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Macrophage Colony-Stimulating Factor Drives Cord Blood Monocyte Differentiation into IL-10^{high}IL-12^{absent} Dendritic Cells with Tolerogenic Potential

Geling Li, Young-June Kim, and Hal E. Broxmeyer

Immature dendritic cells (DCs) induce tolerance and mature DCs induce inflammatory immune responses. However, the likelihood of maturation of immature DCs in vivo limits its potential application for suppression of unwanted immune reactions in vivo. The aim of this study was to generate DCs with anti-inflammatory properties in both the immature and mature states. GM-CSF combined with IL-4 drives monocyte differentiation into DCs. As M-CSF is a critical cytokine in development of the monocytic lineage and its level is dramatically elevated in immunosuppressive conditions, we investigated whether M-CSF could replace GM-CSF and generate DCs with distinct functions from umbilical cord blood monocytes. Highly purified umbilical cord blood monocytes cultured with M-CSF and IL-4, in a GM-CSF-independent fashion, differentiated into IL-10^{high}IL-12^{absent} cells with a DC phenotype (termed M-DC). Single time stimulation with immature DCs (both M-DCs and DCs) derived from cord blood induced hyporesponsive and regulatory CD4^{+} T cells. In contrast to mature DCs, mature M-DCs induced decreased Th1 differentiation and proliferation of naive CD4^{+} T cells in both primary and secondary allogeneic MLR and showed tolerogenic potential. These results demonstrate an unrecognized role for M-CSF in alternative differentiation of monocytes into anti-inflammatory M-DCs and suggest that M-CSF-induced DCs may be of use for suppressing unwanted immune responses. The Journal of Immunology, 2005, 174: 4706-4717.
establishing and maintaining a long-term DC line from murine epidermis (31). We have previously shown that monocytes grown in the presence of M-CSF and IL-4 gradually lose markers of the monocyte/macrophage lineage, but gain markers of DCs (32). However, it was not known whether M-CSF could induce monocyte differentiation into DCs with unique functions.

Cord blood has been successfully used as a transplantable source of hematopoietic stem cells with a relatively low incidence of graft-versus-host disease (GVHD) (33–34). DCs, as the most efficient APCs, have the potential to induce immune activation or tolerance and play a critical role in the pathogenesis of GVHD (1, 41–44). Monocytes constitute a large pool of DC precursors in cord blood and give rise to DCs under the influence of GM-CSF and IL-4 (45). Cord blood monocyte-derived DCs demonstrate markedly reduced efficiency in secreting IL-12 and inducing Th1 differentiation (46, 47). Study of DCs in cord blood may help to elucidate ways to decrease the incidence of GVHD in cord blood, as well as in bone marrow transplantation. Physiologically, cord blood monocytes might have a greater chance to be exposed to M-CSF and IL-4 because higher serum levels of M-CSF have been detected in cord blood than in adult blood (23, 24) and pregnancy is associated with a skewed Th2 cytokine (IL-4) profile (48). Therefore, we chose to use cord blood to study whether M-CSF, in combination with IL-4 could induce cord blood monocyte differentiation into suppressive DCs. Our results demonstrate that IL-10^high^IL-12^absent^ accessory cells with DC features (termed M-DCs) are induced from highly purified umbilical cord blood CD14^+^ monocytes in the presence of M-CSF and IL-4. We have found that both immature and mature M-DCs induce low responsiveness of naïve cord blood CD4^+^ T cells.

Materials and Methods
Isolation of monocytes and CD4^+^ T cells from cord blood
Heparinized umbilical cord blood was collected according to institutional guidelines. PBMC were isolated by density gradient centrifugation on Ficoll Paque Plus (1.077 g/ml; Amersham Pharmacia Biotech). RBC were lysed using PUREGENE RBC lysis solution (Gentra Systems). Monocytes and CD4^+^ T cells were purified from PBMC using MACS CD14^+^ magnetic beads and CD4^+^ T cell isolation kit (Miltenyi Biotec), respectively. Enriched CD4^+^ T cells from different cord blood samples were used fresh or were frozen until use. Isolated monocytes and T cells were >98% and >95% pure populations, respectively, as analyzed by flow cytometry.

Cell culture
CD14^+^ monocytes (5 × 10^5^ cells/ml) were cultured in RPMI 1640 medium (BioWhittaker) supplemented with 10% heat-inactivated FBS (HyClone Laboratories), t-glutamine, HEPES, 50 mM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin for 5–6 days in the presence of 20 ng/ml recombinant human IL-4 with 400 U/ml recombinant human GM-CSF (Immunex/Amen) to generate DCs and 100 ng/ml recombinant human M-CSF to generate M-DCs. Culture medium was changed every 3 days until the end of culture.

Assays for cytokine/chemokine production
To detect cytokine secretion, washed cells (2 × 10^6^/ml) were stimulated with LPS (1 μg/ml) in culture medium in 24-well plates for 24 h; supernatants were frozen at −80°C until assayed for cytokines. Cytokine levels in supernatants were measured in duplicate with human IL-10 and human IL-12 (p70) ELISA kits (BD Biosciences). For intracellular analysis of cytokine production, CD4^+^ T cells (5 × 10^5^/ml) were stimulated with allogeneic DCs as previously described, were activated with 50 ng/ml PMA (Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich) in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich) for 5–6 h. Cells were then fixed, permeabilized (Cytofix/Cytoperperm kit; BD Pharmingen), and stained with FITC-labeled IFN-γ and PE-labeled anti-IL-4 (BD Biosciences). For intracellular analysis of MIP-1β production, LPS (1 μg/ml; Sigma-Aldrich) was added into day 5 M-DC and DC cultures in the presence of Golgistop (BD Biosciences) for an additional 16 h. Cells were harvested, fixed, permeabilized before being stained with PE-labeled anti-MIP-1β (BD Biosciences). Samples were then analyzed by flow cytometry (FACS-Calibur flow cytometer and CellQuest software; BD Biosciences).

Analysis of DC surface phenotype by flow cytometry
Harvested cells were washed twice with PBS supplemented with 1% BSA. Fc receptors on cells were preblocked with excess IgG (Sigma-Aldrich) on ice for 15 min. Cells were stained for 30 min at 4°C with the following FITC-conjugated Abs: anti-CD14, anti-CD16, anti-CD40, anti-CD83, anti-CCR5 (BD Pharmingen); anti-HLA-ABC, anti-CD1a (eBioscience); anti-CD80, anti-CD86, anti-CD14, anti-CD1a-DR (Caltag Laboratories) and with the following PE-conjugated Abs: anti-CD1a (Caltag Laboratories), anti-B7-H1 (eBioscience), anti-CR7 and anti-CD83 (BD Pharmingen). Murine IgG1-FITC, IgG1-PE, IgG2a-FITC, and IgG2a-PE from Caltag Laboratories or BD Pharmingen were used as isotype controls. After washing, cells were analyzed by flow cytometry.

FITC-dextran uptake experiments
To measure the endocytic activity of DCs, 5 × 10^4^ DCs were incubated with 0.1 ng/ml FITC-dextran (Sigma-Aldrich) in 100 μl of culture medium at 37°C and 0°C (as a control) for 1 h. Cells were then washed with ice-cold PBS supplemented with 1% BSA four times before analysis by flow cytometry.

Expansion of CFSE-labeled CD4^+^ T cells
CD4^+^ T cells were suspended in PBS at 5 × 10^5^/ml. A final concentration of 20 μM CFSE (Molecular Probes) was added to the cell suspension and incubated for 5 min at room temperature. Cells were washed three times in RPMI 1640 with 5% FBS. CFSE-labeled CD4^+^ T cells (5 × 10^5^/ml) were cocultured with mature DCs or M-DCs (5 × 10^5^/ml) in X VIVO-20 serum-free medium in flat-bottom plates. T cells were harvested on day 6 and analyzed by flow cytometry.

Primary and secondary MLR
Primary and secondary MLRs were done in either RPMI 1640 culture medium with 10% FBS or X VIVO-20 serum-free medium (Invitrogen Life Technologies). Primary MLR was established in 96-well U-bottom cell culture plates (Costar) with 1 × 10^5^ responding cord blood CD4^+^ T cells per well and allogeneic stimulators at E:T target cell ratios ranging from 1:10 to 1:2500 in triplicate. Cells were pulsed with [methyl-3H]thymidine (1 μCi/well; Amersham Pharcmaica 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).

For secondary MLR, 4 × 10^4^ naive CD4^+^ T cells were first stimulated with 4 × 10^5^ allogeneic stimulators in six-well plates for 6 days. Cells were then collected, washed, and rested for 48 h in cultured medium supplemented with 2 U/ml human IL-2 (eBioscience). Subsequently, viable T cells (5 × 10^5^/well) were restimulated with 5 × 10^5^ DCs generated from the same donor as used for the first culture in the presence or absence of 50 U/ml IL-2 in U-bottom 96-well plates. T cell proliferation was measured on day 3 or day 5 after a 16-h pulse with [methyl-3H]thymidine (1 μCi/well) before the end of culture.

Alternatively, 4 × 10^4^ naive CD4^+^ T cells (donor A) were cultured with 4 × 10^5^ allogeneic M-DCs or DCs (donor B) in six-well plates for 7 days. T cells were harvested and enumerated by trypan blue exclusion. Harvested T cells (5 × 10^5^/well for A or 5 × 10^4^/well for B) were restimulated with 1 × 10^5^ IL-4-induced mature DCs (donor B) at an E:T ratio of 1:5. T cell proliferation was measured by pulsing cells with [methyl-3H]thymidine (1 μCi/well) before the end of culture.
Statistical analysis
The two-tailed paired Student’s t test was used to determine statistical significance unless otherwise indicated. Values of \( p < 0.05 \) were considered significant.

Results
M-CSF in synergy with IL-4 induces monocyte differentiation into accessory cells with DC features

M-CSF and GM-CSF induce monocyte differentiation into macrophages with distinct phenotype and functions (20). IL-4 inhibits macrophage development and, when used in combination with GM-CSF, reprograms monocyte differentiation into DCs instead of macrophages (49, 50). We examined cord blood monocyte differentiation induced by M-CSF/IL-4 in comparison with GM-CSF/IL-4. Cord blood CD14 \(^+\) monocytes (>98% purity) were cultured with M-CSF alone, or with M-CSF or GM-CSF in the presence of IL-4 for 5 days. In the presence of M-CSF alone, monocytes became strongly adherent and elongated with spindle-like morphology, a typical macrophage morphology as previously reported (Fig. 1, upper left panel) (51). M-CSF-induced macrophages expressed the monocyte/macrophage markers CD14 and CD64 and a low level of HLA-DR (Fig. 2). When cultured with GM-CSF and IL-4 (Fig. 1, middle left panel), monocytes gave rise to typical DCs. Compared with GM-CSF/IL-4-induced DCs, monocytes cultured with M-CSF and IL-4 floated less and had dendrites or protruding projections (Fig. 1, lower left panel) and no osteoclast-like cells were generated in 1 wk of cell culture as assessed by Giemsa staining (data not shown). As assessed by phenotype, increasing doses of IL-4 skewed monocyte differentiation into a novel type of CD14\(^-\)CD64\(^-\)HLA-DR\(^{high}\) cells instead of CD14\(^+\)CD64\(^+\)HLA-DR\(^{low}\) macrophages (Fig. 2). Upon LPS stimulation, cells grown with M-CSF/IL-4 responded to LPS in a way quite different from DCs grown with GM-CSF/IL-4; they tended to form large aggregates surrounded by single cells displaying very long dendrites or spindle-like protrusions (Fig. 1, lower right panel), a morphology similar to LPS-activated M-CSF-induced macrophages (Fig. 1, upper right panel). In contrast, such long dendrites were absent in LPS-stimulated DCs, and LPS-activated DCs formed relatively small aggregates (Fig. 1, middle right panel), as previously reported (50). Monocytes cultured with M-CSF/IL-4 were CD1a\(^+\) (Fig. 3A). Upon activation with LPS or TNF-\(\alpha\), they significantly up-regulated surface expression of CD83, a human DC marker, at a slightly lower or similar level as DCs induced by GM-CSF/IL-4 (Fig. 3B). For simplicity, we termed monocytes driven by M-CSF/IL-4 as M-DCs, in contrast to DCs induced by GM-CSF/IL-4. M-DCs, regardless of their maturation state, expressed the functional molecules CD86, B7-1H, HLA-DR, HLA-ABC, and CD40, as did DCs with only quantitative differences. Before maturation, M-DC had a higher surface expression of HLA-DR, HLA-ABC, and CD86 than DCs. After LPS stimulation, DCs significantly up-regulated expression of HLA-DR and CD86 to a level similar to that of LPS-stimulated M-DCs (Fig. 3, A and B). In addition, M-DCs expressed lower amounts of CD40 and B7-H1 on their surface than DCs regardless of their maturation states (Fig. 3, A and B). Generation of M-DCs appeared to be independent of GM-CSF. Daily addition of neutralizing anti-GM-CSF (1 \( \mu \)g/ml) to DC cultures completely blocked CD1a up-regulation induced by GM-CSF (data not shown). However, anti-GM-CSF failed to block

![FIGURE 1. Morphology of M-DCs in comparison with DCs and M-CSF-induced macrophages (M-Mac).](image-url)
generation of M-DCs as analyzed by morphology and surface staining (data not shown).

Chemokines and their receptors have been known to play an important role in DC migration and function (52–55). As shown in Fig. 3A, immature DCs expressed CCR5 and a very low or absent level of CXCR4. LPS stimulation up-regulated the expression of CXCR4 and induced the production of a high amount of MIP-1β as well as a significantly reduced level of CCR5 on DCs (Fig. 3B), possibly due to the internalization of the CCR5 by the produced MIP-1β. In comparison with DCs, M-DCs expressed a minimal level of CCR5 and a detectable level of CXCR4 before and after maturation (Fig. 3A and B). M-DCs also produced a large amount of MIP-1β upon LPS stimulation (Fig. 3B).

As M-DCs and DCs had different expression of CD1a, we investigated whether M-CSF competed with GM-CSF in generation of M-DCs. GM-CSF (40 U/ml) increased the percentage of CD1a+ cells in M-DC cultures; this effect peaked at 200 U/ml GM-CSF (Fig. 4A). However, if GM-CSF was added into day 4 M-DC cultures, GM-CSF failed to induce the appearance of CD1a+ DCs (Fig. 4B). Therefore, it appears that GM-CSF competes with M-CSF in generation of CD1a+ DCs before the M-DC differentiation program initiates.

Immature DCs are well known for their high efficiency in Ag uptake and cytokine production in response to maturation stimuli. As shown in Fig. 5A and B, incorporation of FITC-dextran by immature M-DCs and DCs was highly efficient. Upon LPS stimulation, the ability to take up FITC-dextran by both M-DCs and DCs decreased to a similar level.

M-DCs as IL-10highIL-12absent accessory cells

M-CSF-induced macrophages are known to produce increased levels of IL-10, but to turn off IL-12 production compared with GM-CSF/IL-4–induced DCs (21). We compared the cytokine secretion profile of M-DCs with DCs. We found that LPS-induced M-DCs produced much higher levels (up to 26-fold) of the anti-inflammatory cytokine IL-10 than DCs (Fig. 6). M-DCs produced nondetectable IL-12 upon LPS stimulation (detection limit, 4 pg/ml). There was great variability of IL-12 production among individual cord blood samples. In three cord blood samples we tested, we detected 385, 73, and 7.2 pg/ml IL-12p70 in LPS-activated DCs. However, M-DCs produced a lower level of IL-12p70 than DCs (Fig. 6). Therefore, with IL-4 included in the culture, M-DCs still produced high amounts of IL-10, but no IL-12 production, a pattern similar to M-CSF-induced macrophages (21).

M-DCs have lower alloreactivity than DCs after maturation

A key feature of mature DCs is their high capacity for priming T cells. Therefore, we further compared alloreactivity of LPS-matured M-DCs with DCs. In an allogeneic MLR, mature M-DCs induced significantly less proliferation of allogeneic naive CD4+ T cells than mature DCs (Fig. 7A). When we cultured naive CD4+ T cells with either allogeneic mature M-DCs or mature DCs in six-well plates, we consistently noticed that the subset of T cells cultured with DCs proliferated vigorously to form large clumps noticeable to the naked eye (data not shown). However, for M-DCs, these big clumps were either quite few or absent. Therefore, we labeled naive CD4+ T cells with CFSE and stimulated those CFSE-labeled T cells with either allogeneic mature M-DCs or DCs. We consistently noticed that a small subset of T cells had undergone profound expansion and therefore CFSE intensity declined the most (Fig. 7B, gate R1). This small subset of T cells was absent or sparse when cultured with M-DCs (Fig. 7, B and C), which might account for the decreased thymidine uptake by T cells primed with mature M-DCs.
FIGURE 3. Phenotypic characterization of M-DC before (A) and after (B) its maturation by LPS. Enriched monocytes from the same cord blood were cultured side by side with M-CSF/IL-4 (M-DCs) or GM-CSF/IL-4 (DCs) for 5–6 days. Cells were stained with indicated surface molecules (gray histogram) and isotype controls (black histogram). Data are representative of three to four independent experiments.
Mature M-DCs induce less Th1 differentiation than mature DCs

IL-10 production by DCs has been reported to be involved in the induction of Th2 differentiation. We compared the two types of mature DCs in driving T cell differentiation by analyzing intracellular levels of IFN-γ, IL-4, and IL-10 in T cells. We failed to detect significant amounts of IL-4 and IL-10 (<5%; data not shown) by T cells primed with both types of DCs (Fig. 8) by intracellular staining. IFN-γ production with regard to the percentage of IFN-γ-producing T cells and its amount of median fluorescence intensity (MFI) secreted by T cells were consistently lower for mature M-DCs than for mature DCs regardless of the presence or absence of IL-12 (p < 0.05) (Fig. 8). For both types of DCs, IL-12 did not increase the percentage of IFN-γ-producing T cells, but dramatically enhanced the MFI of cells in the right (R) quadrant in Fig. 8A. These results suggest that mature M-DCs induce less Th1 differentiation than mature DCs. IL-12 increased the amount of IFN-γ production per cell, but failed to enhance the percentage of IFN-γ-producing T cells. Therefore, IL-12 could not fully compensate for the differences in IFN-γ production observed between mature M-DCs and mature DCs.

M-DCs induced decreased secondary MLR and might be tolerogenic even in mature form

As M-DCs were IL-10highIL-12absent cells and failed to induce T cells to produce significant amounts of Th2 cytokine IL-4, we evaluated the tolerogenic potential of M-DCs. For this purpose, we analyzed CD4+ T cells stimulated with M-DCs for their capacity to respond to secondary stimulation by alloantigen in comparison with DCs. Naive CD4+ T cells, after being stimulated by M-DCs or DCs in primary MLC, were harvested, rested for 2 days, and then restimulated by allogeneic DCs generated from the same donor as that used in the primary MLC. Both M-DCs and DCs before their maturation induced hyporesponsiveness of T cells in secondary MLR in comparison with mature DCs on day 3 and day 5 (Fig. 9A). We further examined whether mature M-DCs could induce hyporesponsiveness of CD4+ T cells. Secondary MLRs were done in both serum-free and serum-containing medium. We found that mature M-DCs induced significantly lower proliferation of T cells in secondary MLR than mature DCs (Fig. 9B). This effect varied among individuals and secondary MLR of T cells primed with mature M-DCs during primary MLR decreased 30, 62, and 68% (for serum-containing medium) and 20, 42, and 63% (for serum-free X VIVO-20 medium) compared with those preprimed with mature DCs. Interestingly, we found in some cord blood samples,
even mature DCs poorly induced optimal T cell proliferation in secondary MLR, and IL-2 addition dramatically enhanced the rate of T cell proliferation (Fig. 9B; lower panel). This might be due to special characteristics of cord blood DCs, such as their defect in IL-12 production (46). This low responsiveness of T cells primed by M-DCs could not be blocked by neutralizing anti-IL-10 Abs and was not alloantigen-specific as T cells primed with mature M-DCs in primary MLC also responded less efficiently to allogeneic DCs generated from a third-party donor (data not shown).

Single time stimulation with cord blood immature DCs (both M-DC and DC) induce CD44+ regulatory T cells

Because repetitive stimulation of naive CD4+ T cells with immature monocyte-derived DCs induce tolerance and regulatory T cells (9), we examined whether immature DC (M-DC and DC) generated in our hands could induce regulatory T cells. For this purpose, naive CD4+ T cells were cultured with immature M-DC and DC for 7 days, respectively. For simplicity, we termed T cells at the end of culture as T1 (cultured with immature M-DC) and T2

**FIGURE 6.** M-DCs are IL-10highIL-12absent cells. Equal numbers of M-DCs and DC were stimulated with LPS for 24 h and supernatant was harvested in duplicate for detection of IL-10 (left) and IL-12 (right) in duplicate by ELISA. Three independent experiments are shown. ND, Not detectable (detection limit, 4 pg/ml).

**FIGURE 7.** Mature M-DCs are less efficient in induction of MLRs than mature DCs. A, Comparison of M-DC and DC after maturation in MLR. Mature M-DCs and DCs were cultured with allogeneic naive CD4+ T cells. Proliferation of allogeneic naive CD4+ T cells was determined by thymidine incorporation after culture with graded doses of DCs for 5 days. Data are representative of three independent experiments. *, p < 0.05. Statistically significant difference. B, Mature M-DCs are less efficient in presentation of alloantigens to CD4+ naive T cells than mature DCs. T cells were labeled with CFSE and cultured with allogeneic M-DCs or DCs at 10:1 ratio for 6 days before analyzed by flow cytometry. Percentage of cells in each gate (R1–R3) was shown in histogram (representative of three independent experiments). C, Results of three individual experiments as described in B. Percentage of CFSE+ cells in gate R1 as shown in (B).
As shown in Fig. 10, T1 and T2 were added into an independently set-up MLR consisting of naive CD4^+ T cells and GM-CSF/IL-4-induced DC to examine whether T1 and T2 could suppress MLR. At a T1 or T2 to naive T cell ratio of 1:2, T1 and T2 efficiently switched off MLR of naive T cells by 87% (Fig. 10), 75%, and 85% (data not shown) for T1 and by 75% (Fig. 10), 85%, and 83% (data not shown) for T2. These results demonstrate that single time stimulation of naive CD4^+ T cells with both types of immature DCs derived from cord blood induced the appearance of regulatory T cells.

Single time stimulation with mature M-DC induce hyporesponsive or CD4^+ regulatory T cells

We investigated whether mature M-DC could induce regulatory T cells. Therefore, naive CD4^+ T cells were cultured for 7 days with mature M-DC and mature DC, respectively. T cells harvested at the end of culture were called T3 (cultured with mature M-DC) and T4 (cultured with mature DC), respectively, for simplicity. First, T3 and T4 were restimulated with GM-CSF/IL-4-induced DC in a 2'MLR to confirm the results observed in Fig. 9B that T3 could induce hyporesponsiveness in T cells in 2'MLR. Next, T3 and T4 were then added into an independently set-up MLR containing naive CD4^+ T cells and GM-CSF/IL-4-induced DCs to assay whether T3 could suppress MLR. Results from four donors revealed individual variation. Half of the donor-derived mature M-DCs induced regulatory T cells (Fig. 11, left), whereas the other half of the mature M-DCs induced T cell anergy, but no regulatory T cells (Fig. 11, right). As shown in Fig. 11, T3 after priming with mature M-DC does not respond well to restimulation by GM-CSF/IL-4 (Fig. 11, top). Furthermore, T3 efficiently suppressed the independently set-up MLR by 70% (Fig. 11, left) and 74% (data not shown), demonstrating that T3 contains regulatory T cells for the two donors. For the other two donors, as shown in Fig. 11, T3 after priming with mature M-DC did not respond well to restimulation by GM-CSF/IL-4-induced DC in 2'MLR (Fig. 11, top). However, T3 only suppressed the independently set-up MLR by 13.4% (Fig. 11, right part) and 7.4% (data not shown), and T3 and naive T cells proliferated at the same level as

(cultured with immature DC). As shown in Fig. 10, T1 and T2 were added into an independently set-up MLR consisting of naive CD4^+ T cells and GM-CSF/IL-4-induced DC to examine whether T1 and T2 could suppress MLR. At a T1 or T2 to naive T cell ratio of 1:2, T1 and T2 efficiently switched off MLR of naive T cells by 87% (Fig. 10), 75%, and 85% (data not shown) for T1 and by 75% (Fig. 10), 85%, and 83% (data not shown) for T2. These results demonstrate that single time stimulation of naive CD4^+ T cells with both types of immature DCs derived from cord blood induced the appearance of regulatory T cells.

Single time stimulation with mature M-DC induce hyporesponsive or CD4^+ regulatory T cells

We investigated whether mature M-DC could induce regulatory T cells. Therefore, naive CD4^+ T cells were cultured for 7 days with mature M-DC and mature DC, respectively. T cells harvested at the end of culture were called T3 (cultured with mature M-DC) and T4 (cultured with mature DC), respectively, for simplicity. First, T3 and T4 were restimulated with GM-CSF/IL-4-induced DC in a 2'MLR to confirm the results observed in Fig. 9B that T3 could induce hyporesponsiveness in T cells in 2'MLR. Next, T3 and T4 were then added into an independently set-up MLR containing naive CD4^+ T cells and GM-CSF/IL-4-induced DCs to assay whether T3 could suppress MLR. Results from four donors revealed individual variation. Half of the donor-derived mature M-DCs induced regulatory T cells (Fig. 11, left), whereas the other half of the mature M-DCs induced T cell anergy, but no regulatory T cells (Fig. 11, right). As shown in Fig. 11, T3 after priming with mature M-DC does not respond well to restimulation by GM-CSF/IL-4 (Fig. 11, top). Furthermore, T3 efficiently suppressed the independently set-up MLR by 70% (Fig. 11, left) and 74% (data not shown), demonstrating that T3 contains regulatory T cells for the two donors. For the other two donors, as shown in Fig. 11, T3 after priming with mature M-DC did not respond well to restimulation by GM-CSF/IL-4-induced DC in 2'MLR (top). However, T3 only suppressed the independently set-up MLR by 13.4% (Fig. 11, right part) and 7.4% (data not shown), and T3 and naive T cells proliferated at the same level as

(cultured with immature DC).
naive T cells alone (Fig. 11, bottom right), demonstrating that T3 only induced hyporesponsiveness, but not regulatory T cells. In contrast, T4 prestimulated with mature DC proliferated well in 2°MLR and failed to become hyporesponsive T cells.

Discussion
In this study, we demonstrate that M-CSF, combined with IL-4 induces highly purified CD14+ cord blood monocytes to differentiate along an alternative pathway into a type of accessory cell with DC features including DC phenotype and high efficiency for FITC-dextran uptake. Monocytes cultured with M-CSF/IL-4 express decreased levels of CD40, a critical cognate molecule for DC activation (56) and Th1 (Tc1) immune responses (57, 58), and decreased levels of B7-H1, a costimulatory molecule for T cell proliferation (59); their mature form has decreased alloreactivity compared with DCs grown with GM-CSF and IL-4 and induced decreased responsiveness of T cells in secondary MLR. Different from typical macrophages, they do not express monocyte/macrophage markers CD14 and CD64 and possess low phagocytosis (G. Li and H. E. Broxmeyer, unpublished observations), likely due to the known inhibitory effect of IL-4 on macrophage function (60). Because they are different from typical macrophages as well as DCs, we have termed this type of accessory cell M-DC in contrast to GM-CSF/IL-4-induced DCs and M-CSF-induced macrophages. Consistent with our previous report (61), no osteoclast-like multinucleated giant cells appeared in 1 wk of culture with M-CSF and IL-4. M-CSF/IL-4 has been reported to induce the generation of a large amount of multinucleated giant cells in 2 wk of cell culture (51). However, our culture system used much lower concentrations (around 500-fold lower) of M-CSF than the previous report (51).

M-CSF-induced macrophages produced increased levels of IL-10, but no IL-12 (21). As IL-4 is required for differentiation into M-DCs, we compared production of IL-10 and IL-12 between M-DCs and DCs. In contrast to DCs, M-DCs produce high IL-10, but...
FIGURE 11. Single time stimulation with cord blood mature M-DC induces CD4+ regulatory T cells (left) or hyporesponsiveness in naive CD4+ T cells (right). T3 and T4 were naive CD4+ T cells (donor A) prestimulated with mature M-DC and DC (donor B) for 7 days, respectively. T3 and T4 (2.5 × 10⁶) restimulated with GM-CSF/IL-4-induced DC in 2°MLR, and T3 but not T4 induced hyporesponsive T cells (top). In another experiment, T3 and T4 (2.5 × 10⁶) were added into another MLR containing naive CD4+ T cells (5 × 10⁴; donor A), and GM-CSF/IL-4-induced DC (5 × 10⁴; donor B) (bottom). Proliferation of T cells was determined by [³H]thymidine incorporation after 6 days of culture. Data are two representative of four independent experiments. Percentages shown represent a decrease.

no IL-12, a pattern similar to previously reported M-CSF-induced macrophages. Therefore, IL-4 does not neutralize production of IL-10 and IL-12 by M-CSF-induced macrophages. This finding is in contrast with GM-CSF-induced macrophages producing minimal or no IL-12, although GM-CSF/IL-4-induced DCs produce high levels of IL-12 (21).

The M-DC phenotypes were reproducible in adult monocytes cultured with M-CSF and IL-4 with regard to the expression of CD83, CD40, CD86, and HLA-DR, CD14, CD64 (data not shown). Consistent with our previous report (32) and described in this study (our unpublished observations) on adult blood monocytes, cord blood M-DCs before their maturation did not show obvious deficiencies in induction of MLR and in driving Th1 differentiation in comparison with cord blood DCs (data not shown). Stimulating once with both types of cord blood-derived DCs (M-DC and DC) potently induced the appearance of regulatory T cells (Fig. 10). However, after receiving maturation signals, M-DCs act as anti-inflammatory accessory cells and induce hyporesponsiveness in T cells in contrast to mature DCs. These features of M-DCs might make them a more effective and safer candidate for the purpose of induction of tolerance. Although immature DCs cultured with GM-CSF and IL-4 induce anergy (9), the tolerogenic ability of immature DCs may not be applicable for induction of tolerance in vivo because immature DCs could receive maturation signals in vivo and transform into mature DCs, therefore inducing immune reactions instead of tolerance. M-DCs produce large amounts of IL-10 upon activation signals. Furthermore, their mature form induces hypo- or low responsiveness in T cells and would be less likely than those of GM-CSF-derived DCs to induce inflammatory T cell responses. By producing IL-10 and inhibiting efficient T cell responses to foreign Ag, M-CSF-induced DCs might provide an immune environment favorable for blocking the host immune responses to foreign Ags. These features of M-DCs make them a possible candidate for the purpose of induction of tolerance for the treatment of transplantation rejection. Our study also supports the hypothesis that there is a subset of high IL-10-producing DCs that could induce tolerance regardless of their maturation state, and M-CSF might be an important cytokine for the development of such types of DCs.

IL-10 is a multifunctional cytokine acting on many hematopoietic cell types. The primary function of IL-10 appears to be inhibition of inflammatory immune responses. IL-10 treatment of DCs induces a state of anergy in alloantigen or peptide Ag-activated T cells (62). IL-10-producing DCs have been identified in Pey er’s patches and liver in mice; this population of DCs is associated with development of either T cell anergy or Th2 responses (63, 64). In vitro CD34+ stem/progenitor cell-derived DCs fail to produce IL-10, and CD14+ monocyte-derived DCs only produce low levels of IL-10 (65, 66). M-DCs produce large amounts of IL-10; this population of accessory cells could be a novel type of DCs similar to the in vivo-identified IL-10-producing DCs capable of induction of T cell anergy in vivo (10, 11).

M-CSF-induced monocyte-to-DC differentiation appears to be GM-CSF-independent. In contrast to GM-CSF, M-CSF is constitutively detected in serum of healthy donors and its level is greatly elevated in serum, most tissues and especially in the uterus (1000-fold increase) during pregnancy (22, 23). Furthermore, higher serum levels of M-CSF are detected in neonatal cord blood than in adult blood (23, 24). Pregnancy correlates with skewed Th2-type cells and suppressive immune responses that favor the development of maternal T cell tolerance (48). The unique characteristics of the cord blood immune system also leads to a relatively low incidence of GVHD when cord blood is used in transplantation (40). High M-CSF levels detected in pregnancy and in cord blood might induce the appearance of more M-DCs with tolerogenic potential as described in our study, thus suppressing immune reactions during pregnancy and the neonatal period. We hypothesized that because cord blood monocytes have been pre-exposed to a rich cytokine environment (M-CSF-high and IL-4) during pregnancy and might have been preprogrammed to differentiate along the M-DC pathway before being cultured in vitro with GM-CSF and IL-4 to generate DCs, this might lead to defects in IL-12 production and induction of Th1 differentiation by cord blood-derived DCs in comparison with adult blood monocyte-derived DCs.

M-CSF released by renal cell carcinoma cell lines inhibits differentiation of CD34+ progenitors into DCs. The combination of M-CSF and IL-6 also inhibits DC differentiation. Interestingly, IL-4 reverses the inhibitory effect of M-CSF on the phenotypic and functional differentiation of CD34+ progenitors into DCs (28, 67). Our results suggest an alternative mechanism of M-CSF in DC differentiation. Instead of blocking DC differentiation, M-CSF, in combination with IL-4, might induce generation of suppressive DCs, resulting in nonresponsiveness of the immune system to tumors.

DC function is intimately linked to their capacity to migrate (52). Due to the rarity of human DCs in vivo, most studies took
advantage of in vitro GM-CSF-generated DCs to characterize DC migration properties (55). Consistent with previous reports on adult monocyte-derived DCs, immature DCs expressed CCR5, low amounts of CXCR4, but no CCR6 (55, 68). Maturation induced the up-regulation of CXCR4 and the production of CCR5 ligand, MIP-1β and rapidly down-modulated expression of CCR6 (68), possibly due to internalization of CCR5. In contrast to regular DCs, M-DCs expressed a minimal level of CCR5 regardless of maturation state. Immature M-DCs expressed a decreased level of CCR5 in comparison with DCs. Therefore, due to the DC heterogeneity, M-DCs might locate distinctly from DCs and function to suppress efficient immune reactions. These locations might include tumor stroma, placenta, bone marrow, etc. in which M-CSF is constitutively produced (22–24, 28, 69). It is noted that both M-DC and DC, after LPS-induced maturation, failed to express CCR6 and CCR7 (n = 3, data not shown), chemokine involved in homing of DCs in lymphoid organs (52–55). Whether this is a special feature, an intrinsic homing defect of cord blood DCs, or a mechanism of nonefficient immune responses in cord blood are not yet known.

Using cord blood monocytes, cells minimally or not exposed to pathogens, tumors, and the aging process, we have noted a novel pathway of monocyte differentiation and have identified and characterized a special type of IL-10high-producing anti-inflammatory accessory cells with DC features. As mentioned, adult blood monocytes can be induced with M-CSF and IL-4 into phenotypic M-DCs. It remains to be determined whether adult bone monocytes have a similar capability for differentiation into functional M-DCs. This finding may be of relevance to DC-based therapeutic strategies that are expanded by c-Kit ligand and yield pure dendritic cell colonies in the presence of granulocyte/macrophage colony-stimulating factor and tumor necrosis factor α. J. Exp. Med. 182:1111.


