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Tolerance induction between two different strains of parental mice prevents graft-versus-host disease in haploidentical hematopoietic stem cell transplantation to F1 mice

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ABSTRACT

Haploidentical hematopoietic stem cell transplantation (Haplo-HSCT) has been employed worldwide in recent years and led to favorable outcome in a group of patients who do not have human leukocyte antigen (HLA)-matched donors. However, the high incidence of severe graft-versus-host disease (GVHD) is a major problem for Haplo-HSCT. In the current study, we performed a proof of concept mouse study to test whether induction of allogeneic tolerance between two different parental strains was able to attenuate GVHD in Haplo-HSCT to the F1 mice. We induced alloantigen tolerance in C3H mice (H-2k) using ultraviolet B (UVB) irradiated immature dendritic cells (iDCs) derived from the cultures of Balb/c bone marrow cells. Then, we performed Haplo-HSCT using tolerant C3H mice as donors to F1 mice (C3H × Balb/c). The results demonstrated that this approach markedly reduced GVHD-associated death and significantly prolonged the survival of recipient mice in contrast to the groups with donors (C3H mice) that received infusion of non-UVB-irradiated DCs. Further studies showed that there were enhanced Tregs in the tolerant mice and alloantigen-specific T cell response was skewed to more IL-10-producing T cells, suggesting that these regulatory T cells might have contributed to the attenuation of GVHD. This study suggests that it is a feasible approach to preventing GVHD in Haplo-HSCT in children by pre-induction of alloantigen tolerance between the two parents. This concept may also lead to more opportunities in cell-based immunotherapy for GVHD post Haplo-HSCT.

1. Introduction

Haploidendtical hematopoietic stem cell transplantation (Haplo-HSCT) has been accepted and increasingly employed worldwide and showed favorable outcome in patients who do not have human leukocyte antigen (HLA)-matched donors [1–6]. However, one of the major concerns in Haplo-HSCT is the high risk of severe graft-versus-host disease (GVHD) [7–9]. To overcome this problem, intensive global immunosuppressive therapy is routinely administered post transplantation. While global immunosuppression can largely mitigate the GVHD, the severe side effects caused by immunosuppressive drugs sometimes may be lethal to the recipients. Therefore, it is urgent to discover new ways that prevent GVHD without causing severe side effects.

Alloantigen-specific tolerance induction is an ideal approach in preventing GVHD, and has attracted extensive investigations in the field of allogeneic transplantation [10–16]. Given that the disparity between recipient (son or daughter) and a parent donor in Haplo-HSCT is from another parent, we hypothesize that induction of alloantigen tolerance between the two parents before Haplo-HSCT to their offspring would effectively prevent GVHD. We tested this hypothesis using Haplo-HSCT mouse model, and found that induction of alloantigen tolerance between Balb/c (H-2d) and C3H (H-2k) mice significantly prolonged the survival of F1 mice receiving bone marrow transplantation from C3H mice being tolerized to Babl/c antigens. To our knowledge, this is first to describe a concept on tolerance induction between two parents for Haplo-HSCT to their offspring. This offers a feasible approach to preventing GVHD in child Haplo-HSCT via inducing alloantigen tolerance between both parents.

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2. Materials and methods

2.1. Mice

8–10 weeks old Balb/c (H-2d), C3H (H-2k), F1 (Balb/c × C3H), and C57BL/6 were purchased from Charles River Animal facility in China (Beijing), and housed in specific pathogen free animal facility in Capital Medical University, Beijing. All mice were maintained under specific pathogen-free conditions, and used following the Chinese governmental and Capital Medical University guidelines for animal welfare. This study was The Capital Medical University Animal Ethics Committee approved this study.

2.2. Dendritic cell culture and preparation

Bone marrow derived (BM)-DC cultures and DC isolation were performed using our previously reported method [17].

2.3. Tolerance induction between Balb/c (H-2d) and C3H (H-2k) mice

As previously reported [17], Balb/c immature BM-DCs (iDCs) were prepared from male mice as described above, and irradiated by ultraviolet B (UVB) (1200 mJ/cm^2). C3H male mice received intravenous injection of 2×10^5/mouse UVB-irradiated immature BM-DCs once week for 4 weeks. Two control groups received iDCs and LPS-matured BM-DCs (mDC), respectively following the same schedule as UVB-irradiated iDC injection.

2.4. Measurement of serum anti-donor antibody by flow cytometry

Flow cytometric assay for anti-donor antibodies were performed using the method previously reported [17]. In brief, the plasma samples were harvested from the mice described above after four weekly injections were completed. Spleen cells prepared from Balb/c male mice were incubated with CD4-PerCp (BD BioScience) and the plasma samples from different groups. After wash, the cells were incubated with anti-mouse IgG-FITC (BD Biosience). To eliminate the influence of antibody Fc binding on B lymphocytes, we only analyzed the intensity of FITC on CD4+ T cells using FCS express software (De Novo software, Vancouver, Canada) to reflect the levels of anti-donor Ig in the plasma. The plasma samples from the control group could serve as isotype antibody control.

2.5. In vivo allo-rejection assay

C3H mice including tolerant and non-tolerant mice received Balb/c spleen cells (1×10^7/mouse) pre-labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen). Twenty-four hours later, the recipient mice were sacrificed. The spleen and lymph node cells were prepared and stained by anti-CD4-PerCp, B220-APC and CD8-PE antibodies. CFSE+CD4+, C8+ T cells and B220+ B cells were analyzed by flow cytometry (FACScaliber, BD).

2.6. Haplo-HSCT between parental mice (C3H) and F1 (C3H × Balb/c) mice and monitoring post transplantation

Recipient F1 mice were irradiated by ^{60}Co using a dose of 1100 cGy. Six hours later, recipient mice received intravenous injections of spleen cells (2×10^7/mouse) and bone marrow cells (1×10^7/mouse) from C3H donors receiving different pre-treatments of Balb/c UVB-iDC, iDC, mDC and PBS as described above. One group without cell injection served as an additional control to confirm a sufficient irradiation dose had been employed, and an additional group was chosen to use F1 mice as donors (syngeneic). Thereafter, recipient mice were monitored every other day for the first two weeks. The recovery of white blood cells (WBC) was also monitored once a week until WBC counts were in a normal range. The mice with severe body weight loss (loss 35% or more) were deemed moribund, and euthanized in a CO_2 chamber after counted.

2.7. T cell proliferation assay

Freshly prepared spleen cells were labeled with CFSE following the instructions from the manufacturer (Invitrogen). The CFSE-labeled spleen cells were stimulated with allogeneic mDCs or third party mDCs in the ratios of 20:1. Five days later, the cultured cells were collected and labeled with CD4-PerCp. The T cell proliferation was examined by analyzing the dilution of CFSE-labeled CD4 T cells.

2.8. Intracellular cytokine staining

Spleen cells prepared from mice in different treatment groups were stimulated with allogeneic mDCs at ratio of 20:1, respectively for 4 days. At the last 4 h, the above cultures were incubated with Leukocyte-activation cocktail with GolgiPlug following the protocol from the manufacturer (BD Bioscience). Thereafter, the cells were harvested, stained for CD4 and intracellular cytokine staining for IFN-γ and IL-10 following the protocol from the manufacturer (BD BioSciences). In part of the cultures, spleen cells were pre-stained with CFSE for the detection of cytokine-producing cells in proliferating T cells in response to the stimulation of allogeneic DCs.

2.9. Regulatory T cell examination

Regulatory T cells were examined by CD4 and intracellular Foxp3 staining following the protocol from the manufacturer (eBiosciences), and analyzed by flow cytometry.

2.10. Statistical analysis

The survival analyses were performed using Log-Rank test. Student *t* test was used for two parametric data analysis. ANOVA analysis was used for the data from multiple groups. Prism Graphpad software, version 6 was employed for the above analyses.

3. Results

3.1. Successful induction of alloantigen tolerance between Balb/c (H-2d) and C3H (H-2k) mice

Using the method we previously reported [17], we intravenously injected 2×10^5 Balb/c BM-derived iDCs with or without irradiation by ultraviolet B (200 mJ/cm^2) or BM-derived mDCs into C3H mice once a week for 4 weeks. The following week, we collect blood samples from all mice, and the levels of anti-donor antibodies were measured by flow cytometry. In another set of experiments, we treated C3H mice with intravenous injection of UVB-iDCs and mDCs as described above. Then, all mice received intravenous injection of CFSE-label Balb/c spleen cells. Twenty-four hours later, the mice were sacrificed and the injected CFSE-labeled cells in spleen and lymph nodes were examined using flow cytometry. The same as our previous report [17], we found that C3H mice receiving Balb/c UVB-iDCs failed to develop any levels of anti-Balb/c antibodies, as the levels were comparable to those of naïve C3H mice (Fig. 1A, B, and E). Both iDC and mDC treated mice developed high levels of anti-donor antibodies (Fig. 1C and D). Mature DCs, in particular, triggered even higher levels than did iDC (Fig. 1D). In line with the above findings, we observed that CFSE-labeled Balb/c spleen cells failed to be rejected in UVB-iDC treated mice 24 h after injection. Whereas, those CFSE-labeled cells when injected into mDC-treated mice, al-

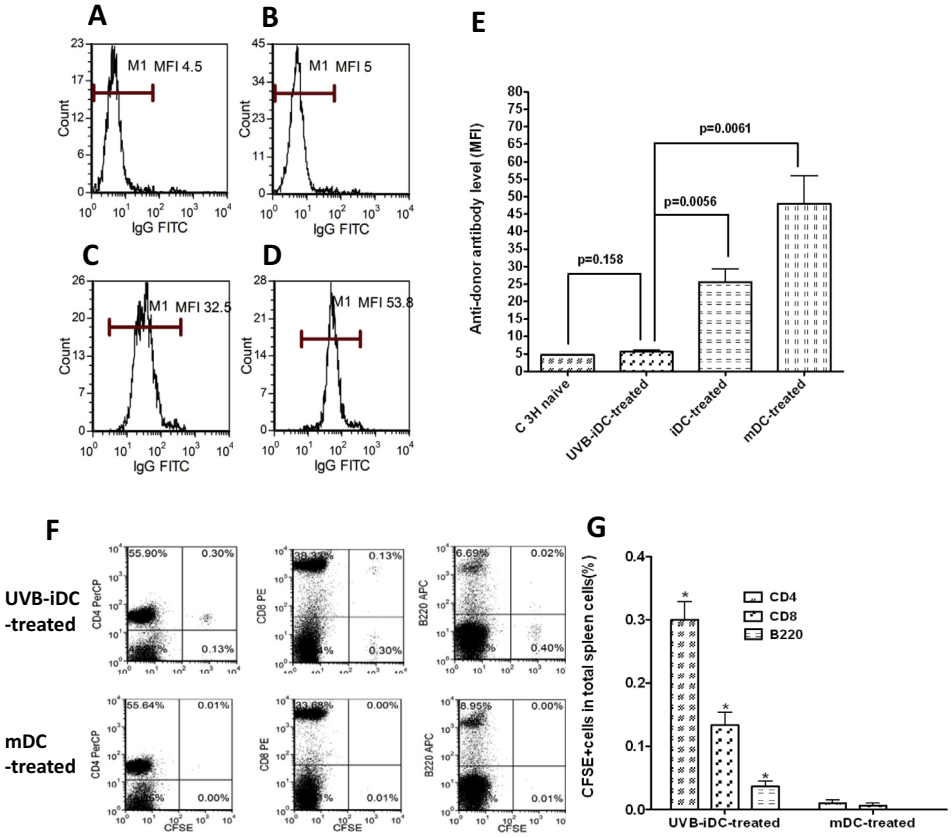

Fig. 1. Tolerance induction between two different strains of mice. C3H mice were treated with 4 weekly injections of Balb/c UVB-iDCs (B), iDCs (C), mDCs (D). The naïve C3H mice served as controls (A). Thereafter, the plasma levels of anti-Balb/c antibodies were measured by flow cytometry. (E) The summary of antibody levels in different groups was shown. (F) C3H mice were treated with 4 weekly injections of Balb/c UVB-iDCs or mDCs. Thereafter, Balb/c spleen cells (1×10^7) pre-labeled with CFSE were injected into the above pre-treated mice. Twenty-four hours later, the recipient mice were sacrificed and CFSE+CD4+, CD8+ and B220+ cells in the spleen were examined by flow cytometry. (G) The summary of CD4+, CD8+ and B220+ CFSE-labeled cells from two different groups was shown. The data shown were from a representative of three mice. $^*p < 0.0001$.

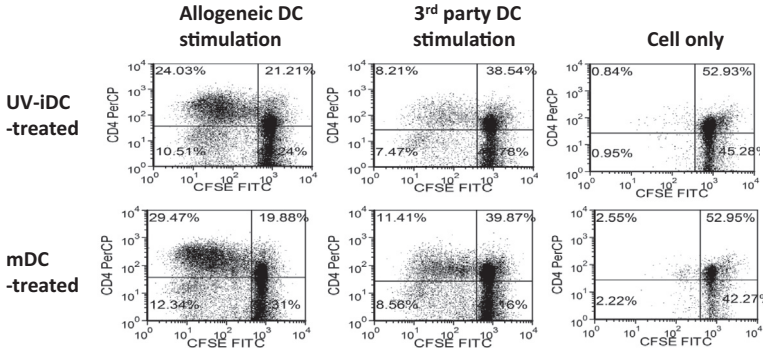

Fig. 2. Spleen cells from tolerant mice exhibit normal immune response to alloantigen stimulation. Spleen cells from the C3H mice pre-treated with Balb/c UVB-iDCs or mDCs as described elsewhere were stained with CSFE. CFSE-labeled spleen cells (1×10^6/well) were incubated with Balb/c mDCs or C57BL/6 mDCs (3rd party) or medium only for 4 days. Triplicated wells were used for the cultures. The cells were harvested from the cultures and stained for CD4, and analyzed by flow cytometry. Similar results were obtained in all 3 mice of each group.

most completely disappeared in the spleen (Fig. 1F and G) and lymph nodes (data not shown) after 24 h.

3.2. T cells from tolerant mice have similar proliferative capability in response to alloantigen stimulation but with more IL-10-producing T cells, and Foxp3+ T regulatory cells

To assess T cell tolerance, we stimulated CFSE-labeled spleen cells from the C3H mice receiving pretreatment of Balb/c UVB-iDCs and mDCs described above by Balb/c mDCs at the ratio of 20:1 for 5 days. Thereafter, the cells were harvested and stained with anti-CD4-PerCp. The T cell proliferation was assessed by analyzing the CFSE dilution using flow cytometry. Surprisingly, the T cell proliferation was comparable between the tolerant and immunized mice (Fig. 2). These findings seem to be conflict with the in vivo findings shown above (Fig. 1). However, when we further characterized the proliferating T cells by examining the IFN-γ- and IL-10-producing CD4+ T cells, we found that UVB-iDC treatment induced more IL-10-producing T cells than did mDC treatment (Fig. 3A). There was no significant difference in IFN-γ-producing T cells between

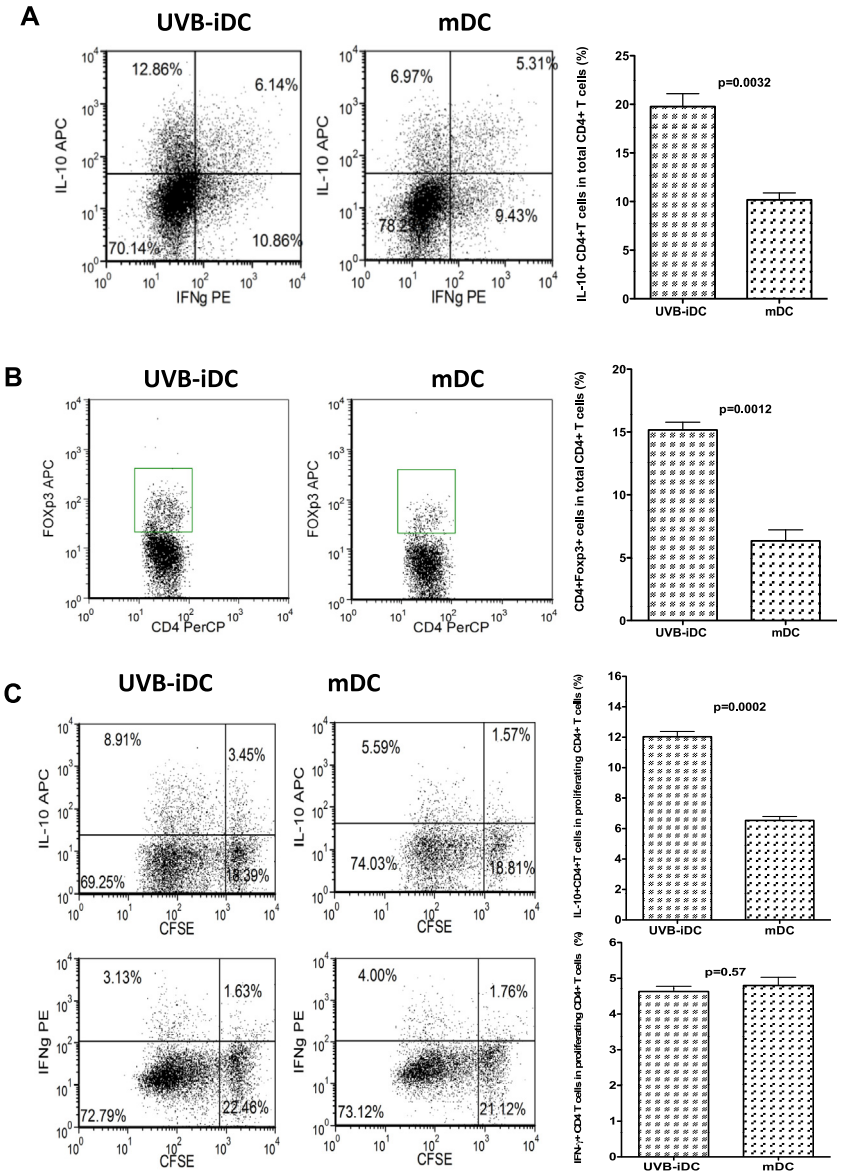

Fig. 3. Foxp3+ regulatory T cells and alloantigen-specific IL-10-producing Tr1 cells are induced in tolerant mice. (A) Spleen cells from the C3H mice pre-treated with intravenous injection of Balb/c UVB-iDCs or mDCs were stimulated with PMA and ionomycin and GolgiPlug (BD Bioscience) for 4 h. Then, the spleen cells were stained with CD4, IL-10 and IFN-γ with the fluorescent antibodies. Cytokine-producing CD4+ T cells were analyzed by gating on CD4+ T cells. The summary of cytokine-producing CD4+ T cells from 3 mice of each group were shown in left panel. (B) Spleen cells were stained for CD4 and Foxp3 fluorescent antibodies and examined by flow cytometry. CD4+Foxp3+ T cells were analyzed by gating on CD4 T cells. Results (left panel) shown were from 3 different mice. (C) C3H mouse spleen cells from different groups as indicated were stained with CFSE, and then stimulated with Balb/c mDCs in a ratio of 20:1 for 4 days. Then, the cells were harvested and stained for CD4, IL-10 and IFN-γ fluorescent antibodies according to the intracellular cytokine staining protocol (BD Bioscience), and IFN-γ- or IL-10-producing CD4+ T cells were analyzed by gating CD4+ T cells. The left panel shows the summary of IL-10- and IFN-γ-producing CD4+ T cells of proliferating cells from 3 different mice of each group. IFN-γ-producing CD4+ T cells are comparable between the two groups ($p > 0.05$).

two groups (Fig. 3A). In addition, we found that Foxp3+ Tregs were higher in UVB-iDC treated than in mDC-treated mice (Fig. 3B). Furthermore, when tested for the alloantigen-responding T cells, UVB-iDC-treatment induced significantly higher IL-10-producing CD4+ T cells than did mDC-treatment (Fig. 3C). Again, we did not observed differences in IFN-γ-producing alloantigen-responding CD4+ T cells (Fig. 3C).

3.3. Prolonged survival and reduced GVHD of F1 mice receiving Haplo-HSCT from tolerant parental C3H donors

To determine whether donor parental strain being tolerized to the alloantigen of another parent strain of mice confers more protection in F1 mice receiving Haplo-HSCT, we performed Haplo-HSCT in F1 mice using donor C3H mice pretreated with Balb/c UVB-iDC, iDC, mDC. Additional three control groups included syngeneic HSCT (F1–F1), F1 mice receiving Haplo-HSCT from naïve C3H donor mice, and F1 mice receiving γ-irradiation preconditioning only. All groups did not receive any immunosuppressive therapy post transplantation. As expected, syngeneic transplantation led to full protection of the recipient mice (Fig. 4D), and F1 mice with γ-irradiation preconditioning alone all died within 8 days post irradiation (data not shown), suggesting HSCT process was successful, and the dose of irradiation was sufficient, respectively. In the group using mDC-treated mice as the donors, the recipient mice developed severe acute GVHD with some mice presenting severe diarrhea. Recipient mice receiving iDC-treated donors showed clinical presentations similar to mDC

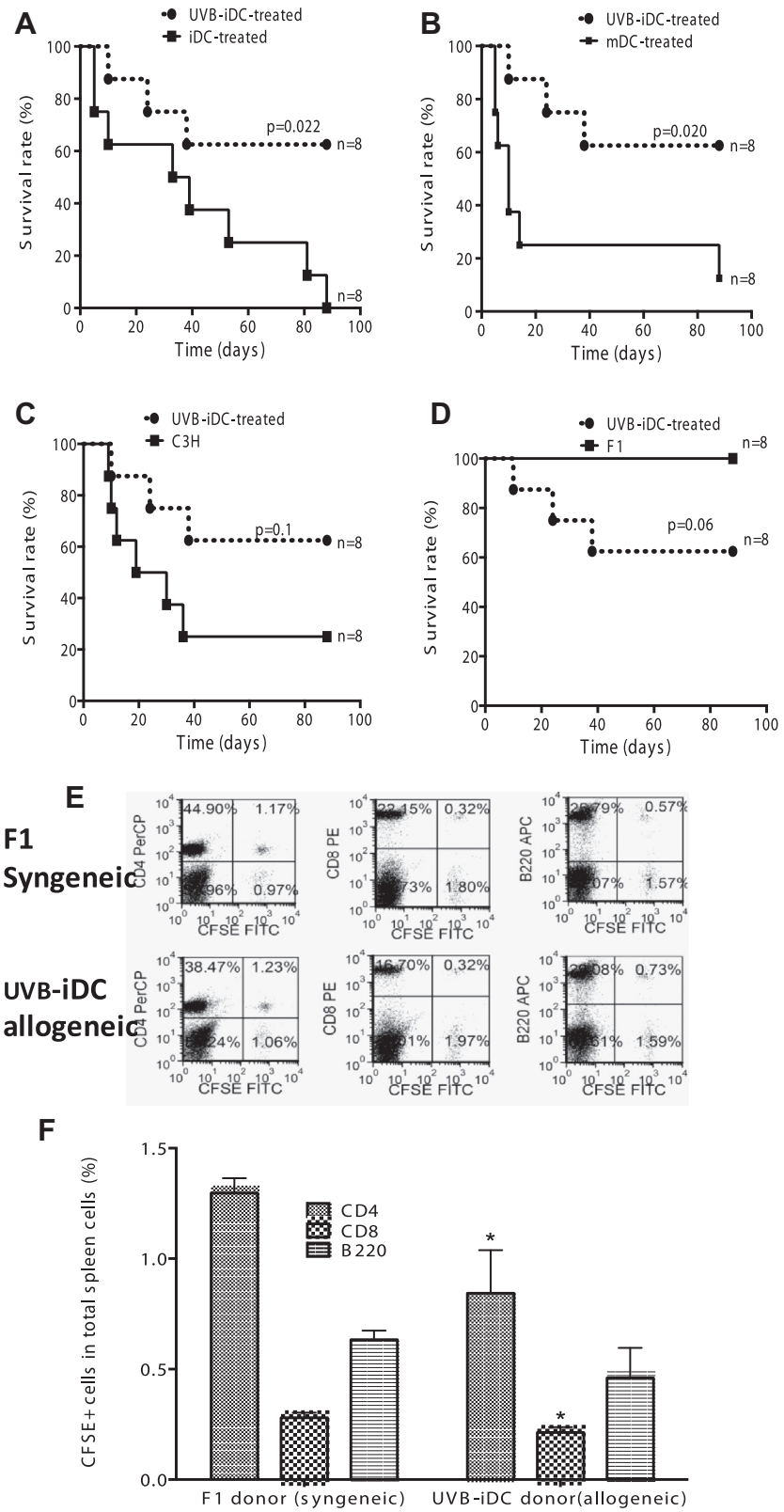

Fig. 4. Pre-induction of alloantigen-specific tolerance in donor parental strains significantly prolongs the survival of F1 recipients in Haplo-HSCT. The donor C3H mice were left alone or treated 4 weekly intravenous injections of Balb/c UVB-iDCs, iDCs, mDCs. Syngeneic HSCT was performed using F1 as donor. Recipient F1 mice were irradiated by 1100 cGy, and then received donor's bone marrow cells (1×10^7/mouse) plus spleen cells (2×10^7/mouse). The recipient mice were monitored every day in the first two weeks and then twice a week until the end of experiment. The comparison of survival rate between UVB-iDC group and each of other groups was analyzed and shown in A, B, C, and D, respectively. (E) Three mice from the UVB-iDC group and 5 mice from the F1 syngeneic mice received injection of CFSE-labeled F1 spleen cells (1×10^7 cells/mouse) to trace their rejection from recipients. Twenty-four hours later, CFSE+ cells in the spleens were examined by flow cytometry after staining with CD4, CD8 and B220 fluorescent antibodies. Flow cytometric data from one representative mouse in each group were shown. (F) The summary of each cell subset from all mice was illustrated. The comparison between two groups was performed for CD4+, CD8+, B220+ CFSE+ cells, respectively. $^*p < 0.05$.

group, and by 90 days post HSCT, 0/8 and 1/8 of recipient mice in these two groups were alive, respectively (Fig. 4A and B). Strikingly, in UVB-iDC group, the survival was significantly improved. By 90 days post HSCT, 5/8 mice were alive (Fig. 4) without using any immunosuppressive therapy. Although the difference was not significant, the survival rate of UVB-iDC group was much higher than the group with naïve C3H mice being donors (Fig. 4C).

3.4. Alloantigen tolerance is maintained in the F1 recipients after hematopoietic reconstitution

To determine whether recipients with tolerant donors maintain tolerance to the F1 alloantigen long time after engraftment. We injected CFSE-labeled F1 spleen cells into the recipient mice (F1–F1 group), or Balb/c UVB-iDC group in the above Haplo-HSCT experiment (Fig. 4A–D). Twenty-four hours later, we sacrificed the mice, and CFSE+ cells in the spleens were examined by flow cytometry. As shown in Fig. 4E and F, F1 recipients reconstituted with C3H donors pre-tolerized by Balb/c UVB-iDC treatment remained tolerant to F1 alloantigen three months post transplant, despite the levels of CFSE-labeled CD4+, CD8+ and B220+ cells in UVB-iDC group were lower than their counterparts in syngeneic group (F1–F1). It appeared that the UVB-iDC group recipients developed new tolerance to B cells (Fig. 4E, lower panel), which was not observed before transplant in the tolerant donors (Fig. 1F).

4. Discussion

Haplo-HSCT is an effective therapy for the patients who need allogeneic HSCT but do not have HLA-matched donors, and makes almost all patients needing allogeneic HSCT able to find their donors [7,18,19]. However, because of the HLA-mismatch between the donor and recipient, the incidence and severity of GVHD in Haplo-HSCT tend to be higher than HLA-matched allogeneic HSCT [8,9]. To minimize the severity of GVHD, the T cell depletion as well as intensive immunosuppressive therapy is usually employed during Haplo-HSCT, which, however, renders the patients susceptible to secondary infections and tumors, or the relapse of primary tumors [8,9]. Thus, it is imperative to discover effective ways to prevent GVHD in Haplo-HSCT without causing severe side effects. Tolerance induction is a promising approach and hopeful for solving this issue in Haplo-HSCT. Given the fact that in Hapo-HSCT between parental donor and child recipient, half the HLA antigens (from another parent) are incompatible, we put forward an idea that if the donor parent were induced to be tolerant to the MHC antigens of another parent, Haplo-HSCT would potentially become "syngeneic" HSCT. In the current study, we executed a proof-of-concept animal study to test the feasibility of this idea. Firstly, we successfully induced allogeneic immune tolerance in C3H mice (H-2k) by intravenous injection of UVB-iDCs generated in vitro from bone marrow of Balb/c mice (H-2d). As we reported previously [17], the anti-donor antibodies could be considered the antibodies developed against donor MHC antigens. The higher antibody levels indicate the stronger allogeneic reaction. Mice receiving UVB-iDC treatment failed to develop any levels of antibodies against Balb/c antigens (Fig. 1A and B). In our previous report, we demonstrated that those tolerant mice were highly tolerated to the alloantigen re-challenge [17]. We also confirmed the tolerance by injection of CFSE-labeled Balb/c spleen cells to the tolerant, or immunized C3H mice (Fig. 1F). The results demonstrated that injected CFSE labeled Balb/c spleen cells remained in the lymphoid tissues including spleen (Fig. 1F) and lymph nodes (data not shown) of tolerant mice but disappeared in the immunized mice 24 h after injection, suggesting that C3H (H-2k) mice receiving Balb/c UVB-iDC treatment has formed immune tolerance to Balb/c antigens, likely MHC antigens (H-2d). Of interest, the tolerance to the alloantigens on CD4 and CD8 T cells appears more potent than that to the alloantigens on B cells, because CFSE-labeled B cells remain in the recipients at significantly lower levels (Fig. 1F). This finding suggests that injection of UVB-iDCs may induce more complete tolerance for MHC-I than MHC-II antigens, because T cell only expresses MHC-I, whereas B cell expresses not only MHC-I but also MHC-II antigens. It is unlikely that B cells may be homing to the lymphoid organs more slowly than T cells because the adoptive transfer of syngeneic spleen cells allows B cells to migrate into lymphoid organs similar to T cells (Fig. 4E, upper panel).

In our Haplo-HSCT model, we injected donor bone morrow cells along with spleen cells to the F1 recipients. The concomitantly injected splenic T cells are the major immune cells to cause acute GVHD. The severity of GVHD is determined by the functional state of injected T cells specific to the alloantigens of the host. As described above, donors receiving UVB-iDC treatment developed alloantigen tolerance (Fig. 1). Consistent with this, our Haplo-HSCT results demonstrate that the survival of F1 recipients receiving C3H donors pre-treated with Balb/c UVB-iDCs is significantly prolonged, showing that by 90 days post HSCT, 5 out of 8 recipients survived without receiving any immunosuppressive therapy (Fig. 4). The above findings further support that pre-induction of alloantigen tolerance between two parent strains is a feasible way to mitigate GVHD in Haplo-HSCT to F1. The reduction of GVHD as well as the protection of recipients is likely due to the increased levels of alloantigen-specific regulatory T cells including IL-10-producing Tr1 cells and Foxp3+ regulatory T cells induced in the donors pre-treated by UVB-iDCs (Fig. 3).

As described above, pre-induction of alloantigen tolerance by injection of allogeneic UVB-iDC for the donors markedly attenuates GVHD. We also found that at 90 days post-HSCT, the blood lineages of recipients were all from the donors' origin (data not depicted). Further studies showed that the tolerance was maintained even 90 days post Haplo-HSCT (Fig. 4E and F). This long-lasting effect against GVHD may be associated with the expansion of alloantigen-specific regulatory T cells in the F1 recipients, as we show IL-10-producing CD4+ T cells can be expanded in vitro when spleen cells from the tolerant mice are stimulated by allogeneic mDCs (Fig. 2). Nevertheless, GVHD is not completely abrogated in the UVB-iDC group. This could be associated with incomplete tolerance induced by UVB-iDC treatment, as the rejection was different for different subsets of lymphocytes of injected allogeneic spleen cells (Fig. 1F). These findings suggest that the tolerance may need to be further strengthened post Haplo-HSCT. Because B cells share MHC-I with T cells, but additionally express MHC-II, the above findings suggest that UVB-iDCs treatment may be insufficient to induce tolerance to allogeneic MHC-II antigens. To further improve the effect of pre-induction of alloantigen tolerance on preventing GVHD, further modification is needed for the current approach probably with a focus on induction of tolerance to allogeneic MHC-II antigens.

It is worth noting that we have not tested whether reduction of GVHD by inducing alloantigen tolerance will lead to attenuation of graft-versus-tumor effect. Further studies are needed to address this issue as well.

This study provides a plausible approach to prevent GVHD through inducing alloantigen-specific tolerance between both parents for Haplo-HSCT to children. Given that expansion of alloantigen-specific CD4+CD25+ Foxp3+ regulatory T cells has been proposed for GVHD in allogeneic HSCT and shown the great potential for future clinical application [20–22], the strategy presented in the current study could also be potentially employed for post-HSCT cellular immunotherapy, for example, in vitro expansion of alloantigen-specific regulatory T cells between the two parents for prevention of GVHD in Haplo-HSCT post transplant. Allogeneic HSCT is prevalently exploited in hematopoietic malignancies. As demonstrated in the current study, Haplo-HSCT provides a perfect

clinical setting for pre-induction of alloantigen tolerance between the two parents as well as for post-HSCT cellular immunotherapy against GVHD.

Conflict of interest

The authors declare that there is no conflict of interest.

Authors' contribution

C.Q.X. designed the study and wrote the manuscript; Y.G. performed the most experiments. Zhang L. and Wu Y. performed part work in animal observation, and spleen and bone marrow cell preparation; Sun X. performed flow cytometric assays; Wan S. assisted flow cytometry data analysis. Yu X.Z. participated in the design and discussions for this study.

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