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*J Immunol* 2007; 179:2153-2162; doi: 10.4049/jimmunol.179.4.2153
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Induction of FoxP3$^{+}$/CD4$^{+}$/25$^{+}$ Regulatory T Cells Following Hemopoietic Stem Cell Transplantation: Role of Bone Marrow-Derived Facilitating Cells$^{1,2}$

Kendra N. Taylor, Vivek R. Shinde-Patil, Evan Cohick, and Yolonda L. Colson$^{3}$


The establishment of donor cell lineages following allogeneic bone marrow transplantation is frequently associated with the development of graft-vs-host disease (GVHD). The identification of cell populations that are capable of supporting allogeneic stem cell (SC) engraftment and the induction of tolerance without inducing GVHD could expand the use of this therapy. CD8$^{+}$/TCR$^{-}$ facilitating cells (FC) have been shown to promote allogeneic SC engraftment with resulting transplantation tolerance across complete MHC barriers without inducing GVHD. Although donor reconstitution in SC plus FC recipients is associated with the induction of regulatory T cell-associated factors, it is not known whether an induction of regulatory T cells and subsequent tolerance is a direct effect of the FC. The current study demonstrates that 1) SC plus FC transplantation results in the induction of donor CD4$^{+}$/25$^{+}$ regulatory T cells and that FC are present in the spleen of recipients before the induction of these cells, 2) activation of FC with CpG-oligodeoxynucleotide promotes CD4$^{+}$/25$^{+}$ T cell differentiation into CD4$^{+}$/25$^{+}$ regulatory T cells in vitro, as demonstrated by cytokine and forkhead/winged helix transcription factor (FoxP3) gene and protein expression, and 3) direct contact between FC and CD4$^{+}$/25$^{+}$ T cells is required for FoxP3$^{+}$/CD4$^{+}$/25$^{+}$ regulatory T cell induction and is dependent on CD86 expression on FC. This is the first report to demonstrate a mechanism for FC in the induction of regulatory T cells following allogeneic SC plus FC transplantation. The transplantation of donor FC may provide an alternative approach to permit clinical SC engraftment and induction of transplantation tolerance in the future.

B one marrow transplantation (BMT)$^{4}$ is an established therapy for the treatment of many hematologic malignancies. However, the clinical application of this therapy is limited to those patients with a closely matched donor. Allogeneic BMT across multiple major histocompatibility (MHC) disparities is characterized by the high mortality and morbidity associated with the development of severe graft-vs-host disease (GVHD) and engraftment failure. The severity of these complications has prevented the application of clinical allogeneic BMT to many non-malignant hematologic maladies and to the induction of solid organ transplantation tolerance ($^{1,2}$).

Studies using murine BMT models have demonstrated that these limitations can potentially be overcome with the use of purified cell populations. Facilitating cells (FC), isolated as CD8 positive (CD8$^{+}$) and $\alpha\beta$TCR negative ($\alpha\beta$TCR$^{-}$) or Tr1 regulatory T cells (15–18). We hypothesize that the donor FC population may have a similar function following transplantation. Although similarities between BM-derived FC and p-preDC have been described, recent findings have identified markers on the FC population that are not present on p-preDC (5, 19, 20). These findings showed that FC are distinct

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Received for publication April 3, 2007. Accepted for publication May 31, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

$^{1}$ This research was supported by the National Institutes of Health Grant R01 HLO74150 (to Y.L.C.).

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$^{4}$ Abbreviations used in this paper: BMT, bone marrow transplantation; MHC, multiple major histocompatibility; GVHD, graft-vs-host disease; FC, facilitating cells; BM, bone marrow; SC, stem cell; FoxP3, forkhead/winged helix transcription factor; DC, dendritic cell; p-preDC, plasmacytoid precursor DC; Tr1, T regulatory 1; TBM, T BM cells; ODN, oligodeoxynucleotide; CSM, cell sort media.

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from p-preDC in that the FC population expresses TCRβ and CD3ε and p-preDC do not. These receptors are also required for FC function in vivo. The TCRβ-chain expression is unique to the FC, in that instead of expressing the conventional αβ TCR heterodimer, FC express a unique heterodimer composed of the TCRβ-chain disulfide linked to a 33kDa protein named FCp33 (19). Furthermore, in a fully allogeneic murine model of SC plus p-preDC or SC plus FC transplantation, p-preDC are much less efficient at facilitating SC engraftment than the FC population (5). These findings demonstrate that there are functional characteristics unique to the FC population. However, FC and p-preDC may share the ability to induce regulatory T cells.

Numerous reports have documented CD4⁺CD25⁺ regulatory T cell involvement in the promotion of hematopoietic cell reconstitution (21), GVHD suppression (22–24), and the establishment of self-tolerance (25–28). Specifically, CD4⁺CD25⁺ regulatory T cell transplantation resulted in allogeneic hematopoietic cell engraftment with increased long-term multilineage donor chimerism in sublethally irradiated murine recipients (21). These findings demonstrate a role for CD4⁺CD25⁺ regulatory T cells in allogeneic hematopoietic cell reconstitution across fully mismatched MHC barriers. Although the mechanisms of regulatory T cell function are not completely clear, the development and activation of CD4⁺CD25⁺ regulatory T cells have been linked to interactions with pDC.

The present study investigates the development of regulatory T cells following allogeneic SC plus FC transplantation in vivo and assesses the ability of FC to directly induce naïve CD4⁺CD25⁻ T cell differentiation into CD4⁺CD25⁺ regulatory T cells in vitro. We report that 1) FoxP3⁺CD4⁺CD25⁺ regulatory T cells are induced within the spleen of SC plus FC recipients postarrival of FC to the spleen, 2) activated FC induce CD4⁺CD25⁺ T cell differentiation into FoxP3⁺CD4⁺CD25⁺ regulatory T cells in vitro, and 3) direct cell-cell contact between FC and CD4⁺CD25⁺ T cells is required for FC-mediated regulatory T cell development in vitro. These findings provide the first mechanistic evidence that FC can induce the generation of FoxP3⁺CD4⁺CD25⁺ regulatory T cells and may have a role in the subsequent development of transplantation tolerance in vivo.

Materials and Methods

Animals

Six- to 8-wk-old female C57BL/6 (B6) and B10.BR mice were purchased from The Jackson Laboratory. All animals were housed in a specific pathogen- and virus-free facility at the Dana-Farber Cancer Institute under the guidelines of the National Institutes of Health for care and use of laboratory animals.

BM cell preparation

BM preparation was performed as previously described (19, 20). In brief, BM was isolated from the long bones of mice by flushing with cold HBSS (Invitrogen Life Technologies). After washing with HBSS, BM cells were resuspended in sterile cell sort medium (Invitrogen Life Technologies; cell sort media: HBSS without phenol red, 2% FBS, 2 μg/ml HEPES buffer, and 30 μg/ml gentamicin).

Monoclonal Abs

The following mAbs were used to sort CD8⁻TCR⁻ FC: anti-CD8α (53-6.7) PE, anti-TCRβ-chain (H57–597) FITC, and anti-TCRγδ chain (GL3) FITC. To sort spleen CD4⁺ or CD4⁺CD25⁺ cells, the following were used: anti-CD4 (GK1.5) FITC and anti-CD25 (PC61) aliphycocyanin. To sort B220⁺CD11b⁻CD11c⁺ p-preDC, the following were used: anti-CD45R/B220 (RA3-6B2) allophycocyanin, anti-CD11b (M1/70) FITC, and anti-CD11c biotin, with streptavidin-PE-Cy5. To sort Sca-1⁻/CD4⁻/CD11c⁻ ‘Lin’ stem cells, Lin⁻ were all FITC conjugated as follows: B220, CD8α, Gr-1, MAC-1, αβ TCR, and γδ TCR. Purified anti-mouse CD3ε (145-2C11) and CD28 (37.51) were used in the proliferation assays. FITC-conjugated anti-mouse H-2K⁺ (AF6–88.5) and H-2K⁻ (36-7.5) were used for donor chimerism analysis. CD86 (GL1) PE-Cy5 and FoxP3 (FJK-16s) PE with isotype control Rat-IgG were purchased from eBioscience, San Diego, CA. All other sort mAbs were purchased from BD Biosciences, San Jose, CA.

Purified p-preDC and T cell isolation

p-preDC from BM and CD4⁺CD25⁻ or CD4⁺CD25⁺ T cells from spleen were sorted as previously described (5, 20). In brief, cells were incubated for 30 min at 4°C with mAbs. After incubation, the cells were washed twice with CSM and resuspended to a final concentration of 2 x 10⁶ cells/ml in CSM before multiparameter sterile live cell sorting within the conventional lymphoid gate on a MoFlo flow cytometric cell sorter (DakoCytomation). Post-sort purity was determined with respect to forward and side scatter parameters and the designated cell surface markers. Postsort purity for p-preDC was 90–95% and spleen T cells was >95%.

Purified SC and FC isolation and transplantation

SC, FC, and BM T cells (TBM) were isolated from donor BM as described previously (3, 4, 19, 20). SC were sorted on a MoFlo cell sorter as Sca-1⁻/CD4⁻/CD11c⁻/Lin⁻ (Lin: B220, CD8α, Gr-1, MAC-1, αβ TCR, and γδ TCR) cells within the lymphoid cell gate. FC and TBM were also sorted within the lymphoid gate as CD8⁻/αββ⁺TCR⁻ and CD8⁺/αββ⁺TCR⁺ populations, respectively. The range for postsort purity was 88–98%. Lethally irradiated (950cGy) B10.BR recipients were reconstituted with 10,000 B6-derived SC alone, or together with 50,000 B6-derived FC (SC plus FC) or TBM (SC plus TBM). Ablated B6 mice transplanted with 2,000 B6-derived SC served as syngeneic controls. Allogeneic SC reconstitution was determined by PBL typing of donor (H-2⁺) MHC Ag expression 28 days following BMT and expressed as percentage of donor chimerism.

RNA isolation and reverse transcription

Total RNA was isolated from sorted cell populations or whole spleen using the RNaseasy System, (Qiagen) and converted to cDNA, (Ambion), as recommended by the manufacturers’ instructions.

Conventional PCR

The primer sequences for IL2 (internal constitutive control): (sense, CT AGGCCACAGATGGAAGATC and anti-sense, GTAGGTGGAAT TCTAGCATCCTC) and GFP (sense, AAGTTCATCTGCACCCAGC and anti-sense, TCTTGGAGAAGATGGTCG) were used for analysis of gene transcripts. Conventional PCR was performed for 40 cycles for 1 min at 94°C, followed by 1-min incubations of 60°C and 72°C, with a final 10-min extension at 72°C. Products were visualized on a 1.5% agarose gel stained with ethidium bromide.

Real-time quantitative PCR

Real-time PCR was performed using 3–10 μl cDNA mixed with diethyl pyrocarbonate-treated water, PCR master mix (Applied Biosystems), and the primer pairs. The gene-specific, real-time PCR products were measured for 40 cycles using the Gene Amp 5700 Sequence Detection System. Non-template controls and dissociation curves were used to detect nonspecific amplification and the formation of primer-dimers. All experiments were run in duplicate and gene expression was normalized to the expression of the housekeeping gene, GAPDH.

cPpG-oligodeoxynucleotide (ODN) and FC stimulation assay

Phosphorothioate-modified CpG-B ODN 2006: 5’ TCTGCTGTGTCGTTTTGTGCT 3’ (18) was obtained from Invitrogen Life Technologies (modified nucleotides are underlined). FC were cultured in absence of CpG-ODN at 10 μg/ml. Cultures were harvested for analysis of gene expression or cytokine production by ELISA and flow cytometry.

ELISA

Cell-free supernatants of 48-h cultures of FC in the presence or absence of CpG-ODN were collected. The amount of cytokines produced was determined by ELISA multiplex for IL1, IL2, IL3, IL4, IL5, IL6, IL7, IL10, IL12p70, IL13, MIPα, MIP2, IFN-γ, and TNF-α. Supernatants from two independent experiments were analyzed using Pierce Diagnostic Services.

Flow cytometry

Cell suspensions were incubated at 4°C with fluorophore- or biotin-conjugated primary mAbs directed against designated surface markers. Biotinylated mAbs were detected with streptavidin-PE-Cy5 secondary mAbs. For analysis of extracellular expression of CD86, FC cells were sorted as previously described, stimulated with CpG-ODN and harvested in FACS buffer (HBSS with 0.05% BSA and 0.05% sodium azide). Cell suspensions
of CpG-stimulated and unstimulated FC were incubated with the mAb for CD86 (anti-CD86 PE-Cy5) or isotype control (Rat-IgG PE-Cy5) for 30 min at 4°C. The cells were washed twice with FACS buffer and then fixed with 2% paraformaldehyde in PBS. For analysis of FoxP3 protein, intracellular labeling was used. Cells were first labeled for extracellular CD4 and CD25 receptors, followed by fixation of cells in 2% paraformaldehyde, permeabilization using 0.05% Triton X-100 in PBS, and then labeled for intracellular FoxP3 with anti-FoxP3 mAb. The isotype control was Rat-IgG. For all studies, fluorescence of 10,000 cells within the lymphoid gate was assessed on a FACSCalibur flow cytometer (BD Biosciences).

T cell functional assays
CD4+CD25+ and CD4+CD25− cells were purified from splenocytes via cell sorting. CD90+ depleted cells were recovered from naive spleens by magnetic depletion (Miltenyi Biotec), irradiated at 3000Gy and used as accessory cells. Cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), streptomycin (100 mg/ml), 2 mM l-glutamine, (all from Invitrogen Life Technologies) and 50 μM 2-ME (Sigma-Aldrich). Purified cell populations were tested for proliferative potential in presence of anti-CD3 mAb, anti-CD28 mAb, and rIL-2 (PeproTech) as described previously (29). CD4+CD25+ regulatory T cell suppressive function was tested by coculturing CD4+CD25− T cells with increasing ratios of CD4+CD25+ cells (1:0, 1:0.1:0.25 and 1:1) in the presence of anti-CD3 mAb and accessory cells in flat-bottom 96-well plates. Cultures were maintained in a humidified incubator at 37°C, 5% CO2, for 72 h and labeled with [3H] (8-h pulse) to ascertain the level of proliferation. Data are expressed as mean ± SEM cpm.

In vitro stimulation of naive CD4+CD25− T cells
Spleen CD4+CD25− T cells were incubated with FC or p-preDC at a 10:1 ratio in 96-well plates with or without CpG-ODN in medium (RPMI 1640 supplemented with 10% FBS, and 2 mM l-glutamine) for up to 6 days. As negative controls, naive CD4+CD25− T cells were cultured alone, with FC, or with CpG. The cocultures were harvested and the generated CD4+CD25− T cells were sorted and analyzed for FoxP3 and cytokine gene transcripts using real-time PCR. CD86 blockade assay
In vitro stimulation of CD4+CD25− T cells was performed as previously described in the absence or presence of anti-CD86 Ab at 1 μg/ml. As negative controls, naive CD4+CD25− cells alone or with FC were cultured with anti-CD86 Ab. The cells were cocultured up to 6 days, harvested, and the generated CD4+CD25− T cells were sorted and analyzed for FoxP3 gene expression.

Statistical analysis
Survival fractions were determined using Kaplan-Meier analysis. Means and variances for real-time PCR data were estimated using JMP statistical software (SAS Institute). Significant differences (p ≤ 0.05) were ascertained via Student’s t test.

Results
SC plus FC recipients are characterized by an in vivo induction of donor FoxP3+CD4+CD25+ regulatory T cells
It is well established that FC promote purified allogeneic SC engraftment and subsequent transplantation tolerance across complete MHC barriers without clinical or histological evidence of GVHD (3–6). However, the mechanisms associated with FC function are not yet clear. Given that donor SC engraftment and tolerance following SC plus FC transplantation parallel the outcome of SC plus FC recipients express FoxP3 and display a functional regulatory phenotype.
of CD4+CD25+ regulatory T cell-mediated engraftment of allogeneic hemopoietic cells and subsequent tolerance, we hypothesized that FC function may result in an induction of CD4+CD25+ regulatory T cells. The current studies first investigated CD4+CD25+ T cell development following SC plus FC transplantation. CD8+ TCR+ FC vs BM-derived CD8+ TCR+ TBM were used in a fully allogeneic (C57BL/6 → irradiation conditioned B10.BR) mouse model. TBM recipients develop GVHD and fail to survive beyond 7 wk. Splenocytes and BM from recipients were assessed for CD4 and CD25 coexpression using flow cytometric analysis. As reflected in the representative panel in Fig. 1A, a significantly higher frequency of CD4+CD25+ T cells was present in the lymphoid populations of whole spleens (2.03 ± 0.28%) and BM (1.03 ± 0.29%) in SC plus FC recipients (n = 12, p = 0.01) compared with spleens (1.07 ± 0.29%) and BM (0.35 ± 0.1%) of surviving SC plus TBM recipients (n = 7). This represents a nearly 2- and 3-fold increase in CD4+CD25+ frequency in the spleens and BM of SC plus FC recipients, respectively. Given that an increased incidence of recruited or proliferating non-T cell types could also decrease the frequency of CD4+CD25+ T cells in SC plus TBM, CD4+CD25+ T cells were also assessed as a fraction of the total CD4+ cell pool. CD4+CD25+ T cells accounted for 11.3 ± 1.3% and 5.7 ± 0.9% of the total splenic CD4+ cell pool in SC+FC and SC+TBM recipients respectively, reflecting a 2-fold increase in the number of CD4+CD25+ splenic cells in SC plus FC recipients. Therefore, in contrast to TBM recipients that develop GVHD, FC recipients show a significant induction of CD4+CD25+ cells within the spleen.

Given that the hemopoietic system of the recipient is ablated with total body irradiation before transplantation, it is not surprising that the majority of CD4+CD25+ cells in SC plus FC recipients are of donor origin, as demonstrated by the flow cytometric analysis.

FIGURE 2. Donor FC precede FoxP3+ CD4+CD25+ regulatory T cell induction within the spleen of SC plus FC recipients. Conventional and real-time PCR were performed using cDNA from splenocytes derived from 2-wk-old (n = 3) and 4-wk-old (n = 3) B10.BR recipients of C57BL/6-derived SC and GFP+ FC. A, GFP transcripts were amplified from spleen samples using conventional PCR. GFP+ and GFP− splenic cDNA were used as controls. PCR products were visualized by ethidium bromide staining. Product sizes are 173 bp (GFP) and 324 bp (IL2; internal control). B, FoxP3 gene expression was analyzed using real-time PCR and is shown as percentage of GAPDH gene expression. The data are representative of average values of duplicate samples with SEM of three independent experiments.

of H-2b surface expression. The percentage of CD4+CD25+ T cells of donor origin in SC+FC vs SC+TBM recipients was 72.3 ± 1.8% and 86.9 ± 7.0% in the spleen and 74.9 ± 2.6% and 57.8 ± 7.6% in the BM, respectively. However, it is important to confirm that these CD4+CD25+ cells are regulatory T cells and not just an activated phenotype. Given that FoxP3 plays a key role in CD4+CD25+ regulatory T cell development, and serves as the most reliable identification marker for these cells, intracellular staining for FoxP3 protein was assessed. As demonstrated in Fig. 1B, FoxP3+ cells represent >50% of splenic CD4+CD25+ T cells in SC plus FC recipients. Therefore, FC-mediated facilitation of allogeneic SC engraftment results in an increase in donor CD4+CD25+ T cells as a percentage of total CD4+ cells. Moreover, more than half of the CD4+CD25+ population expresses FoxP3 suggesting the induction of regulatory T cells following FC transplantation.

CD4+CD25+ T cells derived from SC plus FC recipients are anergic and suppress the activity of CD4+CD25− T cells in vitro

We next investigated the functional regulatory activity of CD4+CD25+ T cells sorted from the spleen of SC plus FC recipients. CD4+CD25+ and CD4−CD25− cells isolated from SC plus FC spleens were incubated with purified anti-CD3 mAb alone, or in combination with IL2 or anti-CD28 mAb. As shown in Fig. 1C, the CD4+CD25− T cells are not anergic; rather, these cells are functional and capable of proliferation in the presence of CD3 mAb alone. Furthermore, the functional capability is enhanced by the addition of IL2 or anti-CD28 mAb. In contrast, the CD4+CD25+ T cells are anergic, exhibiting a decreased proliferative response to anti-CD3 mAb, even in the presence of IL2. Importantly, these CD4+CD25+ cells are potent functional regulatory T cells, as evident by their ability to exhibit a nearly 100-fold inhibition of CD4+CD25− T cell proliferation during coculture at a 1:1 ratio (Fig. 1D).

Donor FC are present in the spleen of recipients before FoxP3+ CD4+CD25+ regulatory T cell induction

Given the current demonstration that regulatory T cells are induced within the spleen of SC plus FC recipients, we investigated whether FC were also present in recipient spleens following transplantation. To identify donor FC within an in vivo model of allogeneic transplantation, B6-derived GFP+ FC were transplanted with GFP− SC into irradiation-conditioned B10.BR recipients. Splenocytes were harvested from SC plus GFP+ FC recipients 2 and 4 wk following transplantation. GFP transcript was detected by conventional PCR. Amplification of the IL2 transcript was used as an internal control for PCR conditions. As demonstrated in Fig.
FIGURE 4. Analysis of FoxP3 in CD4<sup>+</sup> T cells generated following FC coculture. CD4<sup>+</sup> T cells from the spleen of C57BL/6 mice were cocultured with FC alone, CpG alone, or CpG and FC in medium for up to 6 days. The resulting CD4<sup>+</sup> T cells were sorted by flow cytometry. A, The number of CD4<sup>+</sup> T cells generated from the coculture of CD4<sup>+</sup> T cells with FC plus CpG were significantly greater than cocultures without CpG. *Statistically significant differences (p = 0.001). The data are shown as percentage of total CD4<sup>+</sup> cells recovered. Cell recovery from CpG-stimulated FC T cell cocultures ranged from 1300 to 3600 CD4<sup>+</sup> cells and 90,000 to 200,000 CD4<sup>+</sup> <sup>+</sup> cells. B, The CD4<sup>+</sup> T cells were analyzed for gene expression of FoxP3 using real-time PCR. The positive control for regulatory T cell induction was CD4<sup>+</sup> T cells cocultured with p-preDC plus CpG. As expected, the positive control generated CD4<sup>+</sup> T cells that express the FoxP3 transcript. The T cell cocultures with FC plus CpG generated CD4<sup>+</sup> T cells that also expressed the FoxP3 transcript. The data are representative of average values of duplicate samples with SEM of three independent experiments. C, FoxP3 protein expression was analyzed in cocultures of FC and CD4<sup>+</sup> T cells in the presence or absence of CpG. Cocultures in the presence of CpG generated CD4<sup>+</sup> T cells that expressed higher levels of FoxP3 protein compared with cocultures without CpG. The average values of three independent experiments are shown. D, The FC-generated CD4<sup>+</sup> T cells expressed higher levels of IL2 and significantly higher levels of IL4 than p-preDC-generated or freshly isolated native CD4<sup>+</sup> T cells. *Statistically significant differences (p = 0.047). Gene expression levels for IL10, IL15, TGF-β, and TNF-α were similar for all samples. The data are representative of average values of duplicate samples with SEM of three independent experiments.

2A, GFP<sup>+</sup> donor FC are readily detected in recipient spleens as early as 2 wk posttransplantation. Importantly, concurrent analysis of FoxP3 gene expression in these samples by real-time PCR revealed that the induction of FoxP3 gene expression did not occur until 4 wk following FC transplantation (Fig. 2B). The current findings demonstrate that FoxP3<sup>+</sup> CD4<sup>+</sup> regulatory T cell induction and/or recruitment follows the arrival of donor FC within the recipient spleen.

**FC induce the generation of CD4<sup>+</sup> regulatory T cells in vitro**

Given that the identification of FC within the spleen of SC plus FC recipients is followed by an induction of regulatory T cells of donor origin, we addressed the possibility that FC directly induce the development of CD4<sup>+</sup> regulatory T cells. The basis is formulated from the demonstration that the FC population contains a subset of cells that is B220<sup>+</sup>CD11c<sup>+</sup>, a phenotype that is characteristic of p-preDC. In vitro studies have demonstrated that activation of TLR9 on p-preDC result in their differentiation and ability to induce naive CD4<sup>+</sup> T cell differentiation into regulatory CD4<sup>+</sup> or Tr1 regulatory T cells (15–18). We have chosen to use a similar assay to test the FC ability to induce CD4<sup>+</sup> regulatory T cells in vitro as a possible mechanism for the observed induction of CD4<sup>+</sup> regulatory T cells following SC plus FC transplantation. As demonstrated in the current studies, the majority of splenic CD4<sup>+</sup> T cells of SC plus FC recipients are of donor origin. Therefore, we have analyzed the interactions between donor cell types and our subsequent in vitro studies used a syngeneic in vitro model for analysis of FC-mediated induction of CD4<sup>+</sup> regulatory T cells. For these studies, FC and CD4<sup>+</sup> T cells were isolated from the bone marrow and spleen of C57BL/6 mice. It is our hypothesis that similar to p-preDC the in vitro activation of TLR9 on FC in coculture with CD4<sup>+</sup> T cells results in T cell differentiation into CD4<sup>+</sup> regulatory T cells. We began our investigation by contrasting TLR9 gene expression within the FC population to p-preDC, mature DC, NK, and T cells. In this study, we demonstrate that purified FC and p-preDC have similar TLR9 gene expression levels (Fig. 3) and, as expected, the gene expression within these populations is greater than expression levels within mature DC and NK cells that are other potential immune-modulating cell populations (5). Therefore, in subsequent studies, FC were stimulated with the TLR9 ligand CpG-ODN.
2006, a B-type ODN previously shown to stimulate p-preDC in coculture with CD4\(^{+}25^{-}\) T cells (18, 30–33). CD4\(^{+}25^{-}\) T cells from the spleen of B6 mice were cocultured with FC alone, CpG alone, or FC and CpG in medium for up to 6 days. The T cells cultured with FC alone generated few CD4\(^{+}25^{+}\) cells as represented by 0.4 ± 0.2% of total CD4\(^{+}\) T cells recovered (Fig. 4A). In contrast, CpG-stimulated FC T cell cocultures resulted in increased T cell differentiation into CD4\(^{+}25^{+}\) T cells as represented by 1.82 ± 0.10% of total CD4\(^{+}\) T cells recovered (p = 0.001). Therefore, the current findings demonstrate a nearly 5-fold difference in the number of generated CD4\(^{+}25^{+}\) T cells. Because FoxP3 is preferentially expressed in CD4\(^{+}25^{+}\) regulatory T cells and is associated with both the development and suppressive function of these cells (34), FoxP3 gene and protein expression were evaluated within CD4\(^{+}25^{+}\) T cells generated from cocultures using real-time PCR and flow cytometric analysis, respectively (Fig. 4, B and C). The real-time PCR analysis revealed that the CD4\(^{+}25^{+}\) T cells generated from CpG-stimulated FC in CD4\(^{+}25^{+}\) T cell cocultures expressed the FoxP3 transcript at similar levels to T cell cocultures with CpG-stimulated p-preDC. The T cell cocultures with p-preDC and CpG were used as positive controls for the assay. In contrast, the CD4\(^{+}25^{+}\) T cells generated from the coculture of CD4\(^{+}25^{+}\) T cells with FC alone expressed very little to no FoxP3 transcript. The gene expression data were supported with analysis of FoxP3 protein expression. As demonstrated in Fig. 4C, of the total CD4\(^{+}25^{+}\) T cells generated from CpG-stimulated FC-T cell cocultures, 20 ± 3% expressed the FoxP3 protein. In contrast, 3.3 ± 1% of the CD4\(^{+}25^{+}\) T cells generated from FC T cell cocultures without CpG-expressed FoxP3 protein. These findings demonstrated that CpG-stimulated FC induced the generation of FoxP3\(^{+}\)CD4\(^{+}25^{+}\) T cells. The FC-generated FoxP3\(^{+}\)CD4\(^{+}25^{+}\) T cells were further analyzed for gene expression of cytokines associated with the regulatory T cell phenotype. Interestingly, real-time PCR analysis of cytokine transcripts within the FC generated CD4\(^{+}25^{+}\) T cells revealed a significantly higher level of IL4 transcript in contrast to expression within freshly isolated native CD4\(^{+}25^{+}\) T cells or p-preDC induced CD4\(^{+}25^{+}\) T cells (Fig. 4D). These findings support previous reports demonstrating that the CD4\(^{+}25^{+}\) T cells generated from the induction of naive CD4\(^{+}25^{+}\) T cell differentiation in the spleen express higher levels of IL4 when compared with native CD4\(^{+}25^{+}\) T cells (35, 36). Importantly, although FC have previously been characterized as having

**FIGURE 5.** CpG-ODN-stimulated FC produce cytokines associated with DC activation and the induction of regulatory T cells. FC were cultured in medium in the presence or absence of CpG. Cytokine production by CpG-stimulated FC was assessed using ELISA. A and B, CpG stimulation of FC induces protein expression of TNF-\(\alpha\) and markedly high expression of IL6 with nearly no difference in IL12p70 production compared with unstimulated FC samples. C, The cytokine expression of IL10 and IFN-\(\gamma\) are increased but not IL2 and IL4 following CpG stimulation of FC. The graphs are representative of duplicate samples in two independent experiments.
CpG-stimulated FC express cytokines associated with the induction of CD4\(^+\)25\(^+\) regulatory T cells

We have demonstrated that the in vitro stimulation of FC in coculture with CD4\(^-\)25\(^-\) T cells results in T cell differentiation into FoxP3\(^+\)CD4\(^+\)25\(^+\) regulatory T cells. We next investigated the mechanism of this FC-mediated induction by assessing whether CpG-stimulated FC express cytokines that are associated with the induction of regulatory T cells as similarly demonstrated with p-preDC. Supernatants from CpG-stimulated and unstimulated FC were collected and assayed via ELISA for the presence of cytokines. Fig. 5, A and B, show an induction of IL6, TNF-\(\alpha\), and IL10. However, IL2 and IL4 were not detected following stimulation of the FC population. In addition, FC produced high levels of MIP1\(\alpha\) but did not produce IL1, IL5, and IL13 (data not shown). Prior studies have demonstrated that CpG-stimulated p-preDC secrete inflammatory cytokines such as IL6, TNF-\(\alpha\), and IL12 mostly for autocrine function. Our observation that CpG-stimulated FC also secrete these inflammatory cytokines suggest a requirement not only for FC differentiation but also for subsequent FC-mediated induction of CD4\(^+\)25\(^+\) regulatory T cells. The observed production of cytokines supports the induction of a cytokine milieu previously implicated in the differentiation of p-preDC and p-preDC-mediated induction of regulatory T cells.

**FC-mediated differentiation of CD4\(^+\)25\(^+\) T cells requires direct cell interaction**

To assess whether FoxP3\(^+\)CD4\(^+\)25\(^+\) regulatory T cell induction was merely due to cytokine production via CpG-stimulated FC or if direct cell-cell interaction between FC and CD4\(^+\)25\(^+\) T cells was required, CD4\(^+\)25\(^+\) T cells were cocultured with FC plus CpG or separated from FC plus CpG by transwell membranes. As shown in Fig. 6A, in FC-T cell cocultures 2.26 ± 0.44% of total CD4\(^+\) T cells recovered were CD4\(^+\)25\(^+\) T cells. In contrast, only 0.84 ± 0.11% of total CD4\(^+\) T cells recovered from the separated culture were CD4\(^+\)25\(^+\) T cells, demonstrating that the separation of FC and CD4\(^+\)25\(^+\) T cells in culture by transwell resulted in a nearly 3-fold decrease in the number of generated CD4\(^+\)25\(^+\) T cells (\(p = 0.008\)). Of note, the total numbers of CD4\(^+\) T cells recovered were similar for both cocultures and separated cultures. Importantly, the CD4\(^+\)25\(^+\) T cells generated from separated cultures did not express the FoxP3 transcript (Fig. 6B), suggesting that cell-cell contact is required for the development of FoxP3 regulatory T cells.
expressing CD4+CD8− regulatory T cells. These findings demonstrate that the cytokines produced from stimulated FC are sufficient to induce the generation of some CD4+CD8− T cells albeit at reduced numbers, whereas direct contact between FC and CD4+CD8− T cells is required for the sustained development of FoxP3+CD4+ regulatory T cells.

**CD86 expression on FC is required for the development of FoxP3+CD4+ regulatory T cells**

Given the current demonstration that FC and CD4+CD8− T cell direct interaction is required for the development of FoxP3+CD4+ regulatory T cells in vitro, we hypothesized that the B7 receptor, CD86, would be required for FC function. Therefore, we evaluated the expression of CD86 on the CpG-stimulated and unstimulated FC population. As demonstrated in Fig. 7A, flow cytometric analysis revealed that the expression of CD86 was increased within CpG-stimulated FC. Furthermore, the protein expression data were supported with real-time PCR analysis of CD86 transcript. An increase in the gene expression of CD86 was detected following CpG-stimulation (Fig. 7B).

To investigate the direct role of CD86 in FC-mediated T cell differentiation and the subsequent development into regulatory T cells, CD4+CD8− T cells were cocultured with FC plus CpG in the presence or absence of anti-CD86 mAb to block any CD86-mediated interaction between FC and the CD4+CD8− T cells. As expected, the CD4+CD8− T cells generated from CpG-stimulated FC T cell cocultures in the absence of anti-CD86 Ab expressed the FoxP3 transcript (Fig. 7C). In contrast, the CD4+CD8− T cells generated from cocultures in the presence of anti-CD86 Ab did not express the FoxP3 transcript. Importantly, these findings demonstrate that FC-T cell interaction is required and dependent on CD86 expression on the FC for the development of FoxP3+CD4+ regulatory T cells.

**Discussion**

FC are currently characterized by their unique ability to promote the reconstitution of purified allogeneic hematopoietic SC in completely MHC-disparate recipients, without inducing clinical or histological evidence of GVHD, and while conferring transplantation tolerance (3–6). Therefore, FC may have a key role in the induction of immune responses. Previous reports have suggested that the transfer of FC lead to the development of a regulatory T cell response that is potentially associated with the induction of tolerance following transplantation (4, 5). However, until now, there has been no direct evidence to link FC function to a possible role in the development of regulatory T cells. In the current studies, we have demonstrated: 1) the induction of donor FoxP3+CD4+CD8− regulatory T cells within the spleen of recipients following FC transplantation in vivo, and 2) the ability of activated FC to induce CD4+CD8− T cells to develop into FoxP3+CD4+CD8− regulatory T cells during coculture, and 3) the requirement for direct cell-cell contact between FC and CD4+CD8− T cells for FC-mediated regulatory T cell development. These findings provide new insights to understanding the immune regulatory effects of FC transplantation and the potential therapeutic application in the induction of transplantation tolerance.

Recent emphasis has been placed on the characterization of cells that permit transplantation tolerance in mouse models with the intention of developing markers for the clinical induction of tolerance and molecular targets for immune-modulating therapies. CD4+CD8− regulatory T cells have a critical role in maintaining immune tolerance and have been described as having a protective role in GVHD by inhibiting the proliferation and cytokine secretion of CD4+ and CD8+ effector T cells (22, 24, 37–40). In allogeneic transplantation, only donor CD4+CD8− regulatory T cells and not host can provide protection against GVHD (22). Therefore, it is important to explore the mechanisms associated with CD4+CD8− regulatory T cell function following allogeneic hematopoietic cell transplantation. Given that FC transplantation results in increased donor SC engraftment and induction of tolerance without GVHD similar to that described for CD4+CD8− regulatory T cell transplantation (22), we hypothesized that the transplantation of FC may result in the induction of CD4+CD8− regulatory T cells. In line with these studies using a fully allogeneic (C57BL/6 → irradiation-conditioned B10.BR) mouse model of SC plus FC transplantation we have shown that donor CD4+CD8− T cells are increased in recipients following SC plus FC transplantation when compared with recipients of SC plus TBM that fail to exhibit long-term donor SC engraftment and also suffer from GVHD. Of note, the current studies demonstrated that over half of the CD4+CD8− T cells that were detected in the spleen of SC plus FC recipients expressed FoxP3 protein, thus providing evidence of a regulatory phenotype following FC transplantation (Fig. 2A). Moreover, we have demonstrated that the CD4+CD8− T cells isolated from the spleen of SC plus FC recipients dramatically inhibit T cell proliferation in vitro (Fig. 2B). These findings suggest that FC transplantation results in the activation of a regulatory immune response through the induction of CD4+CD8− regulatory T cells.

One way that the immune system can achieve tolerance is through the induction of peripheral CD4+CD8− regulatory T cells (41, 42). The induction of Tr1 and CD4+CD8− regulatory T cells, within peripheral tissue such as the spleen, mediated by activated p-preDC has been reported (15–18, 43). Although nearly 60% of the FC population expresses the B220 and CD11c markers that are also present on p-preDC (5, 20), previous reports have shown that FC are distinct from p-preDC. These findings demonstrated that although FC express CD3ε and the TCRβ-chain, the BM-derived p-preDC population does not (19, 20).

Given that the spleen represents a primary site for plasmacytoid DC-mediated peripheral regulatory T cell induction, we hypothesized that if the FC population is directly involved in CD4+CD8− regulatory T cell induction in situ, donor FC should be present within the spleen of SC plus FC recipients. The current studies have demonstrated that the induction of peripheral CD4+CD8− regulatory T cells is preceded by the presence of the donor FC within the recipient spleen as early as 2 wk post-transplantation (Fig. 3).

Importantly, the majority of splenic CD4+CD8− T cells of SC plus FC recipients are of donor origin and the overall donor chimerism of each recipient was greater than 95%. Therefore, the interactions between donor cell types are important during immune responses and our subsequent in vitro studies used a syngeneic in vitro model for analysis of FC-mediated induction of CD4+CD8− regulatory T cells using FC and CD4+CD8− T cells from C57BL/6 mice. Given that following transplantation, donor FC are present within the spleen during CD4+CD8− regulatory T cell induction, we investigated the possible direct role of FC in the differentiation of naive CD4+CD8− T cells into CD4+CD8− T cells. The establishment of a cell population that is distinct from p-preDC yet capable of inducing the development of regulatory T cells will be important to understanding the role of various cell populations in the activation of immune response following transplantation. We now have evidence to support a direct role of FC in the generation of CD4+CD8− regulatory T cells, a function previously described as a characteristic of p-preDC.

The function of p-preDC cells is largely dependent upon the signals that induce their activation and differentiation (7). Activation and maturation signals for p-preDC are cytokines such as IL6, TNF-α and IL12, or TLR activators that include bacterial and viral
product such as LPS and nucleic acids. In the current model of SC plus FC transplantation, it is likely that TLR9 activation on FC occurs as a result of recipient irradiation-conditioning that makes nucleic acid products available, such as nonmethylated DNA, for association with the TLR9 receptor. Therefore, similar to p-preDC, cytokines and TLR receptor stimulation are important for FC activation and function. As demonstrated in Fig. 4, CpG-stimulated FC in coculture with CD4+25+ T cells result in the generation of CD4+25+ T cells that express FoxP3. Further analysis of the FC-generated CD4+25+ T cells revealed a cytokine gene expression profile that is consistent with reports that showed CD4+25+ regulatory T cells generated from naive CD4+25+ T cells in the periphery expressed higher levels of IL4 when compared with native CD4+25+ regulatory T cells (35, 36). Similarly, IL4 gene expression is higher in FC-generated CD4+25+ T cells than p-preDC generated T cells. Thus, arguing that FC are not merely p-preDC but in fact BM-derived FC and p-preDC populations are distinct.

We next examined the production of cytokines by CpG-stimulated FC. An increase in the production of TNF-α and a markedly high production of IL6 was observed (Fig. 5, A and B). Therefore, similar to p-preDC that produce TNF-α and IL6 for autocrine function, FC may differentiate in the presence of TNF-α and IL6. There was no increase in IL12p70 protein, which consists of the p35 and p40 subunits (Fig. 5A). This may be due to the high production of IL6, as studies have demonstrated that high IL6 expression inhibits IL12p70 production in DC (44–46). Secondly, FC may be similar to thymic-derived plasmacytoid DC that do not up-regulate IL12p70 expression upon CpG stimulation (47). In fact, not only are FC present in the spleen, FC have been noted to migrate to the thymus where they have been thought to play a role in self-nonself discrimination (48). IL10 is an important immunoregulatory cytokine with a role in the down-regulation of immune responses. IL10 produced by DC can be used for autocrine function in the alteration of DC maturation for the development of tolerogenic DC or for the induction of regulatory T cells (49–51). Results from the present study indicate an increase in production of IL10 by FC following CpG stimulation.

Evidence from similar studies demonstrated that TGF-β converts naïve CD4+24− T cells to CD4+25+ regulatory T cells as demonstrated by the induction of FoxP3 expression (42). We have observed that TGF-β gene expression is increased in CpG-stimulated FC when compared with unstimulated cells (data not shown). The findings of the current study demonstrate that activated FC produce cytokines required for the subsequent generation of CD4+25+ regulatory T cells.

We next examined whether the FC-mediated induction of FoxP3+CD4+25+ regulatory T cells requires direct cell contact or if it is merely the production of cytokines by activated FC. Using transwell cocultures, we established that CpG-stimulated FC produce a cytokine environment that facilitates naïve CD4+25− T cell differentiation into CD4+25+ T cells. However, without FC-T cell contact, fewer of these cells were generated and none expressed the FoxP3 transcript. Thus, demonstrating that direct FC-CD4+25− T cell interaction is required for the generation of FoxP3+CD4+25+ T cells.

The B7 receptors, CD80 and CD86, are required for maintenance or development of a stable population of CD4+25+ regulatory T cells as studies have demonstrated that B7−deficient mice have marked reduction in the number of CD4+25+ regulatory T cells (25, 52). It is interesting that the regulatory T cell induction function of the FC is dependent on the direct FC-CD4+25− T cell contact and suggests a possible costimulatory requirement. Therefore, the requirement for CD86 was explored. We have demonstrated that CD86 expression on FC is increased following CpG-stimulation. Furthermore, CD86 expression is required for FC-mediated generation of FoxP3+CD4+25+ regulatory T cells. The current in vitro findings have established that the FC population can differentiate into an active phenotype and is capable of providing the cytokine milieu and the increased CD86 expression as required for naïve CD4+25− T cell differentiation into FoxP3+CD4+25+ regulatory T cells. Therefore, a mechanism of FC-mediated peripheral FoxP3+CD4+25+ regulatory T cell induction during SC plus FC reconstitution is suggested. For the first time, we have linked the function of FC previously described as a promoter of purified allogeneic SC engraftment in vivo, to the direct induction of FoxP3+CD4+25+ regulatory T cells. These findings suggest that the transplantation of FC result in immune responses that are associated with the induction of splenic FoxP3+CD4+25+ regulatory T cells. Although we have demonstrated that FC directly induce the generation of FoxP3+CD4+25+ regulatory T cells in vitro, the induction of these cells following SC plus FC transplantation may not solely be due to FC, as there are other immune responses and cell-cell interactions that may parallel FC function and result in the induction or maintenance of regulatory T cells. Furthermore, other regulatory cells such as Tr1 have been described as having a role in transplantation tolerance and may be induced following FC transplantation.

In summary, the current studies demonstrate that purified FC are capable of inducing the generation of FoxP3+CD4+25+ regulatory T cells from naïve CD4+25− T cells. Thus, suggesting that the induction of transplantation tolerance in SC plus FC recipients may be linked to an induction of FoxP3+CD4+25+ regulatory T cells. Therefore, a mechanism for the role of FC in the induction of transplantation tolerance following SC plus FC transplantation has been suggested. Although the direct transplantation of donor CD4+25− regulatory T cells offers great promise, a large number of unmanipulated fresh donor regulatory T cells and/or ex vivo manipulation are required before transplantation (22, 24, 53), thus potentially limiting the use of this therapy. Therefore, the transplantation of limited numbers of donor FC to facilitate allogeneic SC engraftment and induce FoxP3+CD4+25+ regulatory T cells in vivo can potentially offer a useful and clinically feasible approach to induce transplantation tolerance and avoid GVHD while achieving SC engraftment across major histocompatible barriers.

Acknowledgments
We thank the staff at the Smith Animal Facility/Dana Farber Cancer Institute (DFCI) for outstanding animal care. We also thank Peter Schow at the Dana-Farber Cancer Institute Flow Cytometry Core Facility for cell sorting and technical assistance.

Disclosures
The authors have no financial conflict of interest.

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