Plasmacytoid precursor dendritic cells facilitate allogeneic hematopoietic stem cell engraftment

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Bone marrow transplantation offers great promise for treating a number of disease states. However, the widespread application of this approach is dependent upon the development of less toxic methods to establish chimerism and avoid graft-versus-host disease (GVHD). CD8⁺/TCR⁺ facilitating cells (FCs) have been shown to enhance engraftment of hematopoietic stem cells (HSCs) in allogeneic recipients without causing GVHD. In the present studies, we have identified the main subpopulation of FCs as plasmacytoid precursor dendritic cells (p-preDCs). FCs and p-preDCs share many phenotypic, morphological, and functional features: both produce IFN-γ and TNF-α, both are activated by toll-like receptor (TLR)-9 ligand (CpG ODN) stimulation, and both expand and mature after Flt3 ligand (FL) treatment. FL-mobilized FCs, most of which express a preDC phenotype, significantly enhance engraftment of HSCs and induce donor-specific tolerance to skin allografts. However, p-preDCs alone or p-preDCs from the FC population facilitate HSC engraftment less efficiently than total FCs. Moreover, FCs depleted of p-preDCs completely fail to facilitate HSC engraftment. These results are the first to define a direct functional role for p-preDCs in HSC engraftment, and also suggest that p-preDCs need to be in a certain state of maturation/activation to be fully functional.
type 1 IFN-producing cells due to their potent capacity to produce IFN-α in response to virus or microbial stimulation with toll-like receptor (TLR)-9 ligands such as CpG ODN (23–27). The phenotype of murine p-preDCs has now been characterized as a B220+/CD11cdim/CD11b– cell population with a plasmacytoid morphology (24, 28). p-preDCs can induce the development of either a Th1 or a Th2 immune response, depending on the dose and/or the nature of antigen exposure (19). In HSC transplantation, a direct functional role for p-preDCs has not yet been defined. In the present studies, we report that the majority of FCs share functional characteristics with p-preDCs in their ability to secrete IFN-α, TNF-α, and other cytokines and mature by up-regulating activation markers, after activation by CpG ODN. Flt3 ligand (FL), a key cytokine for p-preDC development (25, 28, 29), similarly regulates FCs in that FCs can be generated from FL-supplemented BM cell cultures, as well as expanded and mobilized in vivo in FL-treated mice. The majority of FL-mobilized FCs exhibit a p-preDC phenotype. Additionally, these mobilized FCs facilitate long-term HSC engraftment and induce tolerance in allogeneic recipient mice. Because of the similarities between p-preDCs and FCs, we examined whether p-preDCs and p-preDCs from the FC population contribute directly to HSC facilitation in vivo. We show for the first time that p-preDCs significantly facilitate HSC engraftment. However, facilitation by the p-preDCs as well as preDC FCs is significantly less efficient than that for total FCs. Moreover, when p-preDCs are depleted from the FC population, facilitation of HSCs is lost. These data demonstrate that p-preDCs play a key role in facilitation of HSCs by FCs and suggest that FCs consist of p-preDCs that act in concert with other collaborative cell types to allow optimal HSC engraftment. A clear definition of FC phenotype and mechanism of action may provide a promising cell-based approach to enhance engraftment and tolerance while avoiding alloreactivity.

RESULTS

CD11c+ cells are the predominant cell type within the FC population

To better characterize FCs, we analyzed markers expressed on the sorted CD8α+/TCR− FC population by FACS analysis. Approximately 65–70% of FCs express CD11c+ (Fig. 1A), and 75–88% of FCs express B220 (Fig. 1A). Among the subpopulations negative for B220 expression, 4–6% were NK (NK1.1+ and DX5+), 6–7% were granulocytes (Gr-1+), and 2–4% were monocytes (CD14+; Fig. 1A). Among the B220+ subpopulation, only 15% were B cells (CD19+; Fig. 1A), and 65% were DCs (CD11c+). The CD19+/B220+ FC subpopulation was also positive for intracytoplasmic IgM (unpublished data), confirming a B cell phenotype. Taken together, these data demonstrate that there are distinct subpopulations within the sorted FCs, with a majority as DCs. In addition, the sorted FCs exhibited a heterogeneous mor-

Figure 1. CD8α+/TCR− FCs: a heterogeneous population. (A) CD11c+ FCs are the predominant subpopulation in sorted FCs. BMCs stained with anti-αβ-TCR FITC, anti-γδ-TCR FITC, and anti-CD8α−PE were isolated from the lymphoid gate (intermediate forward scatter and lower side scatter, R1) and sorted for CD8α+/TCRαβ−/TCRγδ− (FC gate). The sorted FCs were blocked using the anti-Fc receptor Ab and stained with anti-B220-PerCP and anti-Gr−1 FITC, or anti-B220−PerCP, anti-NK1.1 FITC and anti-DX5 FITC, or anti-B220−PerCP with anti-CD19 APC, or anti-B220−PerCP with anti-CD11c APC, or anti-B220−PerCP with anti-CD14 APC, or anti-B220−PerCP with anti-CD8α−PE. Isotype-specific controls were performed. Flow cytometric profiles are representative of at least three experiments in B6, two experiments in B10, and two experiments in B10.BR. The reanalysis of sorted FCs stained with different isotype Ab allowed us to verify the purity of the population (>95%) and the absence of T cell contaminants (<1%). Morphology of sorted CD8α−/TCR− FCs was examined after (B) Wright-Giemsa staining with optical microscopy at two different magnifications and (C) by transmission electron microscopy.
Morphology of the majority of sorted CD8− profiles are representative of at least two separate experiments in B6. Population was analyzed for B220 and CD11b expression. Flow cytometric transmission electron microscopy, after Wright-Giemsa staining under optical microscopy (Fig. 1B), and this CD4+ p-preDCs have been shown to express the CD4 antigen in the presence of CD4 on sorted FCs, as at least 70% of BM was related phenotypically to p-preDCs, we analyzed the known subtypes of DCs present in the BM (24, 30). We therefore determined whether FCs resemble p-preDCs in response to CpG ODN stimulation.

Because p-preDCs mature after CpG ODN stimulation, we analyzed whether FCs resemble p-preDCs in maturation (activation) (28, 30, 31). After overnight exposure with medium or CpG ODN, FCs were analyzed for MHC-class II, CD80, and CD86 expression (Fig. 3 D). Class II and CD86 were highly up-regulated on FCs (from 18 ± 8% to 74 ± 6.5% and from up to 7 ± 19% to 86 ± 12%, respectively, n = 4), and, to a lesser extent, CD80 (from 10 ± 3.7% to 23.5 ± 8%, n = 4; Fig. 3 D). Similarly, CD86 is up-regulated on p-preDCs (from 12 ± 9% to 85 ± 13%, n = 2) in a similar amount to FCs, but class II up-regulation on p-preDCs (from 10 ± 2% to 39 ± 4%, n = 2) did not increase as much as it did on FCs. The increase of CD80 (from 7 ± 1% to 12.5 ± 1%, n = 2) on p-preDCs was only slight.

FL is a key cytokine for FC expansion and maturation in vitro

Coculture of BMCs with FL increases the frequency of p-preDCs (28, 29). We therefore determined whether FCs can be propagated using similar culture conditions. After 10 d in culture with FL, FCs (FL-derived FCs), and p-preDCs (FL-derived p-preDCs) were sorted. We observed a seven-fold increase in FL-derived FCs (n = 8) and 17-fold in FL-derived p-preDCs (n = 4) from the cultured BM (Fig. 4 A).

Interestingly, FL-derived FCs were in a more activated state than fresh FCs, as evidenced by their morphology (Fig. 4 B). Dendrites were already beginning to appear on FL-derived FCs after overnight culture, and their appearance was amplified after exposure to CpG ODN. FL-derived FCs also produced significant amounts of IFN-α after overnight culture (Fig. 4 C), perhaps reflective of their activation state. FL-derived p-preDCs also produced IFN-α after overnight culture. Stimulation with CpG ODN overnight further increased the IFN-α secretion, as well as TNF-α and IL-12p70 production.

The effect of FL on FCs or p-preDCs was further evaluated by analyzing activation marker expression after over-
night culture. Interestingly, both FL-derived FCs and FL-derived p-preDCs significantly up-regulated expression of MHC class II, CD80, and CD86, as compared with FCs and p-preDCs sorted from fresh BM cells (BMCs; Fig. 4 D). Indeed, 40% of FL-derived FCs expressed class II versus 18% of fresh FCs, 47% expressed CD80 versus 11% of fresh FCs, and 51% expressed CD86 versus 17% of fresh FCs. Similarly, FL-derived p-preDCs up-regulated the level of class II, CD80, and CD86. CpG ODN exposure overnight further increased the level of expression of these activation markers on both FL-derived FCs (73% of class II, 48% of CD80, and 77% expression of CD86) and FL-derived p-preDCs (88% of class II, 63% of CD80, and 87% of CD86; Fig. 4 D).

In conclusion, FCs as well as p-preDCs expanded from FL-supplemented BMC cultures are in a more advanced activation state than freshly isolated cells. Nevertheless, they display similar cytokine secretion and activation marker up-regulation after exposure to CpG.

In vivo FL-mobilized FCs facilitate HSC engraftment in allogeneic recipients

FL treatment in vivo expands DCs, including the p-preDCs subtype (25, 32). We showed previously that mice treated with FL demonstrate a significant expansion of FCs in PB, BM, and spleen, with the peak production at 10 d (33). Here, we characterized the influence of FL administration on the different subtypes in the FC population in PB and analyzed the functional potential of purified blood FL-mobilized FCs (FL-FCs) to facilitate HSC engraftment. Approximately 85–90% of FL-FCs in the PB express CD11c (Fig. 5 A), and 5–7% express NK1.1, but none express CD19 or CD14 (unpublished data). Interestingly, there were clearly two distinct DC populations: CD11cdim and CD11cbright. Further analysis showed that the 60% CD11cdim population, characteristic of an immature DC phenotype, was exclusively the p-preDCs phenotype (B220/H11001/CD11cdim/CD11b/H11002). The 20% CD11cbright population, characteristic of mature DCs, contained a majority of mature lymphoid DCs (B220/H11002/CD11cbright/CD11b/H11002), and all expressed the CD86 marker (unpublished data). Therefore, FL mobilization induced a significant increase in the CD11c population, and dramatically decreased the B cell and monocyte populations (unpublished data).

We next asked whether purified FL-FCs from PB maintained their ability to facilitate HSC engraftment in allogeneic recipients. HSCs were sorted from the marrow of untreated B10.BR mice and FCs from the PB of FL-treated B10.BR mice after 10 d of treatment. B10-recipient mice were ablatively conditioned and reconstituted with 5,000 HSCs plus 30,000 FL-FCs. Control B10 mice received 5,000 HSCs alone or 5,000 FCs from untreated B10.BR donor mice. FL-FCs were functional, as evidenced by 87% long-term survival (>180 d; Fig. 5 B). All mice receiving FCs from untreated mice with HSCs survived longer than 180 d. In contrast, none of the mice receiving allogeneic HSCs alone survived > 170 d. Thus, the FL-FCs from PB exhibited significant facilitation of engraftment of HSCs in allogeneic recipients.
We examined recipients of HSCs plus FL-FCs for donor chimerism and multiple hematopoietic lineages 3 mo after transplantation. All surviving animals tested showed >95% donor chimerism for multiple lineages, including T cells, NK cells, B cells, macrophages, and granulocytes (Fig. 5 C).

To test whether these FL-FCs plus HSC chimeras were functionally tolerant, skin grafts from B10.BR (HSC donor) or BALB/c (third-party) mice were performed. Donor-specific skin grafts were accepted by the chimeras (median survival time [MST] >100 d), while third-party (BALB/c) grafts were promptly rejected (MST = 15 d; Fig. 5 D). In conclusion, FL treatment significantly expanded FCs in PB that consist of 85–90% CD11c^+ cells, with 20% of these being mature DCs, and the distinct majority (60–65%) resembling the p-preDCs phenotype. Most importantly, FL-FCs enhance HSC engraftment and tolerance induction in allogeneic recipients.

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p-preDCs play the main role in the facilitating function but could not replace total FCs.

**DISCUSSION**

HSC chimerism has the potential to induce tolerance to organ transplants and restore self-tolerance for treatment of autoimmune diseases. However, the widespread application of this promising therapy in the clinic is dependent upon reducing the toxicity associated with conventional BMT. A great deal of attention has been focused on identification of cells with facilitating potential to enhance HSC engraftment and avoid the toxicity of BMT. One such subpopulation is p-preDCs, which were found to enhance engraftment of purified allogeneic HSCs, as shown by chimerism and tolerance to donor antigens, but with somewhat less efficiency than FCs.

**Depletion of p-preDCs FCs abrogates facilitation**

To determine whether the p-preDCs in the FC population were the critical subpopulation responsible for facilitating function, we coinjected HSCs with FCs depleted of the p-preDCs population (Fig. 7 A) and followed the kinetic of engraftment, but less efficiently than the FCs total. Donor-derived hematopoietic cells were present at levels >95% in all alive animals receiving HSCs + p-preDCs at 3 mo after transplantation (Fig. 6 B), indicating that the chimerism was significantly high and consistent between all recipients. Multilineage production was present in animals transplanted with HSCs + p-preDCs at 3 mo after transplantation (Fig. 6 B).

To test whether the chimerism achieved with transplantation of HSCs + p-preDCs induced donor-specific tolerance, skin grafts from B6 (HSC donor), and BALB/c (third-party) mice were performed. Donor-specific skin grafts were accepted by chimeras (MST > 90 d), whereas third-party (BALB/c) grafts were promptly rejected (MST = 13.5 d; Fig. 6 C). In conclusion, p-preDCs enhance engraftment of purified allogeneic HSCs, as shown by chimerism and tolerance to donor antigens, but with somewhat less efficiency than FCs.
the CD8^+/TCR^− FCs that enhance engraftment of purified allogeneic HSCs without causing GVHD (8, 10). Until now, the precise characterization of FCs has remained controversial due to the heterogeneity of the CD8^+/TCR^− population and the infrequency of the various components. In the present studies, we demonstrate that the major component of the FC population resembles p-preDCs, making them likely candidates for the biologic function of facilitation. We show for the first time that p-preDCs significantly facilitate HSC engraftment in allogeneic recipients and induce tolerance without causing GVHD.

Previously, the heterogeneous CD8^+/TCR^− FC population was described as sharing phenotypic characteristics with CD8α^+ lymphoid DCs (8). At that time, the phenotype for murine p-preDCs was unknown, making an assessment of the relative contribution of this DC subset to facilitation impossible. We have now demonstrated that the B220^+ /CD11c^dim/CD11b^- cells in the FC gate exhibit a morphology and a phenotype that closely resembles p-preDCs. Unlike mouse BM p-preDCs, most of which are CD8α^+, all of the B220^+/CD11c^dim/CD11b^- FCs express the CD8α antigen. CD8α expression on mouse p-preDCs has been demonstrated to vary according to tissue source and state of activation, increasing from 10 to 30% on resting BM p-preDCs to 70 to 100% after activation (24, 30, 34). We therefore hypothesized that the B220^+/CD11c^dim/CD11b^- FCs in the BM represent rest BM CD8α^+ p-preDCs. This hypothesis is reinforced by the fact that isolated FCs from fresh/untreated BM exhibit a low expression of costimulatory molecules (i.e., MHC class II, CD80, CD86), CD40 (unpublished data), and no CD205 expression (unpublished data), as has been reported for p-preDCs (24, 35). Although other cells, including B cells, NK cells, granulocytes, and monocytes are also present in the FC population, the paucity of these cells in the functional FL-mobilized PB-FC population (unpublished data) led us to conclude that these cells do not play a significant role in facilitation and reinforces our hypothesis that this recently described rare population of p-preDCs are central to facilitation.

In the present studies, we have shown that FCs share many features with p-preDCs, including their response to CpG ODN with: (a) secretion of similar cytokines and chemokines; and (b) maturation. The hallmark of p-preDCs is the capacity to produce high amounts of IFN-type I, consisting of IFN-α, and IFN-β, in response to appropriate stimulation (19, 23). Mouse p-preDCs, as their human counterparts, respond preferentially to ligands for TLR7 and TLR9 and only poorly to ligands for TLR2, TLR3, or TLR4 (36). Notably, FCs produce IFN-α after stimulation with CpG ODN, and none after stimulation with LPS (TLR4 ligand; unpublished data). Besides IFN-α, FCs produce other cytokines and chemokines, including MIP-1α, MCP-1, TNF-α, RANTES, IL-6, IFN-γ, and IL-12p70. p-preDCs are potentially “tolerogenic” under selected circumstances, and have been shown to induce anergy in an antigen-specific CD4^+ T cell line (37), differentiation of naive CD4 and CD8 T cells into Th2 cells (38), and induction of regulatory T cells in vitro (31, 39, 40). Moreover, a recent publication has shown that the adoptive transfer of...
p-preDCs are the key cell subtype in the FCs population.

Figure 7. p-preDCs are the key cell subtype in the FCs population. (A) Sort of p-preDC FCs and FCs without p-preDCs. From an FC gate (CD8^+/TCR^+), CD11c^+/B220^+ cells were isolated and represented the p-preDC FC. The FCs without p-preDCs are all cells from the FC gate remaining after removal of the CD11c^+ /B220^+ cells. (B) Survival of allogeneic recipients transplanted with HSCs and FCs without p-preDCs or p-preDC FCs (B10.BR → B10). B10 mice were conditioned with 950 cGy TBI and given 5,000 HSCs alone (HSC group, – – – ) or in combination with 30,000 purified FCs [HSC + FC group, – – – – – – – – ] or FCs without p-preDCs from B10.BR mice (-----). Some recipient mice were used as irradiation controls (-----). The cumulative survival of recipients is represented by the Kaplan-Meier method. Animals were followed for 4 mo. *, P = 0.0165 between the HSC + FC group and the HSC group; **, P = 0.0006 between the HSC + FC group and the HSC + FC without p-preDC group.

To date, there has been only indirect evidence to demonstrate a sustained tolerogenic effect for p-preDCs in vivo. Although prolongation of solid organ allografts has been demonstrated in mice treated with enriched p-preDCs graft, sustained donor-specific tolerance has not been achieved (44). Similarly, although GVHD was decreased in occurrence and severity in BMT recipients who had an increase in p-preDCs in the donor marrow inoculum, a direct biologic role remains to be defined (20, 22). We show for the first time that p-preDCs exhibit a significant and durable graft-enhancing ability for HSCs in mismatched recipients. Notably, p-preDCs significantly enhanced engraftment of HSCs without causing GVHD. Moreover, recipients of p-preDCs facilitated HSC transplants exhibited donor-specific tolerance to skin allografts. Therefore, the tolerogenic effect of this cell population was maintained in vivo as it relates to establishing chimerism and tolerance. The fact that depletion of the p-preDCs component from the FCs total results in loss of facilitation confirms that p-preDCs are the primary component in facilitation.

It is interesting that the total FC population exhibits a significantly greater engraftment-enhancing effect on HSCs than do p-preDCs. A number of hypotheses could explain this observation. p-preDC-like cells in the FC gate may be the rare, CD8α^+ subpopulation of the p-preDCs found in the BM, and only CD8α^+ p-preDCs may be able to fully replace FCs in this functional biological assay. Moreover, p-preDCs facilitate less efficiently than total FCs, pointing to a requirement for collaborative cells and/or signals in the FC population. p-preDCs may also not be in an appropriately activated state. Given the heterogeneous nature of cells in the FC gate, it is possible that another collaborative cell population (i.e., NK cells) is required for optimal function of...
p-preDCs. In support of this mechanism is the fact that activation of p-preDCs by NK cells has been reported in vitro (45). It is also possible that p-preDC phenotype cells within the FC gate are distinct from the bulk population of BM p-preDCs (other than activation or known maturation status). The fact that FCs produce IL-10, whereas p-preDCs do not in humans or mice, would point to an additional collaborative cell or signal in the FC population. Studies are underway to evaluate this mechanism.

In conclusion, CD8^+ /TCR^− FCs will have a significant impact for the clinical application of HSC-induced chimerism since tolerance can be promoted, GVHD avoided, and safe transplants allowed in mismatched recipients (10, 46, 47). Notably, we show for the first time a direct in vivo effect for p-preDCs in facilitating HSC engraftment and inducing durable tolerance to transplanted grafts. The identification of the cells in the FC gate, and the mechanism by which they mediate a full facilitation of HSC engraftment, will lead to novel cell-based therapeutic strategies to optimize the composition of the graft in order to reduce the morbidity of HSC transplants in mismatched recipients.

MATERIALS AND METHODS

**Mice.** 5–10-wk-old male B10.Br/SgSnJ (B10.Br, H-2^d^), C3H/HeJ (C3H, H-2^b^), C57BL/10SnJ (B10, H-2^b^), BALB/c (H-2^d^), or C57BL/6J (B6, H-2^b^) mice were purchased from Jackson ImmunoResearch Laboratories, except those labeled with APC-Cy7 from eBioscience) were used. LPS-stimulated BMCs or FL-mobilized peripheral blood, with a purity ranging from 94 to 98%, were incubated with Fc receptor block (anti-Cd16/CD32) before staining with lineage-specific markers, including anti-CD4, CD11c, CD220, NK1.1, CD45R/B220, CD8 alpha/beta, Gr-1, and Ter-119. Sorted BMCs were stained with anti-CD16/CD32 (FcgammaRII/II receptor), CD11c (53–6.7) PE labeled, anti-TCRg chain (H57-597), anti-TCRdelta chain (GL3) FITC labeled. To sort B220^+ /CD11c^dim/CD11b^− p-preDCs: anti-CD11b (M1/70) APC labeled, anti-CD45R/B220 (RA3-6B2) APC-Cy7 labeled, anti-CD11c (HL3) PE labeled, and anti-TCRbeta chain (H57-597), anti-TCRgamma chain (GL3), anti-CD14 (rmC5-3), anti-CD19 (1D3), anti-pan-NK cells (DX5), and anti-NK1.1 (PK136), all FITC labeled. To sort p-preDCs in the FC population (p-preDC FCs): anti-CD8α (53–6.7) PE labeled, anti-TCRbeta chain (H57-597), anti-TCRgamma chain (GL3) FITC labeled, anti-CD45R/B220 (RA3-6B2) APC-Cy7 labeled, anti-CD11c (HL3) PE labeled. To sort HSCs: anti-TCRbeta chain (H57-597), anti-TCRgamma chain (GL3), anti-Ly-6G (Gr-1), anti-CD11b, Mac-1 (M1/70), anti-CD8alpha/beta (53–6.7), and anti-CD45R/B220 (RA3-6B2), all FITC labeled, anti-Ly-6A/E (Sca-1; E13-161.7) PE labeled and anti-e–Kit (CD117; 2B8) APC labeled. Reanalysis of sorted FCs: purified FCs were stained with anti-CD16/CD32 (FcγRII/II receptor); 2.4G2), anti-CD11c (HL3), anti-CD4 (RM4-5) and anti-CD19 (1D3) all APC labeled, anti-CD45R/B220 (RA3-6B2) PerCP labeled, and anti-pan-NK cells (DX5), anti-NK1.1 (PK136), anti-CD14 (rmC5-3), and anti-Ly-6G (Gr-1) FITC labeled. To analyze the activation state: anti-CD80 (B7-1; 16-10A1), anti-CD86 (B7-2; 16-10A1), and anti-I-A^b^ (APβ; AF6-120.1) FITC labeled. For BPL typing and multilineage chimerism: anti–H-2K^a^ (AF6-88.5) PE or FITC labeled, anti–H–2K^b^ (36–7–5) PE or FITC labeled, anti–TCRbeta chain (H57-597), anti-NK1.1 (PK136), anti-Ly-6G (Gr-1), anti-CD11b, Mac-1 (M1/70), and anti-CD45R/B220 (RA3-6B2) all FITC labeled.

**BMC preparation.** BMC preparations were performed as described previously (9). In brief, BMCs were obtained by flushing femurs and tibias from mice with cold media 199 (GIBCO BRL) containing 30 μg/ml Gentamicin (GIBCO BRL; referred to hereafter as chimera media [CM]). After washing with CM, the BMCs were resuspended to 100 × 10^6^ cells/ml in sterile cell sort medium (CSM: Hank’s balanced salt solution without phenol red, 2% heat-inactivated fetal calf serum, 2 μg/ml Hepes buffer, and 30 μg/ml of Gentamicin [all from GIBCO BRL]).

**Culture of BMCs with FL.** BMCs were resuspended at 10^6^ cells/ml in culture medium consisting of RPMI 1640 (GIBCO BRL), 10% FBS (GIBCO BRL), 1 mM sodium pyruvate (GIBCO BRL), 10 mM Hepes (GIBCO BRL), 2 mM L-glutamine (GIBCO BRL), 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO BRL), and 10^{-8} M 2-mercaptoethanol (Sigma-Aldrich), supplemented with human FL (100 ng/ml, gift from Amgen). After 5 d of culture, half of the medium was replaced by fresh cytokine-supplemented culture medium according to a protocol described previously (28).

**Flow cytometry.** The following mAbs (all from BD Biosciences, except those labeled with APC-Cy7 from eBioscience) were used. To sort CD8^+ /TCR^− FCs: anti-CD8α (53–6.7) PE labeled, anti-TCRbeta chain (H57-597), and anti-TCRgamma chain (GL3) FITC labeled. To sort B220^+ /CD11c^dim/CD11b^− p-preDCs: anti-CD11b (M1/70) APC labeled, anti-CD45R/B220 (RA3-6B2) APC-Cy7 labeled, anti-CD11c (HL3) PE labeled, and anti-TCRbeta chain (H57-597), anti-TCRgamma chain (GL3), anti-CD14 (rmC5-3), anti-CD19 (1D3), anti-pan-NK cells (DX5), and anti-NK1.1 (PK136), all FITC labeled. To sort p-preDCs in the FC population (p-preDC FCs): anti-CD8α (53–6.7) PE labeled, anti-TCRbeta chain (H57-597), anti-TCRgamma chain (GL3) FITC labeled, anti-CD45R/B220 (RA3-6B2) APC-Cy7 labeled, anti-CD11c (HL3) PE labeled. To sort HSCs: anti-TCRbeta chain (H57-597), anti-TCRgamma chain (GL3), anti-Ly-6G (Gr-1), anti-CD11b, Mac-1 (M1/70), anti-CD8α/beta (53–6.7), and anti-CD45R/B220 (RA3-6B2), all FITC labeled, anti-Ly-6A/E (Sca-1; E13-161.7) PE labeled and anti-e–Kit (CD117; 2B8) APC labeled. Reanalysis of sorted FCs: purified FCs were stained with anti-CD16/CD32 (FcγRII/II receptor); CD14, CD45R/B220, CD8alpha/beta, CD11c, and CD19 conjugated–specific markers for further analysis. To validate the specificity of staining, we used various conjugates of the same antibody, including CD11c, CD4, and CD19 conjugated to FITC and APC, as well as B220 conjugated with PerCP, APC, or FITC (unpublished data). To analyze the subtypes of DCs in the sorted FC population, sorted FCs were blocked and stained with CD11bFITC, CD11c APC and B220 PerCP to determine the presence of myeloid preDCs (CD11c^{+}/B220^{-}/CD11b^{+}), p-preDCs (CD11c^{+}/B220^{-}/CD11b^{-},), and mature lymphoid DCs (CD8alpha/beta^{+}/B220^{-}). The cells were washed twice in CSM and analyzed on a FACScalibur using CellQuest Software (Becton Dickinson).

**Cpg oligodeoxynucleotides stimulation.** Sorted p-preDCs or FCs were cultured for 18 h at 10^6^ cells/200 μl in 96-well round-bottom culture plates in culture medium in the presence or absence of toll-like receptor (TLR)-9 ligand, Cpg ODN 1668 (TCCATGACGTTCGGATGCT; GIBCO BRL Custom Primers) at 1 μM, TLR4 ligand, LPS from Escherichia coli (Sigma-Aldrich) at 10 μg/ml, as described previously (28). Cpg or LPS-treated or -untreated cells were subsequently assayed for: (a) the expression of DC activation/maturity cell surface markers by FACS; and (b) morphological appearance by Wright-Giemsa staining on cytospins. The
supernatant of these cultures were collected for analysis of the production of different cytokines by ELISA.

**Cytospins.** Cells (30,000 to 60,000) were centrifuged for 5 min at 300 rpm. The slides were air dried, fixed with methanol, and dried at room temperature. Wright-Giemsa staining was performed using the kit Hema3 according to the manufacturer’s protocol (Fisher Scientific).

**Transmission electron microscopy.** Cells were pelleted at 1,000 g, fixed in situ as a pellet in 2.5% glutaraldehyde, and processed for transmission electron microscopy using standard methods. Sections of 70 nm were cut with a Reichert Ultracut S mounted on copper grids and counterstained with uranyl-acetate (2%) and lead citrate. Observations were performed using a Joel 100 cx electron microscope.

**Cytokine production by ELISA.** In brief, the cell-free supernatants of 12, 18, or 24 h cultured cells (FCs or p-preDCs) with or without CpG ODN or LPS were collected and kept frozen at −80°C. The amount of cytokine produced was determined by (α) ELISA kits for mouse IFN-α (R&D Systems) and mouse TNF-α (BioSource International); and (β) multiplex for MIP-1α (CCL3), GM-CSF, MCP-1 (CCL2), RANTES (CCL5), IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-9, and IL-13 on 18 h incubation supernatants from three different experiments performed by Linco Diagnostic Services.

**Reconstitution of allogeneic recipients with HSCs from untreated marrow and FL-mobilized FCs.** HSCs were sorted from untreated B10.BR mice (H-2b). FCs (CD8+/TCR+) were sorted from PB of 10 d FL-treated B10.BR mice, or from untreated B10.BR mice as controls. Recipient B10 mice (H-2b) were treated with 950 cGy of total body irradiation (TBI) using a 137-cesium source (Gamma-cell 40 Exactor; Nordion International). 6 h after irradiation, 5,000 HSCs were transplanted either alone or in combination with 30,000 FCs by direct tail vein injection. FCs and HSCs were mixed before transplantation. A control group of irradiated mice was also established. The survival was plotted over time.

**Reconstitution of allogeneic recipients with HSC ± BM FCs, p-preDCs, and FC subpopulations.** Recipient mice were given 950 cGy TBI. 6 h after irradiation, recipients were transplanted with 5,000 purified allogeneic HSCs with or without 30,000 FCs or p-preDCs resuspended in CM, or 15,000 FCs depleted of p-preDCs or 15,000 p-preDC FCs via lateral tail vein injection. A group of irradiated mice served as controls. Graft survival was estimated by the Kaplan-Meier method.

**Characterization of donor multilineage engraftment by flow cytometry.** Donor engraftment in the recipient was quantified by peripheral blood cell typing using flow cytometry. Specifically, two-color flow was used to determine the percentage of PBL that express H-2b or H-2b MHC class I antigen as described previously (49). For multilineage analysis, PBL were stained with donor-specific anti-H-2Kb-PE or anti-H-2Kk-PE mAb along with a combination of the following Ab: anti–Gr-1, anti–Mac-1, anti–Iaα/β–TCR, anti–B220, anti–NK1.1, anti–CD11c, and anti–CD19. Cells were washed, acquired, and analyzed on the FACSCalibur.

**Skin grafts.** Skin grafts were performed by techniques published previously (50). In brief, full-thickness skin grafts from the tail of B10.BR, B10.B6, or BALB/c mice were harvested. Full-thickness graft bed were prepared on the lateral thoracic wall. Three skin grafts (syngeneic, donor, and third-party) were placed on each animal. Each graft was separated from the others by a skin bridge of at least 3 mm. Skin grafts were covered by a double layer of petroleum gauze and a cast. The cast was removed after 7 d. Grafts were scored daily for percent rejection. Rejection was defined as complete when no residual viable graft could be detected.

**Significance estimates.** Survival was estimated according to the Kaplan-Meier method and tested with the log rank statistic using SPSS 12.0 for Windows.

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