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HY-Specific Induced Regulatory T Cells Display High Specificity and Efficacy in the Prevention of Acute Graft-Versus-Host Disease

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Naturally derived regulatory T cells (Tregs) may prevent graft-versus-host disease (GVHD) while preserving graft-versus-leukemia (GVL) activity. However, clinical application of naturally derived regulatory T cells has been severely hampered by their scarce availability and nonselectivity. To overcome these limitations, we took alternative approaches to generate Ag-specific induced Tregs (iTregs) and tested their efficacy and selectivity in the prevention of GVHD in preclinical models of bone marrow transplantation. We selected HY as a target Ag because it is a naturally processed, ubiquitously expressed minor histocompatibility Ag (miHAg) with a proven role in GVHD and GVL effect. We generated HY-specific iTregs (HY-iTregs) from resting CD4 T cells derived from TCR transgenic mice, in which CD4 cells specifically recognize HY peptide. We found that HY-iTregs were highly effective in preventing GVHD in male (HY+) but not female (HY-) recipients using MHC II-mismatched, parent–F1, and miHAg-mismatched murine bone marrow transplantation models. Interestingly, the expression of target Ag (HY) on the hematopoietic or nonhematopoietic compartment alone was sufficient for iTregs to prevent GVHD. Furthermore, treatment with HY-iTregs still preserved the GVL effect even against pre-established leukemia. We found that HY-iTregs were more stable in male than in female recipients. Furthermore, HY-iTregs expanded extensively in male but not female recipients, which in turn significantly reduced donor effector T cell expansion, activation, and migration into GVHD target organs, resulting in effective prevention of GVHD. This study demonstrates that iTregs specific for HY miHAgS are highly effective in controlling GVHD in an Ag-dependent manner while sparing the GVL effect. The Journal of Immunology, 2015, 195: 000–000.

A llogeneic bone marrow (BM) transplantation (BMT), as a treatment for leukemias, lymphomas, and myelomas, has historically been hampered by the detrimental effects of graft-versus-host disease (GVHD). Allogeneic T cells within the graft inoculum recognize both major and minor mismatch Ags on leukemic and host tissues, resulting in either beneficial graft-versus-leukemia (GVL) or deleterious GVH effect. Clinicians and scientists still struggle to separate the GVL and GVH responses; among other strategies, the use of naturally derived regulatory T cells (nTregs) has been shown to be a promising approach to effectively control GVHD in animal studies and initial clinical trials. However, isolation and expansion of nTregs still remain a significant obstacle to establishing nTreg therapy as a standard for GVHD treatment. This is due to the low frequency and high number of nTregs needed to effectively control GVHD. Another concern regarding nTreg therapy centers on the loss of the GVL effect. Given that nTregs are nonspecific suppressors, this therapy could result in suppression of allogeneic T cells responding to leukemic cells and therefore increased relapse in patients. Establishing Ag-specific inducible Treg (iTreg) therapy for the treatment of GVHD may solve the previously stated disadvantages of nTreg therapy. First, iTregs can be generated from resting T cells, under specific polarizing conditions, offering a greater number of primary cells for initial expansion. Secondly, we propose, by conferring Ag specificity or Ag education during iTreg generation, we can overcome the high number needed for efficiency as compared with nonspecific nTreg cell therapy. Finally, we propose that drawing the fine line between GVL and GVH responses can be obtained by conferring Ag specificity.

In experimental autoimmune disease models, Ag-specific Tregs are highly effective in controlling autoimmune diabetes, gastritis, and encephalomyelitis (1–3). We and others have initiated studies to evaluate the effects of Ag-specific iTregs in the prevention of GVHD and in the maintenance of GVL activity. We previously generated OVA-specific iTregs by foxp3 transduction or TGF-β...
induction and demonstrated that they persist long-term in vivo and suppress GVHD in nonmyeloablative and myeloablative BMT models when activated by the cognate Ag, either constitutively expressed or introduced via immunization (4, 5). However, we used a nominal Ag to activate Ag-specific iTregs in our preliminary studies, which may not represent clinical settings. Therefore, it is crucial to extend these studies by testing iTregs specific for naturally processed alloantigens, in this case, HY Ag. HY is a minor histocompatibility Ag (miHAg) expressed solely by male recipients. Clinical data show that MHC-matched BMT between female donors and male recipients increased the risk for acute GVHD development (6) and HY-specific alloresponses (7–10). Therefore, due to its clinical relevance, we generated HY-specific iTregs and tested their efficiency, stability, and selectivity in suppressing acute murine GVHD.

**Materials and Methods**

**Mice**

C57BL/6 (B6, H-2b, CD45.2+), BALB/c (H-2a+) and (B6 × DBA2) F1 (BDF1, H-2bd) mice were purchased from the National Cancer Institute. B6 Ly5.1 (H-2b, CD45.1+), B6 bm12 (H-2b), and BALB.b (H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Foxp3gfp knock-in (KI) strain was obtained from A. Rudensky’s laboratory (11, 12). Luciferase-transgenic (Luc–Tg) strain on B6 background was provided by R. Negrin (Stanford University) (13). Anti-HY TCR Tg Marilyn mice (CD4+ Tg, H-2b+, I-Ab restricted) was provided by C. Mainhart (National Institute of Allergy and Infectious Diseases, Bethesda, MD). Marilynn Foxp3gfp knock-in (KI) and (B6 × bm12) F1 strains were produced by cross-breeding. All the mice were housed in a pathogen-free condition at H. Lee Moffitt Cancer Center and Drug Discovery Building at Medical University of South Carolina. All experimental procedures were approved by the Institutional Animal Care and Use Committee.

**T cell purification and iTreg generation**

Total T cells or CD4+CD25+ T cells were purified through negative selection using magnetic beads, as described in our previous work (5, 14). The purity of CD4+CD25+ T cells ranged from 85 to 95%, but CD4+CD25+ cells were always 1% among total CD4+ cells. To generate HY-specific iTregs, CD4+CD25+ T cells from TCR Tg (Marilynn) Foxp3gfp KI mice were seeded at 2.5 × 10^5/ml and stimulated with 0.5 μg/ml HY peptide in the presence of 1.25 × 10^3/ml irradiated syngeneic T cell–depleted (TCD) splenocytes as APCs with 5 ng/ml TGF-β, 5 ng/ml IL-2, and 10 nM retinoic acid (RA) for 6 d.

**Immunofluorescence analysis**

Multiple-color flow cytometry was performed to measure the expression of surface molecules according to standard techniques. Intracellular Foxp3 expression was measured with a Foxp3 detection kit from eBioscience (San Diego, CA), according to manufacturer’s instruction. Intracellular cytokines were measured according to standard techniques, as described in our previous work (15).

**BMT and bioluminescent imaging**

The procedures for induction of acute GVHD were described in previous publications (5, 15). BALB.b mice were exposed to total body irradiation at 850–900 cGy (2 split doses) at day −1. (B6 × bm12)F1, or BDF1 mice were exposed to 1200–1300 cGy total body irradiation (2 split doses). TCD-BM cells alone or in combination with purified T cells from B6 donors were injected via the tail vein into recipients within 24 h after irradiation. Recipient mice were monitored every other day for clinical signs of GVHD, such as ruffled fur, hunched back, lethargy or diarrhea, and mortality. Animals judged to be moribund were euthanized and counted as mortality. Animals judged to be moribund were euthanized and counted as mortality. Animals judged to be moribund were euthanized and counted as mortality.

**Results**

HY-specific iTregs suppress polyclonal T cell response to alloantigens in vitro

iTregs can be generated from conventional CD4 T cells upon TCR stimulation in the presence of TGF-β, and addition of RA further extend these studies by testing iTregs specific for naturally processed alloantigens, in this case, HY Ag. HY is a minor histocompatibility Ag (miHAg) expressed solely by male recipients. Clinical data show that MHC-matched BMT between female donors and male recipients increased the risk for acute GVHD development (6) and HY-specific alloresponses (7–10). Therefore, due to its clinical relevance, we generated HY-specific iTregs and tested their efficiency, stability, and selectivity in suppressing acute murine GVHD.

![Graph showing Treg generation and iTreg isolation.](image)

**FIGURE 1.** Generation and isolation of HY-specific iTregs. (A) CD4+CD25+ cells were purified from spleen and lymph nodes of TCR Marilynn Foxp3gfp KI mice and stimulated with HY peptide (0.5 μM) in the presence of irradiated TCD splenocytes plus IL-2 (5 ng/ml). To generate iTregs, media was supplied with either TGF-β (5 ng/ml) alone or TGF-β and RA (10 nM).

Five to six days after culture, cells were harvested and tested for expression of CD4, CD25, and GFP by flow cytometry. The phenotype of cultured cells under the different condition is shown on gated live CD4+ cells (upper panels). CD4+CD25+GFP+ iTregs and CD25+GFP− cells (controls) were purified by FACS sorting (lower panels). CD4+CD25+ cells were purified from spleen and lymph node of TCR Marilyn, and iTrgs were generated in the presence of TGF-β and RA, as described in (A). Six days after culture, CD4+CD25+ (iTrgs) were isolated by enriching CD4+ cells through negative selection and then purifying CD25+ cells through positive selection using magnetic beads. The phenotype of cultured cells is shown on gated live CD4+ cells before (upper panels) and after (lower panels) iTreg isolation. These results represent accumulative data obtained from >10 experiments. (C) CD4+CD25+ purified T cells from B6 Ly5.1+ mice were labeled with CFSE and stimulated at 2 × 10^5/well with irradiated TCD splenocytes from female (B6 × bm12) F1, mice at 6 × 10^5/well in 96-well plates. Various numbers of HY-specific iTregs were added into culture to achieve indicated Treg/effector T cell (Teff) ratios in the presence of HY (upper panels) or control OVA (lower panels) peptide at 0.5 μg/ml. Six days after cell stimulation, cultured cells were harvested and stained for the expression of Ly5.1 and CD4. CFSE profiles were shown on gated Ly5.1+CD4+ Teffs. These data represent one of three replicate experiments. ND, not done.
increases the generation of iTregs (14, 16, 17). In this study, we selected HY as target Ag, because it is a naturally processed and ubiquitously expressed miHAg with a proven role in GVHD and GVL responses (6–10). HY-specific iTregs were generated from CD4+CD25+ T cells from Marilyn Foxp3gfpKI mice by stimulating with HYabDby, in the presence of IL-2, TGF-β, and RA (Fig. 1A, upper panels) and purified (purity 94 ± 3%) by FACS sorting (Fig. 1A, lower panels).

Foxp3gfp reporter gene allows us to obtain purified Foxp3+ iTregs, but this strategy cannot be applied in humans and GFP KI may affect the function of Tregs (18, 19). Therefore, to exclude any confounding effect, we generated HY-specific iTregs from CD4+CD25+ T cells of Marilyn mice (Fig. 1B, upper panels). CD4+CD25high cells (purity 92 ± 3%) were purified through positive selection for CD25 using magnetic beads (Fig. 1B, lower panels). Thus, iTregs were routinely generated from non-Foxp3gfp CD4+CD25+ T cells and isolated for CD4+CD25high using magnetic beads.

We then tested the suppressive function of HY-specific iTregs in vitro, and found that these iTregs suppressed ~50% proliferation of polyclonal T cells in response to allogeneic APCs at 1:16 ratio of Treg/effectector T cell (Teff) in the presence of HY peptide, but the same iTregs had little suppressive activity in the presence of nominal OVA peptide (Fig. 1C), confirming that the activation of iTregs is required for their suppressive function in vitro.

**HY-specific iTregs prevent GVHD in activation-dependent manner**

Next, we examined whether HY-specific iTregs were able to prevent GVHD induced by polyclonal T cells in a B6→(B6 × bm12)F1 BMT model, in which donor CD4+ Teffs recognize mismatched recipient MHC II alloantigen (H2bm12). In this model, Teffs at the indicated dose induced ~60% GVHD lethality, whereas addition of iTregs at the same time of BMT significantly reduced GVHD lethality in male (p < 0.01), but not in female recipients (p = 0.7) (Fig. 2A, 2B), indicating that recognition of HY Ag by HY-specific iTregs was indispensable for their suppressive function of alloimmune responses in vivo. To assess whether donor reconstitution was impaired by iTreg therapy, 80 d post-BMT, we observed male recipients that received Teffs plus HY-specific iTregs had comparable numbers of total spleen, B, and T cells to those of BM alone (controls without GVHD), whereas the recipients of BM plus Teff (GVHD controls) had significantly reduced numbers of spleen, B, and T cells (vs BM alone, p < 0.05; Supplemental Fig. 1). These results indicate that HY-specific iTregs promoted long-term immune reconstitution and did not cause chronic GVHD in male recipients. We next determined whether infusion of HY-specific iTregs prior to Teffs promotes Treg expansion and increases therapeutic potential of Tregs (20). To this end, we used the same model and infused HY-specific iTregs 3 d prior to Teffs and found that these iTregs completely prevented GVHD lethality in male recipients (p < 0.01) (Fig. 2C, 2D).

In clinical BMT, most patients receive grafts from MHC-matched and multiple miHAg-mismatched donors. In an effort to mimic a clinical scenario, we used the B6→BALB.B (both H2b) model, in which donor and recipient mice differ by at least 29 different miHAg loci (21). HY-specific iTregs were highly effective in preventing GVHD in male (p < 0.05) but not female BALB.B recipients (Fig. 3A, 3B). Likewise, haploidentical transplantation is extensively used in clinic. Utilizing B6→BDF1 model, we further confirmed that HY-specific iTregs were highly effective in preventing GVHD in male (p < 0.05) but not female BDF1 recipients (Fig. 3C, 3D). To further support our long-term data in the B6→BDF1 model, we analyzed pathology scores and found male recipients that received iTregs had significantly reduced pathologic damage within the liver, small intestine, large intestine, and lung compared with all other groups (Fig. 3E; p < 0.01). In agreement with survival data, female recipients receiving iTregs had comparable pathologic injury in target organs to Teff-alone groups, further supporting necessity for iTregs to recognize...
specific Ag to exert their suppressive function. Taken together, these findings support the use of HY-specific iTregs in clinically relevant BMT models.

HY-specific iTregs suppress the expansion, activation, and migration of donor T cells

We next assessed the cellular mechanism by which HY-specific iTregs suppress alloreactive Teffs in vivo. Taking advantage of Luc-Tg mice, the expansion and infiltration of Luc-Tg Teffs can be measured in vivo over time using BLI assay (22). To use this method, we first titrated the dose of T cells that is required for mediating GVHD and found that at least 4-fold lower numbers of Luc-Tg T cells were required to cause GVHD lethality compared to normal B6 donors (Supplemental Fig. 2). These data suggested that Luc-Tg T cells might be significantly more pathogenic in the induction of acute GVHD. Using 0.25 × 10^6 Luc-Tg CD4^+ cells, although there were relatively low signal intensities and no significant difference among groups on day 6 following Teff injection (Supplemental Fig. 3A), throughout later observation periods, the BLI intensity was significantly reduced in male recipients that received HY-specific iTregs compared with recipients with Teff alone (p < 0.001) or in female recipients transplanted HY-specific iTregs (p = 0.02) (Supplemental Fig. 3A, 3B). Furthermore, male recipients that received HY-specific iTreg showed less dispersed BLI signal, mainly confined to the spleen, compared with other recipients (Supplemental Fig. 3B). Similar results were observed in miHAg- or haplo-mismatched BMT models (Supplemental Fig. 3C–F). These data suggest that HY-specific iTregs regulate allo-genic Teff expansion and infiltration into GVHD target organs, such as the gut and liver.

To further evaluate the effect of iTregs on the expansion and migration of Teffs, we transferred Teffs isolated from B6 Ly5.1^+ mice and HY-specific iTregs along with TCD-BM isolated from normal B6 donors into (B6 × bm12) F1 recipients. Seven days after BMT, we measured Teffs (CD4^+Ly5.1^+) and iTregs (CD4^+TCRVb6^+Ly5.1^+) in recipient spleen (Fig. 4A, 4C) and liver (Fig. 4B, 4D). We found that iTregs expanded extensively in the spleen and migrated substantially to the liver of male, but not female recipients (p < 0.01, in spleen and liver), and the number of Teffs in the liver of male recipients was dramatically reduced (Fig. 4D).

To extend these findings to the haploidentical BMT model, we transferred BM and Teffs from B6 Ly5.1^+ mice and HY-specific
iTregs (Ly5.1<sup>+</sup>) into irradiated B6D2F1 recipients. Fourteen days after BMT, analysis of IFN-γ and TNF-α secretion within the spleen of CD4<sup>-</sup> and CD8<sup>-</sup> Teffs showed male recipients had significantly decreased secretion of proinflammatory cytokines (p < 0.01), whereas there was no significant difference between Teff-alone and female recipients (Fig. 5A, 5B). In correlation with our flow data, analysis of the serum cytokine levels, 14 d post-BMT, there were no differences between male or female (B6→F chimera) or on epithelial tissues (F→M chimeras). We then transplanted TCD-BM plus Teffs from B6 donors with or without additional HY-specific iTregs into these lethally irradiated chimeric recipients. In B6→BALB.b (miHAg-mismatched) and B6→BDF1 (haplo-mismatched) BMT models, we found that HY-specific iTregs were highly capable in preventing GVHD, and the efficacy was comparable in either type of chimeric recipients (Fig. 7), indicating target Ag expressed on either compartment is sufficient for iTregs to exert their suppression in GVHD.

**HY-specific iTregs essentially preserve the GVL effect**

To determine the effect of HY-specific iTregs on the GVL activity, we used the clinically relevant B6→BDF1 (haplo-mismatched) BMT model with the injection of p815-luc<sup>+</sup> mastocytoma cell line. One day after lethal irradiation, we injected TCD-BM from B6 donors and HY-specific iTregs into male recipients; 3 d later, we injected B6 Teff cells and p815-luc<sup>+</sup> cells. We observed mice receiving BM plus p815 alone all succumbed to tumor mortality within 20 d post-BMT, as seen by high BLI signal with little weight loss; however, mice receiving an addition of Teff cells died of GVHD, indicated by decreased weight loss with little to no BLI signal (Fig. 8). The addition of HY-specific iTregs significantly increased survival (p < 0.001) and significantly delayed tumor mortality (p < 0.001) as seen by maintained body weight and low BLI signal (Fig. 8).

To better mimic clinical circumstance in which patients have already established tumor, we generated a pre-established tumor model by injecting p815-luc<sup>+</sup> cells to the recipients 3 d prior to irradiation and 7 d prior to Teff infusion. One day after irradiation, male recipients were transplanted with BM plus HY-specific iTregs, and 3 d later Teffs were infused. As shown in Fig. 9, 50% of the recipients of BM plus p815 tumor died within 50 d of BMT without body weight loss and strong BLI signal, indicating tumor relapse (Fig. 9A–C). The recipients of BM plus Teffs also died within 62 d with body weight loss and no detectable BLI signal, indicating GVHD mortality. HY-specific iTreg infusion significantly attenuated GVHD (p < 0.05), reflected by higher percentage of survival and no tumor relapse reflected by no BLI signal (Fig. 9A–C, p < 0.05). Taken together, these data indicate the HY-specific iTregs largely preserved the GVL activity mediated by Teffs.

**Discussion**

Aiming to increase the potency and selectivity of Treg therapy, by using TGF-β–induced Ag-specific iTregs, our previous studies have demonstrated that Ag-specific iTregs, once activated in the recipient, are significantly more effective than expanded polyclonal nTregs in the prevention of GVHD (4, 5). The current study substantially extended our previous work by generating and test-
ing iTregs specific for naturally processed alloantigens. Given the knowledge that female to male transplants occur frequently in the clinic and these patients are at a greater risk of developing GVHD due to miHAg-mismatched Ags, like HY, we strove to provide clinical relevance by generating HY-specific iTregs. We found that monoclonal iTregs specific to HY miHAgs were highly effective in preventing GVHD in an activation-dependent manner. Furthermore, we observed that HY-specific iTregs largely preserve the GVL effect (Figs. 8, 9). Given p815 used in our study is a mastocytoma cell line originally derived from male DBA2 mice (24), it is therefore susceptible to Ag-specific T cell– rather than NK cell-mediated killing. Our results indicate that miHAg-specific iTregs still permit the GVL activity against the tumor that expresses such a miHAg. This observation is important and clini-
cally relevant, because many miHAs, such as HY, are ubiquitously expressed.

Unlike freshly isolated nTregs, iTregs are generated from naive CD4 T cells, and thus, the number of iTregs is essentially unlimited (Fig. 1). In current nTreg cell expansion protocols for clinical application, long culture periods and multiple rounds of expansion are required to reach an optimal number of cells (25, 26) still with potential loss of Foxp3 expression (27). Given iTregs’ rapid expansion potential (28), this will decrease culture times and in turn resolve the fear of Foxp3 loss in vitro; however, there is still concern regarding iTregs’ stability in vivo. Given our results showing iTregs remain highly stable even under extreme inflammatory conditions (Fig. 6), this current work gives strong rationale to move iTreg therapy into the clinic. A potential concern was raised by some studies showing that in vitro generated iTregs were less suppressive than nTregs (29, 30) and failed to prevent GVHD (31, 32). In contrast, there is also substantial evidence in the literature supporting that iTregs were as or more effective than nTregs in suppressing immune responses in vivo (1, 16, 33–39). Consistent with our previous studies using OVA-specific iTregs (4, 5), the current work demonstrated that HY-specific iTregs were highly effective in preventing GVHD in clinically relevant murine models of allogeneic BMT in an Ag-dependent manner (Figs. 2, 3).

The stability and efficacy of iTregs still appear to be controversial with regard to controlling GVHD. However, our results are also supported by the reports from Steinman’s group (40), which demonstrated that iTregs generated with allogeneic DCs in the presence of TGF-β and RA maintained Foxp3 expression and exhibited higher levels of CNS2 demethylation in the Foxp3 gene, a marker for stability. Stability of iTregs generated by us and others may be partially attributed to the presence of RA, which was shown to promote iTregs through increasing histone methylation and acetylation within the promoter and conserved non-coding DNA sequence elements at the Foxp3 gene (38, 41). More strikingly, we interpret that the efficacy of iTregs in the attenuation

FIGURE 7. Effect of HY-Ag distribution on HY-specific iTreg-mediated protection. (A) Male→female or female→male BM chimeras were generated using BALB.b mice, as described in Materials and Methods. These BM chimeras were lethally irradiated and divided into two cohorts, each of which were transferred with TCD-BM alone or plus 25 × 10^6/mouse total splenocytes from normal B6 donors. HY-specific iTregs were also included at 4 × 10^6/mouse into donor graft at the day of BMT for some recipients. Recipient survival is shown, and the data represent five to eight mice in each group. (B) Male→female or female→male BM chimeras were generated using BDF1, mice as described in Materials and Methods. These BM chimeras were lethally irradiated again and divided into two cohorts, each of which were transferred with TCD-BM alone or plus 4 × 10^6/mouse CD25-depleted total T cells from normal B6 donors. HY-specific iTregs were also included at 2 × 10^6/mouse into donor graft at the day of BMT for some recipients. Recipient survival is shown, and the data represent seven to eight mice in each group. Asterisks indicate statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 8. HY-specific iTregs spare the GVL effect. B6D2F1 male recipient mice were lethally irradiated and injected with B6 BM with or without HY-specific iTregs. Three days later, CD25-depleted Teffs plus 5000 p815-luc mastocytoma cells were injected. Mice were monitored for body weight loss (A), survival (B), and tumor mortality (C) using the IVIS200 imager throughout the course of study. The data depicted in (A) and (B) are pooled from two replicate experiments, but the imaging shown (D) is from one of these two replicate experiments. Asterisks indicate statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001.
of GVHD is directly related to TCR-driven activation and expansion in vivo (Figs. 4, 6). The iTregs used in our studies were ~100% Ag specific and able to expand when recognizing cognate Ag, whereas the iTregs used in studies by others were polyclonal, and only a small fraction (e.g., <1%) of them was able to expand when recognizing alloantigens. Our data clearly show that iTregs not activated by cognate Ag were unable to expand and were ineffective in the prevention of GVHD. Along this line, Sela et al. (40) showed that DC-induced, alloantigen-specific iTregs are capable of preventing GVHD. However, the therapeutic efficacy of their iTregs was lower than HY-specific iTregs used in our current study but higher than polyclonal iTregs used in other studies (27–29), indicating that the efficacy directly correlates with the frequency of alloreactive cells among different types of iTregs. Taken together, these data provide direct evidence that TCR-driven activation and expansion of iTregs after infusion are essential for their therapeutic efficacy in the control of GVHD.

Because it is commonly accepted that GVHD development requires that donor T cells recognize alloantigens expressed on epithelial tissues, we hypothesized that Tregs must also recognize Ags expressed on epithelia to prevent GVHD. A recent study by Tawara et al. (42) proposed that the host APCs are necessary and sufficient for GVHD protection by donor Tregs. By creating BM chimeras as recipients in which the alloantigens to be recognized by Tregs are expressed on either hematopoietic cells or parenchymal tissues, we observed that HY-specific iTregs were highly capable in preventing GVHD in either type of chimeric recipients (Fig. 7). These results indicate target Ag expressed on either compartment is sufficient for iTregs to exert their suppression in GVHD.

The current work using TCR Tg T cells clearly provides the evidence that miHAg-specific iTregs were effective in the prevention of acute GVHD. To translate the finding into clinical application, one could generate Ag-specific human iTregs by transducing TCR gene into CD4 T cells and then inducing them into iTregs in vitro. Alternatively and also more practically, miHAg-reactive iTregs could be generated from polyclonal CD4 T cells. In fact, we were able to generate HY-reactive polyclonal iTregs by two rounds of stimulation of CD4 T cells with dendritic cells from normal female B6 mice in the presence of HY peptide (Supplemental Fig. 4A). These iTregs enriched for HY specificity exhibited significantly higher efficiency in suppressing B6 CD4 T cells in response to APCs from BDF1 male mice as compared with polyclonal iTregs generated after anti-CD3 stimulation (Supplemental Fig. 4B). Furthermore, we recently have shown that human iTregs specific for HY miHAg (43) can be extensively expanded ex vivo, which demonstrates the feasibility to acquire sufficient human HY-specific iTregs for clinic trials. In conclusion, the current preclinical study provides strong rationale to apply human miHAg-specific iTregs in the clinic for the prevention of GVHD in patients after allogeneic hematopoietic cell transplantation.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figures:

Fig. S1

**Fig. S1. Effects of HY-specific iTregs on long-term donor immune reconstitution.** Lethally irradiated (B6 x bm12)F1 mice were transplanted with TCD-BM alone or TCD-BM plus CD4^+^CD25^-^ Teffs with or without HY-specific iTregs in male or female recipients as described in figure 1. Eighty days after BMT, survival recipients were euthanatized and their splenocytes were counted and stained for expression of H2K^b^ (donor type), CD4, CD8 and B220. The numbers of total spleen cells, donor B cells (H2K^b^B220^+^), CD4 (H2K^b^CD4^+^) T cells, and CD8 T cells (H2K^b^CD8^+^) were shown. These data represent 1 of 2 replicate experiments.
**Fig. S2.** Pathogenicity of T cells from B6 Luc-Tg mice in GVHD. CD25-depleted total T cells (Teffs) were purified from spleens and lymph nodes of normal B6 or B6 Luc-Tg mice through negative selection. Lethally irradiated BALB/c mice were transplanted with TCD-BM or plus Teffs from normal or Luc-Tg B6 donor at the doses indicated. Recipient survival (A) and body weight changes (B) are shown. The data are pooled from 2 replicate experiments with 8-10 mice per group.
Fig. S3. Effects of HY-specific iTregs on Teff expansion in vivo. The results from experiments using B6→Bm12 as described in figure 2. The BLI signal strength was shown on one representative mouse from each group (A). Average signal intensity from each group of recipients throughout the experimental time points (B). The results were from the experiments using B6 → BALB.b BMT model as described in figure 3 A and B. The BLI signal strength was shown on one representative mouse from each group at day 14 after BMT (C). Average signal intensity from each group of recipients was shown at the time points indicated (D). The results were from the experiments using B6 → BDF1 BMT model as described in figure 3 C and D. The BLI signal strength was shown on one representative mouse from each group (E), and average signal intensity from each group of recipients was shown (F) on day 13 after BMT. The image is shown in one representative mouse in each group. The data represent one of 2 replicate experiments with 5-6 mice per group in each experiment.
**Fig S4.** Education with HY antigen enhances suppressive function of iTregs. HY-TCR transgenic iTregs were generated as described in Fig.1. Polyclonal HY iTregs were generated by isolating WT B6 resting CD4+CD25− T cells and stimulated with syngeneic DCs with HY peptide, IL-2 (5ng/mL), TGFβ (5ng/mL), and RA (10nM) for 5 days. After 5 days, cells were harvested and restimulated with syngeneic DC’s with HY peptide for an additional 5 days. Anti-CD3 polyclonal iTregs were generated by stimulating resting CD4+CD25− T cells with syngeneic APCs and α-CD3 with IL-2 (5ng/mL), TGFβ (5ng/mL), and RA (10nM) for 3 days. Different iTregs were harvested and purified through CD25-magnetic bead positive selection (A). iTreg suppressive function was tested by CFSE labeling CD4+CD25− B6 T cells and stimulating them with BDF1 male APCs. HY transgenic, HY polyclonal, or αCD3 iTregs were then titrated at the indicated ratios. After 5 days culture, cells were harvested and CFSE dilution was assessed through flow cytometry. Percentage of suppression was calculated by using the control (without iTregs) as the baseline for uninhibited T cell proliferation (B).