0.06*   |
| Cohort B                                        | 1.95 (0.67–5.67)      | 0.21    |
| Cohort C                                        | 3.38 (1.13–10.12)     | 0.03    |
| Other significant factors                       |                       |         |
| Mononuclear cell count                          | 0.72 (0.60–0.87)      | 0.001   |
| CD4/CD8 ratio                                   | 1.31 (0.95–1.81)      | 0.09    |

*Two degrees of freedom test.

Discussion

In this prospective trial, we found that low-dose PTCy, rather than high-dose PTCy, is sufficient to alleviate acute GVHD in mouse model, partly owing to the promotion of rapid Treg reconstitution. Moreover, low-dose PTCy could enhance the protective effect of ATG/G-CSF on GVHD both in mouse and human models, without compromising engraftment and GVL effect. Our results suggest that intensified conditioning followed by low-dose PTCy could be a potential novel approach to prevent GVHD and improve outcomes post haplo-HCT.

The main findings from our data, for the first time, indicate that low-dose PTCy alone could alleviate aGVHD, which was not only demonstrated in mouse model but also confirmed by...
our clinical trial. In our allogeneic HCT mouse model, a single dose of 10 mg/kg Cy administered on day 3 was found to significantly improve GVHD clinical score. Notably, improvement in histopathological and general appearance of the mice was also observed. In previous reports, an intermediate dose of 33 mg/kg but not 11 mg/kg administered on days 3 and 4 was found to significantly ameliorate GVHD compared with low-dose PTCy or ATG given alone. Results from clinical cohorts also indicate that compared with contemporary or historical controls, the use of low-dose PTCy significantly reduced the incidence of GVHD. In addition, the time to onset of GVHD in the study cohort was delayed compared with historical control. This was in consistent with Baltimore’s earlier experience. It should be noted that despite the significantly reduced incidence of moderate-to-severe chronic GVHD in the study cohort compared with historical cohort, the incidence remains high compared with that in the Baltimore haploidentical bone-marrow transplant protocol. It may be partly explained by the use of peripheral blood stem cells and donor lymphocyte infusion as well as mostly female donors especially female-to-male pairs in the study cohort. Overall, both our mouse and human model supports the rational that permissible dose reduction of PTCy is feasible for tolerance induction and the combined treatment may represent a reasonable approach. Multi-center studies with larger population in various transplant settings including HLA-matched situation are needed to confirm the results and broaden the practical use of low-dose PTCy in conjunction with other agents in innovative strategies.

Gaining insight into the mechanism of action of low-dose PTCy in conjunction with ATG will allow for the opportunity to explore its use in innovative strategy. Inducing T-cell tolerance, expanding Treg by treating healthy donors with G-CSF and in vivo T-cell depletion and Treg expansion with ATG can achieve stable donor engraftment with acceptable GVHD, as illustrated in the Beijing protocol. Likewise, selectively destroy rapidly proliferating alloreactive T cells as well as persistence and expansion of Tregs by high-dose PTCy was presented by the Baltimore group, USA. Collectively, the potential synergistic effect of low-dose PTCy and ATG/G-CSF at preventing GVHD might rely on some different aspects relevant to inducing immune tolerance although the targeting cells are similar. Besides, the sequential order and timing of drug administration may be critical to the achievement of the synergistic effect. Previous mouse and human models of allo-HCT indicate that restoring the conventional (Tcon)/Treg ratio representing naive, resting, natural Tregs (nTregs); Fr II (CD45RA+ Foxp3 hi) representing activated, effector Tregs (eTregs); and Fr III (CD45RA- Foxp3 lo) representing cytokine-secreting, non-suppressive T cells.

Table 3. In-vivo immune reconstitution.

| Cell type | Cohort A (ATG+PTCy) Median cell counts/μL (range) | Cohort B (ATG) Median cell counts/μL | P-value |
|-----------|--------------------------------------------------|------------------------------------|--------|
| CD4+ T cells | 30d 86.45(0.41–700.29) 60d 798.99(54.40–4941.00) 90d 805.38(52.44–3870.02) | 30d 58.10(0.10–444.68) 60d 671.49(10.12–4264.08) 90d 640.81(41.27–3432.71) | 30d 58.10(0.10–444.68) 60d 671.49(10.12–4264.08) 90d 640.81(41.27–3432.71) | 0.345 |
| CD8+ T cells | 30d 15.90(0.00–238.10) 60d 96.48(22.87–603.45) 90d 136.02(7.29–540.39) | 30d 15.90(0.00–238.10) 60d 96.48(22.87–603.45) 90d 136.02(7.29–540.39) | 30d 15.90(0.00–238.10) 60d 96.48(22.87–603.45) 90d 136.02(7.29–540.39) | 0.630 |
| CD4+CD25 T cells | 30d 3.48(0.00–16.95) 60d 7.11(0.48–70.01) 90d 9.26(1.49–54.70) | 30d 3.48(0.00–16.95) 60d 7.11(0.48–70.01) 90d 9.26(1.49–54.70) | 30d 3.48(0.00–16.95) 60d 7.11(0.48–70.01) 90d 9.26(1.49–54.70) | 0.074 |
| Fr I cells | 30d 0.02(0.00–0.20) 60d 0.06(0.00–0.46) 90d 0.06(0.00–1.15) | 30d 0.02(0.00–0.20) 60d 0.06(0.00–0.46) 90d 0.06(0.00–1.15) | 30d 0.02(0.00–0.20) 60d 0.06(0.00–0.46) 90d 0.06(0.00–1.15) | 0.006 |
| Fr II cells | 30d 0.10(0.00–5.25) 60d 0.65(0.00–5.21) 90d 0.76(0.04–9.92) | 30d 0.10(0.00–5.25) 60d 0.65(0.00–5.21) 90d 0.76(0.04–9.92) | 30d 0.10(0.00–5.25) 60d 0.65(0.00–5.21) 90d 0.76(0.04–9.92) | 0.014 |
| Fr III cells | 30d 0.60(0.00–4.57) 60d 1.83(0.16–12.04) 90d 1.88(0.41–13.59) | 30d 0.60(0.00–4.57) 60d 1.83(0.16–12.04) 90d 1.88(0.41–13.59) | 30d 0.60(0.00–4.57) 60d 1.83(0.16–12.04) 90d 1.88(0.41–13.59) | 0.009 |
| CD4+CD25+Foxp3+ T cells | 30d 0.86(0.00–6.09) 60d 2.94(0.18–14.68) 90d 3.35(0.56–16.50) | 30d 0.86(0.00–6.09) 60d 2.94(0.18–14.68) 90d 3.35(0.56–16.50) | 30d 0.86(0.00–6.09) 60d 2.94(0.18–14.68) 90d 3.35(0.56–16.50) | 0.644 |

Note: fraction I (Fr I) (CD45RA+Foxp3 hi) representing naive, resting, natural Tregs (nTregs); Fr II (CD45RA+Foxp3 hi) representing activated, effector Tregs (eTregs); and Fr III (CD45RA–Foxp3 lo) representing cytokine-secreting, non-suppressive T cells.
receptor binding Tcons deleted by ATG. The assumption was substantiated by the in vivo immune reconstitution analysis. Altogether, our findings indicate that low-dose PTCy-mediated control of alloreactivity and its synergistic effect with ATG/G-CSF at least in part depend on Tregs. Our recent study also confirmed the Treg-dependent mechanism in lower GVHD incidence after NIMA-exposed donor HCT.18

Impressively, the joint use of low-dose PTCy and ATG was safe in terms of engraftment, relapse prevention, or other adverse effects. Low-dose PTCy along with ATG and G-CSF did not influence haematopoietic recovery. ATG was proved to be capable to help prevent the acute rejection of the donor bone marrow by decreasing the immediate alloresponse and thus aid reliable engraftment, which might harbor the potential risk of graft failure due to memory T cells escape from high-dose PTCy.14 As for GVL effect, our mouse and human studies indicate that low-dose PTCy increases Tregs without affecting the levels of CD3+, CD4+, and CD8+ T-cells while previous studies showed that Tregs are highly effective in controlling GVHD without compromising the GV effect.32 thus it is conceivable that low-dose PTCy had little influence on GV effect, which is evidenced by our clinical data that the combined treatment neither increased the relapse rate nor lower survival compared with control cohorts. Although the abrogation of GVHD is a key goal after allo-HCT, the utility of PTCy would be diminished if a more global deletion of cells such as tumor-specific T cells simultaneously took place owing to excessive dose of Cy.13 Moreover, the GV effects could be compensated by ATG at clinically relevant concentrations to kill leukemic blasts.33 In a word, considering the excellent engraftment and low relapse rate from our study, low-dose PTCy is permissible and might obviate the adverse aspects related with high-dose PTCy. In addition, incorporating donor-specific anti-human leukocyte antigen antibodies (DSAs) in the algorithm for haploidentical donor selection is also warranted for engraftment.25 Both animal and human studies are required to investigate dose effect regarding comparison between low-dose PTCy and high-dose PTCy. Although dose-escalating study was not performed in our pilot trial, our findings suggested the additive effect of low-dose PTCy and ATG/G-CSF at preventing GVHD without abrogating engraftment and GVL. Dose-finding studies are necessary before the optimal dose of PTCy can be established with the new combined treatment approach.

In conclusion, low-dose PTCy is sufficient for GVHD abrogation under lymphopenic situation and can enhance the protective effect of ATG/G-CSF on GVHD. Intensified conditioning followed by low-dose PTCy might be a feasible option for patients undergoing haploidentical, T-cell replete HCT. The role of Treg-dependent mechanism and its conjunction with other immune subsets in the GVHD protective activity of low-dose PTCy remains to be further elucidated.

Materials and methods

Establishment of GVHD mouse model administered with low-dose PTCy and ATG

Female C57BL/6 (B6; H-2b) and BALB/c (H-2d) mice were purchased from Beijing Vital Laboratory Animal Technology Company, Ltd., Beijing, China. All animals were maintained in specific pathogen-free conditions and had access to sterilized water and food at the animal facility of Peking University People’s Hospital. Mice were between 6 and 8 week of age at the start of the experiments. All experiments were approved by the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

Mice underwent allo-HCT as described previously.20 Briefly, BALB/c recipients were irradiated at a dose of 8 Gy for 8 minutes from a [60Co] source. On day 0, recipients were transplanted with 1 × 107 BM cells and 4 × 106 spleen cells from C57BL/6 (B6; H-2b) donors. To remove red blood cells, BM cells and splenocytes were lysed in RBC lysing buffer (0.15M NH4Cl, Sigma) to obtain a cell suspension of mononuclear cells. Cell mixtures were injected through tail vein in a final volume of 300µl in sterile 0.9% NaCl (4 to 8 mice per group per experiment). Recipients in control group were only transplanted with 1 × 107 BM cells. ATG (Fitzgerald, IP) was administered to designated groups on day −12, −10 and −8 before allo-HCT, and low-dose PTCy (10mg/kg, IP) was administered on day +3 post allo-HCT.

After allo-HCT, recipients were weighed every 2 days, and the degree of systemic GVHD was assessed by a clinical scoring system including 5 clinical parameters (weight loss, posture, activity, fur ruffling, and skin integrity), as published previously.21 For histopathological analysis, mice were killed and specimens of left lung, liver, intestine and skin were collected post mortem on slides, and stained with hematoxylin and eosin (H&E). Two slides/organs were evaluated and scored by a pathologist blinded to experimental group using a scoring system described previously.22

Single-cell suspensions were obtained from blood, spleen and bone marrow of recipients. For flow cytometric (FCM) analysis, cells were prepared and incubated with appropriate antibodies against cell surface antigens for 15 min at room temperature in the dark. For detection of cytokine production, cells were stimulated for 4 hours with 150ng/ml Leukocyte activation Cocktail (BD) before staining for flow cytometric analysis. For detection of cytokine production, cells were stimulated for 4 hours with 150ng/ml Leukocyte activation Cocktail (BD) before staining for flow cytometric analysis. Cells were assayed for cytokine production by intracellular flow cytometry staining. The following Abs were used for staining: mCD3-FITC, mCD3-PE, mCD4-PE-Cy7, mCD8-V500, mCD25-APC, mCD44-V450, mCD62L-PerCP-Cy5.5, mCD45-PerCP, mIL-17-PE, mIFN-γ-APC, mFoxp3-FITC, mCD45-PerCP and mH2-Kb-FITC(BD Biosciences). Data were acquired using an LSMFortessa (BD Biosciences) and analyzed with FlowJo software (TreeStar).

Study design

The prospective, non-randomized, open-label study included patients recruited at the Peking University People’s Hospital between April 2015 and Aug 2016. All included subjects signed informed consent. The study protocol was in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Peking University. This study was registered at http://clinicaltrials.gov/ NCT02412423.
**Eligibility and exclusion criteria**

The study included consecutive patients, aged 15 to 60 years, with standard-risk hematological neoplasms, which were scheduled to receive a haplo-HCT from maternal donors or collateral relatives. Patients with malignancies were categorized as "standard risk" if they were in the first or second complete remission (CR1 or CR2) of acute leukemia, or in the chronic phase of chronic myelogenous leukemia (CML), or had myelodysplastic syndrome (MDS) with <20% blasts. Exclusion criteria were severe heart, kidney, or liver disease; a prior transplant; and hypersensitivity to rabbit ATG or CY.

**Donor selection and HLA typing**

Donors in the study cohort are either maternal donors or collateral relatives. Patients were eligible for haploidentical HCT if a matched sibling donor or a suitable closely HLA-matched unrelated donor was unavailable or if there was insufficient time for an unrelated donor search due to disease status (acute leukemia in CR2, MDS with >10% blasts or CML in the second chronic phase). For the patients transplanted in CR1, when a suitable donor was available, eligible patients proceeded to HCT after receiving 2–4 cycles of consolidation therapy. Based on the data from our previous reports, young, male siblings or paternal donor is the first choice for haplo-HCT while maternal donor or collateral relative is the last choice. HLA typing was preformed as described previously.

**Subjects**

A total of 28 patients undergoing HCT from maternal donors and 12 from collateral relatives were eligible for this study (Cohort A). Meanwhile, 2 control cohorts were selected: (1) During the study period, a total of 432 patients received haplo-HCT from relatives other than maternal donor or collateral relatives. A matched-pair analysis was designed. For each recipient from the study cohort, 3 recipients were randomly selected from the control cohort and were matched according to the following criteria: age of the patients, sex of the patients, underlying diseases (acute myeloid leukemia, acute lymphoblastic leukemia, CML, or MDS), disease status (CR1/CP1, CR2/CP2, or MDS), and degree of HLA disparity for HLA-A,B,DR (3/6, 4/6, or 5/6) (control cohort, n = 120, Cohort B). (2) Historic control (Cohort C) was selected from among 46 similar subjects receiving allotransplant from maternal donors or collateral relatives at our center from January, 2014 to March, 2015.

**Transplants**

Patients were conditioned with an intensive chemotherapy-based regimen which consisted of: cytarabine, 4 g/m² per day intravenously (i.v.) on days −10 and −9 (transplantation was considered day 0); busulfan, 9.6 mg/kg i.v., given in 12 doses on days −8, −7, and −6; cyclophosphamide (Cy), 1.8 g/m² per day i.v., given on days −5 and −4; simustine (250 mg/m²), given on day −3; and rabbit ATG (Sangstat-Genzyme) 2.5 mg/kg per day i.v., given on days −5, −4, −3, and −2. Two doses of 14.5mg/kg Cy was given on days 3 and 4 after HCT from maternal donors or collateral relatives during the trial period. Allografts were harvested and infused according to our previous protocol.

**Acute GVHD prevention and treatment**

For GVHD prevention, all subjects received cyclosporine (CSA), mycophenolate mofetil (MMF), and methotrexate (MTX). CSA was started on day −9 at 2.5 mg/kg/d i.v. After bowel function had normalized, the dosage was changed to 3–5 mg/kg/d p.o. Whole-blood CSA concentrations were monitored weekly and adjusted to maintain a trough concentration of 150–250 ng/ml. The CSA dose was gradually decreased in subjects without GVHD on day +180, but it was continued in patients with acute GVHD above grade I.

Subjects in the study that developed acute GVHD above grade II received MP at 1 mg/kg/d i.v. Non-responders received basiliximab (Novartis Pharma AG, Basel, Switzerland) at 20 mg/d on day +1 and day +3. Injections were repeated weekly until GVHD was reduced to grade II or lower. Subjects with chronic GVHD received CSA and corticosteroids.

**Cytomegalovirus and Epstein-Barr virus monitoring and prevention**

Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) levels were monitored and infections were treated as described previously.

**Definitions and evaluation**

Engraftment, graft failure, infection, non-relapse mortality (NRM), relapse, disease-free survival (DFS), and overall survival (OS) were defined as described previously. Myeloid recovery was defined as an absolute neutrophil count (ANC) of 0.5 × 10⁹/L or more for 3 consecutive days and platelet recovery was defined as 20 × 10⁹/L or more for 7 consecutive days without transfusion. Acute GVHD was defined and graded based on the pattern and severity of organ involvement. Chronic GVHD was defined and graded according to the National Institute of Health criteria. Relapse was defined based on histological criteria.

**Adjudication of acute GVHD diagnosis**

Case report forms included detailed, structured data regarding organ involvement, which were verified by medical record inspection. Based on these data, a panel of 5 blinded experts determined whether a subject had acute GVHD and, when present, the grade. Discordances were adjudicated by majority rule.

**Immune reconstitution**

Immune reconstitution was assessed as described previously. In addition, as suggested in literatures, to overcome the potential short-coming of using only Foxp3 expression (with or without CD25 and CD127) to assess the in vivo effects of PTCy on Tregs, we used the strategy of Miyara and colleagues and adopted by Luznik, which phenotypically and functionally...
delineated 3 fractions of human CD4\(^{+}\)Foxp3\(^{+}\) T cells on the basis of expression of Foxp3 and CD45RA: fraction I (Fr I) (CD45RA\(^{-}\)Foxp3\(^{+}\)) representing naive, resting, natural Tregs (nTregs); Fr II (CD45RA\(^{-}\)Foxp3\(^{hi}\)) representing activated, effector Tregs (eTregs); and Fr III (CD45RA\(^{-}\)Foxp3\(^{lo}\)) representing cytokine-secreting, nonsuppressive T cells.\(^{30}\) We were able to reproducibly identify these fractions, confirmed that the 2 Treg fractions (Fr I and II) were suppressive and showed differential cytokine production, verifying that Fr I and II were indeed true Treg fractions.

End-points
The study was powered to detect 30% grades II-IV acute GVHD based on a reference rate of 56% at 100d derived using data from our previous reports. The primary study end-point was the incidence of acute GVHD, grades II-IV. Secondary end-points were the engraftment rate; the incidences of acute GVHD grades III-IV, infection, and chronic GVHD; and the cumulative incidences of NRM, relapse, DFS, and OS.

Statistical analyses
Groups and cohorts were compared with the \(\chi^2\) statistic for categorical variables and the Mann–Whitney test for continuous variables. Cumulative incidence curves were used in a competing risk setting, with relapse treated as a competing event, to calculate NRM probabilities, and with death from any cause as a competing risk for GVHD, engraftment, EBV or CMV reactivation, and relapse. Time to GVHD was defined as the time from transplantation to the onset of GVHD of any grade. Probabilities of DFS and OS were estimated with the Kaplan–Meier method. All variables in Table 1 were included into a Cox proportional hazards model. Unless otherwise specified, \(P\)-values were based on 2-sided hypothesis tests. \(\alpha\) was set at 0.05. Analyses were performed with SPSS 19.0 (Mathsoft, Seattle, WA, USA).

Authorship
X.-J.H. designed the research; Y.W., Y.-J. C. and X.-J.H. analyzed the data and wrote the manuscript; L.C., and Z.-L. B. conducted experiments; and all authors, provided patient data and gave final approval for the manuscript.

Financial disclosure statements
This work was partly supported by grants from National Natural Science Foundation of China (Grant No. 81400143 and 81670167 and 81530046 and 81621001), Collaborative Innovation Center of Hematology China, the National Key Research and Development Program of China (Grant No. 2017YFA0104500) from the Ministry of Science and Technology, the Science and Technology Project of Guangdong Province of China (Grant No. 2016B030230003), and the Beijing Municipal Science and Technology Program (Grant No. Z14110000021401).

Disclosure of potential conflicts of interest
The authors declare no conflict of interest.

Acknowledgment
We thank the principal investigators and the skilled teams.

References
1. Apperley J, Niederwieser D, Huang XJ, Nagler A, Fuchs E, Szer J, Kodera Y. Haploidentical hematopoietic stem cell transplantation: A global overview comparing Asia, the European Union, and the United States. Biol Blood Marrow Transplant. 2016;22:23-6. doi:10.1016/j.bbmt.2015.11.001. PMID:26551633
2. Rubio MT, Savani BN, Labopin M, Piemontese S, Polge E, Cicери F, Bacigalupo A, Arcese W, Koc Y, Beelen D, Giliñas Z, et al. Impact of conditioning intensity in T-replete haplo-identical stem cell transplantation for acute leukemia: a report from the acute leukemia working party of the EBMT. J Hematol Oncol. 2016;9:25. doi:10.1186/s13045-016-0248-3. PMID:26980295
3. Di Bartolomeo P, Santarone S, De Angelis G, Picardi A, Cudillo L, Cerretti R, Adorno G, Angelini S, Andreani M, De Felice L, et al. Haploidentical, unmanipulated, G-CSF-primed bone marrow transplantation for patients with high-risk hematologic malignancies. Blood. 2013;121:849-57. doi:10.1182/blood-2012-08-453399. PMID:23165479
4. Lu DP, Dong L, Wu T, Huang XJ, Zhang MJ, Han W, Chen H, Liu DH, Gao YZ, Chen YH, et al. Conditioning including antithymocyte globulin followed by unmanipulated HLA-mismatched/haploidentical blood and marrow transplantation can achieve comparable outcomes with HLA-identical sibling transplantation. Blood. 2006;107:3605-73. doi:10.1182/blood-2005-05-1524. PMID:16380454
5. Wang Y, Chang YJ, Xu LP, Liu KY, Liu DH, Zhang XH, Chen XH, Han W, Chen YH, Wang FR, et al. Who is the best donor for a related HLA haplotype-mismatched transplant? Blood. 2014;124:843-50. doi:10.1182/blood-2014-03-563130. PMID:24916508
6. Wang Y, Liu QF, Xu LP, Liu KY, Zhang XH, Ma X, Fan ZP, Wu DP, Huang XJ. Haploidentical vs identical-sibling transplant for AML in remission: a multicenter, prospective study. Blood. 2015;125:3956-62. doi:10.1182/blood-2015-02-627786. PMID:25940714
7. Wang Y, Liu QF, Xu LP, Liu KY, Zhang XH, Ma X, Wu MQ, Wu DP, Huang XJ. Haploidentical versus Matched-Sibling Transplant in Adults With Philadelphia-negative high-risk Acute Lymphoblastic Leukemia: A biologically phase 3 randomized study. Clin Cancer Res. 2016;22:1347-76. doi:10.1158/1078-0432.CCR-15-2335. PMID:26927664
8. Wang Y, Wang LX, Hai YR, Sun ZM, Wu DP, Jiang M, Liu DH, Xu KL, Liu QF, Liu L, et al. Haploidentical transplant for myelodysplastic syndrome: registry-based comparison with identical sibling transplant. Leukemia. 2016;30:1055-63. doi:10.1038/leu.2016.110. PMID:27133816
9. Mayumi H, Umesue M, Nomoto K. Cyclophosphamide-induced immunological tolerance: an overview. Immunobiology. 1996;195:129-39. doi:10.1016/S0171-2985(96)80033-7. PMID:8877390
10. O’Donnell PV, Luznik L, Jones RJ, Vogelsang GB, Leffell MS, PhelpS M, RhubarB P, Cowan K, Piantadosi S, Fuchs EJ. Nonmyeloablative bone marrow transplantation from partially HLA–mismatched related donors using posttransplantation cyclophosphamide. Biol Blood Marrow Transplant. 2002;8:377-86. doi:10.1038/bmt.2013.223. PMID:12171484
11. Luznik L, Donnell PV, Symons HJ, Chen AR, Leffell MS, Zahrurak M, Gooley TA, Piantadosi S, Kaup M, Ambinder RF, et al. HLA-haploidentical bone marrow transplantation for haematologic malignancies using nonmyeloablative conditioning and high-dose, posttransplantation cyclophosphamide. Biol. Blood Marrow Transplant. 2008;14:641-50. doi:10.1016/j.bbmt.2008.03.005. PMID:18489989
12. Robinson TM, O’Donnell PV, Fuchs EJ, Luznik I. Haploidentical bone marrow and stem cell transplantation: experience with posttransplantation cyclophosphamide. Semin Hematol. 2016;53:90-7. doi:10.1053/j.seminhematol.2016.01.005. PMID:27007332
13. Luznik L, Jalla S, Engstrom LW, Iannone R, Fuchs EJ. Durable engraftment of major histocompatibility complex-incompatible cells after nonmyeloablative conditioning with fludarabine, low-dose total

ORCID
Ying-Jun Chang http://orcid.org/0000-0002-6124-6050
Lan-Ping Xu http://orcid.org/0000-0002-0267-1088
Xiao-Jun Huang http://orcid.org/0000-0002-2145-6643
body irradiation, and post-transplantation cyclophosphamide. Blood. 2001;98:3456-64. PMID:11719388

14. Colson YL, Li H, Boggs SS, Patrenee KD, Johnson PC, Ildstad ST. Durable mixed allogeneic chimerism and tolerance by a nonlethal radiation-based cytoreductive approach. J. Immunol 1996;157:2820-9. PMID:8816385

15. Ross D, Jones M, Komanduri K, Levy RB. Antigen and lymphopenia-driven donor T cells are differentially diminished by post-transplantation administration of cyclophosphamide after haematopoietic cell transplantation. Biol. Blood Marrow Transplant. 2013;19:1430-8. doi:10.1016/j.bbmt.2013.06.019. PMID:23819914

16. Gagulay S, Ross DB, Panoskalsis-Mortari A, Kanakry CG, Blazar BR, Levy RB, Luznik L. Donor CD4+ Foxp3+ regulatory T cells are necessary for post-transplantation cyclophosphamide-mediated protection against GVHD in mice. Blood. 2014;124:2131-41. doi:10.1182/blood-2013-10-525873. PMID:25139358

17. Kanakry CG, Gagulay S, Zahrak M, Bolaos-Meade J, Thoburn C, Perkins B, Fuchs EJ, Jones RJ, Hess AD, Luznik L. Aldehyde dehydrogenase expression drives human regulatory T cell resistance to post-transplantation immunosuppression. Sci Transl Med. 2013;5:211ra157. doi:10.1126/scitranslmed.3006690. PMID:24225944

18. Wang Y, Zhao XY, Xu LP, Zhang XH, Han W, Chen H, Wang FR, Mo XD, Zhang YY, Zhao XS, et al. Lower incidence of acute GVHD is associated with the rapid recovery of CD4+CD25+CD45RA+ regulatory T cells in patients who received haploidentical allografts from NIMA-mismatched donors: A retrospective (development) and prospective (validation) cohort-based study. Oncoimmunology. 2016;5:e1242546. doi:10.1080/2162402X.2016.1242546. PMID:28180031

19. Zhang YY, Liu DH, Liu KY, Xu LP, Chen H, Han W, Wang Y, Huang XJ. HLA-haploidentical hematopoietic SCT from collateral related donors without in vitro T-cell depletion for hematological malignancies. Bone Marrow Transplant. 2014;49:496-501. doi:10.1038/bmt.2013.223. PMID:24510070

20. Mo XD, Zhang XH, Xu LP, Wang Y, Yan CH, Chen H, Chen YH, Han W, Wang FR, Wang JZ, et al. Late-onset severe pneumonia after allogeneic hematopoietic stem cell transplantation: prognostic factors and treatments. Transpl Infect Dis. 2016;18:492-503. doi:10.1111/tid.12553. PMID:27218435

21. Cooke KR, Kobzik L, Martin TR, Brewer J, Delmonte J Jr, Crawford JM, Ferrara JL. An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation.1. The roles of minor H antigens and endotoxin. Blood. 1997;90:3204-13. PMID:9376604

22. Hill GR, Crawford JM, Cooke KR, Brinson YS, Pan LY, Ferrara JLM. Total body irradiation and acute graft-versus-host disease: The role of gastrointestinal damage and inflammatory cytokines. Blood. 1997;90:3204-13. PMID:9376604

23. Wang Y, Liu DH, Liu KY, Xu LP, Zhang XH, Han W, Chen H, Chen YH, Wang FR, Wang JZ, et al. Long-term follow-up of haploidentical hematopoietic stem cell transplantation without in vitro T-cell-depletion for the treatment of leukemia: nine years of experience at a single center. Cancer. 2013;119:978-85. doi:10.1002/cncr.27761. PMID:23097265

24. Wang Y, Fu HX, Liu DH, Xu LP, Zhang XH, Chang YJ, Chen YH, Wang FR, Sun YQ, Tang FF, et al. Influence of two different doses of antithymocyte globulin in patients with standard-risk disease following haploidentical transplantation: a randomized trial. Bone Marrow Transplant. 2014;49:426-33. doi:10.1038/bmt.2013.191. PMID:24292519

25. Chang YJ, Zhao XY, Xu LP, Zhang XH, Wang Y, Han W, Chen H, Wang FR, Mo XD, Zhang YY, et al. Donor-specific anti-human leukocyte antigen antibodies were associated with primary graft failure after unmanipulated haploidentical blood and marrow transplantation: a prospective study with randomly assigned training and validation sets. J Hematol Oncol. 2015;8:84. doi:10.1186/s13045-015-0182-9. PMID:26156848

26. Yan CH, Wang Y, Wang JZ, Chen YH, Chen Y, Wang FR, Sun YQ, Mo XD, Han W, Chen H, et al. Minimal residual disease- and graft-vs-host disease-guided multiple consolidation chemotherapy and donor lymphocyte infusion prevent second acute leukemia relapse after allotransplant. J Hematol Oncol. 2016;9:87. doi:10.1186/s13045-016-0319-5. PMID:27629395

27. Rowlings PA, Przepiorka D, Klein JP, Gale RP, Passweg JR, Henßle-Downey PJ, Cahn JY, Calderwood S, Gratwohl A, Socie G, et al. IBMTR Severity Index for grading acute graft-versus-host disease: retrospective comparison with Glucksberg grade. Br J Haematol. 1997;97:855-64. doi:10.1046/j.1365-2141.1997.1112925.x. PMID:9217187

28. Filipovich AH, Weisdorf D, Pavletic S, Socie G, Wingard JR, Lee SJ, Martin P, Chien J, Przepiorka D, Couriel D, et al. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report. Biol Blood Marrow Transplant 2005;11:945-56. doi:10.1016/j.bbmt.2005.09.004. PMID:16338616

29. Chang YJ, Zhao XY, Huo MR, Xu LP, Liu DH, Liu KY, Huang XJ. Immune reconstitution following unmanipulated HLA-mismatched/haploidentical transplantation compared with HLA-identical sibling transplantation. J Clin Immunol 2012;32:268-80. doi:10.1007/s10875-011-9630-7. PMID:22173879

30. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, Parizot C, Taffin C, Heike T, Valeyre D, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the Foxp3 transcription factor. Immunity 2009;30:899-911. doi:10.1016/j.immuni.2009.03.019. PMID:19464196

31. Mo XD, Zhang XH, Xu LP, Wang Y, Yan CH, Chen H, Chen YH, Han W, Wang FR, Wang JZ, et al. Late-onset severe pneumonia after allogeneic hematopoietic stem cell transplantation: prognostic factors and treatments. Transpl Infect Dis 2016;18:492-503. doi:10.1111/tid.12553. PMID:27218435

32. Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. J Exp Med 2002;196:389-99. doi:10.1084/jem.20020399. PMID:12163567

33. Dabas R, Lee R, Servito MT, Dharmani-Khan P, Modi M, van Slyke T, Luider J, Durand C, Larratt L, Brandwein J, et al. Antithymocyte globulin at clinically relevant concentrations kills leukemic blasts. Biol Blood Marrow Transplant. 2016;22:815-24. doi:10.1016/j.bbmt.2016.01.002. PMID:26779931