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# Ex Vivo Expanded Hematopoietic Stem Cells Overcome the MHC Barrier in Allogeneic Transplantation

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## SUMMARY

**The lack of understanding of the interplay between hematopoietic stem cells (HSCs) and the immune system has severely hampered the stem cell research and practice of transplantation. Major problems for allogeneic transplantation include low levels of donor engraftment and high risks of graft-versus-host disease (GVHD). Transplantation of purified allogeneic HSCs diminishes the risk of GVHD but results in decreased engraftment. Here we show that ex vivo expanded mouse HSCs efficiently overcame the major histocompatibility complex barrier and repopulated allogeneic-recipient mice. An 8-day expansion culture led to a 40-fold increase of the allograft ability of HSCs. Both increased numbers of HSCs and culture-induced elevation of expression of the immune inhibitor CD274 (B7-H1 or PD-L1) on the surface of HSCs contributed to the enhancement. Our study indicates the great potential of utilizing ex vivo expanded HSCs for allogeneic transplantation and suggests that the immune privilege of HSCs can be modulated.**

## INTRODUCTION

Hematopoietic stem cells (HSCs) have been used in transplantation to treat patients with leukemia, lymphoma, some solid cancers, and autoimmune diseases (Bryder et al., 2006). In particular, allogeneic bone marrow (BM) transplantation is potentially curative for both inherited and acquired hematopoietic diseases (Gyurkocza et al., 2010). Two major problems, failure of engraftment and graft-versus-host disease (GVHD), have severely limited the progress in the field, however. Although the inclusion of donor T cells in transplantation enhances donor engraftment and has graft-versus-leukemia effects, it causes life-threatening GVHD. Transplantation of purified allogeneic HSCs diminishes the risk of GVHD but also results in decreased engraftment (Shizuru et al., 1996; Wang et al., 1997). It is not clear why most allogeneic HSCs cannot escape immune rejection and whether the allograft efficiency of HSCs can be improved. The resolution of these questions will promote the understanding of the immunology of HSCs and other stem cells and greatly improve the practice of allogeneic transplantation.

We recently developed an efficient culture system for ex vivo expansion of HSCs (Zhang and Lodish, 2008). This system is based on the use of serum-free culture medium supplemented with several growth factors including SCF, TPO, FGF-1/Flt3-L, IGFBP2, and angiopoietin-like proteins (Angptls) (Huynh et al., 2008; Zhang et al., 2006, 2008). In vivo studies suggested that Angptls are new molecular components of the microenvironment of fetal liver and adult HSCs (Chou and Lodish, 2010; Zheng et al., 2011), and Angptl1 and 2 are essential to HSC development in zebrafish (Lin and Zon, 2008, ASH 50th Annual Meeting, abstract). We and others have used this culture system to expand mouse and human HSCs for transplantation or genetic modification purposes (Akala et al., 2008; Carter et al., 2010; Chen et al., 2009; Drake et al., 2011; Heckl et al., 2011; Huynh et al., 2008; Khoury et al., 2011; Kiel et al., 2007; Stern et al., 2008; Zhang et al., 2006, 2008; Zhao et al., 2010). There are two important features of this HSC culture system: the increased number of repopulating HSCs (Huynh et al., 2008; Zhang et al., 2006, 2008) and the change of surface expression of many surface proteins (Zhang and Lodish, 2005). While the expansion of repopulating HSCs were validated by transplanting cultured HSCs into congeneic or immune-deficient mice in these previous studies, we hypothesized that ex vivo expansion of HSCs may also modulate the immunological properties of HSCs so that they possess an altered ability to cross the immune barrier upon allogeneic transplantation. To test this hypothesis, we started to compare the allograft abilities of freshly isolated HSCs and ex vivo expanded HSCs in allogeneic transplantation models.

## RESULTS

### Ex Vivo Expanded HSCs Have Dramatically Enhanced Allograft Ability

With a well-established mouse model for fully allogeneic transplantation (see Figure S1 available online), we compared the allograft abilities of freshly isolated and ex vivo expanded HSCs from CD45.1 C57BL/6 donors transplanted into lethally irradiated

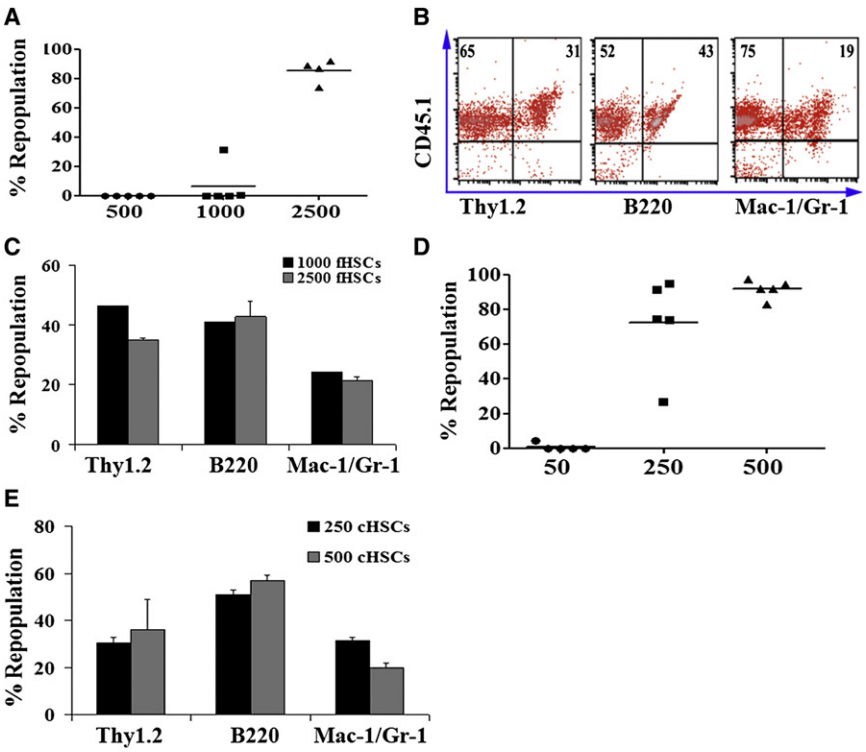


**Figure 1. Ex Vivo Expanded HSCs Overcome MHC Barrier in Noncompetitive Allogeneic Transplantation**

Indicated numbers of freshly isolated Lin$^-$Sca-1$^+$Kit$^+$CD34$^-$Flk2$^-$ HSCs (A–C) or their 8 day cultured progenies (D and E) from CD45.1 C57BL/6 donors were transplanted into lethally irradiated BALB/c (CD45.2) recipients without competitors (n = 4–5). HSCs were cultured in STFIA medium, which allows ex vivo expansion of HSCs (Zhang et al., 2006), and there were ~200-fold increase of total cells after 8 days of culture (with 1.16 ± 0.14 × $10^4$ cultured cells derived from the input 50 cells).

(A and D) Numbers of mice with failed or successful donor engraftment after being transplanted by indicated numbers of freshly isolated HSCs or their cultured equivalents at 16 weeks posttransplant. The 0% repopulated mice included both survived and dead ones.

(B) Representative flow cytometry analysis of the multilineage repopulation of 2,500 freshly isolated HSCs at 16 weeks posttransplant.

(C and E) Multilineage contribution of indicated numbers of freshly isolated HSCs or cultured equivalent at 16 weeks posttransplant, respectively (n = 4–5).

Data are expressed as mean ± SEM. See also Figure S1.

BALB/c (CD45.2) recipients. The culture was performed in our optimized STFIA medium (Huynh et al., 2008; Zhang et al., 2006) for 8 days that allows ex vivo expansion of HSCs. Consistent with previously reported results (Shizuru et al., 1996; Wang et al., 1997), a relatively large number (1,000 or more) of freshly isolated BM Lin$^-$Sca-1$^+$Kit$^+$CD34$^-$Flk2$^-$ HSCs were needed for successful allograft (Figures 1A–1C). By striking contrast, the cultured progeny of 50 or more input equivalent HSCs were capable of achieving the same level of allograft (Figures 1D and 1E). Similar to freshly isolated HSCs, cultured HSCs were capable of multilineage differentiation in allogeneic mice (Figures 1B, 1C, and 1E) and no sign of GVHD was observed. This suggests that ex vivo expanded HSCs have enhanced allograft abilities compared with freshly isolated cells.

The above strategy may result in the death of mice when donor HSCs are not capable of engrafting recipients. To ensure that recipient mice survive after transplantation and to better quantitate the allograft abilities of different donor cells, we performed allogeneic transplantation by including competitors (Figure S1). These competitors are total BM cells freshly isolated from the same type of mice as the recipients; these cells provide short-term radio-protection and serve as internal controls but also significantly enhance the host immune rejection and increase the difficulty of donor engraftment. Figure 2 shows the result of a representative competitive allogeneic transplantation from donor C57BL/6 (CD45.1) to BALB/c (CD45.2) recipients. Although 10,000 freshly isolated CD45.1 C57BL/6 BM Lin$^-$Sca-1$^+$Kit$^+$CD34$^-$Flk2$^-$ HSCs failed to engraft into the BALB/c recipients in the presence of competitors (0%; Figure 2A, left), their cultured progenies had dramatically increased engraftment (55%; Figure 2A, right). Similar results were obtained from the measurement of major histocompatibility complex (MHC) markers of donors and recipients (Figure 2B). This allogeneic reconstitution sustained over time (Figure 2C) and the donor cells repopulated the lymphoid and myeloid lineages (Figures 2D and 2E), attesting to the engraftment of the donor long-term HSCs. Again, no sign of GVHD was observed in the transplanted mice. To test whether allogenic donor HSCs were tolerated in the host, we performed secondary transplantation by isolating BM cells from the primary recipients and transplanting them into secondary BALB/c recipients. We found that the original CD45.1 donor cells successfully repopulated secondary recipients (Figures 2F and 2G). The successful secondary transplantation indicates that the allogeneic donor HSCs were already tolerated after the primary transplantation. This result was further confirmed by the mixed lymphocyte reaction (MLR) experiment, showing that BALB/c T cells were not stimulated by the original donor-derived cells in primary transplanted mice, but reacted to the counterpart cells isolated from CD45.1 C57BL/6 mice (Figure 2H). Therefore, the competitive allogeneic transplantation (Figure 2) gave similar results as the noncompetitive allograft (Figure 1).

We further employed a third transplantation model to compare the abilities of donor HSCs before and after ex vivo expansion to engraft the allogeneic recipient mice with sublethal irradiation. Again the ex vivo expanded HSCs achieved markedly increased allograft compared to their uncultured counterparts (Figure S2A). All these results indicate a dramatic enhancement of allograft ability of HSCs after ex vivo expansion.

Moreover, to directly compare the allograft capacities of HSCs before and after ex vivo expansion, we cotransplanted freshly isolated CD45.2 C57BL/6 HSCs and ex vivo expanded progenies of CD45.1 C57BL/6 HSCs into the same BALB/c recipient

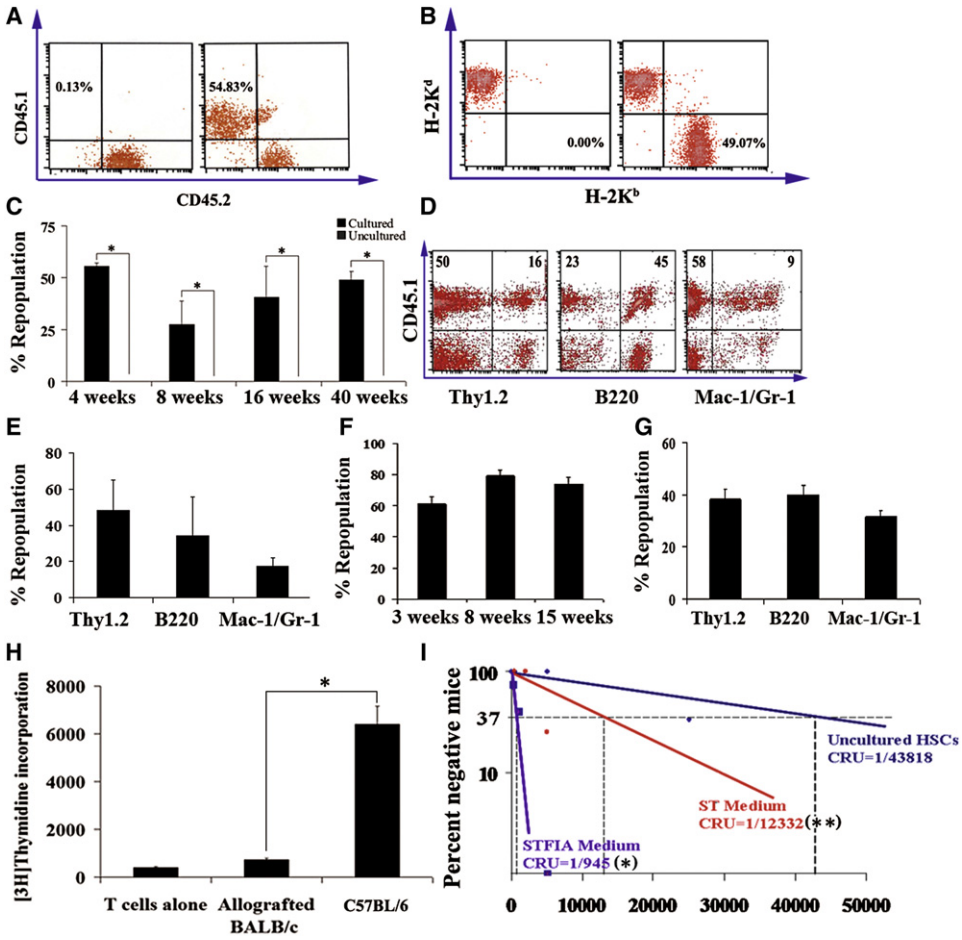


**Figure 2. Ex Vivo Expanded HSCs Overcome MHC Barrier in Competitive Allogeneic Transplantation**

(A–E) Freshly isolated 10,000 Lin$^-$Sca-1$^+$Kit$^+$CD34$^-$Flk2$^-$ HSCs or their 8 day cultured progenies from CD45.1 C57BL/6 donors were transplanted into lethally irradiated BALB/c (CD45.2) recipients along with 100,000 total bone marrow cells freshly isolated from BALB/c mice as competitors. HSCs were cultured in STFIA medium, which allows ex vivo expansion of HSCs. Similar results were obtained in at least two independently repeated experiments.

(A and B) Representative flow cytometry plots show that 10,000 freshly isolated donor HSCs had no engraftment (left), whereas the cultured progeny of 10,000 input donor HSCs (right) had significant engraftment (54.83% CD45.1 or 49.07% H-2K$^b$ donor chimerism in A or B, respectively) in allogeneic recipients.

(C) Summary of donor engraftment in allogeneic recipients at 4, 8, 16, and 40 weeks posttransplant (*$p < 0.05$, n = 6).

(D and E) Multilineage contribution of cultured cells in allogeneic recipients at 16 weeks posttransplant (n = 6).

(F and G) Summary of donor engraftment at 3, 8, and 15 weeks after secondary transplantation into BALB/c mice (n = 5). Multilineage contribution of cultured cells in allogeneic recipients at 15 weeks posttransplant is shown (G).

(H) MLR assay was performed in which splenocytes from C57BL/6 mice stimulated the proliferation of BALB/c T cells (bar 3) whereas splenocytes isolated from cultured C57BL/6 donor HSCs repopulated BALB/c recipients lost the ability to stimulate the proliferation of BALB/c T cells (bar 2). *$p < 0.05$, n = 3.

(I) Comparison of the allograft abilities of freshly isolated and cultured HSCs by limiting dilution analysis. Three types of donor cells, including freshly isolated C57BL/6 CD45.1 Lin$^-$Sca-1$^+$Kit$^+$CD34$^-$Flk2$^-$, progenies after 8-day culture in ST medium (which does not support HSC expansion), and progenies after 8-day culture in STFIA medium (which supports HSC expansion), were compared. Transplantation into lethally irradiated BALB/c mice was conducted along with 100,000 total BM competitors isolated from BALB/c mice. Limiting dilution analysis was performed and L-Calc software was used to calculate the HSC frequency (*, **$p < 0.05$, compared to uncultured HSCs).

Data are expressed as mean ± SEM. See also Figures S1 and S2 and Table S1.

mice (Figure S2B). Ex vivo expanded HSCs demonstrated a clear advantage over freshly isolated HSCs in this direct competitive allograft setting (Figure S2B).

This ability of cultured HSCs to overcome the allogeneic barrier was not restricted to the use of particular allogeneic transplantation models. In addition to using C57BL/6 mice and BALB/c mice as the donor and recipient, respectively, we tested a number of other donor/recipient combinations and reached the same conclusion. For example, ex vivo expanded HSCs isolated from FVB (CD45.1) mice had much greater ability to repopulate CD45.2 C57BL/6 recipients than their freshly isolated counterparts (Figure S2C).

## Both the Increase of HSC Numbers and Expansion-Independent Characteristics Acquired during Ex Vivo Culture Contribute to the Improved Allograft Efficiency

Because we used a culture system that expands HSCs, we sought to determine the contribution of the increase of HSC

numbers during ex vivo expansion to the increased allograft ability by conducting limiting dilution analyses (Huynh et al., 2008; Zhang et al., 2006; Zheng et al., 2011). First, we used competitive syngeneic transplantation to calculate the numbers of repopulating CD45.1 C57BL/6 BM HSCs before and after ex vivo expansion. When we cultured HSCs in the optimized STFIA medium for 8 days, we obtained 11-fold (= 69/6; Table S1A, Figure S2D) expansion in the number of HSCs as determined by limiting dilution analysis in syngeneic transplantation. Next, we quantitated the allograft abilities of these HSCs before and after culture by competitive allogeneic transplantation into BALB/c mice. For freshly isolated donor HSCs, the frequency of allograftable cells was 1/43,818, whereas the frequency in those cells cultured in the STFIA medium was 1/945 of input equivalent cells, determined by the competitive allogeneic transplantation (Figure 2I; Tables S1B and S1C). This represents a ~40-fold (= 43,818/945) increase of allograft ability when cells were ex vivo expanded. Hence, in this experiment, ex vivo expansion led to 11-fold increase of HSC numbers and 40-fold increase of allograft ability. This result is concordant with previous reports that an increased number of HSCs enhances reconstitution of the hematopoietic compartment across the MHC barrier (Shizuru et al., 1996; Wang et al., 1997). Nevertheless, since the ex vivo expansion of HSCs had 40-fold increase of allograft ability and the net increase of HSC number was 11-fold, another ~4-fold increase (= 40/11) should be contributed by culture independent of expansion of HSCs.

To further determine whether culture enhances allograft ability independent of expansion, we cultured HSCs in conditions that do not support HSC expansion and used these cells for transplantation. To this end, we cultured HSCs in serum-free medium supplemented with only SCF and TPO (as ST medium; Figure 2I; Tables S1A–S1C, Figure S2D), based on previous results (Huynh et al., 2008; Zhang et al., 2006; Zhang and Lodish, 2004, 2005) and our syngeneic transplantation (Table S1A), this condition does not support HSC expansion. We determined that the allograft frequency for these cultured but unexpanded HSCs was 1/12,332 input equivalent cells (Figure 2I). This represented a ~4-fold increase (= 43,818/12,332) of allograft ability compared to freshly isolated HSCs. This number is in perfect agreement with the above estimate of a ~4-fold of increase of allograft ability by expansion-independent mechanism(s) based on comparison of results in syngeneic transplantation and allogeneic transplantation. Therefore, increase of allograft ability of HSCs does not necessarily need HSC expansion. In summary, our results indicate that both the increase of HSC numbers and expansion-independent characteristics acquired during ex vivo culture contribute to the improved allograft efficiency.

## Accessory Cells Produced during Culture Does Not Contribute to the Enhanced Allograft Ability of HSCs

To identify the expansion-independent mechanism for cultured HSCs to cross the MHC barrier, we explored two possibilities: the presence of certain accessory hematopoietic or mesenchymal cells and a change of HSC immunogenicity during culture. To test the first possibility, we examined whether facilitating cells (Gandy et al., 1999; Kaufman et al., 1994), regulatory T cells (Taylor et al., 2008), or other cells produced during culture supported allograft. It has been established that unique differentiated BM populations as facilitating cells improve allogeneic reconstitution and result in donor-specific transplantation tolerance across MHC disparities (Gandy et al., 1999; Kaufman et al., 1994). The reported facilitating cells express conventional T cell components such as CD8 but are not T cells because they do not express TCR (Kaufman et al., 1994; Bridenbaugh et al., 2008). Interestingly, facilitating cells induce an increase in numbers of donor regulatory T cells (Treg) (Taylor et al., 2008), which directly facilitate allograft. Although freshly isolated HSCs do not contain $CD3^+$ cells (Figure S3A), after HSCs were cultured for 8 days in STFIA medium, approximately 0.3% of cells possessed the surface phenotype of $CD8^+CD45R^+TCR^-$ (Figure S3B), the same phenotype as the previously characterized facilitating cells (Kaufman et al., 1994). To test whether the phenotypic "facilitating cells" produced in culture supported allograft, we collected these culture-produced $CD8^+CD45R^+TCR^-$ cells by FACS and cotransplanted them with freshly isolated HSCs (1:1 as reported) (Kaufman et al., 1994) for allogeneic transplantation. We did not observe improved transplantation efficiency by including these cultured phenotypic facilitating cells, suggesting that they were not functional facilitating cells. In parallel, we were unable to detect phenotypic Treg ($FoxP3^+CD4^+CD25^+$) cells in the cultures we examined, suggesting the increased allograft of cultured cells was probably not contributed by production of Treg cells. To further test whether differentiated hematopoietic cells affected allograft, we isolated $Lin^+$ cells from the HSC culture and cotransplanted them with freshly isolated HSCs. These $Lin^+$ cells did not alter allogeneic transplantation efficiency (Figure S3C). In addition, there were no apparent adherent cells during our 8-day culture, and a CFU-F assay showed that no meshenchymal stem cells were produced from the cultured HSCs (Figure S3D). These results indicate that there is no engraftment-enhancing effect from mesenchymal stem cells. Taken together, we concluded that the accessory cells produced during the culture did not significantly contribute to increased allograft ability of ex vivo expanded HSCs.

## Upregulation of CD274 during Culture Supports HSC Allograft

Next we tested the possibility that the immunogenicity of HSCs changes during culture by examining the expression of surface immune proteins, including MHC-I, MHC-II, CD274 (B7-H1 or PD-L1), CD275 (B7-H2), CD47, CD80, and CD86. The expression of these surface proteins on freshly isolated and cultured cells, as determined by flow cytometry, are summarized in Figures 3A and 3B. Almost all the freshly isolated HSCs and cultured cells expressed MHC-I and CD47, whereas very few of either population expressed MHC-II, CD275, CD80, or CD86. By contrast, there was a significant increase of surface expression of CD274 upon culture, as evidenced by an increase of $CD274^+$ cells from 61% to 88% (Figure 3A). Importantly, cultured cells contained a new population with more than 10-fold increase of CD274 expression (Figures 3C and 3D, and fold increase of CD274 staining intensity = 10,270/752 in Figure 3C). There was a greater portion of CD274-positive cells in the phenotypic cultured HSCs as $Lin^-Sca\text{-}1^+Kit^+CD48^-$ cells (Noda et al., 2008) than in differentiated cultured cells (Figure 3E), although the expression intensities of CD274 were similar in all fractions of cultured cells (Figure 3F).

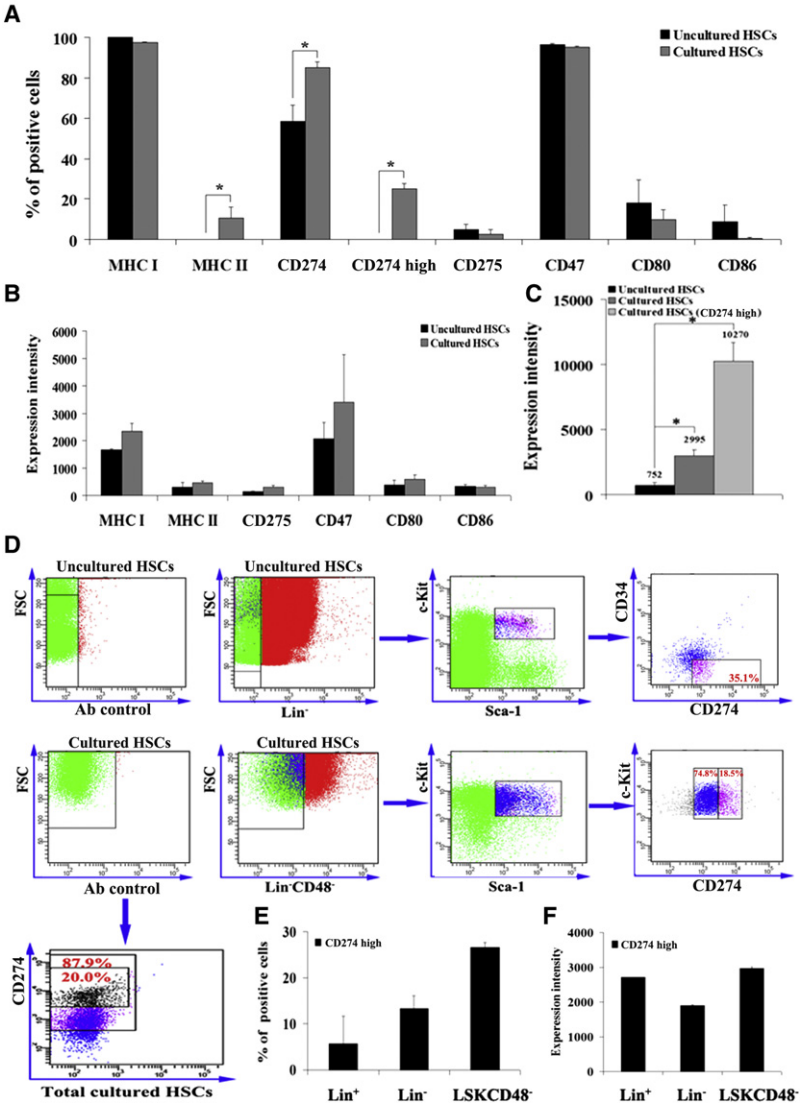


**Figure 3. Altered Expression of Certain Surface Immune Molecules on Phenotypic HSCs during Culture**

(A and B) A summary of the result of flow cytometry analysis of surface expression of indicated immune molecules after 8 days of culture of HSCs in STFIA medium (*$p < 0.05$, $n = 3–5$).

(C and D) CD274 surface expression was increased in cultured cells. In (C), MFI of CD274 expression determined by flow cytometry analysis in freshly isolated and cultured HSCs are shown (*$p < 0.05$, $n = 3–5$). Shown in (D) are representative flow cytometry plots indicating 43.5% freshly isolated HSCs were CD274 positive (as $CD274^{low}$), whereas 69% of cultured cells were $CD274^{low}$ and 20.0% as $CD274^{high}$. Gatings were set based on isotype controls.

(E and F) Percentages of $CD274^{high}$ cells and MFI of CD274 expression in different fractions of cultured HSCs. Data are expressed as mean ± SEM. See also Figure S3.

B7 immune proteins belong to the immunoglobulin (Ig) superfamily, with two Ig-like extracellular domains and short cytoplasmic domains. CD274 is a member of the B7 family that is expressed or induced on dendritic cells or non-antigen-presenting cells and inhibits T cell or innate activation (Francisco et al., 2010; Zou and Chen, 2008). While Figure 3D shows that CD274 might be upregulated on cultured HSCs based on phenotypic analysis, because the exact surface phenotype of cultured HSCs is not defined (Zhang and Lodish, 2005), we used the "gold standard" BM reconstitution analysis to test whether CD274 was expressed on functional HSCs and whether its level was altered upon culture. We first sorted freshly isolated BM cells into fractions negative and positive for immunostaining with antibodies against CD274. The repopulation activities of these fractions were then analyzed in the competitive syngeneic transplantation model. All the repopulating activity was within the CD274-positive fraction (Figures 4A–4C), indicating that all freshly isolated HSCs express CD274 on their surface. Since CD274 level was elevated more than 10-fold on some cultured cells (Figures 3C and 3D), we sought to determine whether surface expression of CD274 was increased on functional repopulating HSCs after culture. To this end, we fractioned the low positive and high positive cultured cells (as $CD274^{low}$ and $CD274^{high}$, respectively) followed by competitive syngeneic transplantation. The repopulating activity was found in both $CD274^{low}$ and $CD274^{high}$ fractions (Figures 4D and 4E; Figure S4). This reveals that indeed a fraction of HSCs increased their surface expression of CD274 more than 10-fold under our culture conditions. Interestingly, different culture conditions did not change the CD274 upregulation (Figure 4F), suggesting that the increase of CD274 expression was induced by general proliferation signals in culture and was independent of HSC expansion.

To determine the role of CD274 in transplantation of HSCs, we utilized mice that are deficient in CD274 (Dong et al., 2004). We showed that CD274-null mice had higher frequency of phenotypic HSCs than wild-type (WT) mice (Figure S5A), and the same number of freshly isolated CD274-null HSCs or ex vivo expanded null HSCs had slightly higher or similar long-term repopulation as WT HSCs in competitive syngeneic transplantation (Figures 5A–5D). These results suggest that CD274 per se does not significantly support the HSC activity in homeostatic and cultured conditions, concordant with the general normal phenotype of the CD274-null mice in homeostasis (Zou and Chen, 2008). By contrast, cultured CD274-null HSCs showed significantly decreased long-term repopulation in the competitive allogeneic repopulation compared to WT HSCs at 16 weeks posttransplant (Figures 5E and 5F). The deficiency of B7-H4, another B7 family immune inhibitor, did not decrease allograft efficiency at 8–16 weeks posttransplant compared to WT HSCs (Figures 5E and 5F). To further confirm that the surface CD274 on cultured HSCs facilitates allograft, we performed noncompetitive allogeneic transplantation and compared the allograft of 1,000 input equivalent WT HSCs, anti-CD274 neutralizing antibody-treated

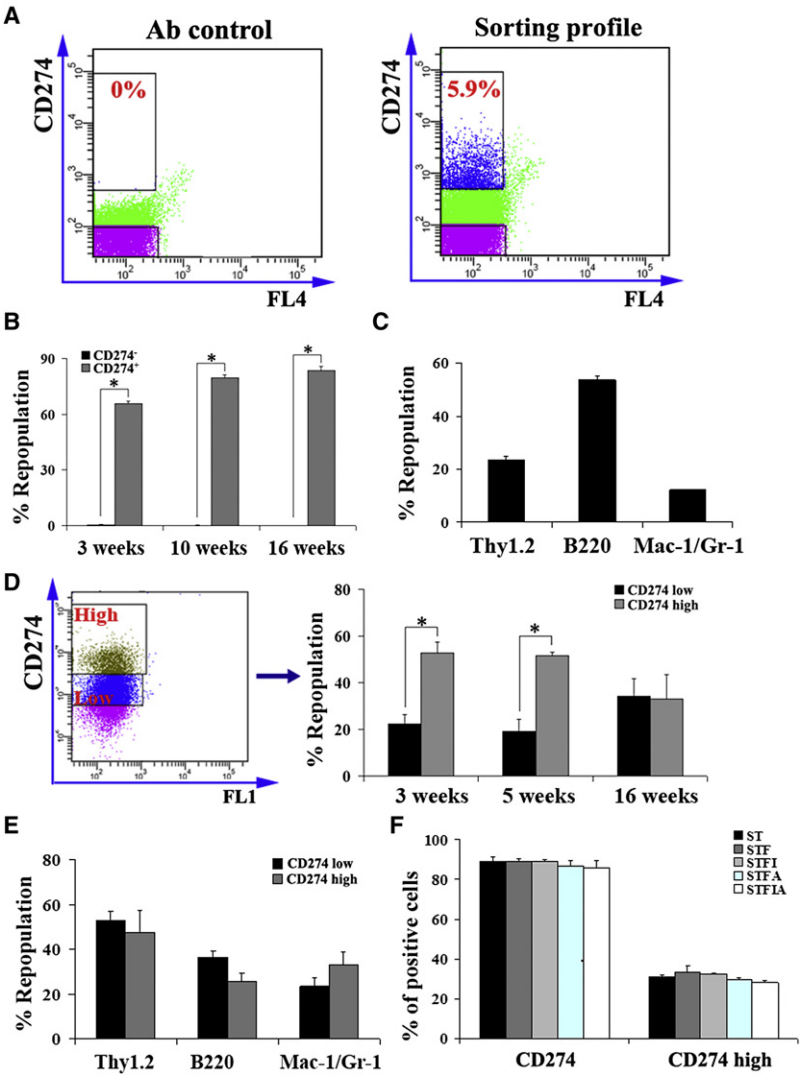


**Figure 4. CD274 Is Upregulated on Repopulating HSCs during Culture**

(A–C) 2 × $10^5$ freshly isolated CD45.2 CD274$^+$ and CD274$^-$ BM cells were transplanted, respectively, together with 2 × $10^5$ CD45.1 competitor cells into lethally irradiated congenic CD45.1 mice (*$p < 0.05$, n = 5).

(A) Gating plots of CD274$^+$ and CD274$^-$ BM cells.

(B) Peripheral blood engraftments at weeks 3, 10, and 16 after transplant.

(C) Multilineage contribution of cultured cells at 16 weeks posttransplant.

(D and E) 9.6 × $10^4$ sorted cultured CD45.2 CD274$^{high}$ and CD274$^{low}$ total cultured cells were transplanted, respectively, together with 1 × $10^5$ CD45.1 competitor cells into lethally irradiated congenic CD45.1 mice (*$p < 0.05$, n = 5).

(D) Peripheral blood engraftments at week 3, 5, and 16 after transplant.

(E) Multilineage contribution of cultured cells at 16 weeks posttransplant.

(F) All tested culture condition induces CD274 expression on the surface of HSCs (n = 3). Shown are the percentages of cells that express CD274 on their surface after 8 days of culture of HSCs in serum-free medium supplemented with SCF+TPO (ST), SCF+TPO+FGF-1 (STF), SCF+TPO+FGF-1+Angptl3 (STFA), SCF+TPO+FGF-1+IGFBP2 (STFI), and SCF+TPO+FGF-1+Angtpl3+IGFBP2 (STFIA).

Data are expressed as mean ± SEM. See also Figure S4.

WT HSCs, and CD274-null HSCs after culture (Figure 5G). Here we used ST medium (that does not support expansion of HSCs) to culture HSCs and specifically evaluate the expansion-independent effect of CD274 on HSC allograft. The 1,000 input equivalent WT HSCs engrafted 5 out of 17 recipients, whereas the anti-CD274 neutralizing antibody treated WT HSCs or the cultured CD274-null HSCs lost donor allograft activity (Figure 5G). Therefore, the deletion of CD274 or treatment with a CD274 neutralizing antibody abrogated the ability of cultured but unexpanded HSCs to cross the MHC barrier. A MLR experiment confirmed that, whereas cultured WT HSCs significantly inhibited allogeneic T cell activation, cultured CD274-null HSCs did not exhibit this inhibitory effect (Figure 5H). Anti-PD-1 was capable of decreasing the late apoptosis of activated T cells cocultured with precultured HSCs (Figure S5B). These results led us to conclude that CD274, a ligand known to inhibit T cell responses, is induced on cultured HSCs and possibly some differentiated cells; PD-1-mediated apoptosis of host T cells is one mechanism by which cultured HSCs overcome the MHC barrier in allograft.

While many studies demonstrated direct evidence that CD274 impedes T cell functions, it was reported that CD274 can also suppress the activation of innate immune cells (Yao et al., 2009). We performed a further experiment to distinguish the possible involvement of T cell-mediated immune response and innate immunity in the cultured HSC-enabled allograft. To this end, we cultured WT and CD274-null HSCs in the STFIA medium, followed by transplantation into sublethally irradiated SCID BALB/c mice (2.5 Gy). These recipient mice do not have functional T cells or B cells but do have normal NK cells. If WT and CD274-null HSCs do not have difference in repopulation in these mice, it would indicate that CD274 mainly works through suppressing allogeneic T cell activation but not innate immunity. Indeed we did not observe difference in allograft abilities of cultured WT and null HSCs in these mice (Figure 5I). Therefore, consistent with previous studies showing that CD274 suppresses T cell-mediated allo-rejection (Francisco et al., 2010; Zou and Chen, 2008), our result suggests that upregulation of CD274 on cultured cells including HSCs inhibited allogeneic T cell response.

## Ex Vivo Expanded HSCs Can Cure the HSC Defective Disease by Allogeneic Transplantation

To test whether ex vivo expanded HSCs can be used to cure genetic diseases, we ex vivo expanded allogeneic HSCs and transplanted these cells into homozygotic DNA-PK 3A/3A knockin mice, in which three phosphorylation sites (Thr2605, Thr2634, and Thr2643) of DNA-PK were eliminated (Zhang et al., 2011). These mice have defective HSC self-renewal during

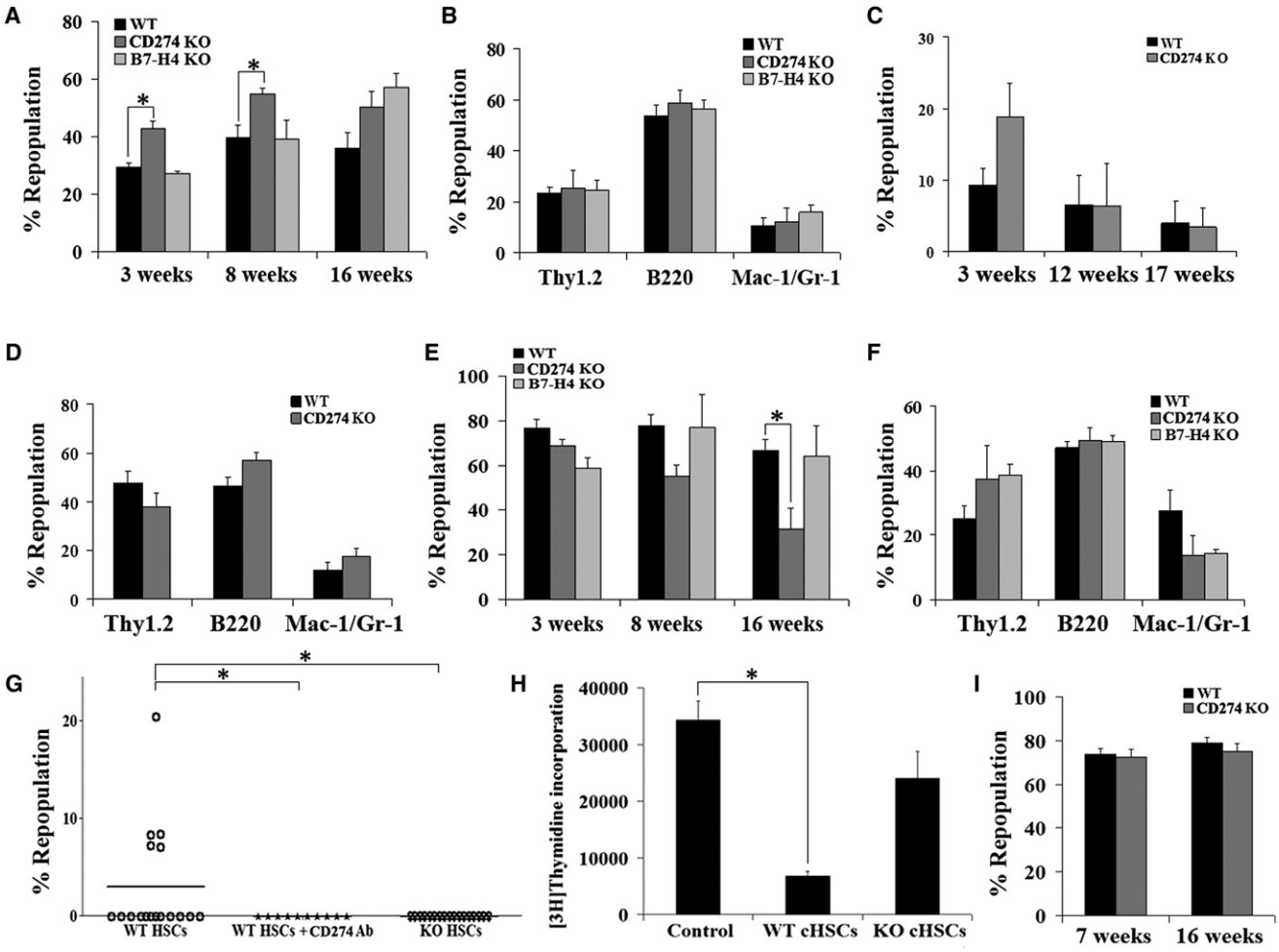


**Figure 5. Elevated CD274 Expression on Cultured HSCs Is Critical to Cross MHC Barrier in Allogeneic Transplantation**

(A and B) CD274 does not affect repopulation of freshly isolated HSCs in syngeneic transplantation. Freshly isolated 1 × $10^5$ BM cells from WT or CD274-null CD45.2 C57BL/6 donors were transplanted into lethally irradiated CD45.1 C57BL/6 syngeneic recipients with 100,000 CD45.1 C57BL/6 total BM competitors (*$p < 0.05$, n = 5).

(A) Donor repopulation at 3, 8, and 16 weeks posttransplant.

(B) Multilineage contribution of cultured cells at 16 weeks posttransplant.

(C and D) CD274 does not affect repopulation of cultured HSCs in syngeneic transplantation. Cultured progenies of 100 $Lin^-Sca\text{-}1^+Kit^+CD34^-Flk2^-$ HSCs from WT or CD274-null CD45.2 C57BL/6 donors were transplanted into lethally irradiated CD45.1 C57BL/6 syngeneic recipients with 100,000 CD45.1 C57BL/6 total BM competitors (n = 5). Cells were cultured in STFIA medium.

(C) Donor repopulation at 3, 12, and 17 weeks posttransplant.

(D) Multilineage contribution of cultured cells at 17 weeks posttransplant.

(E and F) CD274 enhances repopulation of cultured HSCs in competitive allogeneic transplantation. Cultured progenies of input equivalent 10,000 $Lin^-Sca\text{-}1^+Kit^+CD34^-Flk2^-$ HSCs from CD45.2 C57BL/6 donors were cotransplanted with 100,000 freshly isolated BALB/c (CD45.2) BM cells into lethally irradiated BALB/c (CD45.2) recipients (*$p < 0.05$, n = 5). Cells were cultured in STFIA medium. This is an experiment representing two independent experiments that gave similar results.

(E) Donor engraftment at 3, 8, and 16 weeks posttransplantation.

(F) Multilineage contribution of cultured cells at 16 weeks posttransplant.

(G) CD274 enhances repopulation of cultured HSCs in noncompetitive allogeneic transplantation. Cultured progenies of input equivalent 1,000 $Lin^-Sca\text{-}1^+Kit^+CD34^-Flk2^-$ WT or CD274-null HSCs were transplanted into lethally irradiated BALB/c (CD45.2) recipients without competitors. ST medium was used in culture. Shown is donor engraftment at 16 weeks posttransplant (*$p < 0.05$, n = 10–17).

(H) MLR assay was performed in which cultured WT HSCs abrogated the proliferation of allogeneic T cells (bar 2) whereas cultured CD274-null HSCs were unable to do so (bar 3). *$p < 0.05$, n = 3.

(I) CD274 enhances repopulation of cultured HSCs through inhibition of T cell response. Cultured progenies of input equivalent 5,000 $Lin^-Sca\text{-}1^+Kit^+CD34^-Flk2^-$ WT or CD274-null HSCs were transplanted into sublethally irradiated (2.5 Gy) SCID-BALB/c (CD45.2) recipients. Cells were cultured in STFIA medium. Shown are donor engraftments at 7 and 16 weeks posttransplantation (n = 5).

Data are expressed as mean ± SEM. See also Figure S5.

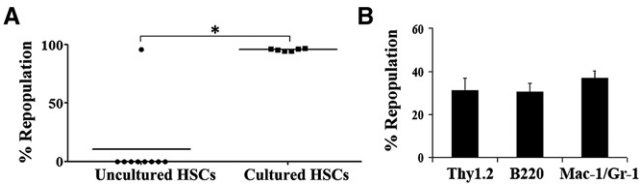


**Figure 6. Culture of Allogeneic WT HSCs Rescues Lethal Phenotype of DNA-PK Knockin Mice**

Freshly isolated 10,000 $Lin^-Sca\text{-}1^+Kit^+CD34^-Flk2^-$ HSCs or their 8-day cultured progenies from CD45.1 FVB donors were transplanted into lethally irradiated C57BL/6/129 CD45.2 knockin mutation at DNA-PKcs T2605 phosphorylation cluster recipients at postnatal day 12 (*$p < 0.05$, $n = 6–9$). HSCs were cultured for 8 days in STFIA medium that allows ex vivo expansion of HSCs. When competitors were used, freshly isolated 2,000,000–4,000,000 $Sca\text{-}1^-$ bone marrow cells isolated from FVB mice were cotransplanted.

(A) Donor engraftment at 16 weeks posttransplant.

(B) Multilineage contribution of cultured cells in rescued DNA-PK knockin mice at 16 weeks posttransplant.

Data are expressed as mean ± SEM.

development and normally die around 1 month after birth (Zhang et al., 2011). Figure 6A shows the result of transplantation of WT FVB (CD45.1) donor into DNA-PK knockin mice of the CD45.2 C57BL/6/129 background. Whereas freshly isolated $Lin^-Sca\text{-}1^+Kit^+CD34^-Flk2^-$ allogeneic HSCs transplanted with 2–4 × $10^6$ $Sca\text{-}1^-$ helper cells (which made the total number of transplanted cells the same as or more than the number of cultured cells transplanted) engrafted only one out of nine recipients, their cultured progeny successfully engrafted and rescued all recipients. The rescued mice had almost 100% donor reconstitution and lymphoid, myeloid, and erythroid lineages were repopulated at 4 months posttransplantation (Figure 6B). Our result demonstrated that ex vivo expanded HSCs can be successfully used in fully nonmatched allogeneic transplantation to rescue the lethal phenotype of genetically mutated mice.

### CD274 Is Induced on Human HSCs upon Culture

It is important to know whether a similar alteration of CD274 occurs on human HSCs upon culture. To this end, we determined the expression of CD274 on freshly isolated and cultured human cord blood HSCs. While only ~10% of freshly isolated human $Lin^-CD34^+CD38^-CD90^+$ cells express CD274 on their surface, the $CD274^+$ population increased to more than 50% after culture (Figures 7A–7E). MLR analysis showed that the elevated CD274 expression on cultured human cord blood HSCs indeed suppressed the proliferation of allogeneic T cells, and this ability was abrogated by the anti-CD274 neutralizing antibody treatment (Figure 7F). When we cultured human cord blood HSCs followed by transplantation into immune-deficient NOD/SCID/gamma(c)(null) (NSG) mice, we observed a stimulating effect of Angptl5 on HSC expansion as previously reported (Figure S6; Drake et al., 2011; Khoury et al., 2011; Zhang et al., 2008). Nevertheless, this enhanced ability to engraft NSG mice was not affected by anti-CD274 neutralizing antibody (Figure S6). This result is similar to what we observed in allograft in SCID BALB/c mice (Figure 5I), suggesting that human CD274 suppresses allogeneic T cell activation but not innate immunity. The upregulation of CD274 on cultured human HSCs may enable these stem cells to possess an enhanced allograft ability.

## DISCUSSION

In this study, we demonstrated that ex vivo expanded HSCs more efficiently overcame MHC barriers and repopulated allogeneic recipient mice than freshly isolated HSCs. As measured by limiting dilution analysis, there was a 40-fold increase in the allograft ability of HSCs cultured for only 8 days compared to that of the freshly isolated HSCs. To identify the underlying mechanisms, we found that both increased numbers of HSCs and cultured-induced elevation of expression of the immune inhibitor CD274 on the surface of HSCs contributed to the enhanced allograft efficiency. As a proof of principle that ex vivo expanded HSCs can be used to cure genetic diseases in allogeneic recipients, we used ex vivo expanded allogeneic HSCs for transplantation and successfully rescued the lethal phenotype of DNA-PK knockin mice.

We used three models of allogeneic transplantation: noncompetitive transplantation into lethally irradiated recipients, competitive transplantation into lethally irradiated recipients, and noncompetitive transplantation into sublethally irradiated recipients. Whereas the first model was well-established and allows fewer numbers of donor cells for engraftment, it may result in the mouse death if donor HSCs cannot repopulate recipients. The second and third models ensure the survival of all recipients and better mimic the human transplantation scenario in which reduced intensity conditioning is often applied. Nevertheless, because of the enhanced host immune rejection, more than 10-fold of freshly isolated allogeneic donor HSCs are needed for successful engraftment in these models. This also underscores the importance of the increased number and MHC matching of donor HSCs in the clinical setting.

Our findings may shed new light on allogeneic transplantation of human HSCs into patients, which cannot be appropriately modeled by xenograft into immune-deficient mouse recipients. Two major problems, failure of engraftment and GVHD, have limited the progress in allogeneic transplantation. A strategy that significantly improves donor engraftment and reduces the risk of GVHD compared to current practice is needed. Transplantation of freshly isolated allogeneic HSCs indeed decreases the risk of GVHD but results in much lower engraftment (Shizuru et al., 1996; Wang et al., 1997). Here we show that ex vivo expanded mouse HSCs possess two advantages: increased HSC numbers and the enhanced immune feature to evade host rejection, therefore having dramatically enhanced allogeneic engraftment. Importantly, similar to freshly isolated HSCs (Shizuru et al., 1996; Wang et al., 1997), no sign of GVHD was observed after allogeneic transplantation of ex vivo expanded HSCs. This is expected because the condition of our (or other) HSC culture supports expansion of HSCs, along with production of differentiated myeloid but not much lymphoid cells. The culture thus does not seem to produce the source cells including T cells that may cause GVHD. Therefore, ex vivo expanded mouse HSCs appear to be an appropriate cell source to solve the problems of allogeneic transplantation in the mouse model. Based on these results of mouse HSCs and the elevation of CD274 on cultured human HSCs, we propose that ex vivo expansion of human HSCs may benefit the practice of allogeneic transplantation for patients. This would apply to nonmatched or low-matched donor human cord blood, BM, or mobilized

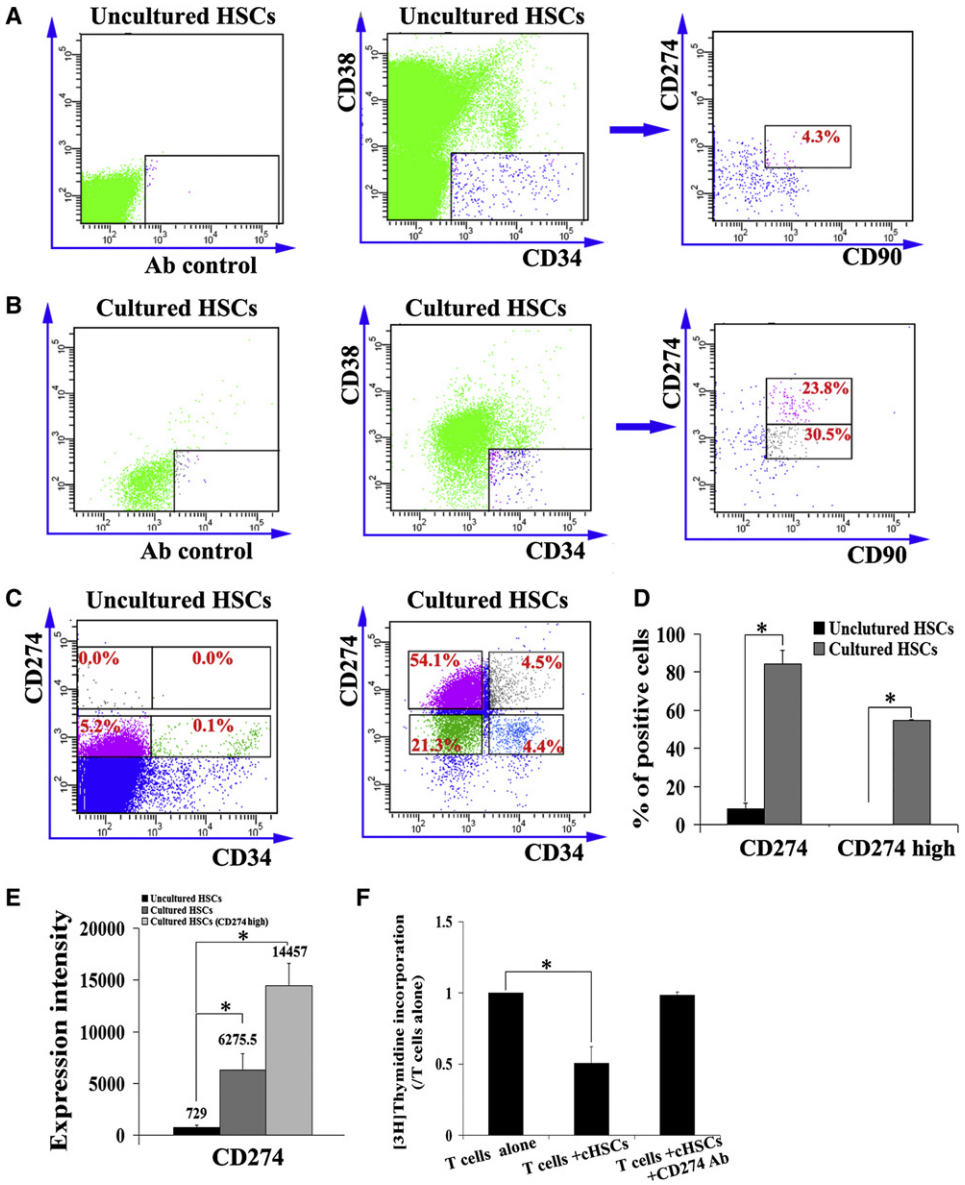


**Figure 7. CD274 Is Upregulated in Cultured Human HSCs**

Human cord blood Lin$^-$CD34$^+$CD38$^-$CD90$^+$ cells were cultured in serum-free medium supplemented with SCF, TPO, and Flt3-L for 8 days.

(A and B) Representative plots of CD34$^+$CD38$^-$CD90$^+$ cells that express CD274 on their surface before (A) and after (B) culture. Gatings were set based on isotype controls.

(C) Representative plot of CD34 and CD274 high or low staining in cultured human cells.

(D) Summary of percentages of CD274$^+$ and CD274$^{high}$ cells in uncultured and cultured human cells (*$p < 0.05$, $n = 5$).

(E) MFI of CD274 expression determined by flow cytometry analysis in freshly isolated and cultured human cells (*$p < 0.05$, $n = 5$).

(F) MLR assay was performed in which cultured human cord blood HSCs abrogated the proliferation of allogeneic T cells (bar 2) whereas CD274 antibody reversed the inhibitory effect of cultured human cord blood HSCs (bar 3). *$p < 0.05$, $n = 6$.

Data are expressed as mean ± SEM. See also Figure S6.

peripheral blood HSCs. If donor human HSCs can be expanded in culture and engraft nonmatched or low-matched patients without GVHD, this strategy will possibly lead to an ultimate solution to problems in allogeneic transplantation.

It is known that some adult stem cells, such as mesenchymal and amnion stem cells, but not embryonic stem cells, are capable of avoiding rejection through production of immunosuppressive molecules and can be used in intra- and even interspecies transplantation (Salem and Thiemermann, 2010; Swijnenburg et al., 2008). Here we demonstrated that the immune inhibitor CD274 is expressed on freshly isolated HSCs and that its expression dramatically increased upon culture. Interestingly, CD274 does not appear to significantly affect the repopulation of long-term HSCs before and after culture as

determined by syngeneic transplantation, suggesting that its main role is not regulation of the regular activity of HSCs, but modulation of immunological properties of these cells. This was confirmed by the result that the deletion of CD274 or treatment with a CD274-neutralizing antibody abrogated the ability of cultured but unexpanded HSCs to cross the MHC barrier. CD274 was shown in previous studies to be expressed on activated immune cells and parenchymal cells and in immune-privileged sites such as eyes and placenta (Francisco et al., 2010; Zou and Chen, 2008). CD274 is also selectively expressed by various cellular components in the tumor microenvironment, where it inhibits tumor-specific T cell immunity by inducing T cell apoptosis and delay rejection (Zou and Chen, 2008). Here we provided an example suggesting that HSCs possess the ability to evade the rejection of the acquired immune system by regulating the expression of their own surface immune inhibitor such as CD274. Besides HSCs, the elevation of CD274 on hematopoietic progenitors produced during culture also might have contributed to the enhanced allograft. However, it is interesting to note that more differentiated $Lin^+$ cells elicit no effect, although they also express CD274. This may be contributed by the different cellular locations of HSCs/progenitors and more differentiated hematopoietic cells home after transplantation. It therefore will be interesting to study where the T cell-mediated immune response occurs for allogeneic transplanted HSCs in the future. In addition, it is noteworthy that CD274 may not be the only immune-suppressor acts on the HSC allograft. This is because that, although CD274-null HSCs behave much worse in allogeneic transplantation than their WT counterparts, they still possess a certain ability for allogeneic engraftment. Consistent with the elevation of the expression of immune inhibitor CD274 upon culture, costimulatory molecules such as CD80 and CD86 lost their expression on some cells after culture. All these observations clearly indicate that ex vivo culture significantly modulates the immunogenicity of stem cells. The identification of additional immune molecules whose alterations can regulate allograft will enable the complete resolution of the issue of immune rejection in allogeneic transplantation.

While our study suggests that the upregulation of CD274 on cultured cells including HSCs inhibited allogeneic T cell response, a related example is surface expression of CD47, which enables HSCs and leukemia cells to evade innate macrophage phagocytosis (Jaiswal et al., 2009). Based on these results, we hypothesize that all homeostatic HSCs express low levels of surface immune suppressors, and the levels of these suppressors can be induced by stress or immune signals. These immune suppressors may thus modulate HSC immunogenicity and, therefore, contribute to the "immune privilege" of HSCs. This regulatable immune privilege should be advantageous to HSCs, because it may allow these important stem cells to rapidly adjust to altered environment or to protect them from the excessive immune activation and even potential autoimmune disorder. Whether the expression of CD274 on HSCs or cancer cells can be regulated in vivo and its biological significance warrants further investigation.

Furthermore, we speculate that a common mechanism exists for regulation of expression of immune inhibitory signals in some other types of stem cells—similar to that in tumor cells. The expression and regulation of immune inhibitors on stem cells per se may allow these cells to survive an unexpected immune attack. It will be interesting to study the immunology of stem cells by investigating the roles of surface immune molecules on embryonic stem cells, induced pluripotent stem cells, other adult stem cells, and cancer stem cells.

In summary, our study demonstrated the great benefits of ex vivo expansion of HSCs for overcoming problems in allogeneic transplantation and revealed the importance of an immune inhibitor on the surface of HSCs. This work should shed new light on understanding the immunology of HSCs and other stem cells and may lead to development of novel strategies for successful allogeneic transplantation of human patients.

## EXPERIMENTAL PROCEDURES

### Mouse HSC Culture

Indicated numbers of BM $Lin^-Sca\text{-}1^+Kit^+$ $CD34^-Flk\text{-}2^-$ cells were isolated from 8- to 12-week-old mice and 150–200 of them were plated into each well of a U-bottom 96-well plate (3799; Corning) with 200 μl of the indicated medium. STFIA medium was defined as Stemspan serum-free medium (StemCell Technologies) supplemented with 10 μg/ml heparin, 10 ng/ml mouse SCF, 20 ng/ml mouse TPO, 10 ng/ml human FGF-1, 100 ng/ml IGFBP2, and 500 ng/ml Angptl3 as described (Huynh et al., 2008), which was used in Figure 2 experiments. In repeated experiments and experiments described in other figures, we refer STFIA medium as the same above medium except with serum-free conditioned medium collected from Angptl2-transfected 293T cells as described (Zhang et al., 2006) (that contains both IGFBP2 [Huynh et al., 2008] and Angptl2) to replace recombinant IGFBP2 and Angptl3. This Angptl2-supplemented medium worked equivalently and reproducibly supported HSC expansion in all experiments. ST medium was defined as Stemspan supplemented with 10 μg/ml heparin, 10 ng/ml mouse SCF, and 20 ng/ml mouse TPO. STF medium was ST medium supplemented with 10 ng/ml human FGF-1. STFA medium was STF medium supplemented with 500 ng/ml Angptl3. STFI medium was STF medium supplemented with 100 ng/ml IGFBP2. Unless otherwise described, cells were cultured for 8 days at 37°C in 5% $CO_2$ and the normal level of $O_2$. The culture duration of 8 days was shorter than that we described in previous studies (Huynh et al., 2008; Zhang et al., 2006). Because a substantially more number of cells were needed for allogeneic transplantation, we plated 150–200 HSCs per well in our experiments, instead of 20 HSCs described previously (Huynh et al., 2008; Zhang et al., 2006) for congeneic transplantation. We typically observed a ~200-fold increase of total number of cells after 8 days of culture. Therefore, a 100 input cells produced $2.32 \pm 0.28 \times 10^4$ total cells after 8 days of culture. This 8-day culture thus allowed us to harvest cells from the culture wells before the expanded cells exhausted the medium. For the purpose of transplantation, we pooled cells from at least 10 culture wells before the indicated numbers of cells were transplanted into each mouse. Flow cytometry analysis was performed to confirm multilineage reconstitution as we described (Simsek et al., 2010; Zheng et al., 2011). Calculation of CRUs in limiting dilution experiments was conducted with L-Calc software (StemCell Technologies) (Huynh et al., 2008; Simsek et al., 2010; Zheng et al., 2011).

### Mouse HSC Allogeneic Transplantation

For allogeneic transplantation without competitors, the indicated numbers of mouse donor cells before or after culture were injected intravenously via the retro-orbital route into each of a group of 6- to 9-week-old recipient mice immediately after irradiation with a lethal dose of 9 or 9.5 Gy for BALB/c or 10 Gy for C57BL/6 mice. Sublethal irradiation of BALB/c mice in Figure S2A and of SCID-BALB/c mice in Figure 5I were performed at a dose of 7.5 Gy and 2.5 Gy, respectively. For competitive allogeneic transplantation, the indicated mouse donor cells before or after culture were mixed with $1–2 \times 10^5$ (as indicated) freshly isolated competitor bone marrow cells before transplantation. When indicated, 1,000,000 bone marrow cells collected from primary recipients were used for the secondary transplantation into lethally irradiated BALB/c mice. The antibody blocking treatment was conducted by incubating

cultured HSCs with 50 μg/ml CD274 neutralizing antibody (Cat# 16-5982-81, eBioscience) for 2 hr followed by washing before transplantation.

### Mixed Lymphocyte Reaction

MLR was performed similarly as we described (Curiel et al., 2003). In brief, for mouse MLR in Figure 5H, BALB/c splenocyte CD90.2$^+$ T cells were plated in 96-well plate (flat-bottom) precoated with 1 μg/ml anti-CD3, followed by coculture with 8 days precultured irradiated C57BL/6 HSCs. For human MLR in Figure 7F, peripheral blood CD3$^+$ cells were plated in the presence of 2.5 μg/ml anti-CD3 and cocultured with 8 days precultured irradiated allogeneic cord blood HSCs. Proliferation was measured at day 3 of incubation at 37°C and 5% $CO_2$ following pulsing with [3H]TdR with a liquid scintillation counter. When indicated, 50 μg/ml anti-CD274 neutralizing antibody (Cat# 16-5983-82) was used to treat the cultured cells for 2 hr.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at doi:10.1016/j.stem.2011.06.003.

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