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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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Abstract

Naturally derived regulatory T cells (nTregs) may prevent graft-versus-host disease (GVHD) while preserving graft-versus-leukemia (GVL) activity. However, clinical application of nTregs has been severely hampered by their scarce availability and non-selectivity. 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 pre-clinical models of bone marrow transplantation (BMT). We selected HY as a target antigen because it is a naturally processed, ubiquitously expressed minor histocompatibility antigen (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 BMT models. Interestingly, the expression of target Ag (HY) on the hematopoietic or non-hematopoietic compartment alone was sufficient for iTregs to prevent GVHD. Furthermore, treatment with HY-iTregs still preserved the GVL effect even against pre-established leukemia.

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Disclosures

The authors have no conflict of interest to disclose.

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 (Teff) 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.

Introduction

Allogeneic bone marrow 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 antigens on leukemic and host tissues, resulting in either beneficial graft versus leukemic (GVL) or deleterious graft-versus host (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 remains 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 non-selective 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 T regulatory (iTreg) cell therapy for the treatment of GVHD may solve the previously stated disadvantages of nTreg therapy. First, iTregs can be generated from naïve T cells, under specific polarizing conditions, offering a greater number of primary cells for initial expansion. Secondly, we propose, by conferring antigen specificity or antigen education during iTreg generation, we can overcome the high number needed for efficiency as compared to non-specific nTreg cell therapy. Finally, we propose 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 TGFβ-induction, and demonstrated that they persist long-term *in vivo* and suppress GVHD in non-myeloablative 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 shows 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-2^b, CD45.2$^+$, BALB/c (H-2^d) and (B6 x DBA2) F1 (BDF1, H-2$^{b/d}$) mice were purchased from the National Cancer Institute. B6 Ly5.1 (H-2^b, CD45.1$^+$), B6 bm12 (H-2^b), BALB.b (H-2^b) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Foxp3^{gfp} knock-in (KI) strain was obtained from A. Rudensky's laboratory (11, 12). Luciferase-transgenic (*Luc*-Tg) strain on B6 background was kindly provided by R. Negrin (Stanford Univ., CA)(13). Anti-HY TCR Tg Marilyn mice (CD4$^+$Tg, H-2^b, I-A^b restricted) was kindly provided by C.R. Mainhart (NIAID, Bethesda, MD). Marilynn Foxp3^{gfp} knock-in (KI) and (B6 x 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 MUSC. All experimental procedures were approved by the IACUC.

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$^-$ cells ranged from 85 to 95%, but CD4$^+$CD25$^+$ cells was always less than 1% among total CD4$^+$ cells. To generate HY-specific iTregs, CD4$^+$CD25$^-$ T cells from TCR Tg (Marilynn) Foxp3^{gfp} KI mice were seeded at 2.5 x 10^5/ml and stimulated with 0.5 μg/ml HY peptide in the presence of 1.25 x 10^6/ml irradiated syngeneic T-cell depleted (TCD)-splenoctyes as APCs with 5 ng/ml TGF-β1, 5 ng/ml IL-2 and 10 nM retinoic acid for 6 days.

Immuno-fluorescence 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 (BLI)

The procedures for induction of acute GVHD were described in previous publications (5, 15). BALB.b mice were exposed to total body irradiation (TBI) at 850–900 cGy (2 split doses) at day −1. (B6 x bm12)F1 or BDF1 mice were exposed to 1200 – 1300 cGy TBI (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 hrs 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 GVHD lethality. To generate BM chimeras, female or male recipients were lethally irradiated and transplanted with TCD-BM from male or female syngeneic donors, and thus HY antigens were expressed only on epithelial tissues (F → M) or hematopoietic cells (M → F). *In vivo* BLI of the recipients transplanted with allogeneic T cells from *Luc*-

Tg B6 donors and BM from non-Tg B6 donors was performed using an IVIS200 charge-coupled device imaging system (Xenogen). For GVL induction, p815-luc mastocytoma cells were injected either on day of transplant with iTregs or three days prior to irradiation (pre-established tumor model).

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 TGFβ, and addition of retinoic acid (RA) further increases the generation of iTregs (14, 16, 17). In this study we selected HY as target antigen, 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 $Foxp3^{gfp}$ KI mice by stimulating with HY^{Ab}*Dby*, in the presence of IL-2, TGFβ, and RA (Fig. 1A, upper panels) and purified (purity 94%± 3%) by FACS sorting (Fig. 1A, lower panels).

$Foxp3^{gfp}$ 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). $CD4^+CD25^{hi}$ 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-$Foxp3^{gfp}$ $CD4^+CD25^-$ T cells and isolated for $CD4^+CD25^{hi}$ 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: 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 x bm12)F1 BMT model, in which donor effector $CD4^+$ T cells (Teff) recognize mismatched recipient MHC II alloantigen ($H2^{bm12}$). In this model, Teffs at indicated dose induced ~60% GVHD lethality while 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 and B), indicating that recognition of HY antigen by HY-specific iTregs was indispensable for their suppressive function of allogeneic responses *in vivo*. To assess whether donor reconstitution was impaired by iTreg therapy, 80 days 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 + Teff (GVHD controls) had significantly reduced numbers of spleen, B and T cells (vs BM alone, $p<0.05$, Fig. S1). 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 utilized the same model and infused HY-specific iTregs 3 days prior to Teffs and found that these iTregs completely prevented GVHD lethality in male recipients ($p<0.01$) (Fig. 2C and D).

In clinical BMT, most patients receive grafts from MHC-matched and multiple mHAg-mismatched donors. In an effort to mimic a clinical scenario, we used the B6 → BALB.b (both $H2^b$) model, in which donor and recipient mice differ by at least 29 different miHA loci (21). HY-specific iTregs were highly effective in preventing GVHD in male ($p<0.05$) but not female BALB.B recipients (Fig. 3A and B). 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 and D). 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 to 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 are required for mediating GVHD and found that at least 4-fold lower numbers of *Luc*-Tg T cells were required to cause GVHD lethality comparable to normal B6 T cells (Fig. S2). This 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 $CD4^+$, although there were relatively low signal intensities and no significant difference among groups on day 6 following Teff injection (Fig. S3A), throughout later observation periods, the BLI intensity was significantly reduced in male recipients that received HY-specific iTregs compared to recipients with Teff alone ($p< 0.001$) or in female recipients transplanted HY-specific iTregs ($p = 0.02$) (Fig. S3A, B). Furthermore, male recipients that received HY-specific iTreg showed less dispersed BLI signal, mainly confined to the spleen, compared to other recipients (Fig. S3B). Similar results were observed in miHAg- or haplo-mismatched BMT models (Fig. S3C–F). These data suggest that HY specific iTregs regulate allogeneic 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 x bm12) F1 recipients. Seven days after BMT, we measured Teffs ($CD4^+Ly5.1^+$) and iTregs ($CD4^+TCRV\beta6^+Ly5.1^-$) in recipient spleen (Fig. 4A and C) and liver (Fig. 4B and D). 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$^-$) into irradiated B6D2F1 recipients. Fourteen days after BMT, analysis of IFNγ and TNFα secretion within the spleen of effector CD4 and CD8 T cells, showed male recipients had significantly decreased secretion of pro-inflammatory cytokines ($p<0.01$) whereas there was no significant difference between Teff alone and female recipients (Fig 5A,B). In correlation with our flow data, analysis of the serum cytokine levels, 14 days post BMT, revealed male recipients had significantly reduced levels of IFNγ and TNFα ($p<0.01$) whereas there was no differences between female recipients and Teff alone groups (Fig. 5C). These cellular findings are consistent with our long-term survival data, proving the recognition of cognate antigen is necessary for iTregs to control Teffs in order to attenuate GVHD.

To address the stability of Ag-specific iTregs after injection, we performed a time course analysis of HY-specific iTregs within male and female recipient's spleen and liver 7, 14 and 21 days after our previously described B6→BDF1 model. The male recipients retained the high number of CD4$^+$Vβ6$^+$Foxp3$^+$ iTregs in their spleens on day 7, 14, and 21 post BMT ($p< 0.001$), whereas the number of HY-specific iTregs declined over time in the female recipients (Fig 6A, B). The male recipients also had higher numbers of CD4$^+$Vβ6$^+$ iTregs than female recipients on day 7 in their livers, although Foxp3 expression was retained similarly at this time point (Fig 6C, D). In the liver, the numbers of iTregs were more strikingly different on days 14 or 21 between male and female recipients. Furthermore, iTregs were highly stable in the male recipients reflected by their Foxp3 expression, whereas iTregs rapidly lost their Foxp3 expression in female recipients ($p<0.001$) (Fig. 6D). Taken together, iTregs were highly stable and expanded extensively after Ag-stimulation, and in turn effectively suppress Teff expansion and activation and migration into GVHD target organs regardless of BMT models

Expression of target antigen on epithelial tissues is not required for iTregs to prevent GVHD

It has been widely accepted that donor T cells have to recognize alloantigens expressed on epithelial tissues in order to cause GVHD in myeloablative BMT models (23). However, it is not clear whether Tregs require expression of target antigen on epithelial tissues in order to suppress GVHD. To address this question, we created 2 types of BM chimeras by transplanting donor (male or female) BM into lethally irradiated syngeneic recipients (female or male), so that the HY antigen was only expressed either on hematopoietic cells (M → F chimeras) 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. 7A,B), indicating target antigen 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 utilized the clinically relevant B6→BDF1 (haplo-mismatched) BMT model with the injection of p815-luc$^+$ mastocytoma cell line. One day after lethal irradiation, we injected TCD-BM from B6 donors and HY-specific iTregs into male recipients, three days later we then injected B6 Teff cells and p815-luc$^+$ cells. We observed mice receiving BM + p815 alone all succumbed to tumor mortality within 20 days post BMT, as seen by high BLI signal with little weight loss, however mice received an addition of Teff cells died from 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).

In order to better mimic clinical circumstance where patients have already established tumor, we generated a pre-established tumor model by injecting p815-luc$^+$ cells to the recipients 3 days prior to irradiation and 7 days prior to Teff infusion. One day after irradiation, male recipients were transplanted with BM plus HY-specific iTregs and three days later Teffs were infused. As shown in figure 9, 50% of the recipients of BM plus p815 tumor died within 50 days of BMT without body weight loss and strong BLI signal indicating tumor relapse (Fig. 9, A–C). The recipients of BM plus Teffs also died within 62 days 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 TGFβ-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 testing 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 antigens, 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 activation-dependent manner. Furthermore, we observed that HY-specific iTregs largely preserve the GVL effect (Fig. 8 and 9). Given p815 used in our study is a mastocytoma cell line originally derived from male DBA2 mice (24), therefore is susceptible to antigen-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 clinically relevant, because many miHAgs, such as HY, are ubiquitously expressed.

Unlike freshly isolated nTregs, iTregs are generated from naïve 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 period 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). On the other hand, 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 (Fig. 2 and 3).

The stability and efficacy of iTregs still appears to be controversial with regards to controlling GVHD. However, our results are also supported by the reports from Steinman's group (40), who 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 (41)(38). More strikingly, we interpret that the efficacy of iTregs in the attenuation of GVHD is directly related to TCR-driven activation and expansion *in vivo* (Fig. 4 and 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 were able to expand when recognizing alloAgs. Our data clearly shows 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 showed that DC-induced, alloAg-specific iTregs are capable of preventing GVHD (36). 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 is essential for their therapeutic efficacy in the control of GVHD.

Since 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 antigens expressed on epithelia in order to prevent GVHD. A recent study by Tawara et al. proposed that the hosts APCs are necessary and sufficient for GVHD

protection by donor Tregs (42). 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 antigen expressed on either compartment is sufficient for iTregs to exert their suppression in GVHD.

The current work using TCR transgenic 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 induce 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 2-rounds of stimulation of CD4 T cells with dendritic cells from normal female B6 mice in the presence of HY-peptide (Fig. S4A). 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 to polyclonal iTregs generated after anti-CD3 stimulation (Fig. S4B). Furthermore, we recently have shown that human nTregs 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 pre-clinical study provides strong rationale to apply human miHAg-specific iTregs in the clinic for the prevention of GVHD in patients after allogeneic HCT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

GVHD	graft-versus-host disease
nTregs	naturally derived regulatory T cells
iTregs	inducible T regulatory cells
GVL	graft-versus-leukemia
BMT	bone marrow transplantation
miHAg	minor histocompatibility antigen
RA	retinoic acid

Teffs Effector T cells

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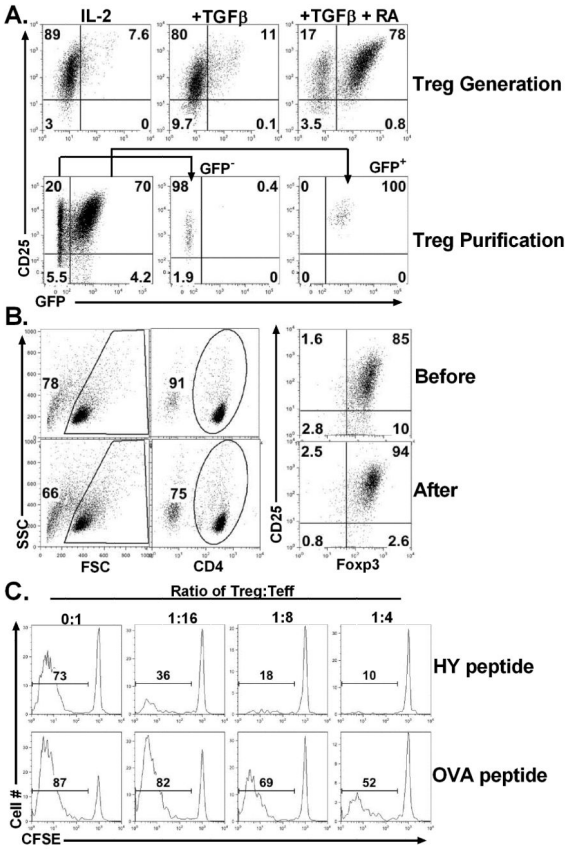

Fig. 1. Generation and isolation of HY-specific iTregs

(A) $CD4^+CD25^-$ cells were purified from spleen and lymph nodes of TCR Marilynn $Foxp3^{gfp}$ 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 TGFβ (5 ng/ml) alone or TGFβ 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). (B) $CD4^+CD25^-$ cells were purified from spleen and lymph node of TCR Marilyn mice, and iTregs were generated in the presence of TGFβ and RA as described in A. Six days after culture, $CD4^+CD25^{hi}$ cells (iTregs) were isolated by enriching $CD4^+$ cells through negative selection and then purifying $CD25^{hi}$ 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 more than 10 experiments. (C) $CD4^+CD25^-$ purified T cells from B6 $Ly5.1^+$ mice were labeled with CFSE and stimulated at 2 x 10^5/well with irradiated TCD-splenocytes from female (B6 x bm12) F1 mice at 6 x 10^5/well in 96-well plates. Various numbers of HY-specific iTregs were added into culture to achieve indicated Treg: 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. CSFE profiles were shown on gated $Ly5.1^{+}CD4^{+}$ Teffs. These data represents 1 of 3 replicate experiments. ND: not done.

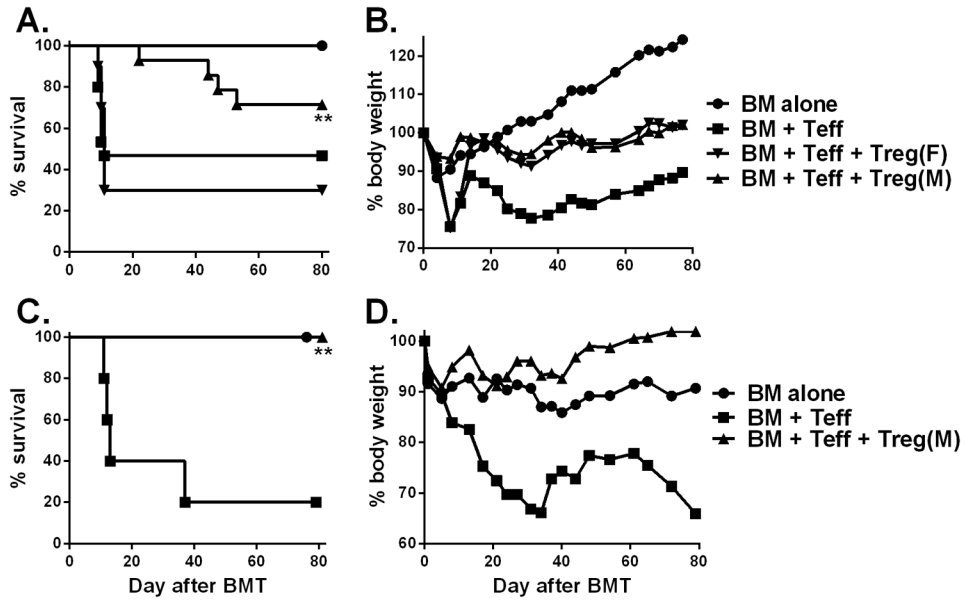

Fig. 2. HY-specific iTreg attenuation of GVHD is antigen dependent

Male or female (B6 x bm12) F1 mice were lethally irradiated and transferred with 5 x 10^6 TCD-BM alone or plus 1–1.5 x 10^6/mouse CD25-depleted $CD4^+$ T cells (Teffs) from B6 donors. HY-specific iTregs were generated and isolated as described in figure 1, were added at 0.5–0.75 x 10^6/mouse into donor graft at the same time of BMT. Overall survival (A) and body weight changes (B) are shown. The data are pooled from 3 replicate experiments with 10–15 mice in each group. In a separate experiment (n = 5–6), male (B6 x bm12) F1 mice were lethally irradiated and transferred with TCD-BM alone or plus 1 x 10^6/mouse HY-specific iTregs on day 0. CD25-depleted $CD4^+$ T cells (Teffs) from B6 donors were injected at 2 x 10^6/mouse on day 3 after BMT. Recipient survival (C) and body weight changes (D) are shown. Asterisk indicates statistical significance: *p<0.05, **p<0.01, ***p<0.001

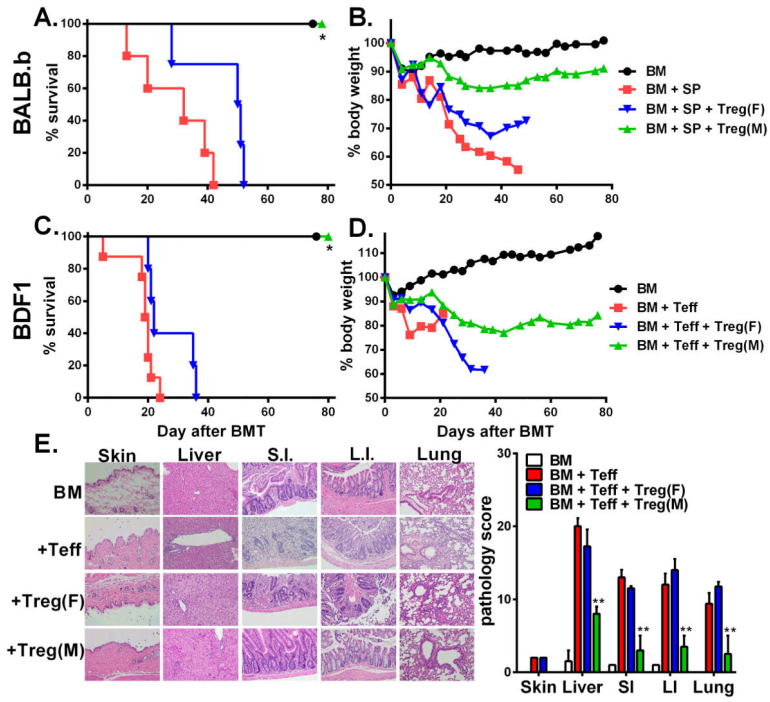

Fig. 3. Effect of HY-specific iTregs in the prevention of GVHD in mHAg- or halpo-mismatched BMT model

Male or female BALB.b mice were lethally irradiated and transferred with TCD-BM alone or plus 4 x 10^6/mouse HY-specific iTregs. On day 3, 25 x 10^6/mouse total splenocytes from normal B6 donors were injected into the recipients previously transferred with BM alone or BM plus iTregs. Recipient survival (A) and body weight changes (B) are shown. The data are pooled from 2 replicate experiments with 9–10 mice in each group. Male or female BDF1 mice were lethally irradiated and transferred with TCD-BM alone or plus at 4 x 10^6/mouse CD25-depleted total T cells from normal B6 donors. HY-specific iTregs were also included at 2 x 10^6/mouse into donor graft 3 days after BMT for some recipients. Recipient survival (C) and body weight changes (D) are shown. The experiments were done 2–3 times for each BMT model, but the data presented are from one experiment with 5–8 mice in each group using cell doses indicated. Seven days post Teff injection, BDF1 recipients as described in the legend were sacrificed and pathology samples of the skin, lung, liver, small and large intestine were collected (E) represents the pathology score of the various organs with 4 mice per group. Asterisk indicates statistical significance: *p<0.05, **p<0.01, ***p<0.001

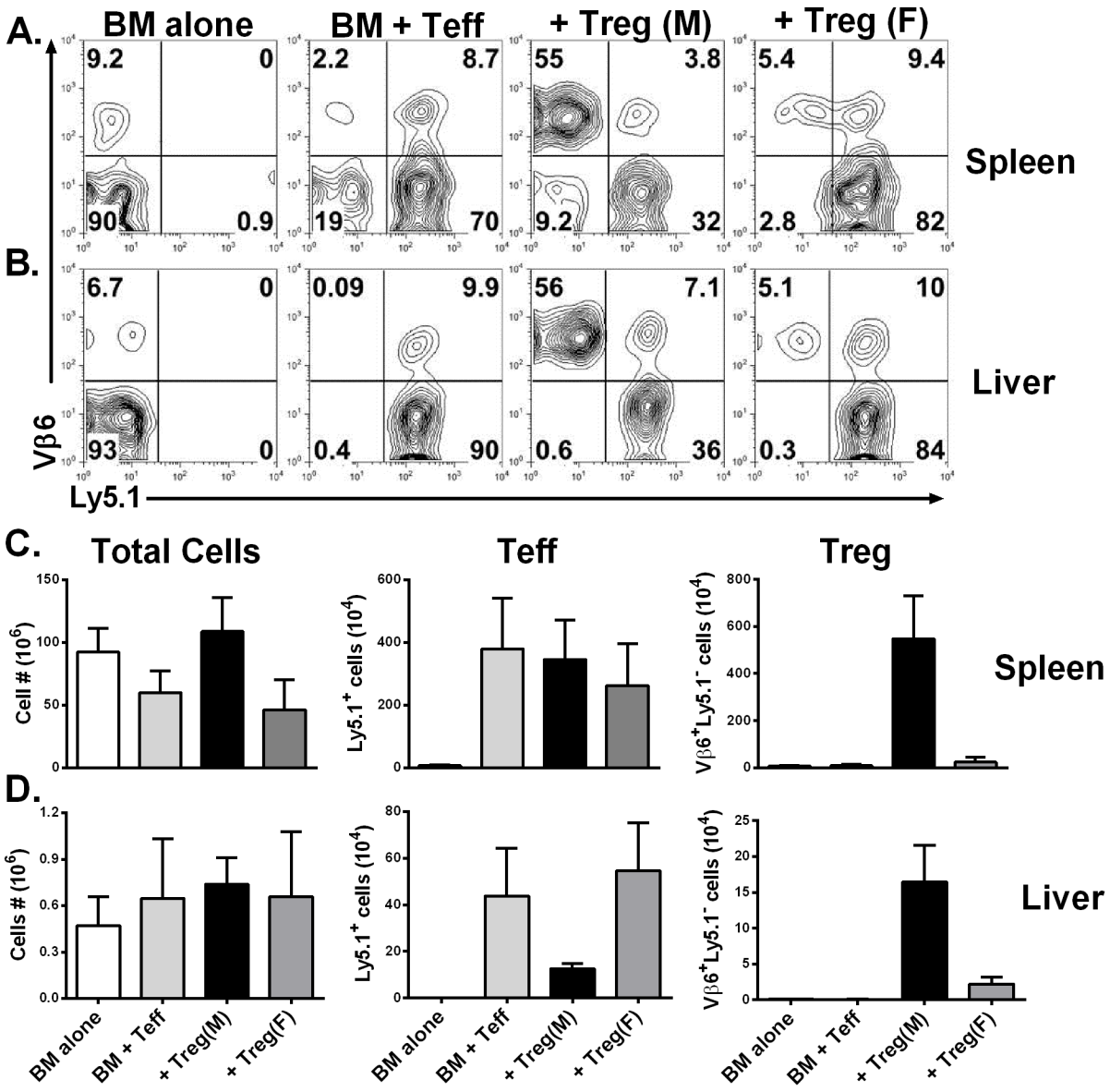

Fig. 4. Stability and Efficiency of HY-specific iTregs

Lethally irradiated male or female (B6 x bm12) F1 mice were transplanted with B6 TCD-BM plus 1.5 x 10^6/mouse Teffs ($CD4^+CD25^-$) isolated from B6 $Ly5.1^+$ mice. At the same time, 0.75 x 10^6/mouse HY-specific iTregs were also included into the donor graft to some recipients. Seven days after BMT, recipient spleen and liver were harvested and measured for expansion and infiltration of iTregs and Teffs. Mononuclear cells were isolated from recipient spleen (A) and liver (B), and expression of TCRVβ6 and Ly5.1 was shown in gated $CD4^+$ live cells. The average numbers of total cells, Teffs ($CD4^+Ly5.1^+$) and iTregs ($CD4^+Vβ6^+Ly5.1^-$) per mouse were shown in recipient spleen (C) and liver (D). Each group includes 3–4 mice, and the data represents 1 of 3 replicate experiments. Asterisk indicates statistical significance: *p<0.05, **p<0.01, ***p<0.001

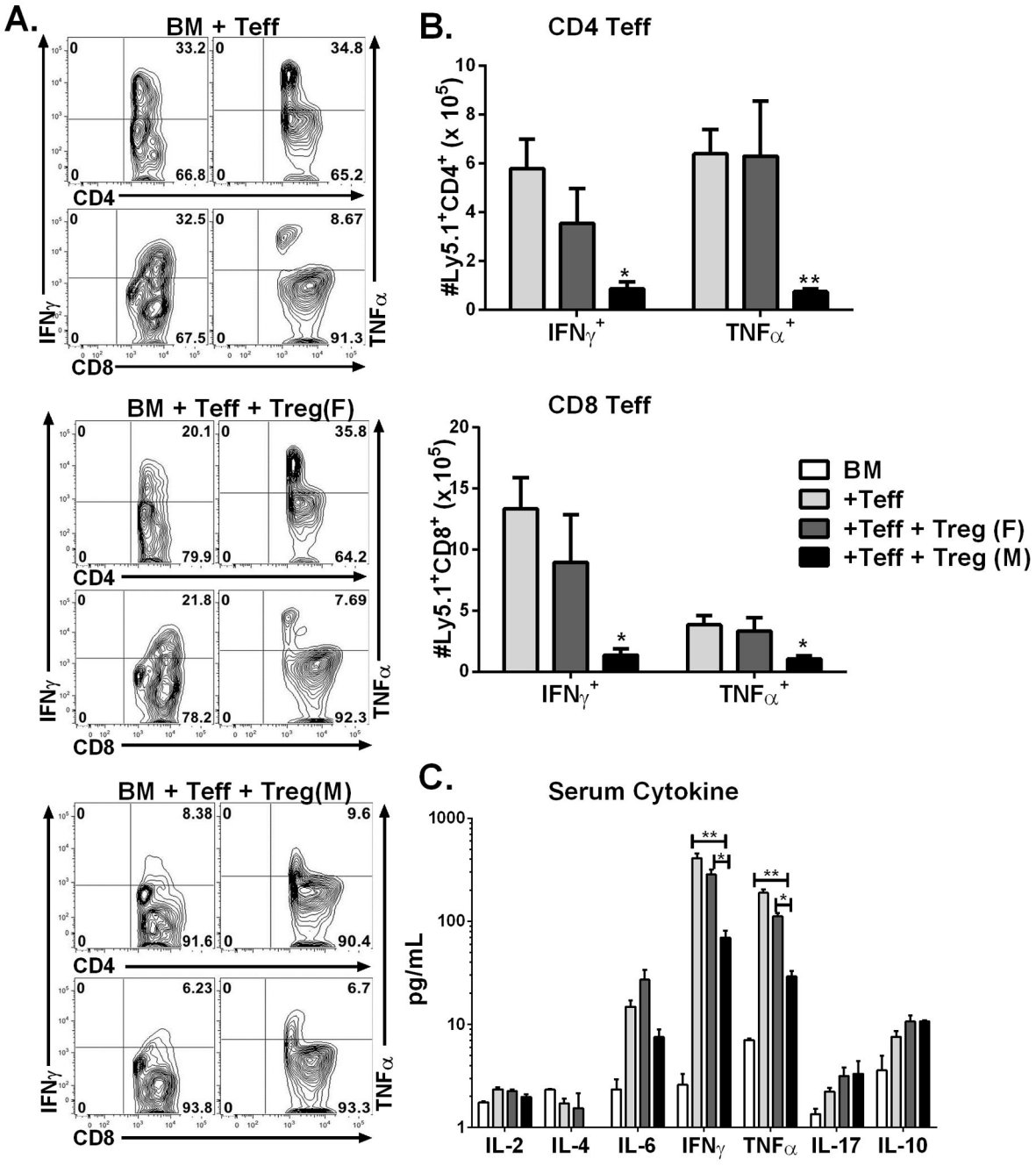

Fig 5. HY-specific iTregs suppress activation and expansion of Teffs

Male or Female BDF1 mice were lethally irradiated and transferred with TCD-BM alone or plus 4 x 10^6/mouse CD25-depleted total T cells from normal B6 Ly5.1$^+$ donors. HY-specific iTregs were also transplanted at 2 x 10^6/mouse on day 0 of BMT. Fourteen days after Teff injection, recipient spleen and liver were harvested and total T cells isolated, 4 mice per group. (A) Representative flow analysis of IFNγ and TNFα with effector CD4 and CD8 T cells. B) Represents the absolute number of CD4 and CD8 Teffs secreting IFNγ and TNFα within the recipient spleen. (C) Serum was collected on day 14 and cytokine levels were

assessed by cytometric bead analysis, one representative analysis of two independent experiments. Asterisk indicates statistical significance: *p<0.05, **p<0.01, ***p<0.001.

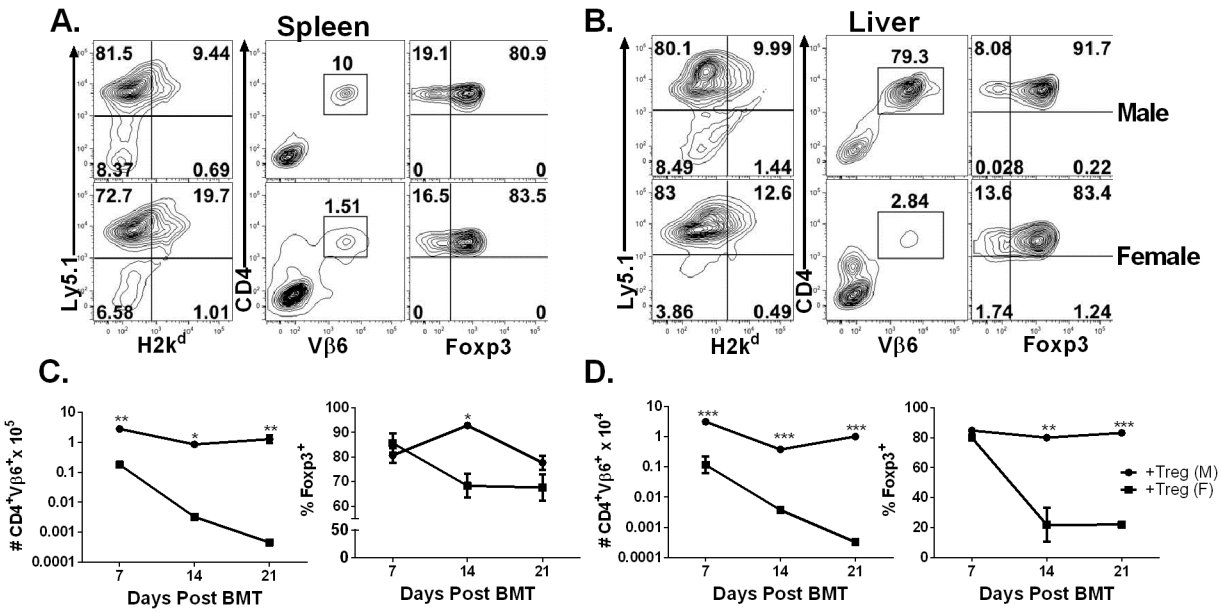

Fig. 6. HY-specific iTregs are highly stable under inflammatory conditions

Male or Female BDF1 mice were lethally irradiated and transferred with TCD-BM alone or plus 4 x 10^6/mouse CD25-depleted total T cells from normal B6 Ly5.1$^+$ donors. HY-specific iTregs were also transplanted at 2 x 10^6/mouse on day 0 of BMT. On days 7, 14, and 21 post BMT spleen (A, C) and liver (B, D) were collected from recipient mice for analysis. Phenotypes of cells isolated from recipient spleen (A) or liver (B) are displayed. Absolute numbers of originally infused iTregs ($H2K^{b+}CD4^+V\beta6^+$) in spleen (C) or liver (D) are depicted on the left panels. Percentages of Foxp3 expression on gated iTregs in spleen (C) or liver (D) were shown on the right panels. Asterisk indicates statistical significance: *p<0.05, **p<0.01, ***p<0.001.

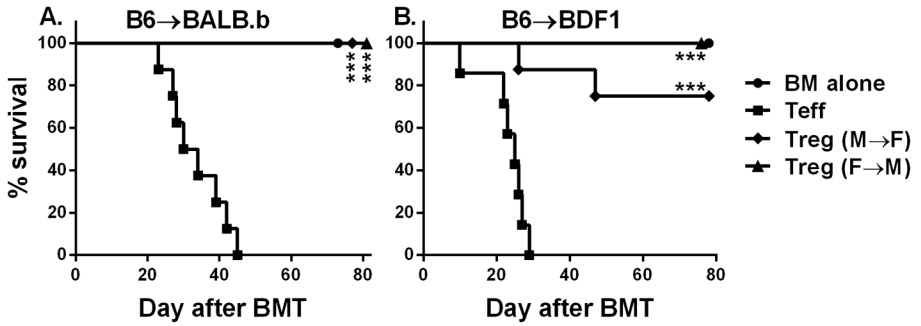

Fig. 7. Effect of HY-antigen distribution on HY-specific iTreg-medicated protection

(A) Male → female or female → male BM chimeras were generated using BALB.b mice as described in "Material and Method". These BM chimeras were lethally irradiated again and divided into 2 cohorts, each of which were transferred with TCD-BM alone or plus 25 x 10^6/mouse total splenocytes from normal B6 donors. HY-specific iTregs were also included at 4 x 10^6/mouse into donor graft at the day of BMT for some recipients. Recipient survival is shown, and the data represent 5–8 mice in each group. (B) Male → female or female → male BM chimeras were generated using BDF1 mice as described in "Material and Method". These BM chimeras were lethally irradiated again and divided into 2 cohorts, each of which were transferred with TCD-BM alone or plus 4 x 10^6/mouse CD25-depleted total T cells from normal B6 donors. HY-specific iTregs were also included at 2 x 10^6/mouse into donor graft at the day of BMT for some recipients. Recipient survival is shown, and the data represent 7–8 mice in each group. Asterisk indicates statistical significance: *$p<0.05$, **$p<0.01$, ***$p<0.001$

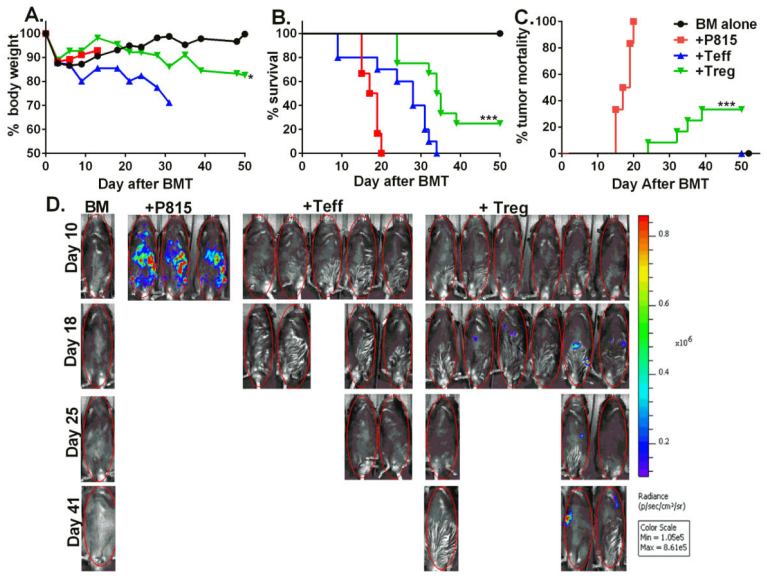

Fig 8. HY-Specific iTregs spare the GVL effect

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 morality (C) using the IVIS 200 imager throughout the course of study. The data depicted in A and B is pooled from 2 replicate experiments, but the imaging shown (D) is from one of these 2 replicate experiments. Asterisk indicates statistical significance: *p<0.05, **p<0.01, ***p<0.001

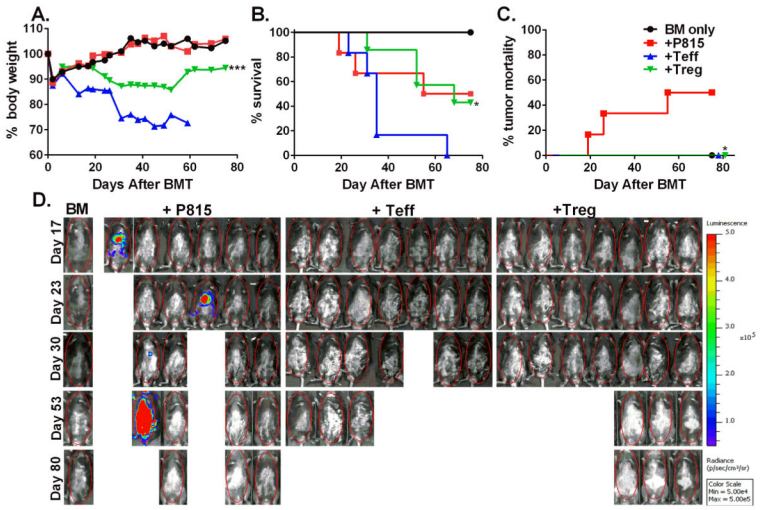

Fig 9. HY-specific iTregs largely preserve GVL effect in pre-established tumor model

Male BDF1 mice injected with 5000 P815-luc cells three days prior to irradiation. Tumor bearing mice were lethally irradiated and transplanted with 5 x 10^6 BM and HY-specific iTregs 2 x 10^6/mouse from B6 donors. Three days later, the recipient mice received 4 x 10^6 CD25-depleted total T cells. Recipient body weight (A) and survival (B) are shown. Data are from one experiment of two replicates with similar but not the same settings. Mice were imaged once weekly and then once every two-three weeks to monitor tumor growth (C and D) using an IVIS 200 imager. Asterisk indicates statistical significance: *p<0.05, **p<0.01, ***p<0.001