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Title

Induction of allograft tolerance by adoptive transfer of donor B cells: an immune regulatory strategy for transplantation using MHC-matched iPS cells

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Abstract

For cellular or tissue transplantation using iPS cells (iPSCs), from the viewpoint of time and economic cost, an use of allogeneic ones is being considered. Immune regulation is one of the key issues in successful allogeneic transplantation. To reduce the risk of rejection, several attempts have been reported to eliminate effects of major histocompatibility complex (MHC) on the iPSCs-derived grafts. On the other hand, we have shown that minor antigen-induced rejection is not negligible even when the MHC's impact is mitigated. In organ transplantation, it is known that donor-specific blood transfusion (DST) can specifically control immune responses to the donor. However, whether DST could control the immune response in iPSC-based transplantation was not clarified. In this study, using a mouse skin transplantation model, we demonstrate that infusion of donor splenocytes can promote allograft tolerance in the MHC-matched but minor antigen-mismatched condition. When narrowing down the cell types, we found that infusion of isolated splenic B cells was sufficient to control rejection. As a mechanism, the administration of donor B cells induced unresponsiveness but not deletion in recipient T cells, suggesting that the tolerance was induced in the periphery. The donor B cell transfusion induced allogeneic iPSCs engraftment. These results suggest for the first time a possibility that DST using donor B cells could induce tolerance against allogeneic iPSC-derived grafts.

40 *Keywords:* transplantation, tolerance, alloimmunity, donor-specific blood transfusion, iPS cell,

41 Runnning head: B-cell-induced tolerance for iPSC transplantation

Introduction

Pluripotent stem cells (PSCs) are thought to be a source of therapeutic cells for transplantation because of the ability to differentiate into any cell types in the body(1) Since induced pluripotent stem cells (iPSCs), a type of PSCs, can be produced by gene introduction into somatic cells(2, 3), it is possible to generate autologous therapeutic cells from each individual who needs transplantation. Although there have been reports that various cell types, such as retinal pigment epithelial cells, were successfully differentiated from autologous iPSCs and transplanted to patients, the time required for the production and quality evaluation of those therapeutic cells may hinder its general application(4–6). On the other hand, when qualified off-the-shelf allogeneic iPSCs are stocked in advance, the duration of the product examination would be shortened(7–9). Because of the advantage, allogeneic iPSCs have been considered as a major source of therapeutic cells. However, allogeneic iPSCs could be a target of immunological rejection by the recipient's immune system.

It has been well established that major histocompatibility complex (MHC) molecular mismatch between the donor and recipient increases the risk of rejection(10). Indeed, studies of kidney transplant recipients have shown that higher number of mismatched MHC caused shorter survival of the transplanted grafts(11). Therefore, in transplantation using iPSCs, it is planned to prepare allogeneic iPSC lines with a matched MHC type for recipients to reduce the risk of rejection(7, 8). Based on this, MHC-type homologous iPSCs that are compatible with

multiple MHC types have been stocked(7, 8). Furthermore, several gene-modified iPSCs which lack MHC molecules to reduce the risk of rejection (so-called universal iPSCs) have been reported(12–15). However, in addition to MHC molecules, minor antigen mismatches also increase the risk of rejection(16, 17). Minor antigens are protein molecules expressed by donor cells whose amino acid sequence differs from that of the recipient and to which recipient T cells react as non-self(18). Minor antigens activate T cells through an indirect pathway presented by recipient antigen-presenting cells, and it is known that a single minor antigen mismatch alone can result in graft rejection(19, 20). In transplantation experiments of iPSC-derived therapeutic cells using non-human primates, it has been reported that the use of MHC-type matched iPSCs attenuated the immune response, but the use of immunosuppressive agents was necessary to maintain graft viability(21–24). These findings suggest that immune control is still essential in allogeneic iPSC-based transplantation even when the impact of MHC is minimized as long as the minor antigen mismatch remains. However, appropriate immune control regimens for iPSC-based transplantation have not been investigated sufficiently.

We have previously developed an experimental mouse skin transplantation model to examine immune responses and develop appropriate immunoregulation protocols for MHC-matched but minor antigen-mismatched transplants(25, 26). This model has shown that it was challenging to control rejection for the long term even with the combination of tacrolimus, mycophenolate mofetil, and steroids, which are commonly used in organ transplantation(26).

Although the effect of the MHC matching was identified in the model, it seemed necessary to develop safe and efficient immunoregulatory protocols, including immune tolerance induction for transplantation using MHC-matched (or universal) iPSCs.

In organ or tissue transplantation, it has been reported that donor-specific transfusion (DST) using spleen or peripheral blood can induce immune tolerance to the donor and control rejection without the use of immunosuppressive agents(27). In clinical practice, DST has been shown to prolong graft survival in human kidney transplantation(28, 29). On the other hand, it has not been clarified whether DST is also effective for iPSCs-based transplantation. To apply DST to iPSCs-based transplantation, it is necessary to prepare hematopoietic cells from iPSCs, but the generation of all blood cell types contained in peripheral blood or spleen is of great technical difficulty and exceeds an affordable cost. For these reasons, it is necessary to identify effective blood cell types to induce immune tolerance and to clarify whether they can be applied to iPSC-based transplantation.

In this study, we have attempted to identify blood cell type which can induce immune tolerance to MHC-matched transplanted grafts by DST. We used the above-mentioned mouse skin transplantation model and also MHC-matched iPSCs transplantation. The results indicated that B cells in the spleen were the cell type that efficiently induced immune tolerance. We analyzed the mechanism of T cell reactivity reduction after donor B cell administration. This information is beneficial when DST is applied for iPSCs-based transplantation.

Methods

Mice

Five to eight-week-old male BALB/c (MHC haplotype: H-2^{d/d}), C57BL/6J (B6) (H-2^{b/b}), B6^{gld/gld} (H-2^{b/b})(30), CBA/N (H-2^{k/k}), and 129X1/Sv (H-2^{b/b}) and female C3H/He (H-2^{k/k}) were purchased from Japan SLC, Inc. C3129F1 (H-2^{k/b}) mice were obtained by breeding female C3H/He and male 129X1/Sv mice in our institute's animal experimental facility. All animal experiments were approved by the Hokkaido University Animal Care Committee (Approval number: 22-0080) and conducted in accordance with the "Regulations Concerning Animal Experiments at Hokkaido University National University Corporation."

Cell Preparation

Splenocytes were obtained by lysing red blood cells from the spleen. B or T cells were isolated from splenocytes using CD19 or CD90.2 MicroBead kits (Miltenyi Biotec), respectively, according to the manufacturer's procedure. The isolated cells were analyzed by flow cytometry to confirm that the purity was >95%. Bone marrow cells were collected from the tibias and femurs and used for induction of bone marrow-derived dendritic cells (BM-DCs) and macrophages (BM-Ms). BM-DC or BM-M were induced by culturing bone marrow cells in RPMI 1640 (Wako) supplemented with 10% fetal bovine serum (Nichirei), 55 μ M 2-

mercaptoethanol (Nacalai Tesque), 1% nonessential amino acids (Nacalai Tesque), 1% sodium pyruvate (Nacalai Tesque), and 1% penicillin and streptomycin (Nacalai Tesque) containing 10 ng/ml granulocyte-macrophage colony-stimulating factor (Biolegend) or 10 ng/ml macrophage colony-stimulating factor (Biolegend), respectively, for 6 days. During induction of BM-DC and BM-M, the medium was changed on days 2 and 4. For BM-DCs, CD11c cells were isolated using the CD11c MicroBead kit (Miltenyi Biotec) according to the manufacturer's instructions. BM-M was collected using a cell scraper in phosphate-buffered saline containing 10 mM ethylenediaminetetraacetic acid (Dojindo).

Donor cell infusion and skin transplantation

As pretreatment for recipient mice, anti-CD4 (clone; GK1.5) and anti-CD8 (53-6.7) antibodies were administered 13 and 8 days before skin transplantation to deplete peripheral T cells. Donor blood cells were administered intravenously in the tail vein (only BM-M was administered intraperitoneally) after 3-Gy total body irradiation of X-rays 7 days before skin transplantation. Dorsal ear skin tissues of donor mice were used as grafts. Recipient mice were anesthetized by intraperitoneal administration of a mixture of Domitor (Nippon Zenyaku Kogyo), Midazolam (Takeda), and Vetorphale (Meiji Seika Pharma), and the skin graft was replaced by excision of the dorsal chest wall skin (approximately 10 mm in diameter)(31). Skin grafts were observed regularly, and we defined the period of graft survival as the time from the

date of grafting to the date when the diameter of the healthy skin graft was less than 30% of its initial size.

Mixed lymphocyte reaction (MLR)

Splenocytes were harvested from recipients 8 weeks after skin transplantation, and B cells were removed using a CD19 MicroBead kit; B cell-depleted splenocytes were labeled with 0.25 nM carboxyfluorescein succinimidyl ester (CFSE) (Dojindo) and used as responder cells. Splenocytes irradiated with 30 Gy of X-rays were used as stimulator cells. Responder cells (2×10^5) and stimulator cells (1×10^5) were co-cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 55 μ M 2-mercaptoethanol, 1% non-essential amino acids, 1% sodium pyruvate, 1% penicillin and streptomycin in 96-well round-bottom plates. Four days after co-culture, cells were analyzed by flow cytometry, and T cells with decreased CFSE fluorescence intensity were evaluated as reacted cells.

Flow cytometry and antibodies

Flow cytometry analysis was performed using FACSCanto II (BD Biosciences), FACS Aria II (BD Biosciences), or FACSCelesta (BD Biosciences), and data were analyzed with FlowJo software V7.6.5 (Tree Star). For fluorescence-activated cell sorting (FACS) analysis, the cell surface was stained with the fluorescent-labeled antibodies listed in supplementary table 1. For

Treg detection, intracellular staining was performed with anti-Foxp3 antibody using Foxp3/Transcription Factor Staining Buffer Set (eBioscience). For data analysis, leukocytes were gated based on forward and side scatters, and cells that incorporated propidium iodide (PI) or 4',6-diamidino-2-phenylindole (DAPI) (Cayman) were excluded from the analysis as dead cells.

RNA extraction for T-cell receptor (TCR) repertoire analysis

CD4⁺ and CD8⁺ T cells were sorted from recipients splenocytes using FACS Aria II at least 100 days after saline or B cell administration. MLR was performed to detect and isolate recipient CD4⁺ and CD8⁺ T cells that reacted against donor splenocytes. In this MLR experiment, CFSE-labeled naive C3129F1-derived splenocytes (2x10⁵) were used as responder cells, and X-ray (30 Gy) irradiated donor splenocytes (8x10⁵) were used as stimulator cells. RNA was extracted from each experimental group using PureLink RNA Mini Kit (ThermoFisher SCIENTIFIC) according to the manufacturer's procedure.

NGS analysis

RNA Integrity number (RIN) value was analyzed using the Agilent RNA6000 Nano kit and the Agilent 2100 Bioanalyzer (Agilent Technologies). The RIN values of all samples were confirmed to be 7.8 or higher. Libraries were prepared from RNA (1300 ng) using the

SMARTer Mouse TCRa/b Profiling Kit (TAKARA). Next generation sequencing was performed using MiSeq Reagent Kit v3 (600 Cycle) with MiSeq (Illumina). The collected data were analyzed after conversion to fastq files.

Data analysis

Sequence reads for each sample were mapped on the TCR gene using MiXCR 3.0.13, the complementary determining region 3 (CDR3) sequence of TCR β was extracted, and the corresponding reads were counted. Read counts were normalized by the total number of reads mapped to CDR3 and converted to T cell clone frequency. Donor-reactive T cells were analyzed according to the method of Reference 31 with some modifications(32), defined as the T cells proliferating in the MLR that show a frequency of 10^{-4} or more and appear five times more frequently than in naïve mice.

Luciferase-expressing iPSC (Luc-iPSC) transplantation

Luc-iPSCs and transplantation using them were established in our previous study(26). Briefly, B6 fetal fibroblast-derived iPSCs were cultured in Advanced-DMEM/F-12 (Thermofisher) supplemented with Neuro Brew-21 (Miltenyi Biotec), N2 supplement (Wako), 1% penicillin-streptomycin, 0.03% L-glutamine, 3 μ M CHIR99021 (Adooq), 1 μ M PD0325901 (Tocris), and in-house recombinant human leukemia inhibitory factor under 37°C, 5% CO₂ conditions on

dishes coated with laminin (Nippi). Recipient mice were anesthetized as described above and inoculated subcutaneously with 1.5×10^6 Luc-expressing iPSCs with an equal number of cells to be administered between groups. Graft monitoring was performed using an IVIS Spectrum Imaging System (Caliper Life Sciences). To detect the luminescent signal, recipient mice were administered 6 mg D-luciferin (Cayman) intraperitoneally, and the signal was measured 20 minutes after the administration by imaging with a 5-seconds exposure time. Data obtained using Living Image software were analyzed to calculate radiance (photons/sec/cm²/sr) and total flux (photons/sec) by region of interest centered on the site where the Luc-iPSC was transplanted.

Statistical analysis

JMP 16.0.0 software (SAS Institute Inc.) was used for statistical analysis. The Student's t-test (unpaired, two-tailed) or Tukey's honest significant difference (HSD) test was used to test for statistically significant differences. Graft survival was analyzed with Kaplan-Meier survival curves and bonferroni-corrected Log-rank tests. Analysis of the frequency of donor-reactive T-cell clones between groups was performed with Fisher's exact test. The number of donor-reactive clones detected in naïve mice was N, the number of donor-reactive clones detected in saline-treated recipients was DT_{saline}, and the number of donor-reactive clones detected in B-cell-treated recipients was DT_{bcell} as [DT_{saline}, N-DT_{saline}; DT_{bcell}, N-DT_{bcell}] 2x2 contingency

213 table was created and statistically analyzed. P values were considered statistically significant

214 when less than 0.05.

215

Results

Administration of donor splenocytes or splenic B cells selectively prolonged the survival of skin grafts

First, we tested whether DST is effective in regulating rejection in an MHC-matched but minor antigen-mismatched transplantation model in order to investigate its applicability to allogeneic iPSC-based transplantation. B6-derived splenocytes were administered intravenously to pretreated recipient mice, and skin transplantation was performed (Fig. 1A). In the control group, which received only saline without donor blood cells, all third-party BALB/c and B6 skin grafts were rejected (Fig. 1B). In contrast, in recipients which had been administered with B6-derived splenocytes, BALB/c skin grafts were rejected by day 58 (median survival time: MST = 18 days), but B6 skin grafts survived for more than 100 days (MST > 100 days) (Fig. 1B). These results indicate that DST is effective for the regulation of minor antigen-mismatched transplantation.

Splenocytes contain various types of blood cells. We next attempted to identify blood cell type that is sufficient for successful DST. In recipient mice administered with B6-derived B cells, BALB/c skin grafts were rejected at early time points, whereas B6 skin grafts survived for more than 100 days (Fig. 1C). The macroscopic observation of the B6 skin grafts was similar to that of syngeneic grafts (Fig. 1D). In recipient mice receiving B6-derived T cells,

BALB/c-derived grafts were all rejected by day 57 (Fig. 1C). In contrast, one out of six B6 skin grafts was rejected on day 13. In addition, two recipients weakened and died on days 20 and 24 after skin grafting (Fig. 1C). It was considered due to transfer-associated GvHD(33, 34) as a result of administering allogeneic T cells to the recipients in an immunocompromised state after pretreatment.

Splenocytes contain a large number of T and B cells but few myeloid cells. Therefore, to evaluate the ability of myeloid cells to regulate rejection, bone marrow-derived dendritic cells (BM-DCs) and macrophages (BM-Ms) were used in the following experiments. Survival of B6 skin grafts was significantly prolonged compared to BALB/c skin grafts when BM-DCs were administered, but half of the grafts underwent rejection (Fig. 1E). When BM-Ms were administered, all BALB/c and B6-derived skin grafts were rejected, with no significant difference in their survival (Fig. 1F). To determine whether the recipients received B cells are tolerant to the donor, second skin transplantation was performed on recipients who had already accepted the B6 skin grafts. Although two out of six B6 skins were rejected, the B6 skin grafts survived significantly longer than the BALB/c skin grafts in the second transplant (Fig. 1G). These results indicate that donor B cell transfer has the ability to control rejection and induce immune tolerance to allografts.

Next, we tested whether the number of B cells needed to regulate rejection could be reduced. When the number of B cells administered was reduced to half (1.5×10^7), B6-derived

grafts were successfully engrafted in 80% of recipients (Fig. S1A). On the other hand, when the B cell number was reduced to $1/10$ (3×10^6), less than 20% of B6 grafts survived longer than 100 days (Fig. S1B). In addition, to verify the necessity of pretreatment, transplantation experiments were performed with B cells administered to non-pretreated recipients. All skin grafts derived from BALB/c and B6 were rejected, with no significant difference in survival between the two groups (Fig. S1C). These results suggest that in allogeneic MHC-matched iPSC-based transplantation, B-cell administration prolongs graft survival in a donor-specific manner without serious side effects. In addition, pretreatment is essential to regulate rejection by the B-cell administration.

***In vitro* T-cell reactivity was selectively reduced with the donor splenic B cell administration**

To detect T cell responses to minor antigens, it is necessary to stimulate T cells by the indirect pathway. Therefore, we performed MLR, which can detect T cell activation by both direct and indirect pathways(25), and analyzed whether T cell responses are suppressed by B cell administration (Fig. S2).

The responsiveness of CD4-positive T cells to syngeneic stimulation cells was not increased by B cell administration, and there was no significant difference in the percentage of proliferated CD8⁺ T cells between the B cell-administrated and control groups (Fig. 2A, B). In

contrast, the percentage of the cells that responded to B6-stimulator cells was significantly lower in the recipients who received B6 B cells compared to the control group (Fig. 2B). These results suggest that the B cell administration has a potential to induce T cell unresponsiveness to the donor. The magnitude of T cell responses to BALB/c stimulator was much higher compared to other stimulators (Fig. 2B), because it includes direct recognition in addition to indirect one. Rather, the B6 B cell-treated group showed higher responses in the BALB/c stimulator settings (Fig. 2B). Since the efficiency of T cell differentiation into memory subsets is enhanced in the presence of B cells(35), it is possible that memory T cells for BALB/c were more frequently present in the B cell-administered recipients than in the saline-administered group.

Donor-derived B-2 cells were mainly distributed in the recipient secondary lymphoid tissues

To elucidate the mechanism of immune tolerance induction, we first analyzed the distribution of donor B cells in the recipient. In peripheral blood and secondary lymphoid tissues, i.e., spleen and lymph nodes, the percentage of donor cells (CD45.1⁺) in total B cell population was highest in the early phase (week 1) after cell administration and decreased by week 3 (Fig. 3A, B). CD45.1⁺ B6 B cells were still detected at week 7 after cell administration, indicating that donor B cells were persistently located in secondary lymphoid tissues at least until week 7 (Fig.

3A, B). Donor B cells were also detected in the primary lymphoid tissue, the thymus, but at a lower rate than in the secondary lymphoid tissue, and some individuals had no donor-derived B cells detected in the thymus at weeks 1, 5 and 7 after treatment (Fig. 3A, B). The central tolerance mechanism of T cells induced in the thymus requires the persistent presence of donor-derived antigen-presenting cells in the thymus(36). Taken together, these findings suggested that the administration of donor B cells may have induced immune tolerance in recipient T cells by a peripheral mechanism.

We then analyzed a subset of B cells distributed in the recipients. The B cell subsets in the spleen, B-1 and B-2 cells are classified as $CD19^+CD43^+$ and $CD19^+CD43^-$ cells, respectively(37) (Fig. 3C, left for WT spleen). Using these markers, we examined the subsets of infused B cells distributed in the recipients as shown in Fig. 3C. The results indicate that the distributed cells retained the preinfused B-1/B-2 cell proportion (Fig. 3D). Therefore, in this experimental setting, administration of donor B cells which mainly contained B-2 subset induced allograft tolerance.

The expression of antigen presentation-related molecules was upregulated on donor B cells in recipient peripheral lymphoid tissues

Next, we analyzed molecular expression changes before and after the B cell administration. Since B cells are known to regulate T cell responses through interactions mediated by

molecules such as Fas ligand (FasL)(38) and programmed death-ligand 1 (PD-L1)(39), we analyzed the expression of these molecules. Although FasL was upregulated in donor B cells in lymph nodes (Fig. S4A) and PD-L1 was upregulated in donor B cells in the spleen (Fig. S4B), results were not consistent between lymph nodes and spleen. In addition, administration of B cells derived from B6^{gld/gld} mice, which are genetically deficient in functional FasL(30), to pretreated C3129F1 recipients maintained long-term survival of B6-derived skin grafts, indicating that the donor B cells induced allograft tolerance in a FasL-independent manner (Fig. S4C).

On the other hand, the expression of MHC class I and II molecules was upregulated in donor-derived B cells distributed in lymph nodes and spleens compared to naïve B6 splenic B cells (Fig. 4A, B). Furthermore, the expression level of CD80/CD86, co-stimulatory molecules for antigen presentation, was similarly elevated in B6-derived B cells in both lymph node and spleen tissues compared to that before administration (Fig. 4C, D). These results suggest that donor B cells have increased antigen-presenting capacity after administration to the recipients. When irradiated (40 Gy) B cells were administered to C3129F1 mice, immune tolerance was not induced and B6 skin grafts were rejected (Fig. 4E). The data indicates that survival of donor B cells is necessary for the induction of immune tolerance.

T cell unresponsiveness is induced by the administration of donor B cells

As a mechanism for the tolerance induction by DST, it has been reported that an increase in the frequency of regulatory T cells(40, 41), a deletion of alloreactive T cells(38, 42), or induction of T cell unresponsiveness(43) occurs in the recipients. Therefore, we tested whether regulatory T cells were changed by the donor B cell administration and found that the percentage of the regulatory T cells was not increased in the spleen, lymph node, and thymus of the recipient mice compared to naïve or saline-treated mice (Fig. 5A). Next, to test whether the deletion of alloreactive T cells contributes to the tolerance induction, we analyzed changes in TCR repertoire frequency before and after donor B cell administration according to the method by Morris et al. (32) (Fig. S3). The frequency of donor-reactive CD4⁺ T-cell clones was not reduced in recipient mice administered donor B cells compared to saline-administered mice. Donor-reactive CD8⁺ T cells were not decreased in the B cell administered recipients compared to the saline administered recipients, but rather significantly increased (Fig. 5B). Taken together with the results of the second transplantation (Fig. 2B), we hypothesized that alloreactive T cells remained after the administration of donor B cells. In summary, in this study, we could not detect any increase in regulatory T cells nor elimination of T cell clones by the administration of donor B cells.

Next, we tested whether unresponsiveness was induced in alloreactive T cells after donor B cell administration using MLR. When CD4⁺ T cells from B cell- or saline-treated recipients were added to MLR in which naive C3129F1 splenocytes were stimulated with

BALB/c cells, the proliferation of C3129F1 CD8⁺ T cells was increased (Fig. 5C, S5). These results indicate that the added CD4⁺ T cell derived from saline- and B cell-administered recipients included BALB/c reactive clones and enhanced CD8⁺ T cell reactivity. The response of C3129F1 CD8⁺ T cells stimulated with B6-stimulator cells was enhanced when CD4⁺ T cells derived from saline-treated recipient mice were added to the MLR. On the other hand, the response was neither enhanced nor suppressed when B cell-treated recipient-derived CD4⁺ T cells were added (Fig. 5C, S5). Taken together, these results suggest that the administration of donor B cells induced alloreactive T cell unresponsiveness in the recipients.

Donor B cell administration promoted engraftment of allogeneic iPSCs

It is known that PSCs form teratomas when transplanted into immunodeficient mice(2). In addition, iPSCs are able to develop teratoma in allogenic settings when the recipients are immunosuppressed(26, 44, 45). Therefore, we tested whether B6-derived iPSC teratoma formation is promoted in recipients treated with B6-derived B cells. In saline-treated recipients, signals were detected in only one out of three recipients 15 days after Luc-iPSC transplantation (Fig. 6A). In contrast, in the B cell-treated recipients, signals were detected in all recipients from day 15 to 50 after transplantation (Fig. 6A). In addition, a significantly higher signal was detected in the B-cell-treated recipients than in the control group from day 15 to 50 post-transplantation (Fig. 6B). On day 50 after the transplantation, teratoma formation was observed

368 in all transplanted recipients in the B cell-treated group, while only one recipient in the saline-
369 treated group had teratoma formation. The weight of the teratoma formed in the B cell
370 transferred group was significantly higher than in the control group (Fig. 6D). These results
371 indicate that donor B cell administration promotes teratoma formation in allogeneic recipients
372 and suggest that B cell-based DST may be effective for immune regulation in iPSC-based
373 transplantation.

374

Discussion

In this study, we have successfully identified a blood cell type for DST sufficient to regulate rejection in MHC-matched allogeneic transplantation. Isolated donor B cells alone were sufficient to control rejection of allogeneic skin grafts and iPSC cell grafts and to induce immune tolerance to the donors. results provide an important perspective for developing new immunoregulatory methods in allogeneic iPSC-based transplantation.

It has been reported that when MHC-type matched donor-derived iPSC cells were used, graft survival was maintained with the use of immunosuppressive agents, but T-cell infiltration of the graft was observed even with the use of immunosuppressive agents(21, 24). Therefore, even if the risk of rejection is reduced by matching MHC types, immune regulation is still essential. In addition, a strategy to avoid direct T cell responses to allografts by MHC knockout of iPSCs is currently being developed and has succeeded in preventing rejection in experimental models(12–15). However, in organ and tissue transplantation, T cells can also be activated through indirect pathways, causing rejection independent of MHC expression in the graft(46–49). Therefore, even if MHC-matched or -knockout iPSCs are used, it is necessary to develop a method to suppress allo-reactive T cells. In the present study, we showed that the administration of isolated donor B cells, particularly living ones, selectively prolonged the survival of donor-type skin and iPSC grafts. Furthermore, T cell activation by the indirect pathway was reduced in recipients treated with the donor B cell administration, suggesting that

394 recipient T cells became tolerant to the donor. These results suggest that the combination of
395 MHC gene-editing technology and the current B cell-based tolerance induction method can be
396 applied to iPSC-using transplantation to control T cell activation through direct and indirect
397 pathways and to prevent graft dysfunction. Previous reports have shown that DST was effective
398 in immunomodulation of many types of grafts(27). In this study, skin tissue and iPSCs were
399 used as grafts, but based on the previous DST reports, it is expected that the B cell-based
400 immunoregulation method will be effective in controlling the rejection of various types of
401 iPSC-derived therapeutic cells.

402 Mechanisms including T cell unresponsiveness, induction of regulatory T cells, and T
403 cell deletion have been reported to contribute to the tolerance induction by DST or B cell
404 administration(38, 40–43). In the present study, the data showed that administration of donor
405 B cells neither increased the percentage of regulatory T cells nor decreased the frequency of
406 donor-reactive T cells. On the other hand, when second skin transplantation was performed on
407 recipient mice treated with donor B cells, some individuals rejected the donor skin grafts. This
408 result indicates that although there was a decrease in reactivity, donor-reactive T-cell clones
409 still remained. It has been reported that apoptotic blood cells were used as a source of donor
410 antigens to induce T-cell tolerance(50, 51). However, administration of X-irradiated B cells
411 does not induce tolerance, indicating that survival of donor B cells is important for the tolerance
412 induction. In fact, it is known that B cells with forced expression of ovalbumin (OVA)

peripherally induce clonal deletion by apoptosis and unresponsiveness of transferred OVA-specific T cells *in vivo*(52). Although, in the present study, T cell clone deletions were not observed, it is possible that T cell unresponsiveness was induced by a similar mechanism. It has long been understood that antigen presentation in insufficient co-stimulatory signals induces T cell unresponsiveness(53). On the other hand, in the present study, donor B cells upregulated expression of CD80/CD86 molecules after transfer to the recipients. However, It has been reported that *in vitro* co-culture of antigen-pulsed B cells and the T cells with cognate TCR induced T cell unresponsiveness even when the expression of CD80/CD86 on the B cells was upregulated(54). Although it was not clear whether donor B cells and recipient T cells interacted directly in this study, it is consistent with this report that upregulation of co-stimulatory molecule expression on the donor B cells would still induce T cell unresponsiveness.

As a limitation of this study, there is a possibility that minor antigen-mismatched donor-reactive T cells could not be detected due to their small population, leading to the failure to detect the change in total Tregs and T cell clones. For the same reason, the low power of detection of minor antigen-mismatched donor-reactive T cells by MLR makes it difficult to analyze changes in reactivity to minor antigen-mismatched donors other than B6. Further analysis is needed to clarify these issues.

When the donor B cell-based immunoregulation is applied to iPSC-using regenerative

medicine, it is necessary to prepare live B cells derived from the same iPSC from which the therapeutic cells are derived. Regarding the generation of B cells from PSCs, it has been reported that B-1 progenitor cell-like cells have been successfully generated(55). On the other hand, most of the splenic B cells used in this study were B-2 cells and most of the donor B cells distributed in the recipient's body were B-2 cells. So far, it has not been clarified which B cell subset is responsible for the tolerance induction. In this study, when the number of cell injections into the recipient was reduced to 1/10, the efficiency of tolerance induction was remarkably decreased (Fig. S1). Therefore, it seems important to establish an efficient differentiation method of B cell subsets from iPSCs which is important for the immune regulation to apply them for the tolerance induction protocol.

In summary, this study demonstrates a basic concept that pretransplant administration of donor-derived live B cells can induce tolerance to MHC-matched allografts, that may be applicable as an immunomodulatory method in iPSC-using regenerative medicine.

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460 **Author Contributions**

461 TM and KS designed the study. TM, RO, AS, and HW performed experiments and data
462 analysis. KT established the experimental conditions for iPSC transplantation. HY and YH
463 performed NGS and TCR repertoire analysis. TM, RO and KS wrote manuscript.

464

465 *Conflicts of interest statement:* the authors declared no conflicts of nterest.

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Reference

1. Siu, J. H. Y., V. Surendrakumar, J. A. Richards, and G. J. Pettigrew. 2018. T cell Allorecognition Pathways in Solid Organ Transplantation. *Front. Immunol.* 9: 1–9.
2. Takahashi, K., and S. Yamanaka. 2006. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell* 126: 663–676.
3. Takahashi, K., K. Tanabe, M. Ohnuki, et al. 2007. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* 131: 861–872.
4. Yamanaka, S. 2020. Pluripotent Stem Cell-Based Cell Therapy—Promise and Challenges. *Cell Stem Cell* 27: 523–531.
5. Mandai, M., A. Watanabe, Y. Kurimoto, et al. 2017. Autologous Induced Stem-Cell–Derived Retinal Cells for Macular Degeneration. *N. Engl. J. Med.* 376: 1038–1046.
6. Blau, H. M., and G. Q. Daley. 2019. Stem Cells in the Treatment of Disease. *N. Engl. J. Med.* 380: 1748–1760.
7. Nakatsuji, N., F. Nakajima, and K. Tokunaga. 2008. HLA-haplotype banking and iPS cells. *Nat. Biotechnol.* 26: 739–740.
8. Taylor, C. J., S. Peacock, A. N. Chaudhry, J. A. Bradley, and E. M. Bolton. 2012. Generating an iPSC bank for HLA-matched tissue transplantation based on known donor and recipient hla types. *Cell Stem Cell* 11: 147–152.
9. Umekage, M., Y. Sato, and N. Takasu. 2019. Overview: an iPS cell stock at CiRA.

487 *Inflamm. Regen.* 39: 17.

488 10. Zachary, A. A., and M. S. Leffell. 2016. HLA mismatching strategies for solid organ
489 transplantation - a balancing act. *Front. Immunol.* 7: 1–14.

490 11. Opelz, G., and B. Döhler. 2007. Effect of human leukocyte antigen compatibility on
491 kidney graft survival: Comparative analysis of two decades. *Transplantation* 84: 137–143.

492 12. Deuse, T., X. Hu, A. Gravina, et al. 2019. Hypoimmunogenic derivatives of induced
493 pluripotent stem cells evade immune rejection in fully immunocompetent allogeneic
494 recipients. *Nat. Biotechnol.* 37: 252–258.

495 13. Gornalusse, G. G., R. K. Hirata, S. E. Funk, et al. 2017. HLA-E-expressing pluripotent
496 stem cells escape allogeneic responses and lysis by NK cells. *Nat. Biotechnol.* 35: 765–772.

497 14. Han, X., M. Wang, S. Duan, et al. 2019. Generation of hypoimmunogenic human
498 pluripotent stem cells. *Proc. Natl. Acad. Sci. U. S. A.* 116: 10441–10446.

499 15. Xu, H., B. Wang, M. Ono, et al. 2019. Targeted Disruption of HLA Genes via CRISPR-
500 Cas9 Generates iPSCs with Enhanced Immune Compatibility. *Cell Stem Cell* 24: 566-578.e7.

501 16. Naesens, M., and D. Ph. 2019. Genomic Mismatch at LIMS1 Locus and Kidney
502 Allograft Rejection . *N. Engl. J. Med.* 381: e16.

503 17. Reindl-Schwaighofer, R., A. Heinzl, A. Kainz, et al. 2019. Contribution of non-HLA
504 incompatibility between donor and recipient to kidney allograft survival: genome-wide
505 analysis in a prospective cohort. *Lancet* 393: 910–917.

506 18. Spierings, E. 2014. Minor histocompatibility antigens: Past, present, and future. *Tissue*
507 *Antigens* 84: 374–360.

508 19. Il Yoo, K., J. Y. Jeon, S. J. Ryu, G. Nam, H. Youn, and E. Y. Choi. 2015. Subdominant
509 H60 antigen-specific CD8 T-cell response precedes dominant H4 antigen-specific response
510 during the initial phase of allogeneic skin graft rejection. *Exp. Mol. Med.* 47: e140-12.

511 20. Chen, Y., Y. Demir, A. Valujskikh, and P. S. Heeger. 2003. The Male Minor
512 Transplantation Antigen Preferentially Activates Recipient CD4 + T Cells through the
513 Indirect Presentation Pathway In Vivo . *J. Immunol.* 171: 6510–6518.

514 21. Kawamura, T., S. Miyagawa, S. Fukushima, et al. 2016. Cardiomyocytes Derived from
515 MHC-Homozygous Induced Pluripotent Stem Cells Exhibit Reduced Allogeneic
516 Immunogenicity in MHC-Matched Non-human Primates. *Stem Cell Reports* 6: 312–320.

517 22. Shiba, Y., T. Gomibuchi, T. Seto, et al. 2016. Allogeneic transplantation of iPS cell-
518 derived cardiomyocytes regenerates primate hearts. *Nature* 538: 388–391.

519 23. Morizane, A., T. Kikuchi, T. Hayashi, et al. 2017. MHC matching improves engraftment
520 of iPSC-derived neurons in non-human primates. *Nat. Commun.* 8: 1–12.

521 24. Aron Badin, R., A. Bugi, S. Williams, et al. 2019. MHC matching fails to prevent long-
522 term rejection of iPSC-derived neurons in non-human primates. *Nat. Commun.* 10: 4357.

523 25. Murata, T., H. Wada, R. Otsuka, et al. 2020. Establishment of an experimental model for
524 MHC homo-to-hetero transplantation. *Sci. Rep.* 10: 1–8.

525 26. Kamatani, T., R. Otsuka, T. Murata, et al. 2022. Evaluation of immunosuppression
526 protocols for MHC-matched allogeneic iPS cell-based transplantation using a mouse skin
527 transplantation model. *Inflamm. Regen.* 42: 4.

528 27. Shaw, B. I., J. R. Ord, C. Nobuhara, and X. Luo. 2021. Cellular Therapies in Solid Organ
529 Allotransplantation: Promise and Pitfalls. *Front. Immunol.* 12: 1–10.

530 28. van Twuyver, E., R. J. D. Mooijart, I. J. M. ten Berge, et al. 1991. Pretransplantation
531 blood transfusion revisited. *N. Engl. J. Med.* 325: 1210–3.

532 29. Jovičić, S., V. Ležaić, R. Simonović, and L. Djukanović. 2011. Beneficial effects of
533 donor-specific transfusion on renal allograft outcome. *Clin. Transplant.* 25: 317–324.

534 30. Nagata, S., and T. Suda. 1995. Fas and Fas ligand: lpr and gld mutations. *Immunol. Today*
535 16: 39–43.

536 31. Garrod, K. R., and M. D. Cahalan. 2008. Murine skin transplantation. *J. Vis. Exp.* 5–6.

537 32. Morris, H., S. DeWolf, H. Robins, et al. 2015. Tracking donor-reactive T cells: Evidence
538 for clonal deletion in tolerant kidney transplant patients. *Sci. Transl. Med.* 7: 272ra10.

539 33. Fast, L. D., C. R. Valeri, and J. P. Crowley. 1995. Immune responses to major
540 histocompatibility complex homozygous lymphoid cells in murine F1 hybrid recipients:
541 Implications for transfusion- associated graft-versus-host disease. *Blood* 86: 3090–3096.

542 34. Dwyre, D. M., and P. V. Holland. 2008. Transfusion-associated graft-versus-host disease.
543 *Vox Sang.* 95: 85–93.

544 35. Ng, Y. H., M. H. Oberbarnscheidt, H. C. K. Chandramoorthy, R. Hoffman, and G.
545 Chalasani. 2010. B cells help alloreactive T cells differentiate into memory T cells. *Am. J.*
546 *Transplant.* 10: 1970–1980.

547 36. Khan, A., Y. Tomita, and M. Sykes. 1996. Thymic dependence of loss of tolerance in
548 mixed allogeneic bone marrow chimeras after depletion of donor antigen. Peripheral
549 mechanisms do not contribute to maintenance of tolerance. *Transplantation* 62: 380–7.

550 37. Wells, S. M., A. B. Kantor, and A. M. Stall. 1994. CD43 (S7) expression identifies
551 peripheral B cell subsets. *J. Immunol.* 153: 5503–15.

552 38. Minagawa, R., S. Okano, Y. Tomita, et al. 2004. The critical role of Fas-Fas ligand
553 interaction in donor-specific transfusion-induced tolerance to H-Y antigen. *Transplantation*
554 78: 799–806.

555 39. Khan, A. R., E. Hams, A. Floudas, T. Sparwasser, C. T. Weaver, and P. G. Fallon. 2015.
556 PD-L1hi B cells are critical regulators of humoral immunity. *Nat. Commun.* 6: 1–16.

557 40. Abe, Y., H. Urakami, D. Ostanin, et al. 2009. Induction of Foxp3-expressing regulatory
558 T-cells by donor blood transfusion is required for tolerance to rat liver allografts. *PLoS One*
559 4: 1–9.

560 41. Zhang, G. Y., M. Hu, D. Watson, Y. M. Wang, J. F. Knight, and S. I. Alexander. 2020.
561 Indirectly Activated Treg Allow Dominant Tolerance to Murine Skin-grafts Across an MHC
562 Class I Mismatch After a Single Donor-specific Transfusion. *Transplantation* 104: 1385–

563 1395.

564 42. Iwakoshi, N. N., J. P. Mordes, T. G. Markes, N. E. Phillips, A. A. Rossini, and D. L.
565 Greiner. 2000. Treatment of Allograft Recipients with Donor-Specific Transfusion and Anti-
566 CD154 Antibody Leads to Deletion of Alloreactive CD8 + T Cells and Prolonged Graft
567 Survival in a CTLA4-Dependent Manner. *J. Immunol.* 164: 512–521.

568 43. Okabe, K., Y. Yamaguchi, E. Takai, et al. 2001. CD45RC- $\gamma\delta$ + T-cell infiltration is
569 associated with immunologic unresponsiveness induced by prior donor-specific blood
570 transfusion in rat hepatic allografts. *Hepatology* 33: 877–886.

571 44. Rong, Z., M. Wang, Z. Hu, et al. 2014. An effective approach to prevent immune
572 rejection of human ESC-derived allografts. *Cell Stem Cell* 14: 121–130.

573 45. Pearl, J. I., A. S. Lee, D. B. Leveson-Gower, et al. 2011. Short-term immunosuppression
574 promotes engraftment of embryonic and induced pluripotent stem cells. *Cell Stem Cell* 8:
575 309–317.

576 46. He, C., and P. S. Heeger. 2004. CD8 T Cells Can Reject Major Histocompatibility
577 Complex Class I-Deficient Skin Allografts. *Am. J. Transplant.* 4: 698–704.

578 47. Valujskikh, A., O. Lantz, S. Celli, P. Matzinger, and P. S. Heeger. 2002. Cross-primed
579 CD8+T cells mediate graft rejection via a distinct effector pathway. *Nat. Immunol.* 3: 844–
580 851.

581 48. Kapessidou, Y., C. Habran, S. Buonocore, et al. 2006. The replacement of graft

endothelium by recipient-type cells conditions allograft rejection mediated by indirect
 pathway CD4⁺ T cells. *Transplantation* 81: 726–735.

49. Honjo, K., X. Y. Xu, and R. P. Bucy. 2004. CD4⁺ T-cell receptor transgenic T cells alone
 can reject vascularized heart transplants through the indirect pathway of alloantigen
 recognition. *Transplantation* 77: 452–455.

50. Singh, A., S. Ramachandran, M. L. Graham, et al. 2019. Long-term tolerance of islet
 allografts in nonhuman primates induced by apoptotic donor leukocytes. *Nat. Commun.* 10:
 1–16.

51. Luo, X., K. L. Pothoven, D. McCarthy, et al. 2008. ECDI-fixed allogeneic splenocytes
 induce donor-specific tolerance for long-term survival of islet transplants via two distinct
 mechanisms. *Proc. Natl. Acad. Sci. U. S. A.* 105: 14527–14532.

52. Werner-Klein, M., C. Dresch, P. Marconi, and T. Brocker. 2007. Transcriptional
 Targeting of B Cells for Induction of Peripheral CD8 T Cell Tolerance. *J. Immunol.* 178:
 7738–7746.

53. Schwartz, R. H. 2003. T cell anergy. *Annu. Rev. Immunol.* 21: 305–334.

54. Höllsberg, P., V. Batra, A. Dressel, and D. A. Hafler. 1996. Induction of anergy in CD8 T
 cells by B cell presentation of antigen. *J. Immunol.* 157: 5269–76.

55. Lin, Y., M. Kobayashi, N. Azevedo Portilho, et al. 2019. Long-Term Engraftment of
 ESC-Derived B-1 Progenitor Cells Supports HSC-Independent Lymphopoiesis. *Stem Cell*

601 *Reports* 12: 572–583.

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604 **A list of all supporting information**

605 **Supplementary Figures**

606 Figure 1: Evaluation of the number of B cells required for immunoregulation and the
607 requirement of pretreatment.

608 Figure 2: Evaluation of PD-L1 and FasL expression on transfused B cells.

609 Figure 3: Comparison of the frequency of donor-reactive T cells between groups

610 **Supplementary Tables**

611 Table 1: List of antibodies used for flow cytometry analysis

612

Figure legends

Figure 1 Donor splenocyte or splenic B cell transfusion promotes long-term survival of skin grafts

(A) The procedures of transplantation experiment. CD4 and CD8-depleting monoclonal antibodies were administered to C3129F1 mice 13 and 8 days before skin transplantation, and total body irradiation was performed seven days before skin transplantation. B6-derived blood cells were transferred intravenously, and skin transplantation was performed one week later. (B) C3129F1 (syngeneic), B6, and BALB/c (third party) derived skin grafts were transplanted into recipients (n=5-9 per group) infused with saline or donor-derived splenocytes (3×10^7 cells), and the graft survival is shown as Kaplan-Meier plots. (C) The skin graft survival in recipient mice administered with isolated B cells or T cells (3×10^7 cells, each). (D) Macroscopic view of skin grafts of B6 B cell-transferred C3129F1 recipients 60 days after skin grafting. (E) The skin graft survival in recipient mice administered with BM-DC (1.5×10^7 cells) or BM-M (2.0×10^7 cells). (F) The survival of second skin grafts to B cell-treated recipient mice. Significance was assessed using the log-rank test (* $p < 0.05$, ** $p < 0.01$).

Figure 2 Anti-donor T cell reactivity is reduced in donor B cell-transfused C3129F1 recipient mice

(A, B) Results of mixed lymphocyte reaction. C3129F1 recipient mice were pretreated with

the same protocol as in Figure 1A and B6-derived B cells (3×10^7) were administered. Eight weeks later, recipient-derived CD19⁺ splenocytes were stained with CFSE and used as responders. The responder cells were co-cultured with irradiated C3129F1, B6, or BALB/c-derived splenocytes for four days and the cells were analyzed using a flow cytometer. (A) Representative FACS plots are shown. (B) The percentage of cells with decreased CFSE fluorescence intensity is shown in the graph. The experiment was performed under the same conditions for three wells, and the graphs represent the mean $\pm$ SD. Statistical analysis was performed by Student's t-test. * $p < 0.05$, ** $p < 0.01$.

Figure 3 Donor B cells were distributed in the C3129F1 recipients' peripheral blood, spleen, and lymph nodes

(A-D) CD45.1⁺ B6 splenic B cells (3×10^7 cells) were administered to C3129F1 (CD45.2⁺) using the same protocol as above, and the percentages of CD45.1⁺ cells in B cells in peripheral blood, spleen, lymph nodes, and thymus at 1, 3, 5, and 7 weeks after cell transfer were shown. Five recipients at each time point were analyzed, and flow cytometry dot plots (A) and mean $\pm$ SE (B) were shown. (C) FACS plots of B cell subsets in WT B6 spleen or recipient's spleen one week after transfusion of donor B cells (CD45.1⁺) are shown. (D) Comparison of B cell subsets 1 week after donor B cell administration ($n=5$ each group). Statistical analysis was performed using student's t-test.

Figure 4 Transfused B cells upregulate expression of MHC and co-stimulatory molecules in lymph nodes and spleen

(A-D) The MFI of H-2K^b (A), I-A^b (B), CD80 (C), and CD86 (D) were analyzed by flow cytometry in lymph nodes and spleen of C3129F1 after one week of CD45.1⁺ B6 splenic B cell administration. Three individual mice were analyzed, and Mean $\pm$ SE is shown. Statistical analysis was conducted by Students' t-test, * $p < 0.05$, ** $p < 0.01$. (E) X-ray irradiated (40 Gy) B6 B cells were transfused to C3129F1 by the protocol described above. One week later, the recipients were transplanted with C3129F1, BALB/c, and B6 skin grafts. Statistical analysis was performed by log-rank test, showing * $p < 0.05$, ** $p < 0.01$.

Figure 5 CD8⁺ T cell help capacity by CD4⁺ T cells is decreased in donor B cell transfused recipients

(A) Percentage of Tregs in recipients' thymus, spleen, and lymph nodes 2-3 weeks after B-cell administration. Statistical analysis was performed by Tukey's HSD test. * $p < 0.05$. (B) T-cell repertoires in the spleen of Naïve, saline, and B-cell administrated recipients were analyzed, and the number of donor-reactive clones detected was compared. Statistical analysis was performed by Fisher's exact test (* $p < 0.05$). (C) The percentage of proliferated CD8⁺ T cells was shown when the CD4⁺ cells from saline or B cell administrated recipients were added into

the MLR cultures of CFSE-labeled naïve mouse CD19⁺ splenocytes (2×10^5) and irradiated donor splenocytes (1×10^5). Student's t-test was performed to compare the two groups in each ratio. Graphs represent Mean+SD or Mean-SD, meaning **p<0.01, *p<0.05.

Figure 6 Donor splenic B cell transfusion promotes long-term survival of iPSC grafts

C3129F1 recipient mice were treated with B6 splenic B cells (3×10^7) as in Fig. 1A. Luc-iPSCs were injected subcutaneously into the recipient mice using Matrigel 3 weeks after B6 B cell transfusion. Recipient mice were intraperitoneally injected with D-luciferin, and luminescence intensity by IVIS was observed at 5, 10, 15, 20, 30, and 50 days after transplantation of Luc-iPSCs. (A-B) The luminescence intensity in each group (n=3 per group) over time (Mean±SE). (C) Photograph of teratoma at 50 days after transplantation of Luc-iPS cells. (D) teratoma weight at 50 days after transplantation of Luc-iPS cells. Statistical analysis was performed using student's t-test at each analysis time point, **p<0.01, *p<0.05.

Figure 1

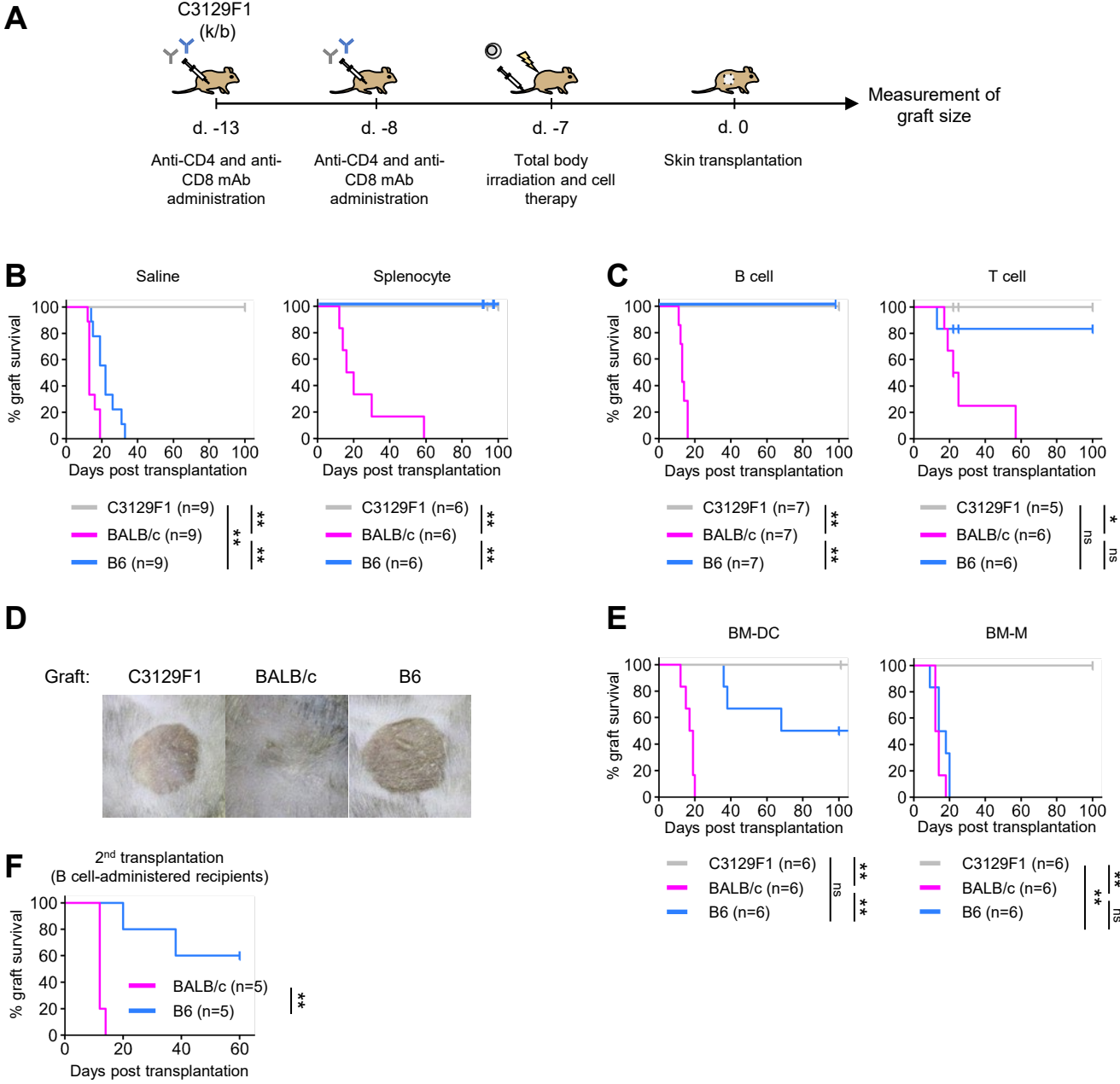


Figure 2

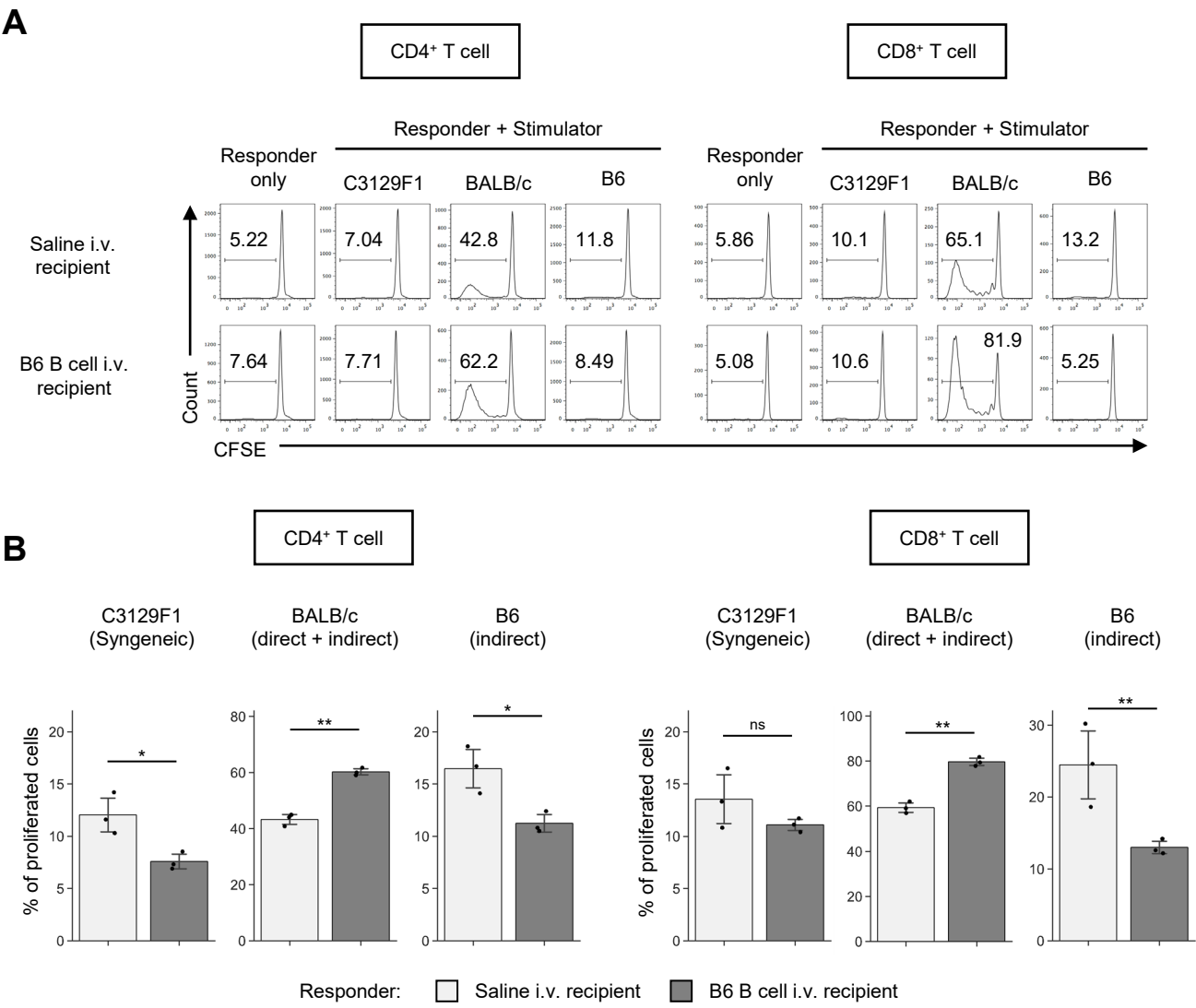
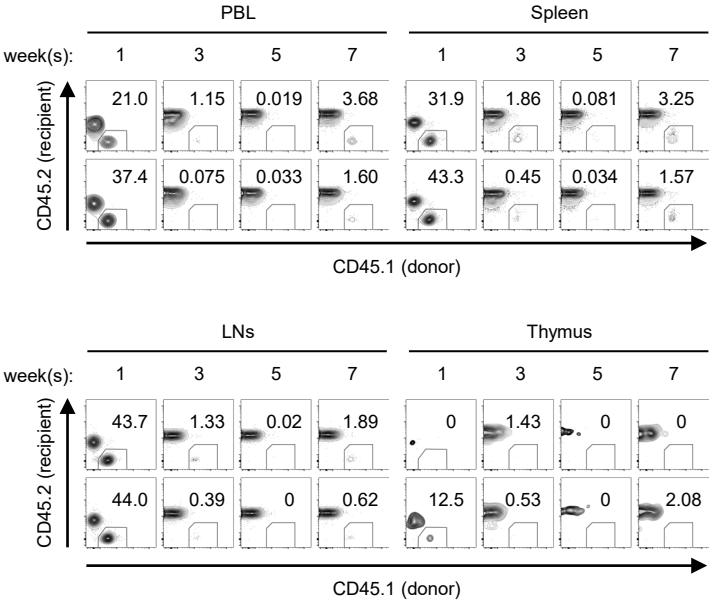
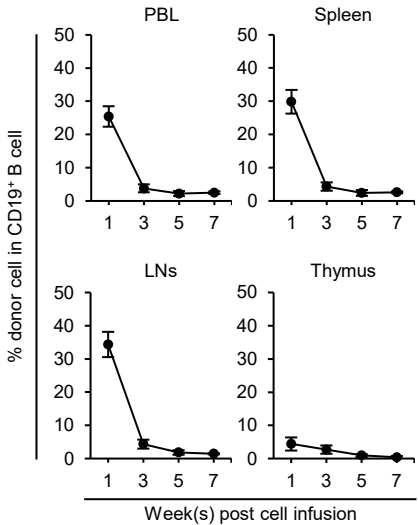


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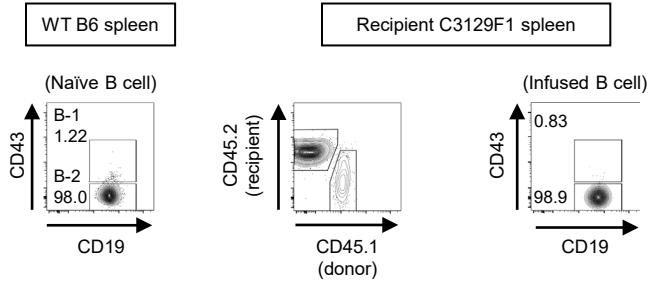
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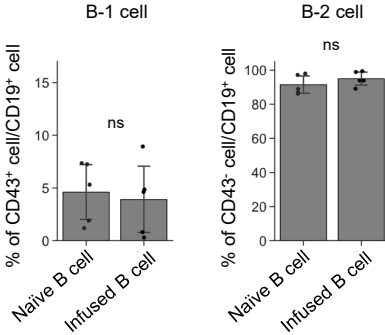


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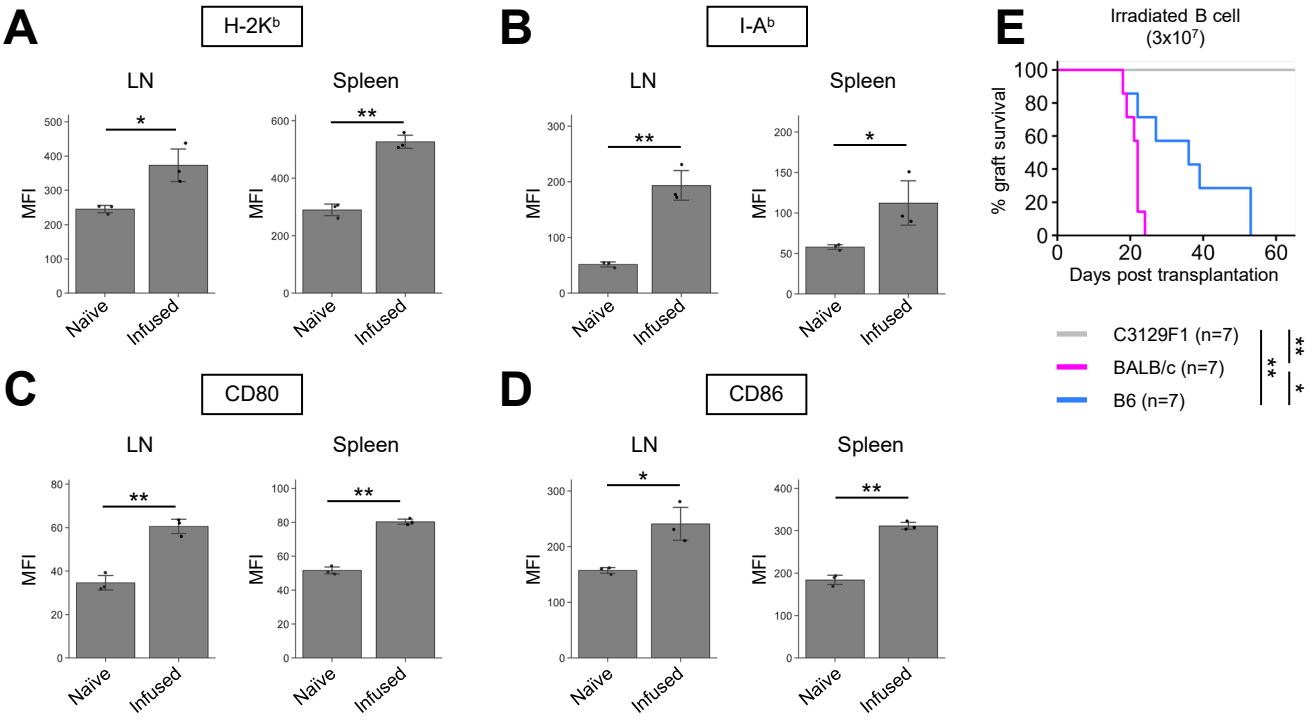


Figure 5

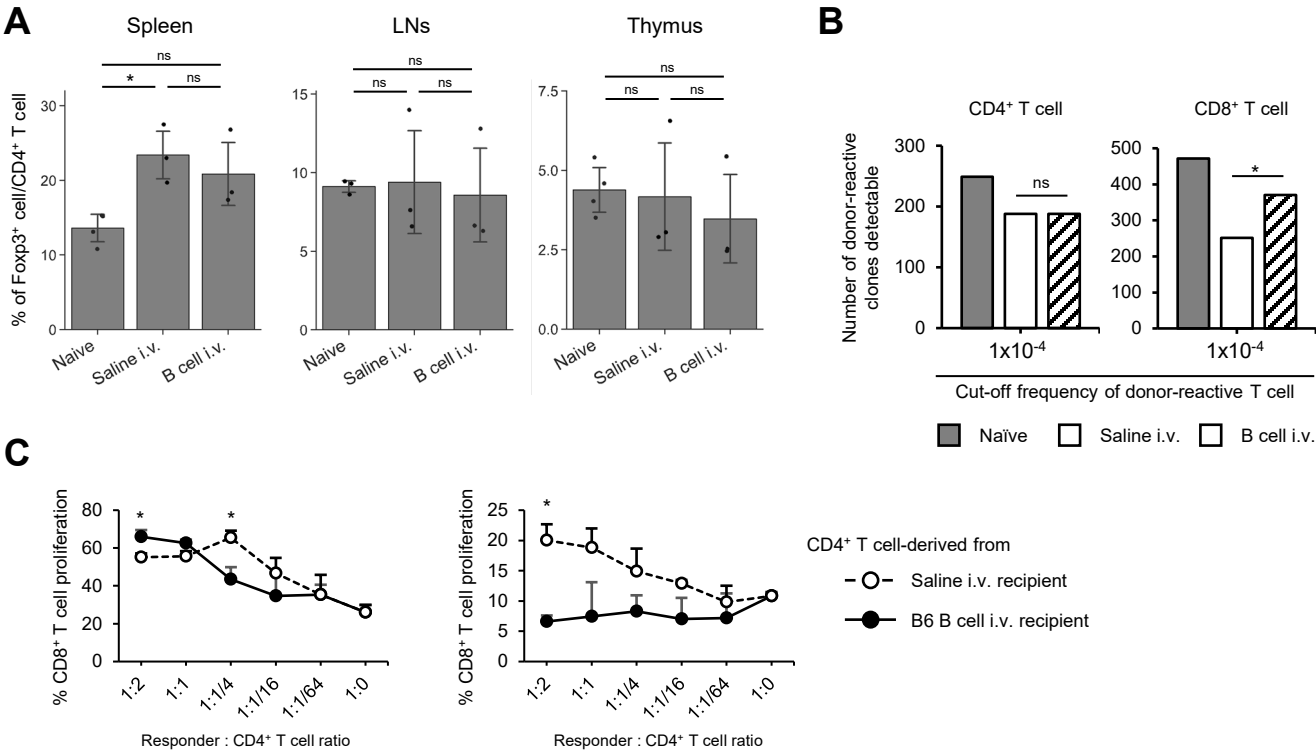


Figure 6

