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Microchimerism, Donor Dendritic Cells, and Alloimmune Reactivity in Recipients of Flt3 Ligand-Mobilized Hemopoietic Cells: Modulation by Tacrolimus

Adrian E. Morelli, Mary A. Antonysamy, Takuya Takayama, Holger Hackstein, Zongyou Chen, Shiguang Qian, Nancy B. Zurowski, and Angus W. Thomson

Flt3 ligand (FL) is a potent hemopoietic growth factor that strikingly enhances stem cells and dendritic cells (DC) in vivo. We examined the impact of infusing FL-mobilized bone marrow (BM) cells on microchimerism and anti-donor reactivity in normal and tacrolimus-immunosuppressed, noncytoablated allogeneic recipients. BM from B10 (H2b) mice given FL (10 μg/day; days 0–8; FL-BM) contained a 7-fold higher incidence of potentially tolerogenic immature CD11c+ DC (CD40low, CD80low, CD86low, MHC IIlow) that induced alloantigen-specific T cell hyporesponsiveness in vitro. C3H (H2k) mice received 50 × 10⁶ normal or FL-BM cells (day 0) and tacrolimus (2 mg/kg/day; days 0–12). On day 15, enhanced numbers of donor (IAb+) cells were detected in the thymi and spleens of FL-BM recipients. Tacrolimus markedly enhanced microchimerism, which declined as a function of time. Ex vivo splenocyte proliferative and CTL responses and Th1 cytokine (IFN-γ) production in response to donor alloantigens were augmented by FL-BM infusion, but reduced by tacrolimus. Systemic infusion of purified FL-BM immature DC, equivalent in number to that in corresponding whole BM, confirmed their capacity to sensitize, rather than tolerize, recipient T cells in vivo. In vitro, tacrolimus suppressed GM-CSF-stimulated growth of myeloid DC from normal BM much more effectively than from FL-BM without affecting MHC class II or costimulatory molecule expression. Infusion of normal B10 BM cells at the time of transplant prolonged C3H heart allograft survival, whereas FL-BM cells did not. A therapeutic effect of tacrolimus on graft survival was observed in combination with normal, but not FL-BM cells. These findings suggest the need for alternative immunosuppressive strategies to calcineurin inhibition to enable the engraftment, survival, and immunomodulatory function of FL-enhanced, immature donor DC. The Journal of Immunology, 2000, 165: 226–237.

The naturally occurring persistence of donor hemopoietic cells in lymphoid and nonlymphoid tissues of organ allograft recipients (microchimerism) (1) is an intriguing immunologic phenomenon that is not well understood and has been the subject of recent debate (2–4). Whether microchimerism represents a contributory factor toward or a consequence of unresponsiveness to donor alloantigens has not been fully resolved. Nevertheless, in spontaneous organ transplant tolerance models, depletion of passenger leukocytes from the donor tissue results in allograft rejection, indicating the potential of these hemopoietic cells to modulate anti-donor reactivity (5, 6). The fact that donor leukocytes are able to integrate and survive within the dominant host immune system for extended periods suggests that they may have the capacity to evade immunologic surveillance or to modulate anti-donor immune reactivity. Indeed, the inherent tolerogenicity of allogeneic hemopoietic cells has long been recognized (7, 8) and has provided a rationale for the use of donor bone marrow (BM) cells in efforts to promote allograft survival in both experimental models (9–13) and humans (14–17).

Flt3 ligand (FL) is a hemopoietic cytokine (18, 19) whose receptor, flt3, is expressed on pluripotent stem and progenitor cells (20, 21). Unlike other hemopoietic growth factors, FL selectively and dramatically increases the number of dendritic cells (DC) and their precursors in BM, peripheral blood, and lymphoid and nonlymphoid tissues (22, 23) without apparent toxicity. Because DC play a crucial role as APC in both initiation and regulation of immune responses (24–26), FL is potentially a valuable tool for the manipulation of these important APC in allograft recipients and for evaluation of their roles as regulators of alloimmunity.

FL can act as either an immunologic adjuvant (27–29) or a promoter of tolerance induction (30). We have shown recently that a short course of FL administered to noncytoablated, transiently immunosuppressed recipients of normal allogeneic BM can dramatically increase microchimerism in host BM and spleen (31, 32). However, this strategy reversed the beneficial effects of donor BM in prolonging cardiac allograft survival, possibly because of immunologic adjuvant effects of FL in the recipient (32). On the other hand, it has been observed that administration of immature BM-derived donor DC capable of inducing alloantigen-specific T cell anergy in vitro (33) can prolong cardiac (34–36), pancreatic islet (37), or skin allograft survival (38). These findings suggest that FL treatment of BM donors to augment both stem cells and immature DC might enhance microchimerism-induced modulation of alloimmunity without direct adjuvant effects of FL on the recipient.

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Received for publication August 16, 1999. Accepted for publication April 13, 2000.

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The work was supported in part by National Institutes of Health Grants DK49745 and AI141011 (to A.W.T.).

A.E.M. and M.A.A. contributed equally to this work and should be considered co-first authors.

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4 Abbreviations used in this paper: BM, bone marrow; DC, dendritic cells; FL, Flt3 ligand; ABC-AP, avidin-biotin complex-alkaline phosphatase.
This study examined the influence of systemic FL administration on the DC population in donor BM. The allostimulatory activity of BM cells from normal and FL-treated donors (FL-BM) and of purified FL-BM DC was evaluated. We also investigated whether infusion of freshly isolated FL-BM cells, either alone or together with tacrolimus, might facilitate donor cell engraftment and survival. Effects on host alloreactive immunity, including graft rejection, were also determined. Our findings indicate that freshly isolated FL-BM contains markedly enhanced numbers of immature DC that can induce alloantigen-specific T cell hypore sponsiveness in vitro. However, infusion of FL-BM cells leads to augmented microchimerism and enhanced anti-donor T cell responses that reflect the capacity of infused purified immature FL-BM DC to sensitize the host. Tacrolimus administration further enhanced microchimerism, accompanied by suppression of anti-donor MLR and CTL responses. In both control and tacrolimus-treated recipients, microchimerism declined as a function of time. In vitro, exposure to tacrolimus inhibited the growth of DC from normal BM or FL-BM in response to GM-CSF and IL-4 without affecting their MHC class II and costimulatory (CD40, CD86) molecule expression.

This study demonstrates the potential of FL-BM, donor immature DC, and tacrolimus to significantly manipulate microchimerism and alloimmunity. It further emphasizes the need for alternative immunosuppressive strategies to calcineurin inhibition to facilitate the long term engraftment of immature donor-derived DC and to inhibit their differentiation into potentially allostimulatory APC. Achievement of this goal may have important implications for the regulation of alloreactive immunity.

Materials and Methods

Animals

Male C57BL/10J (B10; H2\(^b\); IA\(^b\)), C3HHeJ (H2\(^k\); IA\(^k\)), and BALB/c (H2\(^d\); IA\(^b\)) mice, 8–12 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). They were housed in the specific pathogen-free facility of University of Pittsburgh Medical Center.

FL administration

B10 donor mice were given either no treatment or Chinese hamster ovary (CHO) cell-derived recombinant human FL (Immunex, Seattle, WA) in low endotoxin PBS (10 \(\mu\)g/mouse/day i.p.) for 9 consecutive days before use as BM donors.

Phenotypic analyses of DC in freshly isolated BM

BM cells were isolated from femurs and tibias of normal or FL-treated B10 mice. RBC were lysed using 0.83% ammonium chloride. To increase the percentage of DC in the BM, T, B, and NK cells; granulocytes; and erythrocyte precursors were lysed by complement depletion. BM cells were labeled with a cocktail of mAbs (anti-CD3, clone 17A2; anti-CD5R/B220, clone RA3-6B2; anti-NK-1.1, clone PK136; anti-Gr-1, clone RB6-8C5; and anti-TER 119, clone TER-119; all mAbs bind com-

Allogenic BM transplantation

Freshly isolated B10 BM cells (5 \(\times\) 10\(^6\) in 500 \(\mu\)l) were injected via the lateral tail vein into C3H recipients. Following BM transplantation on day 0, recipients were injected i.m. with tacrolimus (formerly FK 506; Fujisawa Pharmaceutical, Osaka, Japan) at 2 mg/kg/day from days 0–12 or received no further treatment.

Immunohistochemistry and quantitation of donor cells in tissues

Mice were killed on day 15 or 30 post-transplant. Samples of spleen, thymus, and skin (ear pinna) were placed in embedding medium (Tissue-Tek OCT compound, Miles, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at \(-80^\circ\)C until further use. Eight-micron cryostat sections were fixed in acetone and incubated successively with 1) 10% (v/v) normal goat serum, 2) avidin-biotin blocking solution (Vector), 3) optimal dilution of biotin-conjugated mouse anti-IA\(^*\) B-chain mAb (clone 25-9-17, Pharmingen), and 4) avidin-biotin complex-alkaline phosphatase (ABC-AP; Vector). AP activity was detected by incubation with the substrate Vector Blue (Vector). Endogenous AP activity was inhibited by addition of levamisole (Vector) in the substrate solution. Sections were counterstained with Fast Red (Vector) and mounted first in Crystal/mount (Biomedex, Foster City, CA) and then in Permount (Fisher Scientific, Pittsburgh, PA). An irrelevant mouse mAb of the same isotype as the primary mAb was used as an irrelevant control. The number of donor MHC class II\(^*\) (IA\(^*\)) cells was counted in three tissue sections (each from different levels) per mouse (three mice per group) by a blinded observer and expressed as the number of positive cells per square centimeter of tissue section. Section surface area was calculated using an ocular grid micrometer. Results are expressed as the mean number of donor positive cells per square centimeter \(\pm\) 1 SD.

MLR and testing for T cell hyporesponsiveness

C3H splenic T cells from normal mice were purified by passage through nylon wool columns and then used as responders (2 \(\times\) 10\(^3\)well in round-bottom 96-well plates) against graded numbers of gamma-irradiated (20 Gy), freshly isolated B10 BM cells, B10 FL-BM cells, B10 FL-BM DC, or splenocytes (B10 or C3H) in one-way MLR. FL-BM DC were positively selected (purity range, 91–93% of DC) by incubating FL-BM cells with a bead-conjugated hamster anti-mouse CD11c mAb (Milenyi Biotec, Auburn, WA) followed by passage through a paramagnetic column (Milenyi Biotec).

To test for T cell hyporesponsiveness, C3H splenic T cells (2 \(\times\) 10\(^3\)well) were incubated initially (primary MLR) at a fixed stimulator:responder cell ratio with gamma-irradiated B10 normal BM cells (1/1), B10 FL-BM cells (1/1), or B10 FL-BM DC (1/10). In some experiments soluble hamster anti-CD28 mAb (10 \(\mu\)g/ml, clone 37.51, Pharmingen) or control mAb IgG was added to the primary MLR. After 2 days, residual responding cells were incubated with FITC-conjugated mouse anti-H2K\(^b\) mAb (Pharmingen), followed by MACS anti-FITC microbeads (Miltenyi). B10 (H2\(^b\)-positive) cells were removed by passage through a paramagnetic col umn (Miltenyi). The C3H T cells were restimulated in medium for another 2 days and then restimulated (secondary MLR) with graded concentrations of gamma-irradiated B10 (allogeneic), C3H (syngeneic), or BALB/c (third-party) splenocytes. In some experiments human rIL-2 (Life Technologies, Grand Island, NY; 20 U/ml) was added at the outset of the secondary MLR.

To test for anti-donor T cell proliferative responses, C3H splenic T cells from normal animals or from recipients of B10 BM, B10 FL-BM, or B10 FL-BM DC injections were used as responders to gamma-irradiated donor strain splenocytes.

In all cases, cultures were maintained in RPMI 1640 (Life Technologies) containing 10% heat-inactivated FBS (Life Technologies), 2 mM l-glutamine, 50 U/ml penicillin and streptomycin, and 2 mM nonessential amino acids (complete medium) for 72 h at 37°C in 5% CO\(_2\). For the final 18 h, individual wells were pulse-labeled with 1 \(\mu\)Ci of \(^{3}H\)thymidine. The amount of radioisotope incorporated was determined using a beta scintillation counter. Results are expressed as the mean cpm \(\pm\) 1 SD.

CTL assay

Spleen cells from C3H recipients of B10 BM were restimulated in vitro for 4 days with gamma-irradiated (20 Gy) B10 donor splenocytes at a 1:1 ratio before use as effectors in CTL assays. The EL-4 (H2\(^b\)) lymphoma cell line (TIB39; American Type Culture Collection, Manassas, VA) was used as a source of specific allogeneic target cells. The P815 (H2\(^b\)) mouse mastocytoma cell line (TIB64, American Type Culture Collection) and the R1.1 (H2\(^b\)) lymphoma cell line (TIB42, American Type Culture Collection) were used as third-party and syngeneic targets, respectively. Target cells were labeled with 100 \(\mu\)Ci Na\(^{51}\)CrO\(_4\) (NEN, Boston, MA), and plated at a concentration of 5 \(\times\) 10\(^3\)/well in 96-well plates. Serial, 2-fold dilutions
of effector cells were added to give E:T cell ratios of 100:1, 50:1, 25:1, and 12.5:1. Following 4-h incubation at 37°C in 5% CO₂, specific 51 Cr release was determined. Supernatants were recovered from the wells using a supernatant collection system (Skatron, Sterling, VA). Maximum 51 Cr release was determined by osmotic lysis of the cells. The percent cytotoxicity was calculated using the formula:

\[ \text{% cytotoxicity} = \frac{\text{experimental (cpm)} - \text{spontaneous (cpm)}}{\text{maximum (cpm)} - \text{spontaneous (cpm)}} \times 100 \]

Results are expressed as the mean ± 1 SD percent 51 Cr release in triplicate cultures.

**Cytokine quantitation**

Supernatants harvested from 72-h MLR cultures were analyzed for mouse IL-4 and IFN-γ levels by ELISA, using reagents and the following procedures recommended by the manufacturer (PharMingen). The limit of detection for both IL-4 and IFN-γ was 19.5 pg/ml.

**Phenotypic and functional analyses of BM DC propagated from normal or FL-treated B10 mice**

DC were propagated from BM cell suspensions using the procedure described initially by Inaba et al. (39), with minor modifications (40). Briefly, 2 × 10⁶ freshly isolated BM cells from normal or FL-treated mice (10 μg/day/mouse for 9 days) were cultured in 24-well plates in 2 ml of RPMI 1640 complete medium containing recombinant mouse GM-CSF (1000 U/ml; a gift from Schering-Plough Research Institute, Kenilworth, NJ) and recombinant mouse IL-4 (1000 U/ml; R&D Systems, Minneapolis, MN) at 37°C in 5% CO₂. To evaluate the influence of tacrolimus on DC growth and differentiation, different concentrations of tacrolimus (0.5, 5, or 50 ng/ml) were added at the start of culture (day 0). On day 5, floating cells (many of which exhibited typical DC morphology) were harvested, and phenotypic and functional analyses were performed. Phenotypic analysis of the cultured DC was conducted as described above, and the allostimulatory activity of BM-derived cells was determined in MLR.

**Heart transplantation**

Intraabdominal cardiac transplantation was performed, and graft survival was monitored as previously described (34).

**Statistics**

Statistical analysis was performed using two-tailed Student’s t test; p < 0.05 was considered significant. Graft survival data were compared by Kaplan-Meier analysis and the log-rank test. Results are expressed as the mean ± 1 SD.

**Results**

**FL administration augments the incidence of immature DC in freshly isolated BM**

We first determined the impact of systemic FL administration on the incidence of DC in freshly isolated B10 BM cell suspensions. Single and multicolor immunostaining followed by flow cytometric analysis were performed to ascertain the incidence of CD11c⁺ DC expressing various differentiation markers. After 9 days of FL administration, a mean 7- to 10-fold increase in the absolute number of CD11c⁺ DC was detected in FL-BM compared with normal control BM (three separate experiments; data not shown). These findings are consistent with previously reported observations of the influence of 10-day FL treatment on numbers of DC in freshly isolated BM (41). Following mAb staining, DC were gated according to forward vs side scatter and CD11c positivity, and their surface Ag expression was analyzed by two-color immunofluorescence. As shown in Fig. 1, only a minor proportion of the CD11c⁺ cells in normal and FL-BM expressed MHC class II (10–26%, respectively). Fewer cells (<5%) were CD40⁺. CD11c⁺ cells in both control and experimental groups were also CD80⁺ and CD86⁺. Sixty to 73% of CD11c⁺ cells exhibited moderate...
staining for CD54 (ICAM-1, a marker up-regulated during DC maturation). Both groups of BM cells also expressed moderate levels of CD11b, a marker expressed predominantly on immature myeloid DC (22). These data indicate that although there were substantial increases in the number and incidence of DC in FL-BM, the DC present in freshly isolated BM of both control and FL-treated groups were at an immature stage of differentiation, as determined by low levels of surface MHC class II, CD54, and costimulatory molecule expression.

**FL administration augments the allogenerative activity of freshly isolated BM cells**

We next compared the T cell allogenerative capacity of freshly isolated control and FL-BM cells in primary MLR. As shown in Fig. 2, unlike normal B10 splenocytes, normal B10 BM cells failed to stimulate naive C3H T cells. B10 FL-BM cells elicited T cell proliferation, but were less effective stimulators than bulk normal B10 spleen cells on a per cell basis, especially at comparatively high stimulator:responder ratios. The stronger allogenerative activity of freshly isolated FL-BM cells compared with that of normal BM cells may be attributed to the presence of a higher incidence of immature CD11c⁺ DC in FL-BM preparations (10–22% DC in FL-BM vs 1–2% in normal BM). Although immature DC are deficient in T cell costimulatory signals, they have the potential, at least in theory, to differentiate in vitro into potent APC. To test this potential, purified DC from FL-BM were employed as allogenerative cells under similar conditions. Lower stimulator:responder ratios were used to maintain a similar range of absolute number of DC to T cells as when bulk FL-BM cells were used as stimulators. Even at high concentrations, FL-BM DC did not exhibit significant allogenerative activity (Fig. 2). These results suggest that purified FL-BM DC were unable to mature into effective APC in vitro, at least under the conditions present in a 3-day MLR.

**Immature DC present in FL-BM induce alloantigen-specific T cell hyporesponsiveness in vitro**

We reported previously that immature DC generated in vitro induced alloantigen-specific T cell hyporesponsiveness/anergy in MLR assays (33). FL-BM cells contain a relatively high proportion of immature DC (Fig. 1). Therefore, we compared the capacity of bulk B10 FL-BM cells and B10 FL-BM DC with that of normal B10 BM cells to induce alloantigen-specific T cell hyporesponsiveness/anergy during primary MLRs. C3H splenic T cells were stimulated initially (primary MLR) with freshly isolated normal B10 BM cells, B10 FL-BM cells, or purified B10 FL-BM DC. Two days later, B10 cells were removed by immunomagnetic beads as

![FIGURE 3](http://www.jimmunol.org/)

Stimulation of T cells in primary MLR with either FL-BM cells or FL-BM DC, but not with normal BM cells, induces Ag-specific hyporesponsiveness upon restimulation in secondary MLR. C3H splenic T cells (2 × 10⁵) were stimulated during a primary 2-day MLR with gamma-irradiated B10 normal BM, B10 FL-BM, or B10 FL-BM DC. Responder T cells were purified, rested for 2 days, and restimulated in a secondary 3-day MLR with graded concentrations of freshly isolated C3H (syngeneic), B10 (alloimmune), or BALB/c (third-party) splenocytes. The results are expressed as the mean cpm ± 1 SD and are representative of three separate experiments.
detailed in Materials and Methods. After resting for 2 days, the primed C3H T cells were restimulated in a secondary MLR with fresh B10 (allogeneic), C3H (syngeneic), or BALB/c (third-party) splenocytes. As shown in Fig. 3A, C3H T lymphocytes stimulated with normal B10 BM cells in a primary MLR proliferated in response to B10 or BALB/c splenocytes in secondary MLR. However, when C3H T cells were exposed to B10 FL-BM cells in a primary MLR, they did not respond upon restimulation with B10 splenocytes, but did exhibit the capacity to proliferate in response to BALB/c splenocytes (third-party) stimulation. This indicated that the T cell hyporesponsiveness was donor specific (Fig. 3B). A similar result was obtained when B10 FL-BM DC were used as stimulators in the primary MLR. This latter observation suggests that the capacity of freshly isolated FL-BM cells to induce hyporesponsiveness/anergy in vitro resides at least partially in immature DC.

It is well known that blockade or absence of costimulation in the presence of TCR ligation induces T cell anergy/deletion (42), and that FL-BM DC lack or express very low levels of CD80 and CD86 (Fig. 1). Therefore, we tested whether addition of exogenous T cell costimulation, using anti-CD28 mAb, to either B10 FL-BM cells or B10 FL-BM DC during the primary MLR could revert the alloantigen-specific T cell hyporesponsiveness detected in secondary MLR. In both cases, addition of anti-CD28 mAb restored the capacity of C3H T cells to respond to restimulation (secondary MLR) with B10 splenocytes (Fig. 4). The hyporesponsive C3H T cells exposed in the primary MLR to B10 FL-BM cells or B10 FL-BM DC responded normally to B10 splenocytes and IL-2 (20 U/ml) added at the outset of the secondary MLR (data not shown).

**Infusion of FL-BM results in an enhanced incidence of donor MHC class II$^+$ cells in primary and secondary lymphoid tissues of normal and tacrolimus-immunosuppressed allogeneic recipients**

We next investigated the in vivo trafficking, microanatomic location, and survival of freshly isolated normal and FL-BM cells following their systemic injection into fully allogeneic, noncytolytically treated recipients. Fifteen days after their i.v. infusion, donor MHC class II$^+$ (IA$^b^+$) cells were detected in small numbers (1:1000) in the spleens and thymi of unmodified C3H mice given normal allogeneic (B10) BM cells (Fig. 5, A and E). The number of donor cells increased significantly in spleen (3-fold; $p = 0.0001$) and thymus (4-fold; $p = 0.003$) when an identical number of FL-BM cells were injected (Fig. 5, C and G, and Fig. 6). Donor IA$^b^+$ cells in both spleens and thymi showed typical dendritic morphology and were present predominantly in the splenic periarteriolar lymphocytic sheaths (T cell areas; Fig. 5C) and in the medulla or cortico-medullary junction of the thymus (Fig. 5G). Recipient treatment with tacrolimus (days 0–12) resulted in a significant, 6- to 7-fold increase in the number of donor-derived cells in spleens of normal BM-infused recipients ($p = 0.0005$; compare Fig. 5, A and B, and Fig. 6). Likewise, a 4-fold increase was seen in recipients of FL-BM and tacrolimus compared with that in mice given FL-BM alone ($p = 0.0006$; compare Fig. 5, C and D, and Fig. 6). The maximum mean relative increase (10-fold) in donor MHC class II$^+$ cells in the spleen was detected in animals given FL-BM and tacrolimus compared with that in animals given normal BM alone (compare Fig. 5, A and D). A similar pattern of change in donor cell numbers was observed in the thymus following...
and C FL-treated mice (and injection. BM cells were prepared from either normal donors (A and B) or FL-treated mice (C and D). Recipient animals were given donor cells without (A and C) or with tacrolimus immunosuppression from days 0–12 (B and D). A, Few donor cells (blue) were evident 15 days after i.v. injection of normal BM cells into normal recipients. Donor MHC class II cells exhibited DC morphology and were located in the perivascular lymphocytic sheaths (PALS), close to arterioles. B, Normal BM-derived cells in tacrolimus-immunosuppressed recipients. The number of donor DC increased significantly. C, donor DC in recipient spleens were substantially augmented when normal mice received BM cells from FL-treated donors. D, Further marked increase in donor DC from BM of FL-treated donors in tacrolimus-immunosuppressed mice. The highest number of DC was detected in this group. Insert, High magnification of a small group of donor DC (indicated by an arrow in D; ×100; ABC-AP). E–H, Identification of allogeneic donor MHC class II+ (IAb+) DC in recipient thymi 15 day after i.v. BM cell administration. BM cells were prepared from normal donors (E and F) or FL-pretreated mice (G and H). Recipient animals received donor cells without (E and G) or with tacrolimus immunosuppression (F and H). E, Few donor cells (positive cells in blue) were detected 15 days after i.v. injection of normal BM cells into normal recipients. Donor MHC class II+ cells showed DC morphology and were located in the thymic medulla or the cortico-medullary junction (indicated by arrowheads in E–H). F, Same situation as in E, but in immunosuppressed recipients. The number of donor DC increased significantly. G, The number of donor DC in recipient thymus was augmented when normal recipients received FL-BM cells. H, Same situation as in G, but in immunosuppressed mice. The highest number of DC was detected in this group. Insert, Higher magnification of a small group of donor DC (indicated by an arrow in H; ×100; ABC-AP). The composite illustrates representative images from three mice per group per time point, analyzed for three different tissue sections.

FIGURE 5. A–D, Identification of allogeneic donor MHC class II+ (IAb+) cells in recipient C3H spleens 15 days after i.v. B10 BM cell injection. BM cells were prepared from either normal donors (A and B) or FL-treated mice (C and D). Recipient animals were given donor cells without (A and C) or with tacrolimus immunosuppression from days 0–12 (B and D). A, Few donor cells (blue) were evident 15 days after i.v. injection of normal BM cells into normal recipients. Donor MHC class II+ cells exhibited DC morphology and were located in the perivascular lymphocytic sheaths (PALS), close to arterioles. B, Normal BM-derived cells in tacrolimus-immunosuppressed recipients. The number of donor DC increased significantly. C, donor DC in recipient spleens were substantially augmented when normal mice received BM cells from FL-treated donors. D, Further marked increase in donor DC from BM of FL-treated donors in tacrolimus-immunosuppressed mice. The highest number of DC was detected in this group. Insert, High magnification of a small group of donor DC (indicated by an arrow in D; ×100; ABC-AP). E–H, Identification of allogeneic donor MHC class II+ (IAb+) DC in recipient thymi 15 day after i.v. BM cell administration. BM cells were prepared from normal donors (E and F) or FL-pretreated mice (G and H). Recipient animals received donor cells without (E and G) or with tacrolimus immunosuppression (F and H). E, Few donor cells (positive cells in blue) were detected 15 days after i.v. injection of normal BM cells into normal recipients. Donor MHC class II+ cells showed DC morphology and were located in the thymic medulla or the cortico-medullary junction (indicated by arrowheads in E–H). F, Same situation as in E, but in immunosuppressed recipients. The number of donor DC increased significantly. G, The number of donor DC in recipient thymus was augmented when normal recipients received FL-BM cells. H, Same situation as in G, but in immunosuppressed mice. The highest number of DC was detected in this group. Insert, Higher magnification of a small group of donor DC (indicated by an arrow in H; ×100; ABC-AP). The composite illustrates representative images from three mice per group per time point, analyzed for three different tissue sections.

FIGURE 6. Administration of FL-mobilized donor BM under cover of low dose immunosuppression, significantly augments the number of donor MHC class II+ (IAb+) cells in recipient spleens and thymi. Donor leukocytes were identified in variously treated recipients’ tissues by immunohistochemistry 15 and 30 days after BM cell infusion. Spleen and thymus sections were stained for donor MHC class II (IAb+), and positive cells were enumerated. Three sections, each from a different level per recipient spleen or thymus were counted, and the data are presented as the mean number of donor class II-positive cells per square centimeter ± 1 SD per section. Counting was performed by a blinded observer using an ocular grid micrometer from three different tissue sections from three different mice per group. Individual means from three animals per group were pooled to obtain a group mean ± SD.

tacrolimus administration, although the incidence of donor cells was substantially lower overall than that in the spleen (Fig. 6).

Donor-derived MHC class II+ cells are reduced substantially with time and following tacrolimus withdrawal

To determine the fate of donor cells at a later time (after the withdrawal of immunosuppression on day 13), IAb+ cells were quantified on day 30, 2.5 wk after cessation of tacrolimus administration. As shown in Fig. 6, donor cells could still be detected in the spleens and thymi of all groups, but in substantially reduced numbers. They were most readily detected in recipients of FL-BM and tacrolimus. No donor MHC class II+ cells were identified in non-lymphoid tissue (skin) on either day 15 or day 30 in any group. These findings clearly indicate that infusion of FL-BM to normal or immunosuppressed allogeneic recipients leads to enhanced microchimerism that subsides as a function of time and following withdrawal of systemic T cell-directed (tacrolimus) immunosuppression.

T cells from recipients of allogeneic FL-BM exhibit enhanced anti-donor proliferative and cytotoxic responses that are suppressed by tacrolimus administration

We next ascertained how these marked changes in microchimerism/numbers of donor DC affected host T cell reactivity to donor by examining the ex vivo proliferative and CTL responses of splenocytes to donor alloantigen. As shown in Fig. 7, mice tested on day 15 after infusion of FL-BM (containing 7- to 10-fold higher numbers of immature DC than normal BM) exhibited markedly augmented ex vivo T cell proliferative responses compared with
animals given normal BM (Fig. 7A). These responses were reduced significantly by low dose tacrolimus immunosuppression (Fig. 7A). Although the anti-donor responses of normal BM recipients were abrogated completely by tacrolimus, some proliferative activity was exhibited by T cells from the immunosuppressed FL-BM recipients. When the cytotoxic activity of T cells from the variously treated groups was examined on day 15 (Fig. 7B), FL-BM recipients showed the most potent CTL responses against donor-specific allogeneic targets. Splenocytes from animals that received normal BM were able to kill allogeneic targets at similar levels, but only at comparatively high E:T cell ratios (50:1 and 100:1). Tacrolimus treatment of mice given FL-BM reduced donor-specific cytotoxic activity by 15–20% (Fig. 7B). By contrast, a more substantial (50–60%) decrease in the ability of normal BM recipients’ T cells to kill donor-specific targets was observed (Fig. 7B). These data reveal that FL-BM recipients are very responsive to donor alloantigen in the absence of immunosuppression, and that immunosuppression is less efficient in inhibiting anti-donor responses induced by FL-BM (containing markedly enhanced numbers of immature DC) compared with normal BM. The findings suggest that exposure to low dose tacrolimus does not prevent the increased numbers of donor DC in FL-BM from exhibiting allostimulatory activity in vivo.

**Exhibition of anti-donor T cell responses following tacrolimus withdrawal**

Anti-donor proliferative and CTL responses were also examined on day 30 post-BM infusion, i.e., 2.5 wk after tacrolimus withdrawal (Fig. 7, C and D). Mice that received either normal or FL-BM in the absence of immunosuppression exhibited similar anti-donor proliferative activities that were reduced, compared with that on day 15, only in the FL-BM group (compare Fig. 7, A and C). As on day 15, tacrolimus-treated animals that received normal BM exhibited markedly reduced anti-donor proliferative responses. However, the suppressive influence of tacrolimus was less pronounced in the FL-BM recipients (Fig. 7C). Mice given normal or FL-BM exhibited similar, but reduced, CTL responses on day 30 (compare Fig. 7, B and D). Although the marked suppressive effect of tacrolimus on CTL responses of normal BM recipients remained apparent, its modest suppressive effect in the
FL-BM group observed on day 15 was lost (compare Fig. 7, B and D). These data are consistent with transient suppression of the potential immunostimulatory function of the enhanced numbers of donor APC observed in secondary lymphoid tissue of FL-BM-infused mice and/or with exhibition of this function after drug withdrawal.

**Splenocytes from recipients of FL-BM secrete higher levels of IFN-γ upon ex vivo restimulation with donor alloantigen**

Supernatants of cultures of ex vivo alloantigen restimulated splenic T cells from the various groups of BM recipients were analyzed by ELISA for levels of IFN-γ and IL-4 production. When examined 15 days after BM infusion, much higher (~7-fold) levels of IFN-γ were secreted by splenocytes from FL-BM recipients compared with those from mice given normal BM (Fig. 8). By day 30, IFN-γ production by cells from the FL-BM group was reduced by ~40%. IFN-γ production was profoundly suppressed by in vivo tacrolimus administration at each time point. IL-4 levels were below the sensitivity limit of the assay (19.5 pg/ml) in all groups. These data clearly indicate that compared with recipients of normal BM, mice given FL-BM containing an enhanced number of immature DC exhibit marked IFN-γ responses, and augmented Th1 cytokine production is inhibited by tacrolimus administration.

**Immature FL-BM DC administered i.v. induce a potent anti-donor T cell response**

As described above, FL-BM cells or their DC fraction induced donor-specific T cell hyporesponsiveness/anergy in vitro (Fig. 3). Conversely, FL-BM cells administered i.v. enhanced the anti-donor proliferative and cytotoxic responses of recipients’ T cells (Fig. 7). A possible explanation of this paradoxical effect may be that immature DC present in FL-BM were unable to mature under MLR culture conditions, but were capable of differentiating into potent APC after in vivo administration. To test this hypothesis, purified B10 FL-BM immature DC were administered i.v. to C3H recipients. Animals were injected either with 0.5 × 10^6 DC (the approximate number present in the normal BM inocula used in this study) or 5 × 10^6 DC (the approximate number in the FL-BM inocula). One week later, and based on the fact that most DC injected i.v. home to the spleen (43), animals were killed, and the splenic T cells were restimulated with C3H (syngeneic), B10 (allogeneic), or BALB/c (third-party) splenocytes. Results illustrate one representative animal (of three per group) and are expressed as the mean cpm ± 1 SD.

**Tacrolimus inhibits the generation of DC from normal and FL-BM, but does not affect DC surface expression of MHC class II and costimulatory molecules**

To address the influence of tacrolimus on the growth and differentiation of DC derived from either normal or FL-BM, freshly isolated BM cells from each group were maintained in GM-CSF and IL-4 with or without tacrolimus (0.5–50 ng/ml) from the start of the culture. Cell growth was inhibited by tacrolimus in a dose-dependent manner. By day 5, the absolute number of cells generated in culture from an original population of 10^6 BM cells was reduced significantly by tacrolimus (50 ng/ml) from 3.68 ± 0.32 to 1.75 ± 0.18 × 10^3/ml (FL-BM; 53% reduction) or from 2.10 ± 0.23 to 1.05 ± 0.09 × 10^3/ml (normal BM; 50% reduction). After 72 h of culture, nonadherent cells were harvested and analyzed by two-color immunostaining and flow cytometry for surface expression of CD11c and MHC class II and costimulatory molecules.
CD11c^+ cells in both cultures to 54 and 15%, respectively. When the influence of tacrolimus on the surface phenotype of these CD11c^+ cells generated from normal or FL-BM was examined, it was found to have had no significant effect on the expression of MHC class II, CD40, or CD86 (Fig. 10B). These data indicate that, although effective in inhibiting the generation of CD11c^+ DC, especially from normal BM, tacrolimus did not significantly modulate the surface expression of key functional molecules on the DC that were recovered from the cultures.

Exposure to tacrolimus abrogates the allostimulatory activity of normal, but not FL-BM-derived, cells

We also evaluated the functional activity of the GM-CSF- plus IL-4-stimulated BM-derived cells, harvested as described above, in 3-day primary MLR (Fig. 11). Cells derived from FL-BM were more effective T cell stimulators than those from normal BM. Exposure to tacrolimus during the generation of DC markedly inhibited the allostimulatory activity of the cultured cells, in concert with inhibition of DC growth (Fig. 10). The stimulatory effect of both normal BM-derived cells and FL-BM-derived cells was diminished substantially with tacrolimus.

FL-BM plus tacrolimus fails to prolong heart allograft survival

To test the influence of FL-BM and tacrolimus on organ allograft survival, C3H recipients of heterotopic B10 cardiac allografts were also given 5 x 10^6 normal or FL-BM cells i.v. at the time of the transplant with or without tacrolimus treatment (2 mg/kg/day) from days 0–12. As shown in Fig. 12, infusion of normal BM cells significantly prolonged graft survival, an effect that was enhanced by tacrolimus. By contrast, neither FL-BM alone nor FL-BM plus tacrolimus prolonged heart graft survival; indeed, there was significant acceleration of rejection.

Discussion

Interest in the infusion of donor hemopoietic cells to facilitate graft survival originated with the studies of Billingham et al. (7, 8), who demonstrated that chimerism and acquired tolerance could be achieved by the infusion of adult hemopoietic cells into immunologically immature mice. Over the past 4.5 decades, donor hemopoietic cells have been used to promote experimental organ graft
survival under a wide variety of experimental conditions. The observation of Starzl et al. (1, 2) that multilineage microchimerism could be detected in long-surviving recipients of organ allografts, and the argument that chimerism was an essential prerequisite for tolerance induction (1, 44) prompted studies to augment natural microchimerism in an effort to promote tolerance induction in humans and reduce or eliminate dependence on immunosuppressive therapy. Recent efforts to enhance natural microchimerism in clinical organ transplantation have centered on the infusion of unmodified donor BM cells, usually at the time of transplantation (16, 45, 46). In principle, an alternative/additional approach is the use of specific growth factors to promote hemopoiesis, as in BM transplantation (47).

In our first efforts to manipulate microchimerism with FL, we found that treatment of allogeneic BM and/or cardiac allograft recipients with FL augmented anti-donor immune reactivity and exacerbated rejection. This indicated that augmentation of both the recipient and the donor hemopoietic cell pool by FL was not an effective strategy for promoting graft survival (32). A related observation had been reported earlier by Monaco et al. (48), who found that administration of recombinant mouse GM-CSF to murine allogeneic BM cell recipients enhanced skin allograft rejection. On the other hand, the same researchers observed that pretreatment of the donor with GM-CSF significantly augmented the capacity of donor BM to prolong graft survival. It was suggested that GM-CSF induced a change in the donor cell population, possibly augmentation of an immunoregulatory myeloid lineage cell that was capable of inducing graft prolongation (48). Studies by Blazar et al. (49) showed that ex vivo exposure of donor BM to recombinant mouse GM-CSF, upon which growth of myeloid DC is dependent (39), augmented engraftment across MHC barriers. FL augments BM stem cells (50, 51) and also markedly increases the number of BM DC (43) with potential for immune modulation. In this study we evaluated the influence of systemic FL on the phenotype and function of BM DC, assessed both in vitro and in vivo. In addition, we examined the impact of FL-mobilized donor BM on microchimerism and anti-donor immune reactivity in non-cytoablated allogeneic recipients.

Freshly isolated BM cell populations from FL-treated donors contained markedly increased numbers of immature DC that induced alloantigen-specific T cell hyporesponsiveness in vitro. This raised the expectation that systemic infusion of FL-BM might lead both to augmented microchimerism and to induction of T cell unresponsiveness to donor alloantigen. Indeed, injection of normal, MHC-mismatched recipients with FL-BM together with a short course of tacrolimus led to substantial increases in microchimerism in both primary (thymus) and secondary lymphoid tissues. Close examination of the donor MHC class II+ cells in the host tissues revealed that most exhibited typical DC characteristics, in particular, restriction to T cell areas, and dendriform morphology. Moreover, on day 15 post-transplant, splenic T cells from animals with markedly enhanced FL-BM-induced microchimerism under cover of tacrolimus showed reduced levels of ex vivo reactivity to donor alloantigen compared with nonimmunosuppressed controls. Both microchimerism and the suppressed alloimmune reactivity seen in FL-BM recipients diminished as a function of time and after tacrolimus withdrawal, suggesting that maintenance of immunosuppression was required for their preservation. Indeed, the potential of immature donor DC purified from FL-BM to prime, rather than render anergic, allogeneic T cells in vivo was amply demonstrated by ex vivo analysis of anti-donor reactivity. Moreover, FL-BM failed to prolong organ allograft survival even when combined with tacrolimus.

Recent studies have examined the influence of recipient treatment with donor BM and FL on the outcome of organ allograft survival in the rat. Heterotopic heart transplants were performed under tacrolimus immunosuppression (1.5 mg/kg/day; days 0–13, 20, and 27) with or without adjunctive donor BM. In an effort to augment levels of microchimerism, subgroups of recipients were also given hemopoietic growth factors (FL, IL-6, G-CSF, or hematopoietic cytokine growth factor). Increases in chimerism and reductions in chronic heart allograft rejection were observed with the addition of FL (200 μg/kg/day; days 0–6) to adjunct donor BM and tacrolimus immunosuppression. The late localization of donor cells (microchimerism) observed in nonlymphoid tissues was attributed to mechanisms of clonal exhaustion and immune indifference (52). Under these conditions, FL had no adverse effect on allograft survival, suggesting that, under the appropriate conditions, it may be of value to positively influence transplant outcome.

Tacrolimus is an immunophilin ligand that inhibits calcineurin (a key enzyme in the T cell signal transduction cascade) activity and nuclear translocation of the gene transcription regulatory protein, NF-AT (nuclear factor of activated T cells). To determine
whether tacrolimus affects DC growth and/or functional matura-
tion, freshly isolated normal and FL-BM cells were cultured in the presence of DC growth-promoting cytokines, GM-CSF and IL-4, with or without tacrolimus. Tacrolimus inhibited the numbers of CD11c+ DC in BM cultures and reduced the maturation and T cell stimulatory activity of BM-derived cells. Taken together with the observed recovery of alloreactivity in vivo following tacrolimus withdrawal, these observations suggest that sustained immunosuppres-
sion with tacrolimus or blockade of costimulation will be re-
quired to prevent FL-BM-derived donor APC from functional ma-
trization in the allogeneic recipient.

It is well accepted that T cell activation by APC requires co-
stimulatory signals, in addition to the primary signal provided by engagement of the TCR (53, 54). Ag presentation in the absence of costimulation promotes T cell anergy or apoptosis. The level of expression of surface MHC and costimulatory molecules on DC is an indicator of their stage of activation/differentiation. Costimula-
tor-deficient, immature DC can induce Ag-specific hyporespon-
siveness in allogeneic T cells (33). Moreover, blockade of co-
stimulation can markedly enhance the ability of DC to induce a
lloreactivity with or without tacrolimus) on allograft survival could not be
demonstrated in the present study. On the other hand, CD40 liga-
tion up-regulates the expression of CD80 and CD86 by DC (56, 57); induces high levels of IL-12, IL-8, TNF-α, and macrophage
inhibitory protein-1α; and enhances their Ag-presenting and co-
stimulatory functions, suggesting a novel target for regulation of DC function (58). To date, there have been several reports that
anti-CD40L (CD154 mAb) and/or the chimeric fusion protein
CTLA4Ig (that blocks B7-CD28 interaction) can promote in-
duction of transplant tolerance in animal models. In this laboratory,
administration of immature donor DC plus anti-CD154 mAb 7
days before transplantation resulted in long term survival of car-
diac allografts (59). Moreover, in a recent study Markee et al. (60)
found that infusion of donor-specific, FL-induced splenic DC to-
gather with a brief course of anti-CD154 mAb prolonged the sur-
vival of allogeneic skin grafts. Pearson et al. (61) used donor BM
and CTLA4Ig to prolong the survival of primary cardiac al-
lografts and secondary skin grafts and demonstrated the presence of
donor hemopoietic cells (microchimerism) in recipient tissues
>200 days post-transplant. Taken together these studies indicate
that functional impairment (by costimulation blockade) of donor-
derived APC in organ allograft recipients can promote donor-spe-
cific tolerance.

In summary, the present study reveals the potential of FL-BM to
markedly promote hemopoietic cell microchimerism, in particular
donor DC, in noncytoablated, tacrolimus-immunosuppressed re-
cipients. The findings provide a basis for evaluation of the impact
of selective enhancement of donor DC in combination with alter-
native forms of immunosuppression, in particular costimulation
blockade, on allogeneic reactivity and graft rejection. Such ap-
proaches, currently under study in this laboratory, may allow ex-
hibition of the tolerogenic potential of the donor hemopoietic cells
that are augmented by FL, with possible therapeutic implications.

Acknowledgments
We thank Immunex Corp. for providing FL. We thank Dr. Adriana
Larregina for advice on statistical analysis. We are grateful to Alison Logar
for expert assistance with flow cytometry, and to Shelly L. Conklin for
secretarial support.


