Long-Lasting Graft-Derived Donor T Cells Contribute to the Pathogenesis of Chronic Graft-versus-Host Disease in Mice

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Chronic graft-versus-host disease (cGVHD) is a major complication in long-term survivors of allogeneic hematopoietic stem cell transplantation (allo-HSCT). Graft-derived T cells (TG) have been implicated in the induction of cGVHD; however, the extent of their contribution to the pathogenesis of cGVHD remains unclear. Using a mouse model of cGVHD, we demonstrate that TG predominate over hematopoietic stem cell-derived T cells generated de novo (THSC) in cGVHD-affected organs such as the liver and lung even at day 63 after allo-HSCT. Persisting TG, in particular CD8+ TG, not only displayed an exhausted or senescent phenotype but also contained a substantial proportion of cells that had the potential to proliferate and produce inflammatory cytokines. Host antigens indirectly presented by donor HSC-derived hematopoietic cells were involved in the maintenance of TG in the reconstituted host. Selective depletion of TG in the chronic phase of disease resulted in the expansion of THSC and thus neither the survival nor histopathology of cGVHD was ameliorated. On the other hand, THSC depletion caused activation of TG and resulted in a lethal TG-mediated exacerbation of GVHD. The findings presented here clarify the pathological role of long-lasting TG in cGVHD.

Keywords: chronic graft-versus-host disease, stem cell transplantation, T cell, T cell subset, immune reconstitution

INTRODUCTION

Chronic graft-versus-host disease (cGVHD) remains a major cause of long-term morbidity, mortality, and poor quality of life following allogeneic hematopoietic stem cell transplantation (allo-HSCT) (1, 2). One hurdle in the development of preventive and therapeutic strategies against cGVHD is the complex origins and maintenance mechanisms of the pathogenic immune cells that are involved in this protracted disease. Despite clinical and experimental evidence that mature T cells in the graft (Tg) are responsible for tissue injury during acute GVHD (aGVHD), the contribution of Tc to the pathogenesis of cGVHD remains poorly understood.

After allo-HSCT, host-reactive Tc are primed by host-type professional antigen-presenting cells (APCs) via the direct allo-recognition pathway in the secondary lymphoid organs, where they expand...
and differentiate into host-reactive effector T<sub>G</sub> (3, 4). Effector T<sub>G</sub> then redistribute to the target organs of aGVHD, such as the skin, liver, intestine, and hematopoietic and lymphoid tissues, where they impair host tissue function. Of note, an indirect allo-recognition pathway in which donor bone marrow (BM)-derived APCs repopulate peripheral tissues, uptake host-type antigens, and present them to donor T cells maximizes GVHD in a CD8<sup>-</sup>-dependent GVHD model (5). Interestingly, in vivo early T cell depletion by antithymocyte globulin (6, 7) or post-transplant cyclophosphamide (8–10) reduces GVHD rather than aGVHD. These clinical observations suggest a role for T<sub>G</sub> in the development of cGVHD, but this is difficult to examine in a human setting, and experimental evidence demonstrating the contribution of long-lasting T<sub>G</sub> to the development of cGVHD is lacking.

Since non-hematopoietic cells in the target organs remain as host-type even after allo-HSCT, T<sub>G</sub> are continuously exposed to cognate antigens, which theoretically induce deletion, anergy, or replicative senescence in T<sub>G</sub> (11, 12). In contrast to T<sub>G</sub>, which lack de novo replenishment, donor HSC-derived T cells (T<sub>HSC</sub>) that have undergone thymic selection are continuously replenished from the thymus, and thus T<sub>HSC</sub> rather than T<sub>G</sub> have been implicated in the pathogenesis of cGVHD (13–15). On the other hand, Hossain et al. (16) have shown that functional T<sub>G</sub> persisted up to 100 days after allo-HSCT in a cGVHD model and that the persisting T<sub>G</sub> confer protection against murine cytomegalovirus infection. This finding suggests that persisting T<sub>G</sub> could be a functional population with a role in the pathogenesis of cGVHD.

In the present study, using a minor-mismatched allo-HSCT model in which the GVHD recipients display histopathology characteristic of cGVHD, we characterized the kinetics, function, and antigen reactivity of T<sub>G</sub> and T<sub>HSC</sub>. Selective depletion of T<sub>G</sub> or T<sub>HSC</sub> in the chronic phase of disease revealed that persisting T<sub>G</sub> suppress the accumulation of T<sub>HSC</sub> in cGVHD-affected organs, whereas T<sub>HSC</sub> suppress the lethal activation of T<sub>G</sub> in affected organs.

**MATERIALS AND METHODS**

**Mice**

Female C57BL/6J (B6; H-2<sup>a</sup>, CD45.2, Thy1.2) mice were purchased from the Jackson Laboratory. B6 background congenic strains were crossed in-house to obtain CD45.1<sup>+</sup> CD45.2<sup>+</sup> Thy1.2<sup>+</sup> and CD45.1<sup>+</sup> CD45.2<sup>+</sup> Thy1.1<sup>+</sup> congenic strains. All animal experiments were conducted in accordance with institutional guidelines with the approval of the Animal Care and Use Committee of the University of Tokyo.

**Transplantation and Assessment of GVHD**

Cell preparation and allo-HSCT were performed as described previously (17, 18) with some modifications. In brief, T-cell-depleted BM (TCD BM) was prepared by depleting Thy1<sup>+</sup> mature T cells from BM using an autoMACS system (Miltenyi Biotec). Splenic T cells were negatively enriched from splenocytes by autoMACS, using antibodies against CD11b, CD11c, B220, Ter-119, NK1.1, and c-kit. Recipients were lethally irradiated (9 Gy, split into two doses given 3 h apart) on day −1, then injected intravenously with 5 × 10<sup>6</sup> TCD BM cells with or without 3–4 × 10<sup>6</sup> splenic T<sub>G</sub> cells on day 0. The development of systemic GVHD was quantified by measuring weight loss and using a clinical GVHD scoring system, as described previously (19).

**Histological Analyses**

For the assessment of pathological changes in tissues, 4–6-µm formalin-fixed paraffin sections were stained with H&E and assessed by a pathologist (Teppei Morikawa; blinded to experimental group) using a scoring system described previously (20–22). For immunohistological analyses, the left lobes of the lung were infiltrated by infusion of 500 µl OCT compound intratracheally before lung tissue was harvested. Acetone-fixed 6- to 8-µm cryosections were incubated sequentially with primary antibodies and the appropriate fluorochrome-labeled secondary antibodies after blocking. Sections were mounted with Prolong Gold Antifade Reagent (Life Technologies) and visualized using an SP-5 confocal microscope (Leica Microsystems).

**Cell Preparation**

Single cell suspensions were prepared from the liver, lung, spleen, BM and thymus after systemic transcardial perfusion with PBS. Liver cells were prepared by pressing liver tissue through a 200-µm stainless steel mesh (23). The right lobe of lung tissue was cut into small fragments and digested for 1 h at 37°C with 0.2% collagenase D (Roche, Penzberg, Germany) and 2,000 U/ml DNase I (Calbiochem, La Jolla, CA, USA). BM cells were flushed from femurs using a needle and syringe. Spleen and thymus tissue were pressed through a 70-µm cell strainer. Non-hematopoietic cells and cell debris were removed by 40% Percoll (GE healthcare) phase separation, and erythrocytes were removed using ACK lysing buffer.

**Flow Cytometry**

Labeled and purified antibodies were purchased from BD Biosciences, BioLegend, or eBioscience (Table 1). Single cell suspensions were incubated sequentially with anti-CD16/32 (to block Fc receptors) then primary antibodies. Data were collected on a Gallios flow cytometer (Beckman Coulter) and analyzed using FlowJo software (Tree Star). For intracellular cytokine analysis, 5–10 × 10<sup>6</sup> leukocytes were restimulated with ionomycin (1 µg/ml) and PMA (25 ng/ml) in the presence of Brefeldin A (10 µg/ml) for 4.5–5 h at 37°C. Following staining for surface antigens, cells were stained for intracellular cytokines using a Cytofix/Cytoperm kit (BD Bioscience), according to the manufacturer’s instructions. For short-term pulse BrdU labeling, mice were injected intraperitoneally with 1 mg/mouse BrdU (Sigma-Aldrich) in 100 µl PBS 1 h before sacrifice. BrdU incorporation was examined using a BrdU flow kit (BD Biosciences), according to the manufacturer’s instructions. For the intracellular staining of Foxp3 and Ki-67, 1 × 10<sup>6</sup> leukocytes were stained for surface antigens and then were fixed and permeabilized with Foxp3 Fix/Perm Buffer (eBioscience) according to the manufacturer’s instructions.
an *in vivo* proliferation assays, CFSE-labeled T<sub>T</sub> and T<sub>HSC</sub> were equally mixed and transferred intravenously into day 49 BMT recipients reconstituted with CD45.2 TCD BM.

### In Vivo T Cell Depletion

To deplete T<sub>T</sub> or T<sub>HSC</sub> selectively, recipient mice expressing various combinations of the congenic markers Thy1.1 and Thy1.2 were injected intraperitoneally with purified rat IgG2b anti-mouse Thy1.2 mAb (clone 30H12, BioXcell) on days 21 (200 µg) and 24 (100 µg) after allo-HSCT, followed by weekly administration (200 µg) from days 31–63.

### Statistical Analyses

All values are expressed as mean ± SEM. All data are representative of results obtained in at least two independent internally controlled experiments, where intergroup effects observed within each experiment were consistent across all experiments. Survival curves for GVHD were plotted using Kaplan–Meier estimates and compared by log-rank analysis (Prism version 5.0; GraphPad Prism Software). Comparisons between two groups were performed by unpaired two-tail Student’s *t*-tests. Multiple comparisons were performed by one-way ANOVA with Dunnett’s post-test. *P*-Values less than 0.05 were considered to be statistically significant.

### RESULTS

#### Establishment of a Clinically Relevant Model of cGVHD

We first established a B6→C3H.SW minor-mismatched cGVHD model to facilitate identification and manipulation of T<sub>T</sub> and T<sub>HSC</sub> populations using CD45 and Thy1 congenic markers. Lethally irradiated C3H.SW recipients were transplanted with TCD BM alone (“BMT group”) or TCD BM with splenic T cells (“cGVHD group”) from B6 donors. In the cGVHD group, body weight loss and GVHD score became progressively worse from day 14 onward (Figures 1A,B), and cGVHD mice had a significantly lower survival rate (Figure 1C). At day 63, the histology of the salivary glands (SG), skin, liver, and lung revealed inflammatory cell infiltration and fibrotic changes, which are the most relevant histological features in the diagnosis of human cGVHD (Figures 1D,E) (24, 25). The pathological scores for these organs were higher in cGVHD mice compared to BMT control mice, reaching statistical significance in the SG and liver (Figure 1F). These results demonstrate that cGVHD mice in the B6→C3H.

SW minor-mismatched allo-HSCT model develop pathology that recapitulates human cGVHD.

#### T<sub>T</sub> Predominate over T<sub>HSC</sub> in cGVHD-Affected Organs

We next examined the kinetics of T<sub>T</sub> and T<sub>HSC</sub> in our cGVHD model using CD45 congenic markers (Figure 2A). The number of CD4<sup>+</sup> CD8<sup>+</sup> thymocytes of CD45.1<sup>+</sup> CD45.2<sup>+</sup> TCD BM origin (T<sub>HSC</sub>) in cGVHD mice increased markedly between days 21 and 35 (Figure 2B). In peripheral tissues such as the liver, lung, and spleen, detectable numbers of T<sub>HSC</sub> appeared from day 21 after...
FIGURE 1 | Continued
HSCT, but within the CD8^+ T cell populations of these tissues, T_G outnumbered T_HSC approximately 10-fold for the duration of our experiments (Figure 2C). A similar trend was observed in CD4^+ T cell populations. Interestingly, T_G were more abundant than T_HSC within CD4^+ Foxp3^+ regulatory T cells in the liver by day 35; however, there was a trend toward T_HSC dominance by day 63 (Figure 2D). Immunofluorescent staining of the liver showed that both Foxp3^+ CD4^+ T cells and Foxp3^+ CD4^+ T_HSC were uniformly distributed throughout the T cell pool at day 63 (Figure 2E). These results suggest that T_G rather than T_HSC persist as the dominant
CD8+ T cell population, while T\textsubscript{HSC} gradually become the dominant CD4+ T cell population, particularly within the Foxp3+ CD4+ regulatory T cell populations in cGVHD-affected organs.

T\textsubscript{G} Remain Functional during cGVHD

We next compared the phenotype and function of T\textsubscript{G} and T\textsubscript{HSC} in the liver at day 63. Compared to their T\textsubscript{HSC} counterparts, CD8+ T\textsubscript{G} expressed high levels of the exhaustion markers PD-1 and LAG-3, the senescence/terminal differentiation marker KLRG-1 (11, 26), and low levels of IL-7R\textalpha, which is inversely correlated with alloreactivity (27) (Figure 3A). Expression levels of the early activation marker CD69 were comparable between T\textsubscript{G} and T\textsubscript{HSC} (Figure 3B). However, we did not observe significant differences in surface marker expression between CD4+ T\textsubScript{G} and CD4+ T\textsubScript{HSC} (Figure 3C). Upon ex vivo stimulation of liver-infiltrating T cells with PMA and ionomycin, a substantial proportion of CD8+ T\textsubscript{G} produced IFN\gamma and TNF\alpha at levels that were equal to those produced by their T\textsubscript{HSC} counterparts (Figure 3C). Because T\textsubscript{G} far outnumbered T\textsubscript{HSC} in cGVHD-affected organs, the majority of cells with potential to produce inflammatory cytokines in cGVHD-affected organs were of T\textsubscript{G} origin (Figure 3D). A similar trend was observed for CD4+ T\textsubscript{G} and T\textsubscript{HSC} (Figures 3C,D). These results suggest that a substantial proportion of CD4+ and CD8+ T\textsubscript{G} persist as a functional population in the target organs of cGVHD.

Interestingly, the proportion of PD-1+ KLRG-1+ cells within the CD8+ T\textsubscript{G} population increased between day 21 and day 63 (Figure 3E). To examine whether these PD-1+ KLRG-1+ cells are functionally distinct from the PD-1+ KLRG-1- cells that make up the majority of CD8+ T\textsubscript{G} in early phase, we analyzed the expression of functional molecules and the proliferation marker Ki-67 by these two populations at day 60. The PD-1+ KLRG-1- population displayed higher IFN\gamma production and LAG3 expression than the PD-1- KLRG-1+ population, whereas the PD-1- KLRG-1+ population displayed higher granzyme B and Ki-67 expression than the PD-1+ KLRG-1+ population (Figures 3E,G). We also compared the functional profiles of the PD-1+ KLRG-1- populations from CD8+ T\textsubscript{G} and T\textsubscript{HSC}. Although expression levels of LAG3 and granzyme B were equivalent between T\textsubscript{G} and T\textsubscript{HSC}, PD-1- KLRG-1- T\textsubscript{HSC} had lower IFN\gamma production and significantly higher expression of Ki-67 than their T\textsubscript{G} counterparts (Figure 3G). These results suggest that among CD8+ T\textsubscript{G} the PD-1+ KLRG-1- and PD-1- KLRG-1+ populations are functionally distinct and that CD8+ T\textsubscript{HSC} have a higher proliferating potential than CD8+ T\textsubscript{G} even when PD-1 expression is equivalent.

Active Proliferation Contributes to the Maintenance of T\textsubscript{G} during the Chronic Phase

We next investigated the proliferative responses of T\textsubscript{G} and T\textsubscript{HSC} by 1 h in vivo BrdU labeling on day 63 after allo-HSCT. The proportion of proliferating (BrdU+) T cells among liver-infiltrating CD4+ T cells was significantly lower within the T\textsubscript{G} population than in the T\textsubscript{HSC} population, whereas there was no significant difference in the proportion of BrdU+ CD8+ T cells (Figure 4A). However, in terms of cell number, CD4+ BrdU+ T\textsubscript{G} were present in equivalent numbers to CD4+ BrdU+ T\textsubscript{HSC} while BrdU+ CD8+ T\textsubscript{G} far outnumbered BrdU+ CD8+ T\textsubscript{HSC} (Figure 4B). To further investigate the proliferative potential of T\textsubscript{G} and T\textsubscript{HSC}, we prepared T\textsubscript{G} and T\textsubscript{HSC} populations from the liver and lung of cGVHD mice and adoptively transferred them into phase-matched BMT recipients on day 49 after allo-HSCT (Figure 4C). A CFSE-dilution assay demonstrated that a significant proportion of CD4+ and CD8+ T\textsubscript{G}, and an even greater proportion of T\textsubscript{HSC} proliferated 3 days after transfer into secondary BMT recipients (Figures 4D,E). These results demonstrate that T\textsubscript{G} maintain proliferative potential during the chronic phase.

T\textsubscript{G} Recognize Host Antigens through both Direct and Indirect Pathways

In the chronic phase of GVHD models, APCs are classified into host radioreistant non-hematopoietic cells or donor BM-derived hematopoietic cells. The former directly present complexes of host-type minor histocompatibility antigen (miHA)-derived peptide and MHC molecule to donor T cells, whereas the latter take-up host-type miHA from host radioreistant non-hematopoietic cells and present miHA-peptide/MHC complexes to donor-T cells through an indirect pathway. To investigate the allo-recognition pathway that contributes to the maintenance of T\textsubscript{G} and T\textsubscript{HSC} populations after allo-HSCT, we cocultured T\textsubscript{G} or T\textsubscript{HSC} with BMT recipient-derived APCs 49 days after allo-HSCT. To examine the direct pathway, we used radio-resistant host-derived non-hematopoietic cells that present endogenous host-type antigens (Figures 4F,G). To examine the indirect pathway, we used donor-derived T cell/NK cell-depleted hematopoietic cells containing professional APCs, which uptake and then present host antigens. We also used untreated C3H.SW mouse-derived hematopoietic cells and non-hematopoietic APCs to examine the direct pathway that is involved in the early induction of effector T\textsubscript{G}. CFSE-dilution assays demonstrated that neither CD4+ nor CD8+ T\textsubscript{G} proliferated in response to coculture with non-hematopoietic cells prepared from BMT recipient or untreated C3H.SW mice. In contrast, both CD4+ and CD8+ T\textsubscript{G} proliferated when cocultured with hematopoietic cells from C3H.SW mice (direct pathway) and to a lesser extent when cocultured with hematopoietic cells from BMT recipients (indirect pathway) (Figure 4G). T\textsubscript{HSC} displayed a similar proliferative response except that a small proportion of CD8+ T\textsubscript{HSC} proliferated in response to coculture with non-hematopoietic cells prepared from BMT recipients (data not shown), these results suggest that indirect host antigen presentation by donor-derived hematopoietic cells may be involved in the maintenance of T\textsubscript{G} in cGVHD-affected organs.

Since inflammatory factors such as IFN\gamma modify antigen processing by inducing expression of immunoproteasomes and MHC molecules (28), we performed similar donor T cell and APC ex vivo coculture experiments with APCs that had been pretreated with 10 U/mL IFN\gamma for 16 h, which induced the expression of MHC class I and MHC class II on both hematopoietic and non-hematopoietic APCs. IFN\gamma pretreatment did not affect antigen presentation by non-hematopoietic APCs (Figure 4H).
However, IFNγ pretreatment of hematopoietic APCs significantly increased the proliferation of CD4+ and CD8+ Tg in both direct and indirect pathway-dependent settings. In contrast, IFNγ pretreatment of hematopoietic APCs significantly suppressed the direct pathway-dependent proliferation of CD4+ ThSC and indirect pathway-dependent proliferation of CD8+ ThSC. These results suggest that IFNγ expression in cGVHD-affected tissues may cause prolonged dominance of Tg among CD8+ T cells.
**FIGURE 4** | Proliferative potential and allo-recognition pathway of T<sub>G</sub> and T<sub>HSC</sub>. (A,B) Recipients were injected with BrdU 1 h before sacrifice to enable measurement of cell proliferation in liver T cells. (A) Percentage of BrdU<sup>+</sup> CD4<sup>+</sup> or CD8<sup>+</sup> T cells within the T<sub>G</sub> or T<sub>HSC</sub> populations on day 63. (B) Time-course analysis of the BrdU<sup>+</sup> T<sub>G</sub> and T<sub>HSC</sub> populations in the liver. (C) Schematic of in vivo proliferation assay. CD45.1<sup>+</sup> Thy1.1<sup>−</sup> T<sub>G</sub> or CD45.1<sup>+</sup> Thy1.1<sup>+</sup> T<sub>HSC</sub> were prepared from the liver and lung of chronic graft-versus-host disease (cGVHD) recipients on day 49. These cells were equally mixed, CFSE-labeled, and transferred to phase-matched BMT recipients that had been reconstituted with CD45.2<sup>+</sup> T-cell-depleted bone marrow (TCD BM). (D) Representative flow cytometry plots showing CFSE dilution in CD45.1<sup>+</sup> donor cells, which consist of Thy1.1<sup>−</sup> Thy1.2<sup>+</sup> T<sub>G</sub> and Thy1.1<sup>+</sup> Thy1.2<sup>−</sup> T<sub>HSC</sub>, in the spleen 3 days after secondary transfer. (E) Percentages of T<sub>G</sub> or T<sub>HSC</sub> that underwent one or more cell division. (F) Schematic of in vitro proliferation assay. T<sub>G</sub> and T<sub>HSC</sub> were cocultured with lung non-hematopoietic cells or spleen CD3<sup>−</sup> NK1.1<sup>−</sup> hematopoietic cells, which were prepared from either untreated C3H mice or BMT recipient C3H mice reconstituted with TCD BM from B6 mice, to determine allo-recognition pathway. (G) Percentage of T<sub>G</sub> or T<sub>HSC</sub> that underwent one or more cell division after 3 days of culture. Data represent mean ± SEM [n = 6 (A,B), n = 4 (E), n = 3 (G,H)] from one of two independent experiments. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001 [(B,E,G); T<sub>G</sub> vs. T<sub>HSC</sub>, (H); and IFNγ (−) vs. IFNγ (+)].
Selective Depletion of T\textsubscript{G} or T\textsubscript{HSC} Failed to Ameliorate the Pathology of cGVHD

To determine the involvement of T\textsubscript{G} and T\textsubscript{HSC} in the pathogenesis of cGVHD, we selectively depleted T\textsubscript{G}, T\textsubscript{HSC}, or both T\textsubscript{G} and T\textsubscript{HSC} from cGVHD mice using Thy1.1/1.2 congenic markers and an anti-Thy1.2 depleting mAb (Figure 5A). This protocol resulted in >95% depletion of Thy1.2\textsuperscript{+} T cells from cGVHD-affected organs from day 35 to the end of our experiments (Figure 5B). Unexpectedly, survival rate, fibrotic changes, and inflammatory cell infiltration in cGVHD-affected organs were not ameliorated by the depletion of T\textsubscript{G} (Figures 5C–E), despite the predominance of functionally competent T\textsubscript{G} in the cGVHD-affected organs of untreated cGVHD mice. These results suggest that T\textsubscript{G} alone are not responsible for the development and/or maintenance of the histopathology of cGVHD. On the other hand, survival was markedly reduced in the T\textsubscript{HSC}-depleted group, in which all mice died within approximately 3 weeks of initial antibody treatment (Figure 5F). This lethal exacerbation of GVHD was not due to the

![Figure 5](https://example.com/figure5.png)

**FIGURE 5** | Both T\textsubscript{G} and T\textsubscript{HSC} are involved in the pathogenesis of chronic graft-versus-host disease (cGVHD). (A) Schematic of the in vivo depletion study. T\textsubscript{G} (T\textsubscript{G}-dep), T\textsubscript{HSC} (T\textsubscript{HSC}-dep), or both T\textsubscript{G} and T\textsubscript{HSC} (T\textsubscript{G/HSC}-dep) were selectively depleted from cGVHD mice using Thy1.1/1.2 congenic markers and an anti-Thy1.2 depleting mAb. Recipient mice received Thy1.1\textsuperscript{+} T-cell-depleted bone marrow (TCD BM) and Thy1.2\textsuperscript{+} splenic T cells, Thy1.2\textsuperscript{+} TCD BM and Thy1.1\textsuperscript{+} splenic T cells, or Thy1.2\textsuperscript{+} TCD BM and Thy1.2\textsuperscript{+} splenic T cells from B6 donors and were then treated with an mThy1.2 antibody or a control Ab on days 21 and 24 after transplantation, and weekly thereafter until day 63. (B) Depletion efficiency of anti-Thy1.2 mAb on Thy1.2\textsuperscript{+} T cells in the liver. (C) Survival data are combined from three independent experiments (n = 12–15). (D) Representative HE images and (E) histological scoring of the salivary glands, skin, liver, and lung from T\textsubscript{G}-dep and cGVHD (n = 5) mice on day 63. Scale bars, 50 µm. (F) Survival of cGVHD, T\textsubscript{HSC}-dep, and T\textsubscript{G/HSC}-dep group mice. Data are combined from three independent experiments (n = 12–15). (G) Number of HSCs (CD48\textsuperscript{−}CD150\textsuperscript{+} LSK) in the BM on day 28 after transplantation (7 days after T\textsubscript{HSC} depletion). (H) Proportion of Foxp3\textsuperscript{+} cells within the liver CD4\textsuperscript{+} T\textsubscript{G} population on day 28 after transplantation. Data in (B,E,G,H) represent mean ± SEM (n = 3 for the cGVHD group; n = 4 for the T\textsubscript{HSC}-dep group) from one of three independent experiments. n.s., not significant; *P ≤ 0.05; **P ≤ 0.01; and ***P ≤ 0.001 (comparisons as indicated).
depletion of Thy1+ HSCs by the anti-Thy1.2+ antibody, because the number of CD48− CD150− lineage− c-Kit+ Sca1+ long-term HSCs was equivalent between the cGVHD and T HSC-depleted groups (Figure 5G). In addition, there was no significant difference in the frequency of Foxp3+ liver-infiltrating CD4+ T cells between the cGVHD and T HSC-depleted groups, suggesting that the lethal exacerbation of GVHD in the T HSC-depleted group could not be attributed to the loss of Foxp3+ regulatory T cells (Figure 5H). Importantly, simultaneous depletion of T G and T HSC rescued cGVHD mice from the lethal exacerbation of disease induced by the depletion of T HSC alone (Figure 5F). These results suggest that neither T G nor T HSC are solely responsible for cGVHD and that the lethal exacerbation of GVHD induced by T HSC depletion is mediated by T G.

T HSC Suppress Activation of T G
The observation that simultaneous depletion of T G and T HSC ameliorated cGVHD while selective depletion of T G or T HSC failed to ameliorate cGVHD suggests that an interaction exists between these two distinct T cell populations. To investigate this interaction further, we examined the effects of T HSC depletion on the number and function of T G. Since T HSC-depleted mice died within 3 weeks of depletion (day 21), analyses were performed on days 28 and 35. There were no significant differences in liver and lung T G numbers between the T HSC-depleted and cGVHD groups (Figure 6A). In addition, the frequency of BrdU+ (proliferating) cells within the T G population and the expression levels of CD44, CD25, and CD69 on T G cells were equivalent between the T HSC-depleted and cGVHD groups (Figures 6B,C). However, the proportion of IFNγ+ and TNFα+ cells among liver-infiltrating CD4+ and CD8+ T G cells was higher in the T HSC-depleted group compared to the cGVHD group on both days 28 and 35 (Figure 6D and data not shown). These results suggest that T HSC, despite being 10–100 times lesser in number than T G at the time of depletion, inhibit inflammatory cytokine production by T G and play a critical role in preventing lethal exacerbations of GVHD.

T G Suppress the Accumulation of T HSC in cGVHD-Affected Organs
Finally, we examined the effects of T G depletion on the number and function of T HSC. In kinetic studies of CD4+ and CD8+ T cell number in the liver, T G rapidly decreased after anti-Thy1.2 mAb treatment in the T G-depleted group, whereas T HSC increased to numbers approximately 10-fold higher than the cGVHD group between days 35 and 63 (Figure 7A). By day 63 after allo-HSCT, the number of T HSC (particularly CD4+ T HSC) in the T G-depleted group had increased to levels comparable to the sum of T G and T HSC numbers in the cGVHD group (Figure 7B).

To determine whether this increase in T HSC number was due to an increase in T HSC proliferation, we performed BrdU labeling on day 63 (Figure 7C). The proportion of BrdU+ cells among liver-infiltrating CD4+ and CD8+ T HSC was significantly higher in the T G-depleted group, outnumbering the sum of BrdU− T G and T HSC in the cGVHD group. There was no significant difference in the frequency of Foxp3+ cells among CD4+ T cells between the cGVHD and T G-depleted groups (Figure 7D). The majority of CD8+ T HSC in the liver and lung, but not in the spleen, of T G-depleted group mice were CD44hi effector T cells and had increased expression of the activation markers CD25 and CD69 (Figure 7E). The total proportion of IFNγ+ or TNFα+ cells among liver-infiltrating CD4+ and CD8+ T cells was equivalent between T G-depleted and cGVHD group mice (Figure 7F). However, the cellular composition of these populations switched from predominately T G in the cGVHD group to predominately T HSC in the T G-depleted group. Collectively, these results demonstrate that the proliferation and activation of T HSC are augmented by T G depletion in cGVHD-affected organs. This change appears to compensate in part for the decrease in T G numbers and is likely to contribute to the histopathology observed in the cGVHD affected organs of the T G-depleted group.

DISCUSSION
Until now, the contribution of T G to the development and maintenance of cGVHD has remained unclear. In this study, we established a model of cGVHD that recapitulates the pathology of clinical cGVHD. We demonstrated that functional T G persist and predominate over T HSC in the organs affected by cGVHD. Furthermore, our depletion studies revealed the existence of an unexpected bidirectional regulatory interaction between T G and T HSC: T G suppress the proliferation and accumulation of T HSC in cGVHD-affected organs, whereas T HSC suppress the activation of T G in affected organs, thereby preventing exacerbations of GVHD mediated by T G.

There are few mouse models of cGVHD that accurately recapitulate the histopathology and disease course of clinical cGVHD. Recently, a B6 (H2b) Recent (H2k) allo-HSCT model, which utilized cyclophosphamide and total body irradiation as a conditioning regimen, was demonstrated to induce fibrotic changes in the lungs, liver, and SG (29, 30). The developmental course of this model recapitulated cGVHD that develops without preceding aGVHD. Mice in our B6/C3H.SW minor mismatched allo-HSCT model also developed several histological aspects of clinical cGVHD, such as fibrotic changes in the skin, liver, lungs, and SG following aGVHD, which may recapitulate the acute to chronic transition in clinical GVHD.

In our cGVHD model, a large number of T G persisted in cGVHD-affected organs up to day 63 after allo-HSCT. On the other hand, peripheral reconstitution of T HSC became obvious from about 4–5 weeks after allo-HSCT and gradually increased thereafter. As a result, both T G and T HSC infiltrated the affected organs in late phase cGVHD with an unexpected predominance of T G. In an aGVHD model, Zhang et al. (31) have described a CD44hi CD62Lhi IL-7Rα+ CD8+ memory stem cell population that maintain CD8+ T G in the recipient for a long period of time. In contrast, in our cGVHD model, the majority of T G in affected organs had a CD44hi CD62Lhi IL-7Rα+ effector phenotype. Despite the higher proportion of PD-1+ or KLRG-1+ phenotypically exhausted or senescent populations within the CD8+ T G population, the potential for inflammatory cytokine production and in situ proliferation was almost equivalent between T G and T HSC. Masopust et al. (32) have reported that KLRG-1+ PD-1+ senescent CD8+ T cells induced by a heterologous prime-boost vaccination...
show strong cytotoxic activity. Thus, KLRG-1+ PD-1− CD8+ T cells may be involved in the cellular immunopathogenesis of cGVHD.

In the cGVHD-affected liver and lung, a significant proportion of CD8+ T cells were actively proliferating even at day 63, suggesting that Tc maintenance is a dynamic process controlled by cell proliferation and cell death. Considering that Tc do not proliferate in response to direct presentation of host antigens by non-hematopoietic cells, it appears that donor HSC-derived APCs capture host alloantigens and indirectly present them to Tc in cGVHD-affected organs. An intriguing question that remains is whether Tc clones that expanded in response to direct presentation in the acute phase cross-react to indirect presentation in the chronic phase, or whether indirect presentation-reactive Tc clones are independently expanded by donor-derived APCs after repopulation of these cells in the peripheral tissues. A better understanding of the clonal response of Tc after allo-HSCT and the pathological significance of this process may facilitate the early diagnosis and treatment of cGVHD.
FIGURE 7 | Continued
The finding that depletion of the small THSC population potentiated the inflammatory cytokine production of TG and caused a TG-mediated acute exacerbation of GVHD and promoting acute to chronic disease transition following allo-HSCT. Impaired inhibition of TG by THSC may account for the GVHD that is refractory to immunosuppressive therapy with a calcineurin inhibitor, which inhibits both TG activation and THSC development (33). However, the molecular and cellular mechanisms underlying the inhibition of TG activation by THSC remain to be elucidated.

Reconstitution of THSC is markedly delayed and suppressed in the presence of GVHD (16). In the early phase of aGVHD, CD4+ TG impair the production of common lymphoid progenitors through the destruction of the hematopoietic niche in the BM, resulting in a severe reduction in T- and B-lymphocyte genesis (17, 34). TG also impair thymopoiesis by disrupting the thymic epithelium (35). Thus, TG may suppress THSC reconstitution by impairing primary lymphoid tissues. In the present study, depletion of TG resulted in a rapid increase in the number of THSC in the liver and lung. Interestingly, increased numbers of THSC in the TG-depleted group partially compensated for the decreased numbers of TG in GVHD-affected organs. This finding points to the possible existence of a niche that provides antigenic signals and survival factors to pathogenic T cells of TG or THSC origin. Such a “pathogenic T cell niche” might have a fixed pool capacity, meaning that TG and THSC compete with each other for space in the niche during cGVHD.

In summary, we have characterized the cellular mechanisms underlying the maintenance of pathogenic T cells in a clinically relevant cGVHD model. Both TG and THSC with the potential to proliferate and produce inflammatory cytokines infiltrated cGVHD-affected organs, with the number and/or activity of TG and THSC being reciprocally regulated by each other. In addition, our depletion studies highlight the importance of reciprocal tuning of the balance between TG and THSC, which requires the successful reconstitution of THSC in the control of GVHD. By elucidating the interactions between TG and THSC, our findings will help guide the development of novel therapeutic strategies for the prevention and treatment of cGVHD.

ETHICS STATEMENT

All animal experiments were conducted in accordance with institutional guidelines with the approval of the Animal Care and Use Committee of the University of Tokyo.

AUTHOR CONTRIBUTIONS

MK-K, SU, and KM participated in research design; MK-K, SU, JA, SS, FHWS, TM, NS, AY, FS, and WY conducted experiments; MK-K, SU, JA, SS, FHWS, TK, MI, TT, and KM wrote or contributed to the writing of the paper.

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