Absence of Clinical GVHD and the In Vivo Induction of Regulatory T cells following Facilitating Cell Transplantation

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Abstract

Graft-versus-host disease (GVHD) and failure of engraftment limit clinical bone marrow transplantation (BMT) to patients with a closely matched donor. Engraftment failure of purified allogeneic hematopoietic stem cells (HSC) has been decreased in various BMT models by including donor BM-derived CD8\(^+\)/αβγδTCR\(^-\) facilitating cells (FC) or CD8\(^-\)/αβTCR\(^+\) T cells in the BM inoculum. To aggressively investigate GVHD potential of these donor CD8\(^+\) populations, a purified cell model of lethal GVHD was established in a murine semiallogeneic parent → F\(_1\) combination. Lethally irradiated recipients were reconstituted with purified donor HSC alone, or in combination with splenic T cells (T\(_{SP}\)), BM-derived T cells (T\(_{BM}\)), or the FC population. In marked contrast to the lethal GVHD present in recipients of HSC plus T\(_{SP}\) or CD8\(^-\) T\(_{BM}\), recipients of donor HSC+FC inocula did not exhibit significant clinical or histologic evidence of GVHD. Instead, HSC+FC recipients were characterized by increased splenocyte expression of TGFβ and the induction of the regulatory T cell genes, CTLA4, GITR and FoxP3. These findings suggest that the FC, which express a unique FCp33-TCRβ heterodimer in place of αβTCR, permits HSC allograftment and prevents GVHD through the novel approach of regulatory T cell induction in vivo.
Introduction

Allogeneic bone marrow transplantation (BMT) currently plays an important role in the treatment of various hematologic maladies such as lymphoma, leukemia, aplastic anemia and severe immunodeficiencies. However, even in the 30% of patients with a matched donor that undergo BMT, clinical success has been limited by graft vs. host disease (GVHD).\textsuperscript{1-3} The initial enthusiasm following the decreased incidence of GVHD noted with the transplantation of T cell depleted (TCD) marrow or purified hematopoietic stem cells (HSC), was tempered by an increase in graft failure and malignant disease recurrence.\textsuperscript{1,3-7} Attempts to add-back titrated doses of mature T lymphocytes to restore engraftment and the graft vs. leukemia effect present in unmanipulated BM, have been hampered in the setting of multiple donor-recipient antigen disparities by the induction of lethal GVHD, even with limited numbers of mature T cells.\textsuperscript{8,9} However, several animal studies have recently shown that BM-derived CD8\textsuperscript{+} populations, T and non-T cell, are an important means to facilitate engraftment of purified HSC.\textsuperscript{6,10,11} We have previously identified such a non-T cell population, known as the donor facilitating cell (FC), which dramatically enhances allogeneic engraftment of a mixed syngeneic and allogeneic TCD BM inoculum in lethally irradiated MHC-disparate murine recipients.\textsuperscript{10} Characterized as CD8\textsuperscript{+}/αβγδTCR\textsuperscript{-} (CD8\textsuperscript{+}/TCR\textsuperscript{-}), the addition of only 30,000-50,000 of these donor BM-derived FC to the mixed BM inoculum reliably facilitated engraftment of the allogeneic HSC across complete MHC barriers.\textsuperscript{10} The incidence of alloengraftment increased from 43\% to 100\% and the average level of donor chimerism rose from 13\% to greater than 90\%. In contrast, supplementation of the inocula with similar numbers of CD8\textsuperscript{+}/αβγδTCR\textsuperscript{+} (CD8\textsuperscript{+}/TCR\textsuperscript{+}) BM-derived T-cells (T\textsubscript{BM}) resulted in failure of allogeneic engraftment with most recipients exhibiting syngeneic reconstitution. Even among T\textsubscript{BM} recipients with low levels of donor chimerism, 75\% exhibited histologic evidence of GVHD. Despite recent demonstrations of tumor-specific CD8\textsuperscript{+} T cells in...
BM from patients with hematologic malignancies and the evidence that BM-derived CD8⁺ T cells can elicit a tumor response in vivo, these findings suggest that supplementation with CD8⁺/TCR⁺ TBM may not improve alloengraftment and may, in fact, prove to be clinically disastrous, regardless of other potential anti-tumor benefits. Therefore, understanding differences in GVHD potential of CD8⁺/TCR⁺ TBM and CD8⁺/TCR⁻ FC donor cell populations in a clinically relevant model of purified HSC transplantation, is critical before attempts at clinical application can be considered.

To date, the induction of GVHD in an irradiated allogeneic host has characteristically required supplementation of the TCD donor BM inocula with large numbers of mature T lymphocytes obtained from donor spleen, thymus, or lymph nodes. The exact composition of each inoculum depends on the thoroughness of TCD and the source and composition of peripheral T cell supplementation. Although extremely helpful in our understanding of GVHD, clinical relevance of these prior models is diminished since GVHD potential is assessed for mature peripheral T cells, while the T cell subsets within the donor BM are removed. Furthermore, the TCD donor inoculum contains a variety of cell types that differ in cell-cell interactions, maturation, activation, and cytokine profiles – all of which may alter engraftment and GVHD potential. Given that the current state of the art in clinical BMT utilizes purified donor HSC inocula, where other donor populations are less available to modulate the graft vs. host response, the question of GVHD effector activity for specific BM-derived populations in the setting of purified HSC transplantation has become clinically very relevant.

To better understand the contribution of individual donor cell subsets, and BM-derived CD8⁺ populations in particular, to the induction of acute GVHD in the setting of purified HSC transplantation, we have established a purified cell-based model of lethal GVHD using a P → F₁ (semiallogeneic) murine combination. Transplantation of purified HSC alone, as the source of donor BM, excludes all other donor hematopoietic cell populations from potentially affecting engraftment and modulating the risk of GVHD. This approach permits one to assess the GVHD potential of various individual donor cell populations by adding back only purified GVHD effectors to the HSC inocula. Utilizing this model, three separate populations of potential effectors, all of which contain CD8⁺ cells, were assessed for the ability to induce
GVHD in lethally irradiated semiallogeneic recipients. The subsets evaluated were: 1) αβTCR⁺ splenic T cells (TSP); 2) CD8⁺/TCR⁺ BM-derived T cells (TBM) and 3) the BM-derived CD8⁺/TCR⁻ facilitating cell (FC) population. The results of the current study demonstrate that the recipients of HSC + αβTCR⁺ TSP or CD8⁺/TCR⁺ TBM develop lethal GVHD, whereas recipients of HSC + CD8⁺/TCR⁻ FC fail to exhibit clinical or histological evidence of significant GVHD. Subsequent gene analysis of recipients following HSC+FC transplantation has shown that an immunoregulatory profile characterized by the expression of regulatory T cell genes and TGF-β is induced. These findings suggest, for the first time, that a regulatory immune state by which GVHD is controlled and HSC engraftment and immune reconstitution is favored can be induced in vivo through the use of FC transplantation.
Materials and Methods

Mice

B10.BR SgSnJ (BR, H-2k), C57BL/6 (B6, H-2b), and B6D2 F1 (F1, H-2b/d) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in sterile microisolator cages and received autoclaved food and acidified water for 2 weeks after BMT. Care was in accordance with guidelines of the Institutional Animal Care and Use Committee at Dana Farber Cancer Institute.

Multiparameter live sterile cell sorting

HSC, FC and T BM were isolated from donor BM as previously described 10. Briefly, BM was isolated from the long bones of mice and washed in Hank’s Balanced Salt Solution (HBSS, Gibco, Grand Island, NY). Monoclonal antibodies (mAbs) chosen to isolate murine HSC were Ly6A/E (Sca-1) PE, c-kit biotin, and a mixture of FITC-conjugated anti-lineage (Lin-) mAbs: B220, CD8α, GR-1, MAC-1, αβTCR, and γδTCR. After 45 minutes of incubation at 4°C, cells were washed, resuspended at 200x10^6 cells/ml, and incubated with streptavidin Cy-chrome for 30 minutes. Cells were washed and resuspended in sterile sort media consisting of HBSS, 2% fetal calf serum (Gibco), and 2 µl/ml HEPES buffer (Gibco) prior to multiparameter sterile live cell sorting for HSC as Sca^+/c-kit^dim/intermediate^-/Lin^- cells within the conventional lymphoid gate on a MoFlo flowcytometric cell sorter (Cytomation, Denver, CO). BM-derived T BM and FC were similarly isolated as CD8^+/TCR^+ and CD8^+/TCR^- populations, respectively, using CD8α-PE, αβTCR-FITC, and γδTCR-FITC. T_sp were isolated from single cell suspensions of donor murine splenocytes following incubation with αβTCR^- FITC mAb. All mAbs are from BD Biosciences Pharmingen (San Diego, CA). Post-sort purity was reanalyzed with respect to forward and side scatter parameters and the designated cell surface markers. Purity for all experimental samples was 85-98%. Cells were aliquoted in 1ml HBSS and injected into lethally irradiated mice.

Bone marrow transplantation

For allogeneic BMT, 10,000 HSC plus 50,000 FC or TBM from B6 donors were transplanted into lethally irradiated (950 cGy) B10.BR recipients via tail vein injection. Semiallogeneic (haploidentical) P → F1
BMT entailed the intravenous transplantation of 2,000 HSC alone or together with 50,000-400,000 FC, CD8⁺ TBM, or αβ TCR⁻ splenocytes (TSP), all sorted from B6 donors, into B6D2F1 recipients conditioned with 1100 cGy in a split dose separated by three hours to limit GI toxicity. To maintain as much homogeneity within a given experiment as possible, recipients were of the same age, same shipment of mice, transplanted with donor populations isolated at the same sorting session, and all controls were performed on the same day. Survival was monitored daily.

**GVHD clinical assessment**

Following BMT, mice were scored for clinical evidence of GVHD in a blinded fashion once a week for the first two weeks and then daily until the animals were sacrificed. A more sensitive indicator of GVHD severity than weight alone, the clinical GVHD score was generated by the cumulative sum of grade 0 (no GVHD) to grade 2 (severe GVHD) for each of 5 clinical parameters: weight loss, posture (hunching), activity, fur texture and skin integrity. Total scores ≤ 2 are indicative of no GVHD, > 4 suggests moderate-severe GVHD and > 7 signifies moribund lethal disease.

**Flowcytometric analysis of allogeneic donor engraftment**

Peripheral blood lymphocytes (PBL) were collected into heparinized vials and analyzed for H-2 antigen expression to determine the extent of donor (parental or fully allogeneic) chimerism. Each PBL sample included a negative control and separate aliquots were stained with H-2b FITC (donor) or H-2k FITC (recipient) for B6 → BR chimeras or H-2b FITC and H-2d PE for confirmation of B6 parental engraftment (H-2ᵇ⁺/H-2ᵈ⁻) in B6D2F1 recipients. Data were collected on a FacScan flowcytometer and analyzed in the lymphoid gate with Cell Quest (Becton Dickinson, San Jose, CA) or WinList (Verity, Topsham, ME) software.

**Tissue procurement and histopathology**

Recipients either at the end of the designated GVHD assessment period of 28 days unless otherwise stated, or when pre-morbid, were sacrificed and small intestine was harvested and placed in 10% neutral buffered formalin. Tissue samples were imbedded in paraffin and 6µm-thick samples were sectioned, stained with hematoxylin and eosin (H & E), and assessed for GVHD in a blinded fashion. GVHD was recognized histologically by the presence of crypt epithelial cell degeneration and apoptosis (Grade 1),
apoptotic crypt abscesses (Grade 2), crypt dropout (Grade 3), and mucosal erosion or ulceration (Grade 4) in accordance with previously described criteria.\textsuperscript{18}

**Statistics**

Statistically significant differences in survival among various treatment groups was determined using Kaplan-Meier analysis. Comparison of GVHD scores was performed with the Mann-Whitney U non-parametric test. Means and variances for Real Time PCR data were calculated with JMP statistical software (SAS Institute, Cary, NC). Student’s t test analysis was used to determine the statistical significance of differential gene expression between HSC+FC and HSC+T\textsubscript{BM} recipients, and geometric fold change analysis was used to determine the extent of differential expression.

**Real time quantitative PCR**

RNA from purified FC, T\textsubscript{BM}, T\textsubscript{SP} or from whole spleen of HSC+FC or HSC+T\textsubscript{BM} recipients 28 days following BMT was isolated using TriReagent (Sigma, St. Louis, MO). RNA was treated with Dnase I and reverse transcribed to cDNA via SuperScript II (Invitrogen, Carlsbad, CA). cDNA was mixed with DEPC-treated water, SYBR Green PCR Master Mix and the primer pair of interest. The specific primer pairs used were designed with Primer Express software (Applied Biosystems, Foster City, CA). Gene-specific real-time PCR products were continuously measured by the Gene Amp 5700 Sequence Detection System (Applied Biosystems) for 40 cycles. All experiments were run in duplicate. The cycle threshold (CT) was determined at the same fluorescent signal intensity during the most exponential phase, was inversely proportional to the copy number of the target template, and was related to the CT of the housekeeping gene GAPDH. The % GAPDH calculations for each gene are: \( \frac{100}{2^{(\text{CT}_{\text{Gene}}-\text{CT}_{\text{GAPDH}})}} \). Log 2-fold change calculations for each gene were: \( \log 2 - \left(2^{-\left(\text{CT}_{\text{Gene}}-\text{CT}_{\text{GAPDH}}\right)}\right) - \left(\text{CT}_{\text{Gene}}-\text{CT}_{\text{GAPDH}}\right) \). Non-template controls and dissociation curves were used to detect primer-dimer formation and non-specific amplification.
Results

Fully allogeneic HSC transplantation: FC required for long-term HSC engraftment without acute GVHD

We have previously demonstrated that donor alloengraftment is markedly improved when the mixed syngeneic and allogeneic TCD inocula is supplemented with donor BM-derived CD8⁺/TCR⁺ FC. We hypothesized that the absence of GVHD in this model may be secondary to suppression of the FC’s GVHD effector activity by other donor or recipient cell populations present in the mixed BM inoculum. Therefore, an allogeneic BMT model incorporating only purified donor HSC and FC was utilized to assess the GVHD effector activity of the FC in the absence of all other non-HSC populations. B10.BR recipients were lethally irradiated and reconstituted with 10,000 purified Sca⁺/ckit⁺/Lin⁻ HSC and 50,000 CD8⁺/TCR⁺ FC, both isolated by rare event cell sorting from B6 donor BM (HSC+FC→B10.BR). All HSC+FC recipients were PBL typed for evidence of allogeneic engraftment and assessed weekly for survival and evidence of acute GVHD for the first 8 weeks. Previous studies have shown that transplantation with HSC and donor CD8⁺ T cells of BM or splenic origin do not facilitate allogeneic HSC engraftment and thus recipients succumb to radiation–induced aplasia in this purified HSC model. Therefore, allogeneic HSC+T_BM and HSC+T_SP recipients were not evaluated in this model, as too few recipients survive to be reliably assessed for evidence of GVHD.

As shown in Table 1, allogeneic HSC+FC recipients exhibited excellent survival (88.9% at 4 weeks) and high levels of fully allogeneic donor engraftment at 3 months (94.1±1.8% donor). Clinical scores for mild to severe GVHD (0 to 10, respectively) were compared to syngeneic HSC recipients as a treatment control for radiation conditioning. Scores were not significantly different between animals undergoing allogeneic HSC+FC (n=8) or syngeneic HSC reconstitution (n=6). The respective peak clinical scores of 1.25±0.3 and 1.0±0.3 are consistent with the absence of clinical GVHD.
Semiallogeneic HSC transplantation: Establishment of a purified cell model of lethal GVHD

The failure of TBM or TSP to reliably rescue recipients of allogeneic HSC across complete MHC barriers, prevented comparison of GVHD potential between FC and T cells in the previous fully allogeneic model of HSC engraftment. Furthermore, radioresistant recipient cells can generate an anti-donor response that suppresses GVHD effector activity of the FC. However, the semiallogeneic parent into F1 recipient combination of B6→B6D2F1, permits unopposed GVHD by alloreactive donor cell populations and HSC engraftment at significantly lower HSC numbers, with or without the FC. Individual donor populations may be added to the HSC inoculum and directly assessed for lethal GVHD potential. Therefore, to assess the potential GVHD effector activity of the FC in a rigorous GVHD model, in the absence of other donor non-HSC populations, a purified cell model of lethal GVHD was developed using semiallogeneic HSC.

B6D2F1 recipients were lethally irradiated and reconstituted with 2,000 purified HSC of B6 origin. The HSC inoculum was supplemented with αβTCR+ splenocytes sorted from B6 donors as positive GVHD effector controls. Recipients of donor HSC alone or HSC plus either 100,000 or 200,000 αβTCR+ splenic T cells (TSP) were assessed weekly for survival and severity of GVHD as described above. As expected, recipients of HSC alone (n=8) exhibited 100% survival without evidence of GVHD as reflected in the peak morbidity score of 1.5 ± 0.7 (Figure 1). In contrast, recipients of HSC plus TSP exhibited a dose dependent effect on GVHD severity. Recipients of HSC plus 100,000 TSP survived with clinical evidence of moderate GVHD occurring between 2 and 3 weeks following BMT. The peak GVHD morbidity score was 3.7 ± 0.3. The addition of 200,000 TSP (n=14) resulted in lethal GVHD marked by decreased survival at one month (35.7%, Figure 2) and increased morbidity. As shown in Figure 1, the peak morbidity scores for this group occurred 3 weeks following BMT and the peak score of surviving animals was 4.5 ± 0.5. Therefore, morbidity and mortality were significantly increased compared to HSC alone (p=0.0005).
HSC supplementation with of CD8⁺/TCR⁺ TBM

Given the mild GVHD seen when mixed TCD inocula were supplemented with TBM in the allogeneic model, and the recent evidence of anti-tumor and pro-engraftment properties evident in CD8⁺ TBM in other models, we hypothesized that TBM supplementation of the purified semiallogeneic HSC inocula may be possible without a significant increase in the risk of GVHD. Irradiated B6D2F₁ recipients were reconstituted with a donor B6 inoculum of 2,000 HSC plus 200,000 CD8⁺/TCR⁺ TBM and assessed for survival and GVHD severity. As with HSC+TSP, recipients of HSC+TBM rapidly exhibited lethal GVHD with decreased survival (30-day mortality of 50%, Figure 2) and significant morbidity. The peak GVHD score of surviving HSC+TBM recipients at 3 weeks was 4.3 ± 0.7 (n=9). This GVHD score and 30-day survival are not statistically different from HSC+TSP recipients.

FC does not exhibit acute or chronic GVHD effector activity

Given the marked increase in GVHD seen with CD8⁺/TCR⁺ TBM in this purified HSC semiallogeneic model, it was critical to determine the GVHD potential of the CD8⁺/TCR⁻ BM-derived FC population. Therefore, lethally irradiated B6D2F₁ recipients were transplanted with 2,000 HSC alone or together with 200,000 FC or 200,000 TBM, all from B6 donors. As demonstrated in Figure 3, recipients of HSC+FC (n=9) failed to exhibit significant morbidity or mortality secondary to GVHD as compared to recipients of HSC plus TBM (n=7). The 6-week survival for HSC+FC recipients
was 100% with a peak score of 1.5 ± 0.2. This score is similar to HSC controls (1.55 ± 0.3; NS) but significantly less than the 3.5 ± 0.8 score in surviving TBM recipients (n=7; p<0.05). The addition of as many as 400,000 FC did not result in adverse clinical scores.

Depletion of donor T cell subsets from the inoculum has been previously demonstrated to result in chronic GVHD in B6 → B6D2F1 models by 12 weeks\textsuperscript{19,20}. To assure that the appearance of GVHD was not merely delayed or converted to chronic GVHD, HSC+FC recipients were followed for 14 weeks without any clinical evidence of chronic GVHD. The average peak clinical score for HSC+FC recipients between 9 and 14 weeks after BMT (n=5) was 0.75 ± 0.16. These results demonstrate that the FC population does not induce acute or chronic GVHD in this model.

**Morbidity and mortality following HSC plus T cell transplantation is not due to engraftment failure**

A limited number of HSC are present in the donor inoculum, therefore, to confirm that the increased mortality and morbidity of TBM or TSP recipients was secondary to GVHD and not engraftment failure, F\textsubscript{1} recipients were PBL typed 28 days following BMT to document donor engraftment. This also provided confirmatory evidence that sufficient donor cells exist for the induction of GVHD in HSC+FC recipients. The level of B6 donor chimerism (H-2\textsuperscript{b+}/H-2\textsuperscript{d-}) was high in all treatment groups with no significant differences evident between groups (Table 2). These results demonstrate that although the FC is required for HSC engraftment across complete MHC barriers, facilitation is not required for HSC engraftment in semiallogeneic recipients.
The histologic absence of significant GVHD in FC recipients

F₁ recipients reconstituted with HSC+T<sub>B</sub>M or HSC+FC were electively sacrificed 28 days following BMT and small intestine was assessed for early histologic evidence of acute GVHD (n=7 each). Histopathologic evidence of severe GVHD was present in HSC+T<sub>B</sub>M, but not HSC+FC recipients. Consistent with the fact that 50% of HSC+T<sub>B</sub>M recipients succumb to lethal GVHD in this model after 4 weeks, 57% of these recipients exhibited histologic evidence of acute GVHD within the gut, as manifest by villous shortening, lymphocytic infiltration and crypt apoptosis (Figure 4A and 4B). Gut GVHD in HSC+T<sub>B</sub>M recipients was moderate to severe in intensity with an average histologic grade of 2.4 ± 0.4 on a scale of 0-4.

In contrast, villous architecture was maintained without evidence of lymphocytic infiltrate or crypt abscess in HSC+FC recipients (Figure 4C). Histologic analysis was independently scored as mild GVHD (occasional crypt apoptosis) or none in 86% of HSC+FC recipients. Only a single HSC+FC recipient developed evidence of crypt dropout, which was non-specific in nature, and was not associated with any clinical evidence of GVHD.
FC recipients characterized by increased gene expression of TGFβ

Several recent studies have demonstrated that acute GVHD can be suppressed by the addition of immunoregulatory T cells. In order to determine the mechanism by which FC recipients avoid GVHD, whereas TBM induce lethal GVHD, splenocyte mRNA isolated from B6D2F1 recipients 28 days following HSC+FC (n=4) or HSC+TBM (n=3) transplantation was analyzed for differences in gene expression of cytokines commonly associated with the immunosuppressive function of regulatory T cells (i.e. TGF-β, IL-4 or IL-10), or are downregulated in the setting of immune tolerance networks (i.e. IL-2, γ-IFN). HSC+FC recipients, as compared to HSC+TBM, exhibited a nearly 2-fold increase in splenocytes TGFβ expression. In contrast, there was little difference in γ-IFN, IL-10, IL-4 or IL-2 gene expression. In figure 5, log-2 transformation was utilized to enhance visualization of up and downregulation patterns of these cytokines with the log-2 transformation of the 1.72 fold change in TGFβ expression being equal to 0.76 ± 0.09. This demonstrates a marked trend towards increased gene expression of the immunosuppressive cytokine TGFβ following FC transplantation (p=0.2 via Student’s t test.). Given the importance of TGF-β in the induction of immunoregulatory tolerance, the trend towards increased TGF-β gene expression in HSC+FC recipients suggested that the FC is a regulatory T cell or that regulatory T cells are induced following FC transplantation.

Fig. 5

![Figure 5](https://www.bloodjournal.org)
The FC does not express regulatory T cell genes

The possibility that the FC was a regulatory T cell was evaluated by analyzing the FC prior to BMT for expression of genes commonly present at increased levels in regulatory T cells, namely glucocorticoid induced TNF receptor (GITR), cytotoxic T lymphocyte-associated antigen 4 (CTLA4) and FoxP3. Although GITR and CTLA4 can also be expressed by activated T cell subsets, FoxP3 is considered essential for regulatory T cell development and function \(^{28-30}\). Therefore, purified FC (n=4) and T\(_{BM}\) (n=4) populations were isolated from donor B6 bone marrow and analyzed via real-time PCR for GITR, CTLA4 and FoxP3 gene expression. A representative experiment is shown in Figure 6. GITR, CTLA4 and FoxP3 expression in FC and T\(_{BM}\) is statistically significantly lower than expression in the characteristic CD\(_4^+\)CD\(_25^+\) regulatory T cells identified in spleen from B6 donors (GITR p=0.0005, CTLA4 p=0.0004, FoxP3 p=0.0003 via Student’s t test analysis). Compared to the non-tolerogenic CD\(_4^-\)CD\(_25^-\) splenocyte population, there is no statistically significant difference in GITR expression by FC (p=0.8734) and FoxP3 and CTLA4 expression are significantly lower (p<0.05), although the absolute change in expression is very small (less than 0.5% GAPDH). These results demonstrate that the donor BM-derived FC does not express the genes classically associated with the regulatory T cell phenotype, and thus the FC is not a regulatory T cell.
FC recipients are characterized by the *in vivo* induction of regulatory T cell genes

We subsequently investigated whether the absence of GVHD in HSC+FC recipients was associated with the *in vivo* induction of regulatory T cell genes. Lethally irradiated B6D2F1 recipients were reconstituted with HSC+FC (n=4) or HSC+TBM (n=3) and electively sacrificed 28 days following BMT. RNA from individual spleens was analyzed via real-time PCR for regulatory T cell marker gene expression. Log 2 fold change was used to determine the extent of differential gene expression and, as evident in Figure 7, GITR, CTLA4, and FoxP3 gene expression are all significantly increased within the spleen of HSC+FC recipients compared to HSC+TBM recipients. FC recipients expressed a 2.06 ± 0.29, 2.45 ± 0.30, and 12.33 ± 2.61 fold increase in respective GITR, CTLA4, and FoxP3 expression over HSC+TBM controls. Student’s t test analysis of these gene expression patterns in HSC+FC and HSC+TBM recipients 28 days following BMT shows a highly statistically significant difference for FoxP3 (p=0.02), GITR (p=0.02), and CTLA-4 (p=0.01) gene expression. These findings indicate that although the FC is not a regulatory T cell, FC transplantation results in the absence of GVHD in the setting of an *in vivo* induction of regulatory T cells within the recipient.
Discussion

We have previously established that failure of allogeneic engraftment across MHC class I and II disparities can be decreased with the assistance of a unique CD8⁺/TCR⁻ population derived from donor BM, known as the facilitating cell. The initial description of the FC has been substantially expanded in the current study by demonstrating the absence of GVHD effector function within the FC and the induction of regulatory T cells following FC transplantation. We have now demonstrated that despite the absence of other non-HSC populations in the BM inoculum, which could potentially downregulate GVHD, HSC+FC recipients exhibit substantial donor chimerism without clinical evidence of GVHD across fully allogeneic MHC barriers. However, it is well established that radioresistant recipient cells may mitigate potential GVHD activity, and GVHD effector activity can be markedly enhanced when recipient alloreactivity is lowered, as in the lethally irradiated P → F₁ models. Prior GVHD models utilizing the B6 → B6D2F₁ combination have reconstituted irradiated recipients with a donor inoculum of 5 x 10⁶ T-cell depleted BM cells and induced lethal GVHD via the addition of 1-2 x 10⁶ peripheral T cells. Given a 0.5% incidence of HSC within murine marrow, a significant number of non-HSC donor cells are included in this TCD inoculum. There has been much discussion of the contribution and need for other cell populations in the initiation and development of acute GVHD and our goal was to specifically assess the GVHD potential of the FC. Therefore, a semiallogeneic P→F₁ model of lethal GVHD was established using only purified donor cells, specifically HSC, T cell and FC. Using only 2,000 HSC as the source of donor BM, cells conventionally included in the TCD inoculum are absent thereby permitting the assessment of potential GVHD effector activity of specific purified cell subsets added in isolation. The current model utilizes only 200,000 purified αβTCR⁺ splenocytes added to the donor HSC inoculum to induce lethal GVHD in a dose dependent manner, with a greater than 50% mortality rate. This represents an approximately 5-fold increase in sensitivity for GVHD effector activity as compared with the TCD model where 1-2 x 10⁶ splenocytes are used.
The current studies have elucidated three fundamental observations with potential clinical significance in relation to GVHD. First, to the best of our knowledge, this is the most clinically relevant model of GVHD, utilizing limited numbers of purified HSC and cells to induce lethal GVHD. This model is particularly important as several recent studies have suggested that donor CD$^+$ T cells decrease the risk of engraftment failure and leukemic relapse $^{6,11-14}$. However, our data demonstrates that surprisingly severe GVHD is elicited by purified TBM in a semiallogeneic model suggesting that BMT supplementation with donor BM-derived CD$^+$ T cells could be clinically devastating, at least in the setting of purified HSC into a myeloblated recipient.

Second, we have determined that the FC does not exhibit GVHD effector activity. Although the ability of the FC to facilitate engraftment of purified HSC has been well documented in a fully allogeneic model, GVHD effector potential of the FC has not previously been assessed in a GVHD sensitive model. Therefore, the FC was evaluated in a semiallogeneic BMT model, where effector activity was not opposed by radioresistant alloreactive recipient cell populations. Furthermore, the ability of HSC to engraft in this semiallogeneic model without the FC permitted a direct comparison of GVHD potential for TBM and FC in vivo. Despite the induction of severe GVHD by limited numbers of αβ TCR$^+$ TSP or CD$^+$ TCR$^+$ TBM, escalation of the number of transplanted FC, from the usual 50,000 required for allogeneic engraftment to as many as 400,000 FC, failed to show clinical or histologic evidence of significant GVHD effector activity. Furthermore, HSC+FC recipients did not develop the clinical appearance, nor did they manifest low levels of donor engraftment or decreased cell numbers, classically present in chronic GVHD.

The third contribution focuses on understanding the mechanism by which severe GVHD is so readily induced by CD$^+$ TCR$^+$ TBM but absent following transplantation of CD$^+$ TCR$^-$ FC. The current findings demonstrate that in marked contrast to recipients of HSC+TBM in whom lethal GVHD develops, FC transplantation is characterized by an increased expression of factors linked to T-cell mediated immunosuppression. HSC+FC recipients demonstrate significant increases in TGFβ transcription, a key
factor necessary for the induction and development of regulatory T cells, as well as a several fold increase in CTLA-4, GITR and FoxP3 gene expression, which are characteristic of regulatory T cells and suppressor T cell function. These factors are not induced in the splenic T cells of HSC+TBM recipients, indicating that FC transplantation avoids GVHD through the \textit{in vivo} establishment of regulatory T cells in the reconstituted recipient. Others have shown that the addition of ex vivo activated regulatory T cells to the donor inoculum can inhibit GVHD and prevent the expansion of alloreactive donor T cells. However, the FC is not a CD4+ CD25+ T cell, nor does it express the established regulatory T cell genes CTLA4, GITR or FoxP3, indicating that the FC is not a regulatory T cell. Instead, FC transplantation induces the development of immunoregulatory T cells \textit{in vivo}, a previously unknown characteristic of the FC and FC-mediated tolerance. This ability to induce regulatory T cells is particularly intriguing since the FC expresses a TCRβ heterodimer containing a novel 33kD protein, FCp33, in place of the conventional αβ and γδTCR heterodimers present on the GVHD effectors, TSP and TBM. The role of the FCp33 receptor and the mechanism by which the FC induces immunoregulatory T cells in the reconstituted host following BMT are important areas of future study.

In summary, the data presented in this manuscript establishes that the purified semiallogeneic HSC model of GVHD is both sensitive and specific, and that GVHD effector activity of individual purified donor cell populations can be accurately assessed in a clinically relevant model of HSC transplantation. Significant GVHD mortality and morbidity were demonstrated following the co-transplantation of HSC and αβTCR+ TSP or CD8+/TCR+ TBM. These findings suggest that the use of CD8+ T cells for adoptive therapy potentially carries a significant risk of GVHD. This may be particularly true in the clinically relevant setting of purified donor HSC transplantation between parent and child, where additional cell populations with the potential to mitigate the GVHD response have been removed. Of greater potential importance however, is the absence of significant GVHD following the transplantation of the CD8+/TCR− FC population. Interestingly, the lack of GVHD following HSC+FC transplantation is associated with the \textit{in vivo} induction of regulatory T cells during immune reconstitution. These findings
demonstrate that induction of immunoregulatory T cells as a means to prevent GVHD can be achieved in vivo, through a novel FC-mediated mechanism, without prior antigen exposure or ex vivo T cell expansion. The ability to decrease engraftment failure and avoid GVHD in allogeneic BMT through the in vivo induction of regulatory T cells, suggests that the donor FC may potentially offer a safer, more efficient and clinically relevant approach to HSC supplementation and donor organ tolerance in the future.
Acknowledgements

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References


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Table 1. The FC facilitates allogeneic HSC engraftment without acute GVHD

<table>
<thead>
<tr>
<th>Engraftment</th>
<th>n</th>
<th>30-day Survival</th>
<th>% Donor Chimerism at 3 months</th>
<th>GVHD Clinical Score</th>
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<tbody>
<tr>
<td>Syngeneic HSC</td>
<td>6</td>
<td>100%</td>
<td>-</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>Allogeneic HSC+T&lt;sub&gt;BM&lt;/sub&gt;</td>
<td>10</td>
<td>10% *</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Allogeneic HSC+FC</td>
<td>8</td>
<td>88.9%</td>
<td>94.1 ± 1.8%</td>
<td>1.25 ± 0.3</td>
</tr>
</tbody>
</table>

*All HSC+T<sub>BM</sub> recipients expired by 3 months
Table 2. Donor Chimerism following semiallogeneic HSC transplantation

<table>
<thead>
<tr>
<th>Donor Inoculum</th>
<th>n</th>
<th>% Donor Chimerism (%) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSC</td>
<td>7</td>
<td>95.7 ± 0.7</td>
</tr>
<tr>
<td>HSC+T&lt;sub&gt;SP&lt;/sub&gt;</td>
<td>7</td>
<td>97.5 ± 1.0</td>
</tr>
<tr>
<td>HSC+T&lt;sub&gt;B&lt;/sub&gt;M</td>
<td>5</td>
<td>97.9 ± 0.9</td>
</tr>
<tr>
<td>HSC+FC</td>
<td>9</td>
<td>97.1 ± 0.5</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. Establishment of a purified cell model of GVHD. Lethally irradiated B6D2F1 recipients reconstituted with B6-derived HSC (n=8) or with HSC plus 200,000 αβTCR⁺ splenocytes from B6 donors (n=14) were assessed for evidence of acute GVHD. Clinical GVHD scores ± SE are displayed for each week post-BMT. The recipients of HSC alone showed no clinical GVHD. In contrast, recipients of 2,000 HSC + 200,000 αβTCR⁺ donor TSP exhibited severe and often lethal GVHD with the peak severity and incidence occurring at week 3 (p=0.0005 vs. HSC alone).

Figure 2. TBM induce severe lethal GVHD. Lethally irradiated B6D2F1 recipients were reconstituted with B6-derived HSC (n=8), HSC plus 200,000 CD8⁺ TBM (n=9) or HSC plus 200,000 TSP (n=14) and assessed for survival. The inoculum containing CD8⁺TBM resulted in mortality from lethal GVHD similar to that seen with αβTCR⁺ TSP used as a positive GVHD control (p=NS). Survival was markedly decreased for TBM and TSP compared to HSC alone (p<0.05 for both groups at 30 days).

Figure 3. Absence of clinical GVHD following FC transplantation. Lethally irradiated B6D2F1 recipients reconstituted with 2,000 B6-derived HSC alone (n=8) or HSC+200,000 FC (n=9) also from a B6 donor, failed to exhibit significant evidence of GVHD even at 3 weeks post-BMT. This is in contrast to HSC+200,000 TBM recipients (n=7) where clinical scores consistent with moderate-severe GVHD were evident (p≤ 0.05). Peak clinical scores ± SE for each group are shown.
Figure 4. **Histopathologic evidence of acute GVHD following donor T_{BM}, but not FC, transplantation.** Small intestine was harvested from HSC+T_{BM} and HSC+FC recipients electively on day 28. Paraffin-embedded sections were stained with H&E and histologically analyzed for evidence of GVHD. A) Small intestine of a representative HSC+T_{BM} recipient (n=7) exhibits villous shortening, crypt dropout, and extensive lymphocytic infiltration of the lamina propria (200X). B) In these same mice, lymphocytic infiltration of crypts with apoptosis (open arrow) and the formation of a crypt abscess (closed white arrow) is observed at higher magnification (400X). These findings are indicative of moderate to severe GVHD. C) Small intestine of a representative HSC+FC recipient (n=7). There is no evidence of GVHD (x200).

Figure 5. **Increased TGF\(\beta\) gene expression in HSC+FC recipients.** Splenocytes harvest 28 days following BMT from HSC+FC (n=4) and HSC+T_{BM} (n=3) recipients were analyzed via real time PCR for cytokine gene expression. Relative differences in expression levels among HSC+FC recipients compared to HSC+T_{BM} recipients are shown as log-2 fold change from two separate experiments.

Figure 6. **T regulatory gene expression is not increased in the FC or T_{BM} populations.** Real time PCR analysis of CD8\(^+\)/TCR\(^-\) FC (n=4) or CD8\(^+\)/TCR\(^+\) T_{BM} (n=4) isolated from donor BM as compared to analysis of unstimulated control B6 splenocytes sorted for the CD4\(^+\) CD25\(^+\) regulatory T cell phenotype, the CD4\(^+\) CD25\(^-\) or CD4\(^-\) CD25\(^-\) non-tolerogenic phenotypes. Individual samples were analyzed for the presence of
mRNA encoding GITR, CTLA4 and FoxP3 and are semiquantitated as a percentage ± SD of baseline GAPDH expression.

**Figure 7. Induction of T regulatory genes following FC transplantation.** Splenocytes isolated from HSC+FC (n=4) and HSC+T<sub>BM</sub> (n=3) recipients 28 days following BMT were individually analyzed via real time PCR for mRNA encoding GITR, CTLA-4 and FoxP3. Log 2-fold change ± SD of gene expression for HSC+FC recipients compared to HSC+T<sub>BM</sub> recipients from two independent experiments are shown.
Absence of Clinical GVHD and the In Vivo Induction of Regulatory T cells following Facilitating Cell Transplantation

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