Donor mesenchymal stem cells trigger chronic graft-versus-host disease following minor antigen-mismatched bone marrow transplantation

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Chronic graft-versus-host disease (cGVHD) is a complication after minor antigen mismatched bone marrow transplantation (BMT) characterized by an autoimmune-type reaction in various organs. Aberration in T cell regulation is involved, with donor mesenchymal stem cells (MSCs) playing a possible role in immunomodulation. In a minor-antigen mismatched mouse BMT model, transplantation of mismatched, but not syngeneic MSCs triggered the onset of cGVHD, and was associated with fibrosis, increased IL-6 secretion, decreased Foxp3$^+$ regulatory T cells and increased Th17 in the peripheral blood. Mismatched MSCs alone were sufficient to induce cGVHD, while removal of donor MSCs rescued mice from cGVHD. RAG2 knockout recipient mice did not suffer cGVHD, indicating that host T cells were involved. Residual host-derived T cells were significantly higher in cGVHD patients compared to non-cGVHD patients. In conclusion, donor MSCs react with residual host T cells to trigger the progression of cGVHD.

Chronic GVHD (cGVHD) has distinct clinical findings, which resemble features of autoimmune disease such as systemic sclerosis or Sjögren's syndrome involving exocrine glands [1,2], and is believed to be distinct from the potentially lethal acute form of GVHD. This autoimmune phenotype has been attributed to donor-derived T cells that escape negative selection by the host thymus damaged by acute GVHD [3,4].

Bone marrow transplantation with 8 week-old donor B10.D2 (H-2d) mice and recipient BALB/c mice (H-2d) has been reported as a MHC-compatible, minor antigen (miHA)–incompatible model of cGVHD (Supplementary Fig. 1a) [5]. The phenotype of the mice closely resembles clinical samples of patients suffering from cGVHD (Supplementary Fig. 1b). Signs of cGVHD appear by 3 weeks after BMT, and progresses to full-blown disease by 8 weeks characterized by low tear volume and excessive fibrosis of the lacrimal gland, conjunctiva, salivary gland, skin, lung, liver and intestine (Supplementary Fig. 1c). Accumulation of donor-derived fibroblasts in fibrotic lesions surrounding exocrine ducts was observed (Supplementary Fig. 1d), which was similar to human patients as shown in our previous report [6]. These results suggested that donor-derived fibroblasts were part of the pathological process leading to cGVHD.

Multipotent mesenchymal stem/stromal cells (MSCs) in the bone marrow differentiate into several mesenchymal lineages such as fibroblasts, adipocytes, osteocytes and chondrocytes [7,8]. However, the *in vivo* dynamics of MSCs after WBMT are still unknown due to the lack of specific markers. Furthermore, a crucial step involving *in vitro* expansion was required to isolate these cells, which may modify their phenotype and function [9]. Most current information on MSCs comes from such *in vitro* studies of adherent cells referred to as fibroblast CFUs (CFU-Fs) [7,8,10,11], which are a heterogeneous population of cells at best. Tracing the fate of MSCs following transplantation is complicated due to these reasons. We have recently succeeded in prospectively isolating

murine MSCs based on their expression of PDGF receptor α and Sca-1 (PDGFRα^{+}/ Sca-1^{+} (PαS) cells) [12]. Isolated PαS-MSCs without *in vitro* expansion can differentiate into hematopoietic niche cells, osteoblasts and adipocytes after systemic *in vivo* transplantation [13]. In this study, we sought to answer the debated role of donor MSCs in the pathogenesis of the autoimmune-like phenotype of cGVHD using prospectively isolated MSCs and HSCs in a mouse model of cGVHD.

RESULTS

Minor antigen mismatched HSC and MSC co-transplantation

In order to elucidate the role of donor-derived fibroblasts, we first established a modified cGVHD model by co-transplanting isolated hematopoietic stem cells (HSCs) and MSCs. PαS-MSCs and side population (SP) HSCs (Supplementary Fig 2a) from B10.D2 bone marrow were individually isolated by cell sorter and systemically co-transplanted into BALB/c mice (Fig. 1a). Unlike the original WBMT model which co-transplanted splenic cells, the graft in our experiments does not include mature, differentiated cells both in the hematopoietic and non-hematopoietic fractions. In addition, there is lineage exclusivity, where all hematopoietic lineage cells are derived from SP-HSCs, while non-hematopoietic cells are of donor PαS-MSC origin [13,14]. This modified co-transplantation model induced systemic fibrosis (Fig. 1b), which was indistinguishable from that of cGVHD induced by the conventional WBMT model (Supplementary Fig 1c). In addition, cells double positive

for the fibroblast marker HSP47 and EGFP were detected in cGVHD tissue when $EGFP^+$ PαS-MSCs were transplanted with wild type B10.D2 SP cells (Fig. 1c, d). Approximately 42.9 ± 7.3% of the $HSP47^+$ fibroblasts in cGVHD tissue were GFP^+, indicating that the majority of fibroblasts were derived from donor PαS-MSCs (Fig.1d). On the other hand, a notable number of cells derived from mismatched donor SP-HSCs also migrated to fibrotic lesions, but none of the cells expressed HSP47 (Fig. 1c, e). This shows that the donor fibroblasts originated from purified MSCs but not HSCs.

Mismatched PαS-MSCs induce cGVHD

In order to determine if either mismatched MSCs or HSCs play a dominant role in cGVHD, we performed the following transplantation. BALB/c mice were transplanted with a combination of BALB/c (syngeneic) HSCs and B10.D2 (mismatched) MSCs, or vice-versa (Fig. 2a). Surprisingly, progressive fibrosis was observed in mice receiving mismatched MSCs combined with syngeneic HSCs (Fig. 2b, Supplementary Fig. 2b, lower). On the other hand, recipients of mismatched HSCs combined with syngeneic MSCs were either normal, or only suffered a forme-fruste form of the disease (Fig. 2b, Supplementary Fig. 2b, upper). Affected lesions in the mismatched MSC transplanted-mice had significantly higher $HSP47^+$ fibroblasts (Fig. 2c, Supplementary Fig. 2c) and lacrimal gland function was reduced as quantitated by tear volume (Fig. 2d). The severity of cGVHD-associated fibrosis triggered by mismatched MSCs was dose dependent on the amount of transplanted

MSCs (Fig. 2e), which taken together, strongly suggest that the presence of mismatched MSCs triggers the onset of cGVHD.

Deletion of PαS-MSCs prevents cGVHD

To confirm the requirement of mismatched MSCs for the onset of cGVHD, we tried removing PαS cells from donor bone marrow (MSC(-)) to see if this prevents the onset of cGVHD. As expected, MSC(-) mismatched WBM transplanted recipients showed minimal pathological changes (Fig. 3a-b, Supplementary Fig. 3a). $HSP47^{+}$ fibroblasts associated with fibrotic lesions were also reduced (Fig. 3c-d, Supplementary figure 3b). Mean tear secretion volume in mismatched WBM-transplanted mice gradually decreased reaching statistical significance at 8 weeks after transplantation, but was preserved in the MSC(-) group (Fig. 3e). Therefore, the histological change and functional loss of the lacrimal gland was rescued by MSC depletion from donor cells, suggesting that mismatched MSCs alone can cause cGVHD.

Host T cells are required for the progression of cGVHD

The progression of cGVHD in this mouse model is T cell-dependent as shown by the lack of fibrotic lesions when BALB/c background nu/nu mice were used as recipients of mismatched B10.D2 MSCs (Supplementary Fig. 4). The next question was to determine whether these pathogenic T cells were of donor origin, or residual host T cells that have lost

self-tolerance. It is conceivable that mismatched donor MSCs integrate into the thymic epithelium and induce donor HSC-derived T cells that recognize host tissue as non-self within this time frame. However, this is unlikely since $EGFP^+$ MSC-derived cells did not integrate into the cortical region of the thymic epithelium 3 weeks after transplantation (Supplementary figure 2d). On the other hand, mature T cells often escape from radiation damage and remain as residual host-derived T cells in the peripheral blood of recipient mice [15]. Therefore, both host-derived T cells and donor HSC-derived T cells are believed to exist in the cGVHD mice. To determine T cell origin, we used BALB/c background RAG2KO mice that lack T and B cells as recipients or donors of HSCs. The former would lack recipient T cells, while the latter would not provide donor T cells after transplantation. At 3 weeks after transplantation, fibrosis due to GVHD was limited, indicating that mismatched MSCs alone do not induce clinical signs of cGVHD without competent recipient-derived T cells (Fig. 4a). On the other hand, when mismatched MSCs and RAG2KO mouse-derived HSCs were transplanted into wild type BALB/c recipients, cGVHD-associated fibrosis was observed (Fig. 4b) also shown by a statistically higher number of $HSP47^+$ fibroblasts (Fig. 4c). These results suggest that purified mismatched PαS-MSCs reacted with recipient mature T cells to trigger cGVHD fibrosis, at least during the early onset of disease.

Activated T-cells in mismatched MSC recipients

The fact that mature host T cells acquire the ability to react to auto-antigens during cGVHD is reminiscent of an autoimmune process, and therefore, the following *in vitro* experiments were done to observe T cell activation. Various sources of T cells (from mismatched MSC-transplanted, autologous MSC-transplanted, wild type B10.D2 or wild type BALB/c mice) were co-cultured with freshly isolated PαS-MSCs or splenic dendritic cells (DC) from BALB/c or B10.D2 mice. Thy-1^{+} T cells derived from mismatched MSC-transplanted recipients proliferated in response to not only mismatched B10.D2 MSCs, but also to syngeneic BALB/c MSCs confirming the auto-reactive nature of recipient T cells (Fig. 5a). In addition, naïve T cells from wild type B10.D2 or BALB/c mice proliferated at basal levels in response to either type of MSCs, but not to DCs. We also detected elevated levels of IL-6 in the supernatant of co-cultures with T cells from mismatched MSC transplanted recipients and B10.D2 or BALB/C MSCs (Fig 5b). In contrast, IL-6 levels remained low when T cells of all sources were co-cultured with autologous or mismatched DC, indicating that antigens expressed in MSCs, but not in DCs, trigger the oligo-clonal auto-reactive T cell response in the recipient. Flowcytometry revealed that both PαS-MSCs and T cells from mismatched MSC-transplanted recipients produce IL-6 when reacted with B10.D2 MSCs *in vitro* (Fig 5c). Serum levels of IL-6 also increased in recipient mice transplanted with mismatched MSCs starting at 3 weeks after transplantation (Fig. 5d), indicating that systemic levels of IL-6 increased with the early onset of cGVHD. These results clearly show that mismatched MSCs can activate

host T cells *in vivo*, leading to the contrasting T cell proliferation and IL-6 production of T cells and MSCs *in vitro*.

Temporal increase of regulatory T-cells is suppressed with mismatched MSC

Increased IL-6 during an autoimmune process leads to the induction of Th17 cells and a decrease in regulatory T cells (Tregs) [16]. We therefore further examined whether similar events occur after mismatched MSCs transplantation. We found that $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs in peripheral blood transiently increased following lethal irradiation and syngeneic transplantation of MSCs or WBMT which recovered to basal levels by 8 weeks (Fig. 5e, f). This transient increase in Tregs, reaching statistical significance at 3 weeks, was not observed after mismatched MSC or WBM transplantation (Fig. 5f). This was followed by an increase of Th17 cells (Fig. 5g) in both the mismatched WBMT and MSC groups reaching statistical significance compared to syngeneic controls at 3 weeks in the WBMT group (Fig 5h-left), and 8 weeks in the MSC mismatch group (Fig 5h-right). These results suggest that mismatched MSCs suppress Treg production during the early phases of cGVHD, leading to the increase of Th17 cells during later stages. The results so far show that the transplantation of mismatched MSCs leads to pathological changes in major organs similar to autoimmune disease with inflammation due to Th17 cells via suppression of transient increase in Tregs.

Detection of recipient-derived T cells in cGVHD patients

In human BMT patients, it is believed that host T cells are completely replaced by donor-derived T cells in the cGVHD phase [17]. However, our results using the cGVHD model mouse showed the role of recipient-derived T cells in the onset of cGVHD. In order to confirm if our findings also applied to human cGVHD cases, we performed FISH for X- and Y-chromosomes using peripheral blood mononuclear cell samples (PBMC) from cGVHD and non-cGVHD patients who received sex-mismatched WBM transplantation (Fig. 6a). The proportion of recipient-derived T cells in PBMC ranged from 5.6 to 16.7% (9.0±4.4%) in 5 patients who developed cGVHD, while recipient-derived T cells ranged from 0.4 to 3.7% (2.1±1.4) in 4 patients who did not develop GVHD (Supplementary Table). In accordance to our hypothesis, the frequency of recipient-derived $CD3^+$ T cells was statistically higher in cGVHD patients compared to non-cGVHD patients (Fig. 6b).

DISCUSSION

The therapeutic potential of MSCs was first reported in a case of acute GVHD transfused with haploidentical MSCs with dramatic improvement of symptoms [18]. This was followed by a phase II trial of MSC infusion for severe acute GVHD anticipating that allogeneic MSCs play an immunosuppressive role following BMT [19]. While the lethal nature of acute GVHD may necessitate such and other therapeutic trials [20], the mechanisms involved are unknown other than reports suggesting the release of immunomodulatory cytokines by

MSCs trapped in the lung [12,21-23]. The pros and cons of MSC therapy should be carefully weighed before further clinical use of these cells since they seem to exert different results in acute and chronic forms of GVHD. Furthermore, since it is now possible to prospectively isolate MSCs, a thorough re-evaluation of previous works that have relied on *in vitro* expansion for the collection of MSCs is required.

We found that massive fibrosis in lacrimal and salivary gland, skin, lung, liver and intestine in a cGVHD mouse model was attributed to donor derived MSCs, and not mismatched HSCs. Our results also suggested that radio-resistant residual recipient T cells, but not donor-HSC derived de novo T cells, were activated following mismatched MSC transplantation. The development of cGVHD in our model started as early as 3 weeks, although at least 4 weeks are required for maturation of T cells to appear in the peripheral blood following purified HSC transplantation [24]. Furthermore, we found a statistically higher ratio of recipient-derived T cells remaining in patients suffering from cGVHD compared to non-cGVHD patients following WBMT. In view of all the supporting evidence, we conclude that residual host T cells are responsible for the pathogenesis of cGVHD in both this cGVHD animal model, as well as in human cGVHD patients. These results are compatible with other reports showing how residual recipient $CD4^+$ T cells regulate cGVHD [15,25].

Previous reports presented data showing the lack of lymphocyte activation by MSC co-cultures as evidence for the immuno-suppressive properties of MSCs [26,27]. In

contrast, our results show that donor-derived MSCs react with host-derived T cells in the pathogenesis of the autoimmune type pathology observed in cGVHD. Thy-1^{+} T-cells isolated from mice after mismatched BMT were activated by PαS-MSCs *in vitro,* as shown by enhanced proliferation and IL-6 secretion. The onset of cGVHD is associated with progressive loss of CD4^{+}CD25^{+}Foxp3^{+} Tregs during episodes of acute GVHD [28]. We found a similar autoimmune response in cGVHD mice characterized by repression of Treg induction, followed by an increase in Th17 effector cells without an episode of acute GVHD. In summary, donor PαS-MSCs are the initial trigger of events leading to increased levels of circulating IL-6, followed by a decrease in Tregs, and conversely an increase in Th17 cells. Activation or maturation of MSC-derived cells is probably also involved since accumulation of donor-derived fibroblasts was not observed in syngeneic transplantation, where cGVHD does not occur (Supplementary Fig. 5).

Since naïve T-cells responded to PαS-MSCs *in vitro* at low levels, we speculate that auto-reactive T cells that are only present in basal levels under normal conditions [29] recognize minor differences between B10.D2 and BALB/c MSCs. The auto-antigen responsible for the autoimmune type reaction still needs to be identified. A common antigen specifically expressed in MSCs of B10.D2 and BALB/c with different isoforms or SNIPs, is one of the most probable candidates. The lack of cGVHD following mismatched HSC transplantation suggests that hematopoietic lineage cells, including DCs, did not express such a molecule. The same cGVHD phenotype was observed when host

and recipient were reversed (ie. BALB/c MSCs transplanted to B10.D2 recipients, data not shown). This implies that both B10.D2 and BALB/c MSCs can be a primary inducer against mismatched recipient T cells. Using MHC matched strains of mice other than B10.D2 and BALB/c is required to further confirm generality. Recently, the role of host non-hematopoietic antigen presenting cells (APCs), and not hematopoietic professional APCs, including DCs, was shown to be responsible for the progression of lethal acute GVHD [30]. These non-professional APCs were suggested to be mesenchymal cells due to the expression of αSMA. Our results clearly showed that donor-derived MSCs were responsible for the onset of non-lethal cGVHD. These findings together strongly challenge conventional paradigms of GVHD. The source of mesenchymal cells (ie. donor or host) responsible for the initial trigger may be used to differentiate the acute and chronic forms of GVHD, which until recently have been differentiated by the timing of onset empirically set at 100 days after transplantation. There are other limitations to the data presented in this study. Namely, although the PαS fraction contains a $2x10^5$-fold higher concentration of clonogenic MSCs compared to WBM, the population is still not "pure". Therefore, it may include progeny of both stimulatory cells that interact with T cells and fibroblasts that directly contribute to tissue fibrosis. In order to clarify this point, further purification of MSCs will be necessary.

The clinical implications of our study cannot be ignored, for cGVHD is a major complication that can severely compromise quality of life in patients receiving bone

marrow transplants. Our data clearly indicated that depletion of MSCs from donor tissue significantly reduced cGVHD-related fibrosis in all organs examined and rescued mice from lacrimal gland dysfunction associated with cGVHD. Targeted therapy against specific cytokines such as IL-6 was reported as a feasible therapy for preventing cGVHD [31]. However, we have identified selective markers for isolating MSCs in humans (unpublished data). With the availability of markers to prospectively isolate human MSCs, excluding the MSC fraction from donor cells may be more effective in preventing the onset of cGVHD. A recent report also supports our hypothesis where the incidence of cGVHD is less following cord blood transplantation [32,33]. Since we have found that MSCs are rarely detected in cord blood (unpublished data), this may explain the lower frequency of cGVHD in CBT. A prospective clinical study investigating the onset of cGVHD in association with the amount of MSCs that were included in the donor BM or cord blood will be necessary to confirm our hypothesis.

In conclusion, transplantation of minor antigen-mismatched MHC-compatible MSCs interact with residual host T cells to induce the auto-immune phenotype observed in cGVHD, and strategic removal of these cells may be an effective way to prevent suffering due to cGVHD.

METHODS

Mice.

B10.D2, BALB/c and Nude mice 8-10 week of age were purchased from Sankyo Laboratory, Inc. (Tokyo, Japan). B10.D2 GFP mouse were obtained by backcrossing of B10.D2 mice with C57BL/6 GFP mice (Japan SLC Ltd). The 10^{th} generation of backcrossed B10.D2 GFP progenies was used for experiments. RAG2KO on BALB/c background were kindly provided from Dr. Shigeo Koyasu (Keio University). The mice were kept under specific pathogen-free conditions in our animal facility at Keio University School of Medicine. All experimental procedures and protocols were approved by the ethics committee of Keio University and were in accordance with the Guide for the Care and Use of Laboratory Animals.

Isolation of SP-HSCs

Bone marrow cells, suspended at $1x10^6$ cells per milliliter in calcium- and magnesium-free Hanks balanced salt solution (HBSS) supplemented with 2% fetal calf serum, 10 mM HEPES and 1% penicillin/streptomycin (HBSS+) were incubated with 5 μg /ml Hoechst 33342 (Sigma Aldrich) for 60 minutes at 37°C. The side population (SP) was sorted as described previously [34].

Isolation of CD45$^-$TER119$^-$PDGFRα$^+$Sca-1$^+$ cells (MSCs)

Purified MSCs were isolated by flowcytometry as described previously [12,13]. Briefly, femurs and tibias were dissected and crushed with a pestle. The bone marrow was gently washed in HBSS+. Collagenase (0.2%) was used to digest the minced tibia and femur in HBSS+ without fetal bovine serum at 37°C for 1 hour. After red cell lysis, the residual cells were stained with FITC-labeled anti-Sca-1 (Ly6A/E), APC-labeled anti-PDGFRα (APA-5), PE-labeled anti-CD45 (30-F-11), and PE-labeled anti-TER119 (TER-119) (all from e-Bioscience). Analysis and sorting were performed on a triple-laser MoFlo flowcytometer (Beckman Coulter). CD45$^-$ TER119$^-$ PDGFRα$^+$ Sca-1$^+$ cells were routinely prepared at 99% purity by this method.

MSCs and HSCs Co-transplantation

In all transplantation experiments**,** 1x10^4 PαS-MSCs and 1x10^3 SP-HSCs (Supplementary Fig. 2a) were intravenously injected into the tail vein of etherized recipient mice that had been lethally irradiated with a dose of 10.5 Gy (200 μl per mouse) as indicated in our previous studies [12,34].

T-cell isolation and co-culture with MSCs

Spleens were obtained from mice that received B10.D2 MSC or BALB/c MSC transplants.

T cells were purified from the spleens by anti-CD90.2 monoclonal antibody (mAb)-conjugated microbeads (Miltenyi Biotec) according to the manufacturer's instructions. The purity was consistently > 98%. T cells were cultured alone or co-cultured with PαS-MSCs at a ratio of 10:1 (T cells: MSCs).

Proliferation assay

MSCs were plated at $1x10^4$ cells/ well into 96-well plates in triplicate, which were irradiated at 52 Gy after adherence. $1x10^5$ purified mouse T cells were added to each well. On the fourth day, 5-bromo-2'-deoxyuridine (BrdU) was added. Twenty-four hours later, BrdU uptake was quantified [35] by cell proliferation enzyme-linked immunosorbent assay (ELISA) using a BrdU Kit (Roche Applied Science). The supernatant of the co-cultures was subjected to IL-6 ELISA using commercial kit (BD Biosciences). T cells and MSCs without irradiation after co-cultures were used for FACS analysis for analyzing the cellular source of IL-6 production.

Serum cytokine analysis

Serum was collected from mice using retro-orbital beads [31]. The concentration of IL-6 in the supernatants was determined by ELISA using a specific kit, according to the manufacturer's instructions (BD Biosciences). The experiments were performed in triplicate.

Flowcytometry analysis for intracellular cytokine IL-6 and IL-17

Two × 10^6 spleen cells were stimulated with 10 ng/mL phorbol myristate acetate (PMA) (Sigma) and 10 ng/mL inomycin (Sigma) in the presence of the Golgi inhibitor Brefeldin (Sigma) (10 μg/mL) for 4 hours. The cells were then stained with FITC-labeled anti-CD4 (GK 1.5, e-Bioscience) and PE-labeled anti-IL-6 (MP5-20F3, BD Pharmingen™) or APC-labeled anti-IL-17 (eBio17B7, e-Bioscience) as described [36]. PE-labeled IgG2a, FITC-labeled IgG1, and APC-labeled IgG2a and IgG2k were used as isotype controls (all from e-Bioscience). Cells were analyzed on a FACScan with Cellquest software (Beckton Dickinson). For the co-cultures with T cells and MSCs, T cells were stimulated with Brefeldin (10 μg/mL) alone for 4 hours.

Foxp3 staining

Whole blood samples were co-stained with anti-CD4-FITC and anti-CD25-PE (PC61.5). After fixation and permeabilization, cells were stained with APC-labeled anti-Foxp3 mAb (FJK-16s) (all mAbs were from e-Bioscience) as described [28]. Rat IgG2a, κ was used as an isotype control.

CD3 immnunostaing and FISH for human PBMCs

cGVHD was diagnosed according to the previously reported criteria [2]. PBMCs from sex

mismatched BMT recipients were obtained by Ficoll-Paque gradient centrifugation. Cytospined $2x10^5$ PBMCs were subjected to FITC-conjugated CD3 immunostaning (OKT3, Biolegend) and Y-chromosome or X-chromosome FISH using a DNA probe (Vysis™, Abbot Japan) with TO-PRO-3 (Molecular Probe) as described [6]. Majority of PBMCs (> 99%) on the slides were confirmed as $CD3^+$ T cells. Two observers (Y.O. and S.S.) blindly evaluated at least 500 non-overlapping T cells on coded slides. The frequency (%) of recipient-derived T cells was calculated according to the following formula; for analyzing the number of X-$FISH^+$ or Y-$FISH^+$ recipient-derived T cells, at least five different fields of two specimens at x200 magnification were analyzed under a confocal laser-scanning microscope (LSM 700, Carl Zeiss). All studies with human sample were under approvals of Institutional Review Board at Keio University for human subjects.

Statistics

Two-tailed Student's t-test (Excel 2007) was used to analyze the number of $HSP47^+$ fibroblasts per field, cytokine serum levels, and cell proliferation and cytokine production in co-cultures. Differences were considered significant when $p < 0.05$. Data presented as mean ± SD.

Additional methods. Whole bone-marrow transplantation, tissue processing, immunostaining, Y-chromosome FISH for tissue section and measurement of tear secretion

volume are described in the **Supplementary Methods**.

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Authors contribution

S.S., H.O., K.T., and Y.M. conceptualized and designed the experiments; Y.O. performed most of the experiments. S.M. discovered the purified MSC markers, leading to this study. Y.O., S.M., S.S., and Y.M. analyzed the data; Y.M. and Y.K. supervised on immunologic field; T.Y. assisted with T cell proliferation and cytokine assay; S.M. Y.Mabuchi. Y.O. and S.Y., performed isolating bone marrow stem cells; S.Suzuki. performed cell sorting and surface marker analysis; T.I. measured the tear volume, and generated GFP B10.D2 GFP mice.

Competing financial interests

The authors declare no competing financial interests.

Figure legends

Figure 1: Modified cGVHD model by co-transplanting isolated HSCs and MSCs

(**a**) By using a cell sorter, PαS-MSCs (PDGFRα^{+}/ Sca-1^{+}) and HSCs (side population (SP) cells) from B10.D2 bone marrow were individually isolated and systemically co-transplanted into BALB/c mice. (**b**) Representative images of systemic fibrosis (Mallory staining, blue) in various organs of PαS-MSCs and SP-HSCs co-transplanted recipients at 3 or 8 weeks. Double arrows in skin indicate the thickness of epidermal region leading to the loss of adipose tissue in the mismatched MSC group. Scale bar, 100 μm (Skin, Lung, Liver, 50 μm). (**c**) Transplantation scheme of EGFP^{+} labeled B10.D2 PαS-MSCs or SP-HSCs. Each was co-transplanted into wild type BALB/c along with unlabeled SP-HSCs or PαS-MSCs respectively. (**d, e**) Fluorescence microscopic images of GVHD target tissues from recipient mice stained for EGFP (green), HSP47 (red) and with DAPI (blue).. The data in **b**, **d** and **e** from two replicate experiments (n = 3 per group). Scale bar, 20 μm.

Figure 2: Mismatched PαS-MSCs induced cGVHD

(**a**) BALB/c mice were transplanted with a combination of BALB/c HSCs and B10.D2 MSCs, or vice-versa. (**b**) Mallory staining of lacrimal gland, liver and intestine from recipient mice either of mismatched or syngeneic MSC at 3 or 8 weeks after transplantation. Data from 5 independent experiments (n = 4-5 per group). Scale bar 100 μm (intestine, 50

μm). (**c**) Semi-quantitative histopathology of $HSP47^+$ fibroblasts in the tissues from Syngeneic MSC (blue) and Mismatched MSC (red) transplanted-mice. Data from five different fields in two replicate experiments. Error bars indicate s.d. $*P<0.05$, $**P<0.01$. (**d**) Lacrimal gland function was quantitated by tear volume in the syngeneic MSC (blue) and mismatched MSC transplanted-mice (red) at 8 weeks after transplantation. Data from two replicate experiments (n=3-5 per group), $*P<0.05$. (**e**) Mallory staining of lacrimal gland tissue sections of BALB/c recipients at 3 weeks after transplantation, which were transplanted with $5x10^2$, $2x10^3$, $8x10^3$, or $1.6x10^4$ mismatched PαS-MSC or $1.6x10^4$ syngeneic PαS-MSC (n=3 per group). Scale bar, 100 μm.

Figure 3: Deletion of PDGFRα$^+$Sca-1$^+$ MSCs prevents cGVHD.

Mallory staining of lacrimal gland (**a**) and salivary gland (**b**) tissue sections of BALB/c mice that received either syngeneic whole bone marrow (WBM), mismatched WBM, or PαS-MSCs depleted mismatched WBM (MSC(-)) at 8 weeks after transplantation. Representative data from three replicate experiments (n=5 per group). Scale bar, 100μm. (**c, d**) Semi-quantitative histology of $HSP47^+$ fibroblasts in mismatched WBMT (red), syngeneic control (blue) and MSC-depleted mismatched WBMT(green). The data in **c** and **d** was collected from 5 different fields in two replicate experiments. Error bars indicate mean ± s.d. $*P<0.01$, $**P<0.001$. (**e**) Tear secretion volume 8 weeks after mismatched WBMT (red) compared with syngeneic controls (blue) and MSC-depleted mismatched

WBMT (green) (n=3 per group). Error bars indicate mean ± s.d. ∗P<0.05.

Figure 4: Host T cells are required for the progression of cGVHD

(**a**) Mallory staining of lacrimal gland, salivary gland, liver and intestine of recipient RAG2KO mice at 3 and 8 weeks after transplantation of mismatched PαS-MSCs and syngeneic BALB/c SP cells (B10 MSC + BALB/c HSC into RAG2KO). Scale bar, 100 μm. (**b**) Typical signs of tissue fibrosis in B10.D2 MSC + RAG2KO SP cell transplanted BALB/c recipient mice. The data in **a** and **b** was collected from two replicate experiments (n = 3 per group). Scale bar, 100 μm ∗P<0.01, ∗∗P<0.001. (**c**) Quantification of $HSP47^{+}$ fibroblasts per field in B10 MSC with RAG2KO HSC into BALB/c group (red) and B10 MSC with BALB/c into RAG2KO group (blue). Data from five different fields in two replicate experiments. Error bars indicate s.d. ∗P<0.01, ∗∗P<0.001.

Figure 5: T cells following mismatched BMT are activated by PαS-MSCs

T cell proliferation (**a**) and IL-6 production (**b**) following co-culture with PαS-MSCs or splenic DCs. Bars indicate; T cells from mismatched PαS-MSC transplanted BALB/c (white), syngeneic PαS-MSC transplanted BALB/c (black), wild-type B10.D2 (dark gray) and wild-type BALB/c (light gray). (**a**) T cell proliferation was evaluated by BrdU uptake 24 hrs after incubation. Results are expressed as the mean value of OD 450 nm (± SD) obtained from triplicate cultures and are representative of two independent experiments.

∗P<0.05, ∗∗P<0.01. (**b**) IL-6 concentration in the supernatants of co-cultures was measured by ELISA at 24 hours after stimulation. Data from triplicate culture of two independent experiments. Error bars indicate s. d. ∗P<0.05, ∗∗P<0.01. (**c**) Representative flowcytometry analysis of intracellular IL-6 in $CD3\varepsilon^+$ T cells and $Sca\text{-}1^+$ MSCs at 24 hours after co-culture. Dot plots of mAb stained sample (black) and Isotype control (light gray) were shown. (**d**) IL-6 serum concentration after mismatched MSC and syngeneic MSC transplantation was measured by ELISA. Data from two independent experiments (n= 3). Error bars indicate s. d. ∗P<0.05. (**e**) Representative flowcytometry analysis of CD25 and Foxp3 in $CD4^+$ splenic cells derived from mismatched WBMT mice (right) or syngeneic control mice (left) at 3 weeks. (**f**) Mean percentage of $CD4^+CD25^+Foxp3^+$ cells after WBMT (left) or MSCT (right) were serially measured by flowcytometry. (**g**) Representative flowcytometry analysis of CD4 and IL-17 for splenic cells from mismatched WBMT or syngeneic control mice. (**h**) Mean percentage of $CD4^+IL\text{-}17^+$ cells after syngeneic or mismatched WBMT (left) and MSCT (right) at 3 and 8 weeks after transplantation was measured by flowcytometry. (**e-h**) Data from 3 independent experiments (n=3-5 per group). Error bars represent SD. *P<0.05, ∗∗P<0.01.

Figure 6: Detection of recipient-derived T cells in cGVHD patients

(**a**) FISH for X- and Y-chromosomes using peripheral blood mononuclear cell samples

(PBMC) from cGVHD and non-cGVHD patients who received sex-mismatched WBM transplantation. Scale bar, 100 μm. Arrows indicate recipient-derived T cells. (X-FISH, two green signals; Y-FISH, one red signal). (**b**) Average amount of recipient-derived T cells in the peripheral blood of cGVHD (n=5) and non-cGVHD (n=4) patients. *P<0.05

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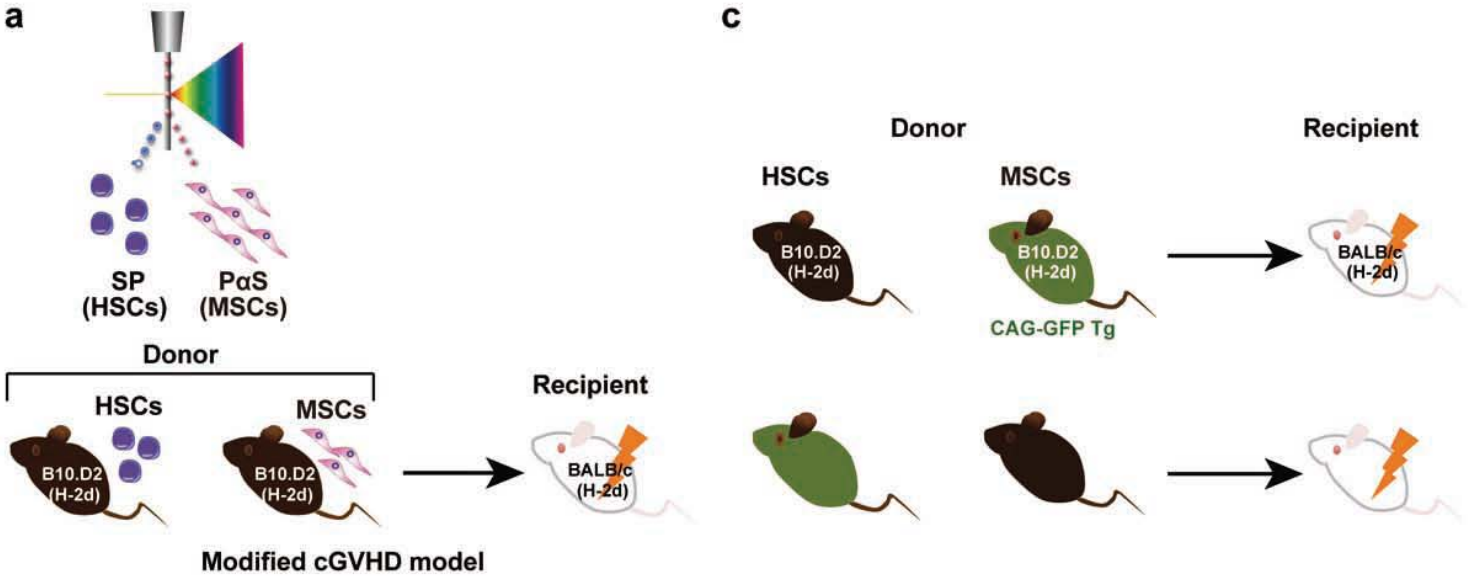

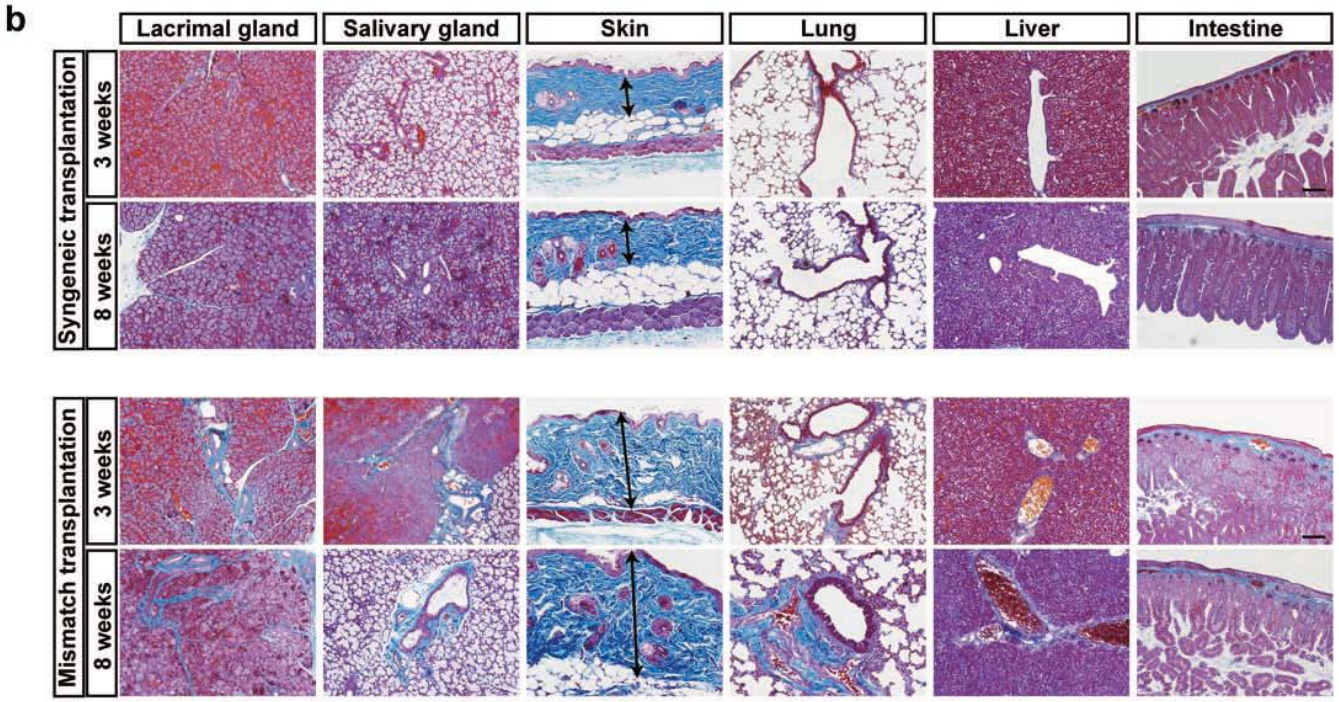

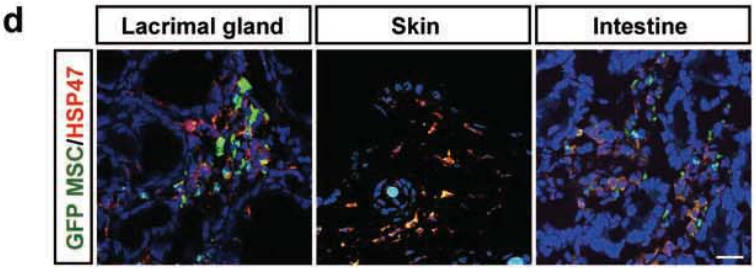

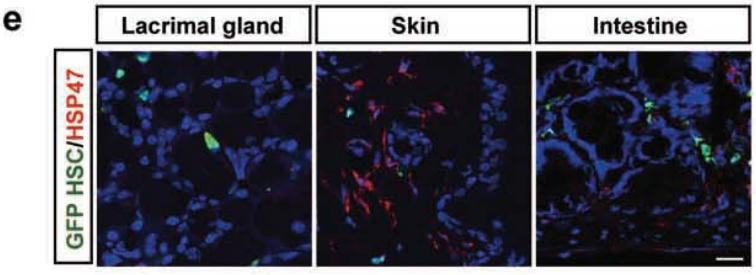

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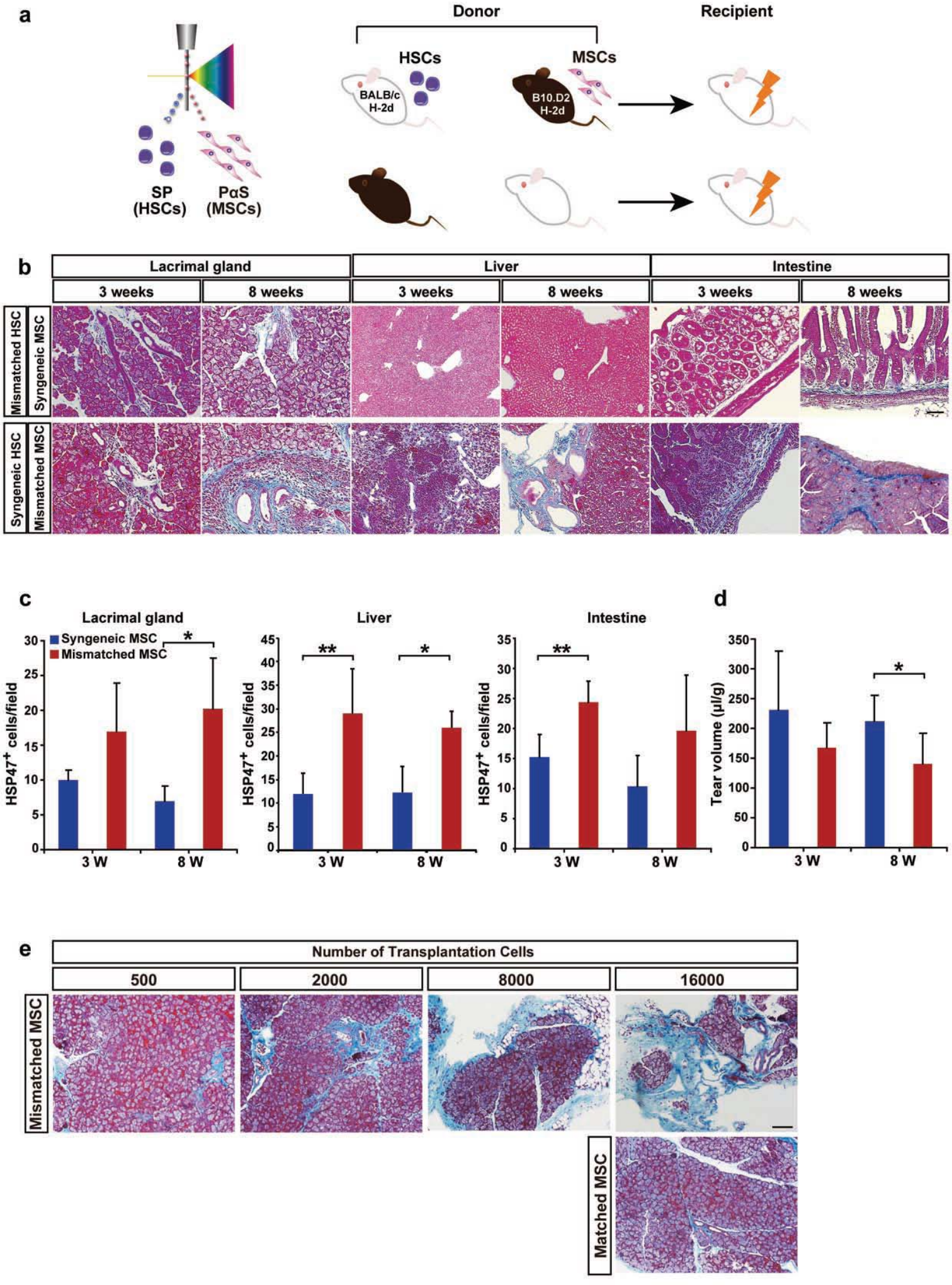

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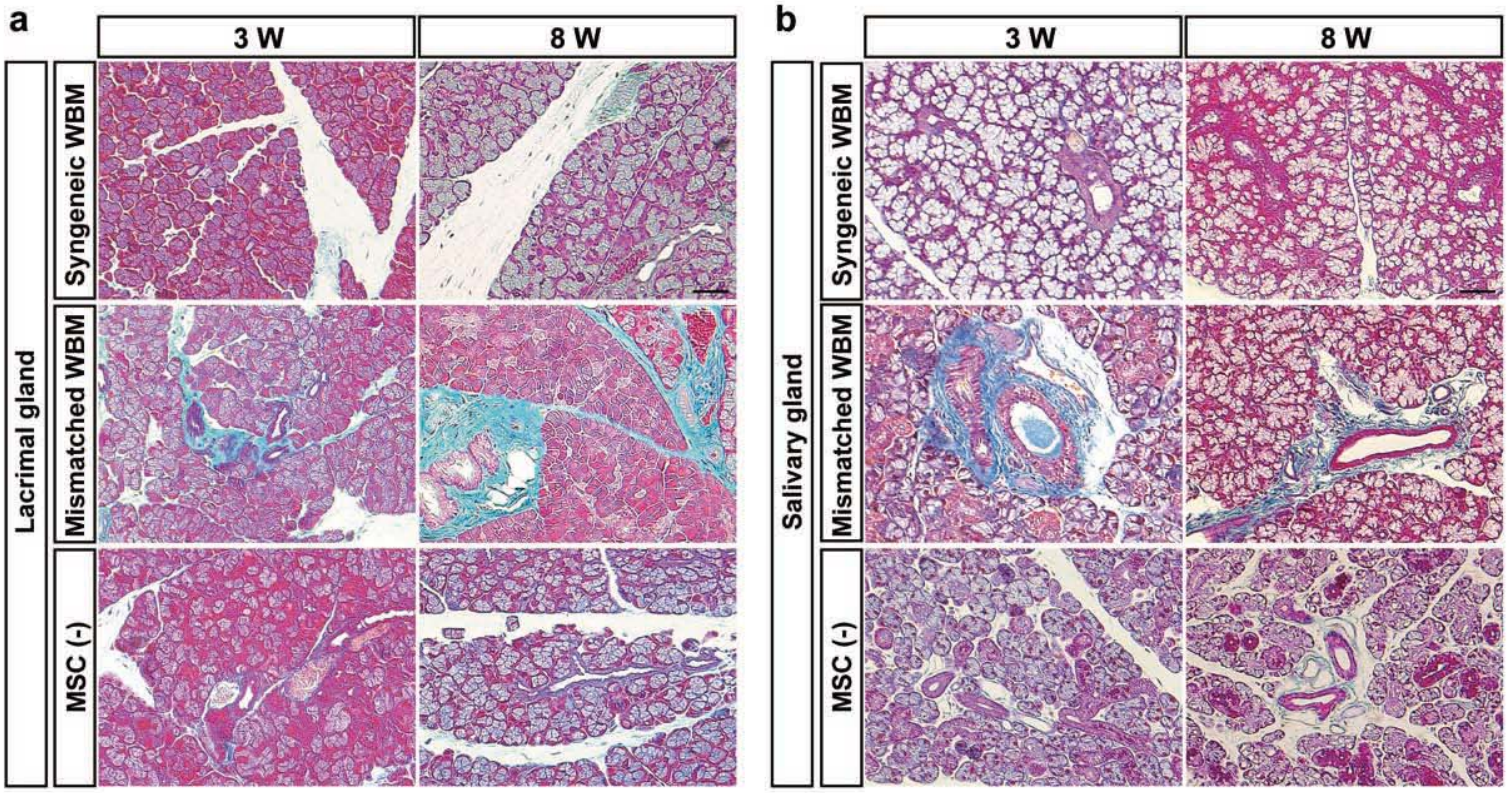

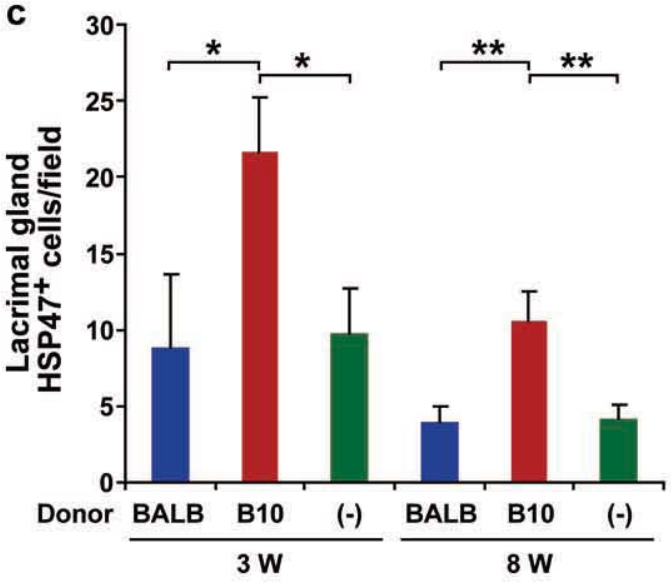

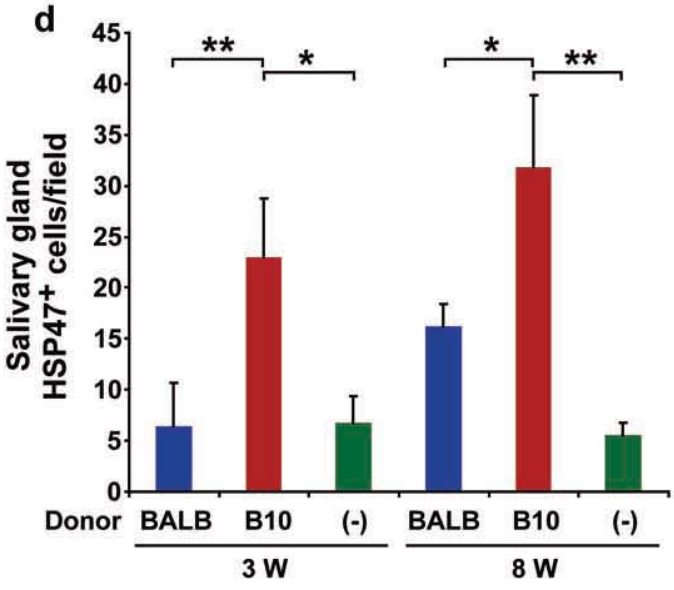

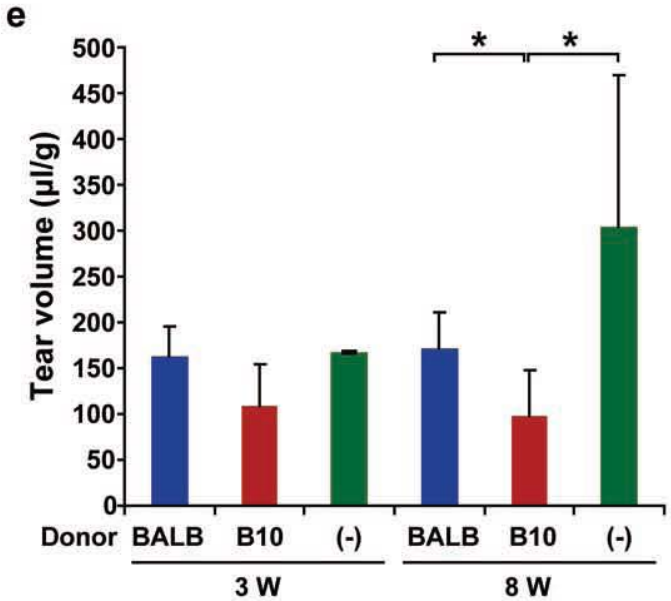

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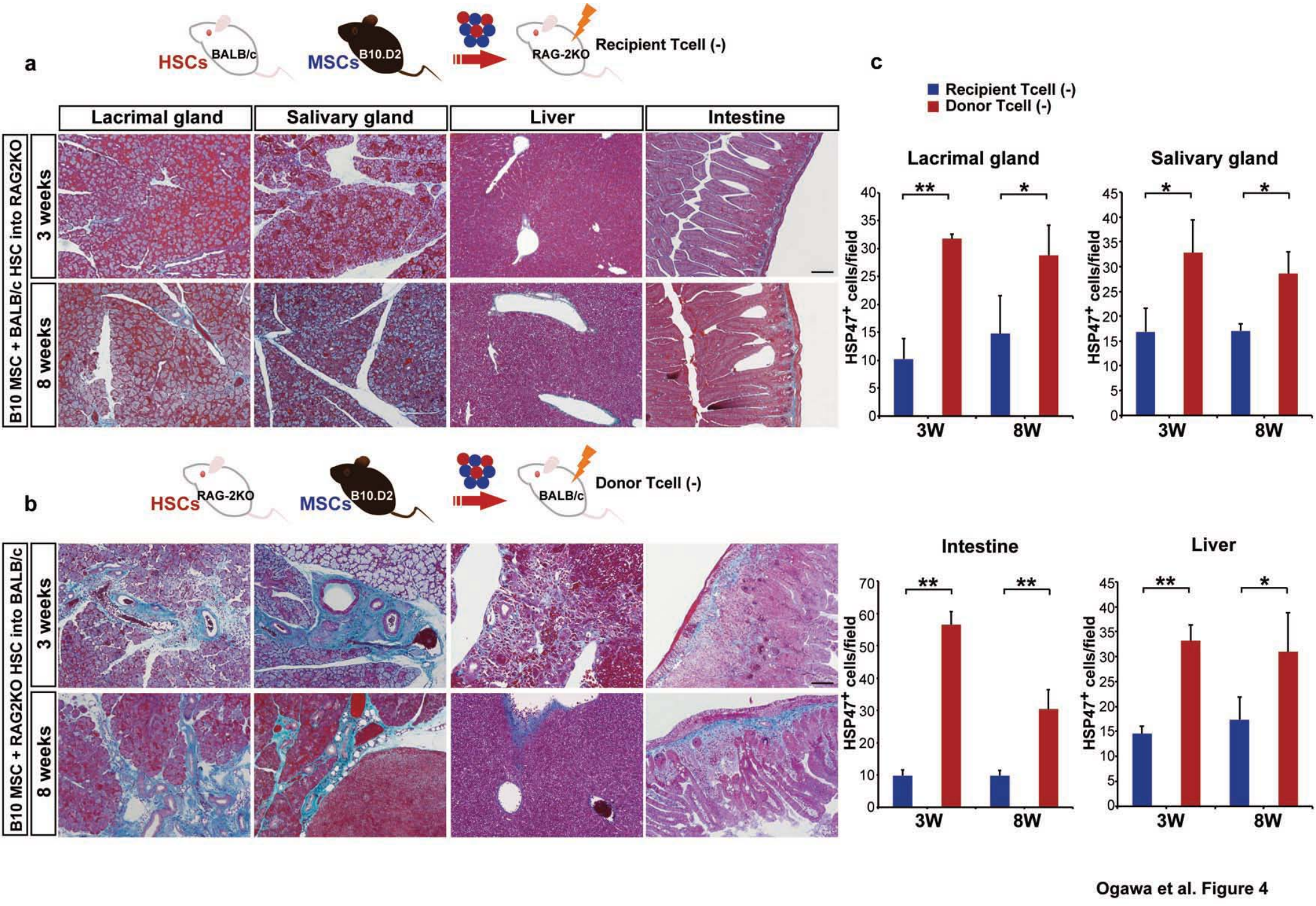

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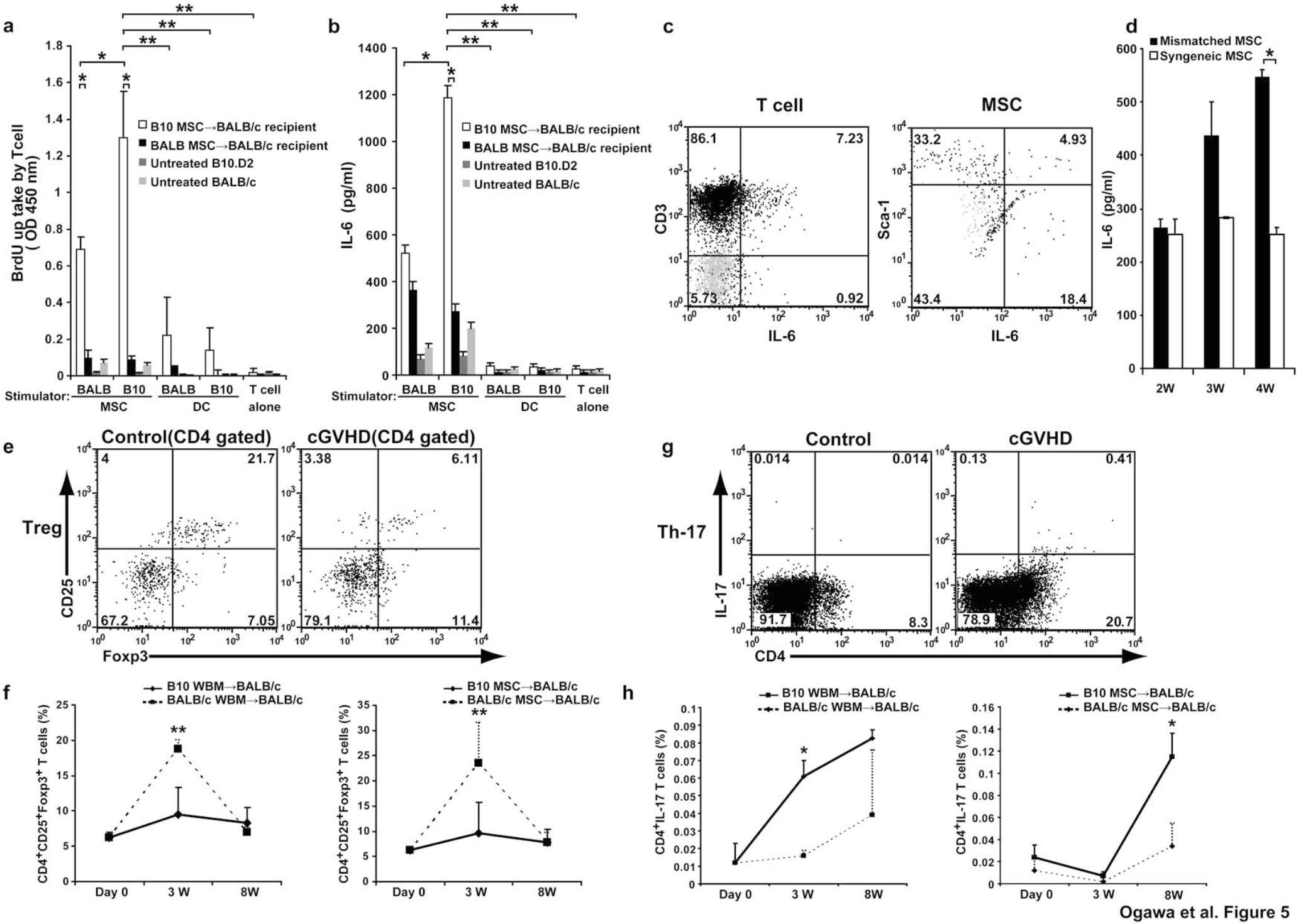

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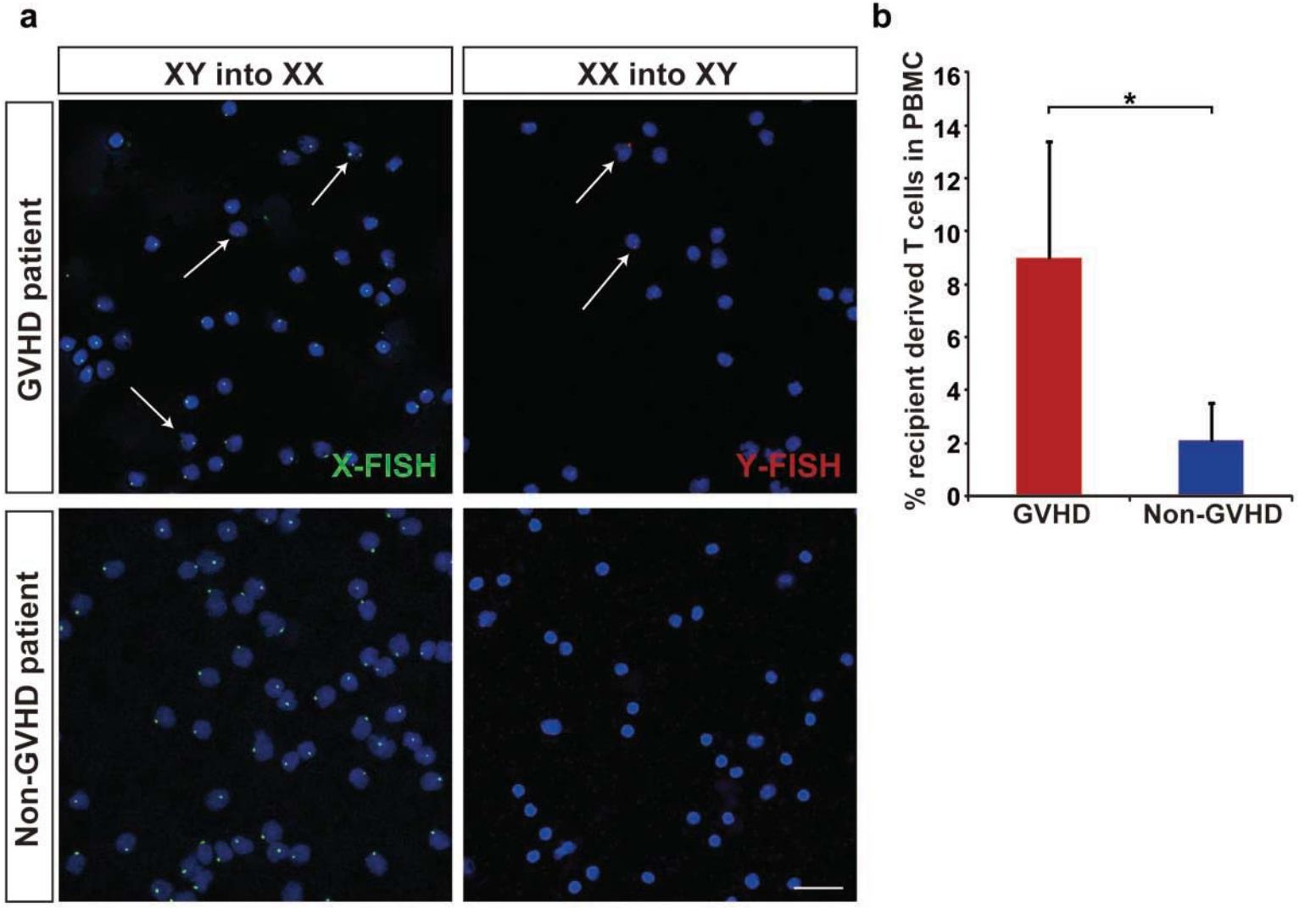

Ogawa et al. Figure 6