TGF-β1 combined with M-CSF and IL-4 induces generation of immune inhibitory
cord blood dendritic cells capable of enhancing cytokine-induced \textit{ex-vivo} expansion
of myeloid progenitors*

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Abstract

Tolerogenic dendritic cells (DC) may be valuable in transplantation for silencing immune reaction. M-CSF/IL-4 induces differentiation of cord blood (CB) monocytes into DC (M-DC) with tolerogenic phenotype/function. We assessed whether factors produced by tolerogenic DC could modulate hematopoiesis. TGF-β1 added to CB M-DC cultures induced bona fide DC morphology (TGF-M-DC), similar to that of DC generated with TGF-β and GM-CSF/IL-4 (TGF-GM-DC). Of conditioned medium (CM) produced from TGF-M-DC, TGF-GM-DC, M-DC, and GM-DC, TGF-M-DC CM was the only one that enhanced SCF, Flt3 ligand and TPO expansion of myeloid progenitor cells ex vivo. This effect was blocked by neutralizing anti-M-CSF Ab, but protein analysis of CM suggested that M-CSF alone was not manifesting enhanced expansion of myeloid progenitors. LPS-stimulated TGF-M-DC induced T cell tolerance/anergy as effectively as M-DC. TGF-M-DC secreted significantly lower concentrations of progenitor cell inhibitory cytokines and were less potent in activating T cells than TGF-GM-DC. Functional differences between TGF-M-DC and TGF-GM-DC included enhanced responses to LPS-induced ERK, JNK, and P38 activation in TGF-M-DC and their immune suppressive-skewed cytokine release profiles. TGF-M-DC appear unique amongst culture generated DC in their capability for silencing immunity while promoting expansion of myeloid progenitors, events that may be of therapeutic value.
Introduction

Dendritic cells (DC) are the most potent antigen-presenting cells and play a pivotal role in modulating immune responses.\(^1\) DC have the potential to induce immune rejection or tolerance and thus play a critical role in transplantation.\(^2\,^3\) Plasmacytoid precursor DC facilitate hematopoietic stem cell (HSC) engraftment in an allogeneic setting without causing graft-versus-host diseases (GVHD).\(^4\) Generation of regulatory DC by treatment of DC with IL-10 induces tolerance across MHC barriers in both human xenogeneic GVHD and murine GVHD models.\(^5\) These results suggest the possibility for potential therapeutic uses of modified DC to achieve transplantation tolerance.

Little is known about potential roles for DC in proliferation, survival and differentiation of hematopoietic stem/progenitor cells. Murine bone marrow-derived DC support colony formation of bone marrow CFU-GM and CFU-megakaryocytes \textit{in vitro}.\(^6\) Whether human DC, especially tolerogenic DC, support hematopoiesis has not been reported. A population of DC that induces tolerance as well as enhancing hematopoietic recovery may be of therapeutic use. In transplantation settings, host conditioning and GVHD lead to systemic and local inflammation that could possibly induce maturation of DC. Maturation signals such as bacterial lipopolysaccharide (LPS) induce DC to upregulate messenger RNA for cytokines and chemokines,\(^7\) which might modulate systematic immune reactions and hematopoietic recovery after transplantation.\(^7\)

We reported that M-CSF and IL-4 induce differentiation of CB monocytes into a population of IL-10\(^\text{high}\)IL-12\(^\text{absent}\) DC (M-DC) with tolerogenic potential.\(^8\) This is different
from standard methods that use GM-CSF and IL-4 to generate DC (GM-DC). TGF-β1 modulates DC differentiation and function, but whether TGF-β1 modulates M-DC immune function and plays a potential supporting role for these cells in hematopoiesis was not known. Maturation of DC is associated with activation of at least 3 members of the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 kinase. MAPK activation associated with maturation plays an important role in regulation of phenotype, cytokine production and T cell regulatory function of human DC. In the present study, we evaluated effects of TGF-β1 in modulating M-DC immune function, enhancing cytokine-induced ex-vivo expansion of myeloid progenitor cells, and a role for MAPK in these effects.
Materials and Methods

Isolation of monocytes, CD34+ cells and CD4+ T cells from CB. Heparinized umbilical CB was collected according to institutional guidelines. Approval was obtained from the committee for the Indiana University School of Medicine’s Institutional Review Board for these studies. Adult blood (AB) buffy coat was obtained from the Indiana Blood Center (Indianapolis, IN). CB and AB mononuclear cells were isolated by density gradient centrifugation on Ficoll Paque™ plus (1.077 g/ml; Pharmacia). Monocytes, CD34+ progenitor cells and CD4+ T cells were purified from CB respectively using MACS CD14+ magnetic beads, CD34 progenitor isolation kit and CD4+ T cell isolation kit (Miltenyi Biotec). CD14+ monocytes were also purified from AB buffy coat. Enriched CD4+ T cells (>95% pure as analyzed by flow cytometry) from different CB samples were frozen until use.

Cell culture. CB or AB CD14+ monocytes (5 × 10^5 cells/ml) were cultured in RPMI 1640 medium (BioWhittaker) as described previously with different combinations of the following amounts of purified recombinant human cytokines: 10 ng/ml Interleukin-4 (IL-4), 100 ng/ml Macrophage Colony Stimulating Factor (M-CSF), 10 ng/ml Transforming Growth Factor-Beta 1 (TGF-β1) (Peprotech) and 10 ng/ml Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) (ImmuneX/Amgen). To induce maturation of M-DC (produced with M-CSF, IL-4±TGF-β) and DC (produced with GM-CSF, IL-4±TGF-β), 1 μg/ml bacterial lipopolysaccharide (LPS) from Salmonella Minnesota (Sigma-Aldrich) was added to cultures on d 5 and cells were incubated for an additional 24 h before cells were washed.
**Surface and intracellular immunostaining.** Harvested cells were washed twice with PBS supplemented with 1% BSA. Fc receptors on cells were pre-blocked with excess human IgG (Sigma-Aldrich) on ice for 15 min. Cells were stained for 30 min at 4°C with the following FITC-conjugated Abs: anti-HLR-DR, anti-CD80, anti-CD86 and anti-CD83 or isotype controls (eBioscience), and PE-conjugated Abs: CD1a (eBioscience) and CD207 (Langerin, BD bioscience). For intracellular staining of Foxp3 expression on T cells, T cells were first stained with anti-CD4-FITC and anti-CD25-PE. Then T cells were fixed and permeabilized before staining with anti-Foxp3-APC (BD Bioscience). Cells were analyzed by flow cytometry.

**Production of conditioned medium (CM).** Cultured M-DC and GM-DC were harvested on day 5 and washed three times to remove residual cytokines. Cells (5 × 10^5/ml) were stimulated with 1 μg/ml LPS for 24 h in RPMI 1640 culture medium. Supernatant was collected and frozen at -80°C. Control CM prepared with 1μg/ml LPS without cells was used as a control for the effects of LPS itself. Control medium are RPMI 1640 culture medium.

**Ex vivo expansion.** For colony assessment of pre-cultured freshly isolated CB, CD34+ cells were plated in triplicate as described in 0.9% methylcellulose culture medium with 30% FBS and a combination of recombinant human GM-CSF (10 ng/ml), interleukin-3 (IL-3, 10 ng/ml), stem cell factor (SCF, 50 ng/ml) and erythropoietin (Epo, 1 U/ml). Different CD34+ cell concentrations were plated in order to find the
concentration of cells that resulted in enough colonies to score accurately and without colony overlap. For ex-vivo expansion, CB CD34+ cells (1 $\times$ 10^4/ml) were cultured in IMDM medium (supplemented with 5% heat-inactivated FBS, 2 mM L-glutamine, 25 $\mu$M Heps, 50 $\mu$M β-mercaptoethanol, 100 U/ml penicillin and 100 $\mu$g/ml streptomycin) and 25-50% v/v CM in the presence or absence of a combination of previously determined optimal progenitor cell expanding growth factors (50 ng/mL SCF, 100 ng/mL Flt-3 ligand (FL), and 100 ng/mL Thrombopoietin (TPO): =SFT). In some experiments, neutralizing goat anti-M-CSF or goat control Ab (20 $\mu$g/ml) (R&D systems) was added into cultures containing CM and SFT. Cells were cultured for 1 week, harvested and counted to determine absolute cell numbers after expansion, stained with CD34-PE to determine percentage of CD34+ cells, and plated at various concentrations in 0.9% methylcellulose culture medium as described above to determine generation of myeloid progenitor cells. Cells were plated in low (5 %) oxygen for ex vivo expansion and for colony assessment. Colonies were scored after 12-14 days in methycellulose culture. Absolute numbers of myeloid progenitor cells were calculated after 1 week culture.

**Assay for cytokine protein levels.** Cells were washed extensively and 2.5 $\times$ 10^5 cells/ml were stimulated with LPS (1 $\mu$g/ml) in culture medium in 24-well plates for 24 h. Washed cells were also incubated with ERK inhibitor U0126 for 30 min before addition of LPS. Conditioned media were sent to Whatman (Sanford, ME) for analysis of multiple cytokines using microspot ELISA technology. Each sample was analyzed in triplicate.
Primary and secondary mixed leukocyte reaction (MLR). The assay was done as described previously\textsuperscript{8} with modifications. In brief, primary MLR was established with 5 x 10\textsuperscript{4} CB CD4\textsuperscript{+} T cells and allogeneic stimulators at effector to target (E:T) cell ratios ranging from 1:10 to 1:2500 (in triplicate). Cells were pulsed with [methyl-\textsuperscript{3}H]thymidine (1 µCi/well; Amersham Pharmacia Biotech) for 16 h before the end of the culture on day 6 and harvested onto glass fiber filter. Incorporated thymidine was measured using a scintillation counter (Microbeta; Wallac, Finland).

Also, 3 x 10\textsuperscript{6} CB CD4\textsuperscript{+} T cells (donor A) were cultured with 3 x 10\textsuperscript{5} different subsets of allogeneic DC (donor B) in six-well plates for 7 days. T cells (donor A) were harvested, washed and restimulated at 2.5 x 10\textsuperscript{4} cells/well with GM-CSF/IL-4-induced mature DC (donor B) at an E:T ratio of 1:5. T cell proliferation was measured by pulsing cells with [methyl-\textsuperscript{3}H]thymidine (1 µCi/well) for 16 h before the end of culture on day 6. In another experiment, harvested T cells (2.5 \times 10^4/well from donor A) were added into an independently set-up MLR containing naive CD4\textsuperscript{+} T cells (5 \times 10^4/well; donor A) and GM-CSF/IL-4-induced mature DC (5 \times 10^3/well; donor B) to examine whether harvested T cells could suppress the independently set-up MLR. T cell proliferation was measured by pulsing cells with [methyl-\textsuperscript{3}H]thymidine (1 µCi/well) for 16 h before the end of culture on day 6.

Preparation of cell lysates and western blotting. M-DC and GM-DC (1 \times 10^6/ml) were stimulated with 1µg/ml LPS in signaling buffer (RPMI medium with 0.5 % endotoxin-free BSA and 25 µM Hepes) for 15 and 30 min. Cell pellets were solubilized in lysis buffer (10 mM Tris.HCl, 1 mM EDTA, 200 mM NaCl, 10 % glycerol, 0.5% NP40, 5 mM
NaF, 0.5 mM NaVO₄, 1 mM DTT, 1 mM PMSF and protease inhibitors) on ice for 30 min. Equal amounts of protein, as determined by Bio-Rad protein assay were electrophoresed on 12% SDF-PAGE gels and transferred to PVDF membranes for western blot analysis. Briefly, PVDF membranes were incubated with TBS super-blocking buffer (Pierce) for 1 h, and then incubated overnight at 4°C with Abs against p-ERK, p-JNK and p-P38 (Cell Signaling). Immunoreactive bands were detected by HRP-labeled goat anti-rabbit Ab (Cell Signaling), visualized by Supersignal West Pico Chemiluminescent Substrate (Pierce) and quantified by densitometry. Membranes were stripped and reprobed with Abs against total ERK as loading controls.

**Statistical analysis.** A two-tailed paired student \( t \) test (unless otherwise indicated) was used to determine statistical significance. Values of \( P <0.05 \) were considered significant.
Results

Effects of TGF-β1 on CB and AB M-CSF/IL-4-induced M-DC differentiation.

We previously reported that CB monocytes cultured with M-CSF and IL-4 (M-DC) differentiated into a tolerogenic population of cells, but with a heterogeneous morphology. In this report, we analyzed effects of TGF-β1 on M-DC differentiation. Addition of TGF-β1 to highly purified CB monocytes cultured with M-CSF and IL-4 (TGF-M-DC) induced homogeneous cell morphology similar to that of cells cultured with GM-CSF and IL-4 (GM-DC), as well as cells cultured with GM-CSF, IL-4 and TGF-β1 (TGF-GM-DC) (Figure 1). TGF-M-DC expressed reduced surface levels of CD86 compared to M-DC. After LPS activation, TGF-M-DC acquired significantly increased expression of CD86, HLA-DR and CD83, although CD86 and CD83 were expressed at a lower level than on M-DC (Figure 2). TNF-α in addition to LPS did not make any noticeable phenotypic changes in the TGF-M-DC (data not shown), suggesting that LPS alone is able to induce activation of TGF-M-DC. TGF-β1 is known to induce Langerhans cells from CD14+ cells. However, CB TGF-M-DC did not show any phenotypic criteria of Langerhans cells in that they did not express either CD1a or langerin on their surface (Figure 2). AB monocytes cultured with M-CSF, IL-4 and TGF-β also gave rise to a homogeneous population of cells with DC morphology. Analysis of phenotype showed the expression of HLA-DR, CD86, and minimal level of CD1a on their surface. LPS acted by significantly upregulating surface level of HLA-DR, CD86 and CD83 on adult TGF-M-DC (data not shown).
Conditioned medium (CM) collected from LPS stimulated CB TGF-M-DC enhances ex-vivo expansion of myeloid progenitors in combination with SCF, FL, and TPO (SFT). To determine if DC could modulate ex-vivo expansion of hematopoietic progenitors by themselves or in the presence of the potent cytokine combination of SFT, CM was generated from the 4 different types of DC described in Figure 1 after maturation of these cells with LPS. CM from these DC were compared for their capacity to expand CB myeloid progenitors in the absence and presence of SFT (Figure 3). Fold expansion of CFU-GM in the presence of SFT using medium conditioned by LPS in the absence of cells (control CM) was similar to that of control culture medium (8.5 vs 7.2 fold expansion; P>0.05) (Figure 3A). Of the CM produced by the four types of DC, only CM from mature TGF-M-DC significantly enhanced expansion of CFU-GM above that of the control medium. None of the CM significantly expanded CFU-GM numbers in the absence of SFT.

In further experiments, CM from LPS stimulated CB and AB TGF-M-DC and TG-GM-DC were compared for effects on ex-vivo expansion of CB CFU-GM. In the absence of CM from DC, the combination of cytokines (SFT) caused a 4.5±0.3 fold expansion of CFU-GM (N=4; compared to control medium with or without LPS, p<0.001). Only CM from CB TGF-M-DC in combination with SFT significantly enhanced ex-vivo expansion of CFU-GM above that of SFT (2.0±0.1 fold; N=2). CM from CB TGF-GM-DC (1.07-1.2 fold; N=2) or AB TGF-M-DC or TGF-GM-DC (0.93-0.98 fold; N=3 each) had no significant enhancing effects above that of SFT. Thus, ex-vivo enhancing effects seem to be confined to CB TGF-M-DC.
Assessment of the types of cells making up the CFU-GM colonies after expansion culture with SFT in the absence and presence of CM from the different types of DC demonstrated neutrophil, macrophage and mixed neutrophil-macrophage colonies. As M-CSF is a cytokine acting on macrophage and mixed neutrophil macrophage progenitors, we added a neutralizing Ab against M-CSF or control Ab at the initiation of the 1 week expansion cultures consisting of SFT and TGF-M-DC CM. Neutralizing anti-M-CSF Ab, but not control IgG, completely blocked TGF-M-DC CM-induced enhancement in expansion of myeloid progenitors (Figure 3B). Also, adding rhM-CSF (100 ng/ml) at the initiation of the 1 week-expansion culture consisting of SFT in the absence of CM significantly enhanced ex-vivo expansion of CFU-GM progenitors. This did not reach the level achieved by TGF-M-DC CM. M-CSF enhanced ex-vivo expansion of CFU-GM was blocked by anti-M-CSF, but not control Ab (Figure 3B). After treatment with anti-M-CSF or control Ab, the cultures expanded with mature TGF-M-DC produced CFU-GM colonies containing mainly macrophages or mixed neutrophils and macrophages. These results suggested that M-CSF present in mature TGF-M-DC CM contributed to expansion of these myeloid progenitors. However these results suggest that M-CSF was not the only cytokine in mature TGF-M-DC CM responsible for the enhanced ex-vivo expansion of CFU-GM.

Cytokine levels in mature TGF-M-DC CM were quantified using microSpot ELISA technology and mature M-DC CM was analyzed as a control. Results of the cytokine analysis are summarized in Table 1. Interestingly, a number of the cytokines analyzed (IL-1β, IL-6, IL-10, GM-CSF and VEGF) were significantly lower in mature TGF-M-DC
CM compared to mature M-DC CM, or were not significantly different between the two groups. Of interest, the amount of M-CSF found in the CM from both sets of mature DC was very low and not significantly different. Thus, while it is clear from the antibody studies shown in Figure 3B that M-CSF was involved in the enhanced expansion of CFU-GM by the mature TGF-M-DC CM, it was not M-CSF alone that was responsible for the enhancement. M-CSF had to be manifesting its effect in combination with one or more cytokines present in the mature TGF-M-DC CM (including possibly factors we did not evaluate in the CM) and/or with SCF, FL, and/or TPO to synergistically enhance *ex-vivo* expansion. Among the cytokines assessed in the DC CM, a number of them (including IL-1, IL-10, TNF-α, and VEGF) are known to suppress proliferation of CFU-GM either directly or through induction of release of other cytokines with suppressive activity.\textsuperscript{19} Since the levels of myeloid suppressive cytokines in the mature TGF-M-DC were below that present in the mature M-DC CM which did not enhance *ex-vivo* expansion, it is possible that decreased levels of some suppressive cytokine might also be responsible at least in part for the enhanced activity of the mature TGF-M-DC CM.

A comparison of CM prepared from mature TGF-M-DC to that prepared from mature TGF-GM-DC also showed no significant difference in release of M-CSF. Here the TGF-M-DC showed significantly less release of IL-1, IL-6, GM-CSF and VEGF, but increased IL-10 and TGF-β compared to that released by TGF-GM-DC. The results suggest again that M-CSF is not the only player in the enhanced expansion capability of the mature TGF-M-DC CM.
**LPS-activated TGF-M-DC induce T cell tolerance/regulatory T cells.** Since a possible advantage of M-DC is that they might have tolerogenic potential even in their mature form, we examined effects of TGF-M-DC in regulation of T cell proliferation and tolerance in their mature form compared to that of mature M-DC. LPS-activated TGF-M-DC displayed similar effect in inducing allogeneic MLR to LPS-stimulated M-DC, both at reduced potency compared to LPS-activated GM-DC (figure 4A).

To analyze whether mature TGF-M-DC could induce inhibitory T cells, naive CD4⁺ T cells were cultured for 7 days with LPS-activated M-DC or TGF-M-DC. T cells harvested at the end of culture were respectively denoted T1 (cultured with LPS-activated TGF-M-DC) and T2 (cultured with activated M-DC). First, T1 and T2 cells were restimulated with GM-CSF/IL-4-induced DC in a 2°MLR. Results shown in Figure 4B, top panel demonstrated that both T1 and T2 cells were hyporesponsive T cells in 2° MLR. Second, T1 and T2 cells were added into an independently set-up MLR containing naive CD4⁺ T cells and allogeneic GM-CSF/IL-4-induced DC to assay whether T1 and/or T2 cells could suppress the MLR. Results shown in Figure 4B (bottom panel) revealed that both T1 and T2 cells significantly suppressed the independently set-up MLR. These results demonstrated that both LPS-activated TGF-M-DC and M-DC induced a population of regulatory T cells from the three donors tested. Therefore, LPS-activated TGF-M-DC had similar potency in induction of T cell tolerance/or regulatory T cells as that of M-DC.

Foxp3, a transcription factor, is known to play a critical role for regulatory T cell function. Thus, we determined Foxp3 expression in the T cells recovered from MLR by
three color-staining with antibodies against CD4, CD25 and Foxp3. Results (Figure 4C, left 2 panels) showed that activated GM-DC derived from CB induced allogeneic CB CD4+ T cells to express the highest level of CD25 and Foxp3 among the three types of DC tested (GM-DC, M-DC and TGF-M-DC). Co-staining of CD25 and Foxp3 indicated that Foxp3 was exclusively expressed in activated CD25+ T cells subsets. Therefore, the expression level of Foxp3 is highly correlated with the activation status of CD4+ T cells. CB T cells cultured with TGF-M-DC and M-DC were less well activated with reduced expression of both CD25 and Foxp3. These results further indicated that CB TGF-M-DC and M-DC were less stimulatory. However, regulatory capacity of TGF-M-DC could not be further confirmed by phenotypic analysis due to the lack of a reliable phenotypic marker for in vitro activated human regulatory T cell. This information is supported by the previous reports that human CD4+ T cells induce Foxp3 expression simply by activation and thus human Foxp3+CD4+ T cells are not necessarily regulatory T cells.21,22 In contrast, TGF-M-DC and M-DC generated from AB were not consistently less stimulatory than adult GM-DC in terms of expression level of CD25 and Foxp3 (Figure 4C, right 2 panels). TGF-M-DC derived from 3 out of 6 AB donors we tested were capable of inducing marked levels of CD25 and Foxp3 expression on CB CD4+ T cells at a comparable level to GM-DC (data not shown).

**LPS-activated TGF-M-DC are less immune active than mature TGF-GM-DC; effects associated with enhanced LPS activation of the MAPK pathway in TGF-M-DC.** We previously reported that M-DC produced in the presence of M-CSF and IL-4 were more anti-inflammatory than GM-DC. We now tested whether in the presence of
TGF-β, such differences between M- and GM-CSF-induced DC still exist. Results in Figure 5 show that TGF-M-DC displayed reduced capability in induction of allogeneic MLR compared with TGF-GM-DC. The LPS induced TGF-M-DC secreted greater amounts of immune suppressive cytokines IL-10, but lower amounts of inflammatory cytokines (IL-1, IL-6, GM-CSF and VEGF) than mature TGF-GM-DC (Table 1B). This suggests that M-CSF-induced DC might generally be more favorable than GM-CSF-induced DC for silencing the immune system, regardless of whether or not these DC populations are produced in the presence of TGF-β.

To begin to understand the potential intracellular molecules that might underlie functional differences between M- and GM-CSF-induced DC in response to maturation signals, we examined activation of the 3 members of MAPK pathway (ERK, JNK, P38) by LPS. Since TGF-M-DC, compared to M-DC, have similar tolerance potential but are homogeneous in morphology, less adherent for easy handling and myeloid promoting, we chose to study the MAPK pathways in M- and GM-CSF-induced DC cultured in the presence of TGF-β. LPS-induced dramatically increased phosphorylation of ERK, JNK and p38 in TGF-M-DC compared to TGF-GM-DC (Figure 6), suggesting that enhanced MAPK activity in TGF-M-DC might play a role in their unique functions. We found that the ERK specific inhibitor U0126 suppressed LPS-induced secretion of TGF-β in TGF-M-DC derived cells (Table 2). In contrast, this ERK inhibitor suppressed TGF-β, IL-6 and IL-1-β, and increased GM-CSF by TGF-GM-DC from the same donors. These results suggest that the ERK inhibitor differently regulates cytokine production by TGF-M-DC and TGF-GM-DC. Thus, within the MAPK pathway, at least enhanced ERK
signaling in TGF-M-DC might be involved in quantitative cytokine differences we observed between mature populations of TGF-M-DC and TGF-GM-DC.
Discussion

In the present study, we demonstrated that TGF-β1, in combination with M-CSF and IL-4 induced CB monocytes to differentiate into a novel type of DC (TGF-M-DC) with the capability of enhancing expansion of myeloid progenitors *ex vivo* and inducing immune cell tolerance in vitro. These observations, combined with our previous reports on M-DC,\(^8\) indicate that CB M-CSF-induced DC might be more appropriate for silencing unwanted immune responses, such as in transplantation or autoimmune diseases. Major differences between M-CSF- and GM-CSF-induced DC lie in their activities upon LPS-induced maturation. M-CSF-induced DC produced enhanced amounts of anti-inflammatory/inflammatory cytokines and more potently induced T cell tolerance upon LPS stimulation. AB M-CSF-induced DC might not be as efficient and consistent as CB M-CSF-induced DC in the context of silencing immune responses in that they could be potent enough to induce T cell activation. It remains unsolved whether the Foxp3\(^+\)CD4\(^+\) T cells generated by coculture with CB TGF-M-DC and M-DC represents prototype Foxp3\(^+\) regulatory T cells. This is due to the fact that Foxp3 could be induced merely by T cell activation, an observation consistent with recent reports.\(^{21,22}\) However, the fact that Foxp3 is upregulated by T cell activation does not necessarily rule out the possibility that Foxp3 expressed in the CD4\(^+\) T cells cocultured with CB TGF-M-DC might act tolerogenically. More reliable phenotypic markers for regulatory T cells would allow us to define the nature of the inhibitory CD4\(^+\) T cells we developed using TGF-M-DC from CB.
M-CSF might be an important growth factor for inhibitory DC development in vivo. In line with this speculation, it was recently reported that M-CSF-deficient mice had reduced numbers of DC.\textsuperscript{23} M-CSF levels are elevated in immune suppressive conditions like pregnancy, neonatal CB and tumors.\textsuperscript{24-27} It is known that immature DC induce tolerance, while mature DC induce T cell activation. Biochemical analysis showed that M-CSF-induced TGF-M-DC respond to LPS with enhanced activation of the three MAPK family members, ERK, JNK and p38. This suggests that the enhanced tolerance function of TGF-M-DC is not due to inability to respond to maturation signals and differentiation into a mature form. MAPK signaling is likely to contribute, at least in part to the functional differences between M-CSF- and GM-CSF-induced DC. ERK inhibitor inhibited TGF-\( \beta \) but increased GM-CSF expression by both TGF-M-DC and TGF-GM-DC. As TGF-M-DC produced increased level of TGF-\( \beta \), but decreased level of GM-CSF, increased ERK signaling in TGF-M-DC might play a role in preferential production of TGF-\( \beta \), but diminished production of GM-CSF.

Cytokines/growth factors regulate hematopoiesis.\textsuperscript{19, 28, 29} Due to the potential therapeutic value of tolerogenic DC in transplantation, we considered it an important goal to investigate the capability of tolerogenic DC to modulate hematopoiesis. Of particular interest, CB TGF-M-DC were the only DC type that released factors that enhanced SFT-induced ex-vivo expansion of myeloid progenitors. The blocking effects of neutralizing Ab against M-CSF suggests that M-CSF is involved in this expansion, but is not the only factor involved. In other words, the very low levels of M-CSF present in the CM may be acting in synergy with other cytokines present in the CM or cultures, or alternatively,
higher levels of M-CSF may be produced endogenously by CD34+ or other cells co-cultured with TGF-M-DC CM during the first 1 week of expansion. Quantitative protein array analysis identified previously known cytokines produced by DC in response to LPS (IL-1, IL-6, IL-10, TGF-β, GM-CSF, M-CSF) as well as cytokines/growth factors not previously reported to be produced by DC (IL-2, SDF-1α and PDGF). Among the cytokine detected, IL-6 and SDF-1 promote the expansion or survival of hematopoietic progenitors, while IL-1 and VEGF could be hematopoietic suppressive. The quantitative cytokine profile of factors released by TGF-M-DC in response to LPS could account for the myeloid supporting activity of CB TGF-M-DC, although all the actual players involved in the expansion are not yet clear. Based on the cytokine profile we analyzed, we reasoned that M-CSF, in synergy with moderate level of hematopoietic supportive factors IL-6 and SDF-1 and minimal level of hematopoietic suppressive factors IL-1 and VEGF detected in TGF-M-DC CM could contribute to overall myeloid expansion effect of TGF-M-DC CM we observed ex vivo in synergy with SFT. Overall, CB TGF-M-DC might be therapeutically desirable accessory cells in CB transplantation by enhancing myeloid expansion of hematopoietic progenitors, thus facilitating myeloid recovery and at the same time, inducing immune tolerance.
Author Contribution

GL and HEB designed and performed the study, analyzed the data, and drafted the paper. TBC, SI, BG-E, and SC performed the study. SA and Y-JK performed the study and analyzed the data.
References


Table 1A. Comparative Analysis of Cytokines Released by LPS activated- M-DC and-TGF-M-DC.

<table>
<thead>
<tr>
<th>Cytokines (pg/ml)</th>
<th>CM: Mature (LPS-Stimulated) M-DC</th>
<th>CM: Mature (LPS-Stimulated) TGF-M-DC</th>
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<tbody>
<tr>
<td>i.) Significant decrease from M-DC to TGF-M-DC</td>
<td></td>
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<tr>
<td>IL-1β</td>
<td>17±2</td>
<td>ND**</td>
</tr>
<tr>
<td>IL-6</td>
<td>12804±3186</td>
<td>4092±987**</td>
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<td>IL-10</td>
<td>3798±736</td>
<td>1218±415**</td>
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<td>GM-CSF</td>
<td>179±10</td>
<td>58±19**</td>
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<tr>
<td>VEGF</td>
<td>588±232</td>
<td>82±25*</td>
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<tr>
<td>ii.) No change from M-DC to TGF-M-DC</td>
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<td></td>
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<tr>
<td>SDF-1α</td>
<td>750±543</td>
<td>325±192</td>
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<td>TGFβ</td>
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<td>IL-2</td>
<td>7±1</td>
<td>7±1</td>
</tr>
<tr>
<td>M-CSF</td>
<td>ND</td>
<td>2±2</td>
</tr>
<tr>
<td>IL-3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-12</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-17</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Conditioned medium prepared from 5x10^5 cells/ml. Data represent average cytokine levels of triplicate ± SEM from 4 different donors. * and ** indicates p<0.05 when using one-tailed or two-tailed t test; ND, non-detectable in all 4 donor samples.
Table 1B. Comparative Analysis of Cytokines Released by LPS activated- TGF-GM-DC and TGF-M-DC.

<table>
<thead>
<tr>
<th>Cytokines (pg/ml)</th>
<th>CM: Mature (LPS-Stimulated) TGF-GM-DC</th>
<th>CM: Mature (LPS-Stimulated) TGF-M-DC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>i.) Significant decrease of TGF-M-DC to TGF-GM-DC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>86±4</td>
<td>3±3**</td>
</tr>
<tr>
<td>IL-6</td>
<td>50305±1282</td>
<td>20661±2251*</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>559±67</td>
<td>40±14**</td>
</tr>
<tr>
<td>VEGF</td>
<td>103±4</td>
<td>15±15*</td>
</tr>
<tr>
<td><strong>ii.) Significant increase when comparing TGF-M-DC to TGF-GM-DC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>195±115</td>
<td>829±251**</td>
</tr>
<tr>
<td><strong>iii.) No significant change from TGF-GM-DC to TGF-M-DC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-β</td>
<td>41±13</td>
<td>27±3</td>
</tr>
<tr>
<td>M-CSF</td>
<td>13±13</td>
<td>34±34</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>242±101</td>
<td>151±151</td>
</tr>
<tr>
<td>IL-2</td>
<td>12±1</td>
<td>22±13</td>
</tr>
<tr>
<td>IL-3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-12</td>
<td>40±40</td>
<td>ND</td>
</tr>
<tr>
<td>IL-17</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Conditioned medium prepared from 2.5x10^5 cells/ml. These samples were from experiments different from those reported in Table 1A, and cells were conditioned in different types of wells in this from those reported in Table 1A. Data represent average cytokine levels of triplicate ± SEM from 2 donors. * and ** indicates p<0.05 when using one (*) or two-tailed (**) paired t-test. ND, non-detectable in all samples.
Table 2. Effects of ERK inhibitor on cytokine production by TGF-M-DC and TGF-GM-DC

<table>
<thead>
<tr>
<th>Cytokines decreased by ERK inhibitor</th>
<th>CM: TGF-M-DC</th>
<th>CM: TGF-GM-DC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pg/ml</td>
<td>pg/ml</td>
</tr>
<tr>
<td>ERK inhibitor</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TGFβ</td>
<td>41±13</td>
<td>27±3</td>
</tr>
<tr>
<td>IL-6</td>
<td>20661±2251</td>
<td>50305±1282</td>
</tr>
<tr>
<td></td>
<td>9757±3613</td>
<td>23300±5558*</td>
</tr>
<tr>
<td>IL-1β</td>
<td>86±4</td>
<td>12±6**</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>725±265</td>
<td>176±135</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cytokines increased by ERK inhibitor</th>
<th>CM: TGF-M-DC</th>
<th>CM: TGF-GM-DC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pg/ml</td>
<td>pg/ml</td>
</tr>
<tr>
<td>ERK inhibitor</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>40±14</td>
<td>559±67</td>
</tr>
<tr>
<td></td>
<td>155±67</td>
<td>720±72**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cytokines unchanged by ERK inhibitor</th>
<th>CM: TGF-M-DC</th>
<th>CM: TGF-GM-DC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pg/ml</td>
<td>pg/ml</td>
</tr>
<tr>
<td>ERK inhibitor</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>VEGF</td>
<td>15±15</td>
<td>103±4</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>151±151</td>
<td>242±101</td>
</tr>
<tr>
<td>IL-1β</td>
<td>3±3</td>
<td>4±4</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>1141±139</td>
<td>1358±159</td>
</tr>
</tbody>
</table>

Cytokine levels were detected on protein array slides in triplicate for each sample treated ± ERK inhibitor U0126. Data represent average cytokine levels from triplicate samples ± SEM from 2 donors. * and ** indicates p<0.05 when using one (*) or two-tailed (**) paired t-test.
Figure 1. TGF-β1 induces a homogeneous DC morphology for M-DC. CB monocytes were cultured with GM-CSF and IL-4 or M-CSF and IL-4 to generate GM-DC and M-DC respectively (Left panels). TGF-β1 was added into GM-DC and M-DC cultures at the beginning of cell culture (right panels). Pictures were taken on day 5 of culture. Results are representative of >10 independent experiments.

Figure 2. Effects of TGF-β1 on phenotypic markers of M-DC. CB monocytes were cultured with M-CSF and IL-4 ± TGF-β1 for 5-6 days. LPS was added the last day of cell culture. Cells were stained with CD1a, CD80, CD86, CD207 (langerin), HLA-DR and CD83. Gray line designates isotype control. The black area represents indicated molecules. The mean fluorescence intensities of indicated molecules are shown on the upper right corner of the histograms. Results are representative of 3-5 independent experiments.

Figure 3. Enhancing effects of conditioned medium produced by mature (LPS-stimulated) TGF-M-DC on expansion of CFU-GM progenitors from CB. (A) CM from TGF-M-DC selectively expands CFU-GM ex vivo in synergy with SFT. One × 10^4 freshly isolated CD34^+ cells/ml were incubated with CM from M-DC, TGF-M-DC, GM-DC, TGF-GM-DC, control CM or control medium ± SFT for 1 week. Cells were harvested and plated in triplicate. Fold expansion of CFU-GM after 1 week culture was determined. Data represent a summary of CM from 8 different CB donors. Average fold-
expansion of CFU-GM is shown on the top of each bar. (B) Neutralizing anti-M-CSF Ab, but not control Ab blocks the ex-vivo myeloid enhancing activity of TGF-M-DC CM. One × 10^4/ml isolated CD34+ cells were cultured with those factors as indicated on the x-axis in the presence of SFT for 1 week. Fold expansion of CFU-GM after 1 week-culture is shown on the y-axis. The average fold-expansion level of CFU-GM is shown on the top of each bar. The data represent an average ± SEM from 4 experiments for the neutralizing anti-M-CSF and 2 experiments for the recombinant M-CSF.

Figure 4. Effect of mature (LPS-induced) TGF-M-DC, in comparison with mature M-DC, in regulation of T cell proliferation and tolerance. (A) Mature TGF-M-DC are similar to mature M-DC in induction of allogeneic MLR, both at lower efficiency than mature GM-DC in MLR. LPS-activated TGF-M-DC, M-DC and GM-DC were stimulated with allogeneic naïve CD4+ T cells for 6 days. T cell proliferation was determined by [Methyl-^3H] thymidine incorporation for the last 16 h of cell culture. (B) TGF-M-DC induce T cell tolerance/regulatory T cells with similar efficiency to that of M-DC. T1 and T2 are naïve CD4+ T cells (donor A) prestimulated with mature TGF-M-DC or M-DC (donor B) for 7 days. T1 and T2 (2.5 × 10^4) were restimulated with allogeneic GM-CSF/IL-4-induced DC in 2°MLR, and both of T1 and T2 were hyporesponsive T cells (top panel). Also, T1 and T2 (2.5 × 10^4) were added to another MLR containing naïve CD4+ T cells (5 × 10^4; donor A) and GM-CSF/IL-4 induced DC (5 × 10^3; donor B) (bottom panel). Proliferation of T cells was determined by [¹H]thymidine incorporation after 6 days of culture. Results are representative of 3 independent experiments. (C) CB CD4+ T cells were cocultured with LPS-activated GM-DC, M-DC and TGF-M-DC derived from both
CB and AB in MLR for 7 days. Cells were harvested and stained with anti-CD4-FITC, anti-CD25-PE and intracellular anti-Foxp3-APC. The top row is CD4+ T cells isolated from CB stained with anti-CD4, anti-CD25 and isotype control IgG for APC. The percentages of gated cell population are shown on the upper right corner of dot-plots. Results are one representative of three CB and six AB.

Figure 5. Mature TGF-M-DC are less efficient in induction of allogeneic MLR than mature TGF-GM-DC. LPS-activated mature TGF-M-DC and TGF-GM-DC were stimulated with allogeneic naïve CD4+ T cells for 6 d. T cell proliferation was determined by [Methy-3H] thymidine incorporation assay. Data are representative of 3 independent experiments.

Figure 6. TGF-M-DC respond to LPS with markedly enhanced phosphorylation of members of MAPK pathway (ERK, JNK and p38) compared to TGF-GM-DC. TGF-M-DC and TGF-GM-DC were either not treated (as a control) or treated side by side for 15 and 30 min with LPS. Cell lysate was subjected to western blotting using rabbit polyclonal antibodies specific for p-ERK, -JNK or -P38. PVDF membranes were stripped and reprobed with a rabbit polyclonal antibody specific for total ERK as a loading control.
Figure 1

GM-CSF + IL-4

- TGF-β1 + TGF-β1

M-CSF + IL-4
Figure 2

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Figure 3

A

CFU-GM fold expansion

Control medium
Control CM
CM:M-DC
CM:TGF/M-DC
CM:GM-DC
CM:TGF-GM-DC

+ SFT

- SFT

30
25
20
15
10
5
0

7.2 8.5 6.5 21.3 4.1 8.0 0.3 0.4 1.0 0.6 0.3 0.9
B


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Figure 4

A

B

T1: T cells cultured for 7 d with mature [LPS-Stimulated] M-DC
T2: T cells cultured for 7 d with mature [LPS-Stimulated] TGF-M-DC
Figure 4C

Control
CB CD4+ T cells

GM-DC

M-DC

TGF-M-DC

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Figure 5

![Graph showing the relationship between APC:T ratio and cpm × 10^3. The graph compares LPS-Stimulated TGF-GM-DC and LPS-Stimulated TGF-M-DC. The y-axis represents cpm × 10^3 ranging from 0 to 350, while the x-axis represents APC:T ratio ranging from 1:10 to 1:250. The graph includes error bars and asterisks indicating statistical significance.]
Figure 6

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TGF-β1 combined with M-CSF and IL-4 induces generation of immune inhibitory cord blood dendritic cells capable of enhancing cytokine-induced ex-vivo expansion of myeloid progenitors

Geling Li, Saeid Abediankenari, Young-June Kim, Timothy B. Campbell, Shigeki Ito, Barbara Graham-Evans, Scott Cooper and Hal E. Broxmeyer