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CD137 is a member of the TNFR family, and reverse signaling through the CD137 ligand, which is expressed as a cell surface transmembrane protein, costimulates or activates APCs. CD137 and CD137 ligand are expressed on small subsets of bone marrow cells. Activation of bone marrow cells through CD137 ligand induces proliferation, colony formation and an increase in cell numbers. Compared with total bone marrow cells, the small subpopulation of progenitor cells that express no lineage markers but express CD117 cells (or Lin⁻, CD117⁺ cells) responds with the same activities to CD137 ligand signaling, but at a significantly enhanced rate. Concomitantly to proliferation, the cells differentiate to CFU granulocyte-macrophage and CFU macrophage, and then to monocytes and macrophages but not to granulocytes or dendritic cells. Hematopoietic progenitor cells differentiated in the presence of CD137 protein display enhanced phagocytic activity, secrete high levels of IL-10 but little IL-12 in response to LPS, and are incapable of stimulating T cell proliferation. These data demonstrate that reverse CD137 ligand signaling takes place in hematopoietic progenitor cells, in which it induces proliferation, an increase in cell numbers, colony formation, and differentiation toward monocytes and macrophages. The Journal of Immunology, 2008, 181: 3923–3932.

An alternative to an ex vivo amplification and potentially differentiation of HSC and progenitor cells is the administration of hematopoietic growth factors to patients. G-CSF is being used to reconstitute the hematopoietic system of cancer patients after chemotherapy or radiation therapy, whereas erythropoietin and thrombopoietin are being used for the treatment of anemia and thrombocytopenia, respectively (4, 5).

The cytokine receptor CD137 (ILA/4-1BB) is a member of the TNFR family and is expressed dependent of activation by T cells (6, 7). Costimulation through CD137 enhances T cell activity, enabling the immune system to reject tumors or allogeneic transplants (8, 9). Interestingly, CD137 agonists can also dampen immune responses and ameliorate autoimmune diseases under certain conditions (10, 11).

The ligand for CD137 is expressed by APCs as a transmembrane protein, and it also can deliver signals into the cells it is expressed on. Therefore, bidirectional signaling exists for the CD137 receptor/ligand system (12). Signaling through CD137 ligand enhances proliferation and Ig secretion of B cells (13) and the expression of costimulatory molecules, cytokines, and cellular adhesion in dendritic cells (DC) (14–16). In peripheral monocytes it induces a specific signaling cascade (17) leading to activation, proliferation of survival, and migration (18–23). Monocytes are generated in the bone marrow, enter the circulation from where they migrate into the tissues, and differentiate to tissue macrophages. There they participate in the regulation of inflammatory and immune reactions, and in physiological processes such as wound healing (24). Peripheral monocytes and macrophages were regarded as not being able to proliferate. However, CD137 ligand signaling induces proliferation and endomitosis in these mature cells, and CD137 is so far the only known factor with this activity (19).

We therefore hypothesized that CD137 ligand signaling may also regulate proliferation of the progenitor cells of monocytes. In this study we show that CD137 ligand signaling induces proliferation and colony formation of hematopoietic progenitor cells and differentiation to CFU granulocyte-macrophage (CFU-GM). From
there, cells differentiate to monocytes and macrophages but not to DC.

Materials and Methods

**Mice**

Female BALB/c mice between 16 and 20 wk of age were used as a source of bone marrow cells. Animals were specific pathogen-free, and kept with free access to food and water in the animal care facility at the National University of Singapore under the institutional guidelines for usage of experimental animals.

**Isolation of bone marrow and lineage-negative (Lin−, CD117+) cells**

The femur bones of BALB/c mice were dissected, and the bone marrow was flushed out by PBS with 2 mM EDTA using a 10-ml syringe and 27-gauge needle aseptically. Total bone marrow cells were passed through a 30-μm filter (Miltenyi Biotec), washed with PBS with 2 mM EDTA, and resuspended in RPMI 1640 (Sigma-Aldrich) with 10% FBS.

The Lin− and CD117+ progenitor cells were isolated by immunomagnetic separation (MACS) using the mouse lineage cell depletion kit and mouse CD117 selection kit (Miltenyi Biotec), following the manufacturer’s instructions. Briefly, the fresh bone marrow cells were labeled with a mix of biotinylated Abs against a panel of Ags (CD5, CD45R (B220), CD11b, anti-Ly-6G (Gr-1), 7–4, and Ter119 Abs) expressed on mature cells, followed by anti-biotin microbeads. The cell suspension was passed through an LD column in a strong magnetic field, and the Lin− cells were collected in the effluent. These Lin− cells were then positively selected for CD117 expression using CD117 microbeads. The purity of Lin−, CD117+ cells was >90% as determined by flow cytometry using a FITC-conjugated anti-CD117 Ab and an allophycocyanin-conjugated anti-biotin Ab (Miltenyi Biotec).

**Recombinant proteins and chemicals**

Recombinant human CD137-Fc protein was purified from supernatants of stable transfected CHO cells by protein G-Sepharose, as previously described (25). The endotoxin concentration in the CD137-Fc protein is 55 IU/mg. Human IgG1 Fc protein was purchased from Accurate Chemical and Scientific. Recombinant murine GM-CSF, IL-4, M-CSF, and G-CSF were purchased from PeproTech. LPS was obtained from Sigma-Aldrich.

**Abs and flow cytometry**

PE-conjugated and unconjugated anti-mouse CD137 Ab (clone 17B5) and anti-mouse CD117 ligand Ab (clone TKS-1) were obtained from eBioscience. PE- or FITC-labeled rat anti-mouse CD3e, CD11b, CD11c, CD14, CD19, F4/80, Ly-6G (Gr-1), CD117 (c-kit), MHC class II, and respective isotype controls (rat IgG2a, rat IgG2b, Armenian Hamster IgG) were purchased from eBioscience. Neutralizing anti-mouse GM-CSF (clone MPI-22E9) and its isotype control rat IgG2a (clone RTK2758) were purchased from BioLegend.

Flow cytometry was performed either on a FACSCalibur (BD Biosciences) with CellQuest data acquisition and analysis software, or on a cyan flow cytometer (DAKOcytometry) with Summit software. Nonspecific staining was controlled by isotype-matched Abs.

**CFSE labeling**

Fresh bone marrow cells at a concentration of 106 cells/ml were labeled with 5 μM CFSE, using the CellTrace CFSE Cell Proliferation kit (Invitrogen), according to the manufacturer’s protocol. Labeled cells cultured in plate precoated with 10 μg/ml Fc or CD137-Fc protein. The cells were harvested on days 7 and 14, and analyzed by flow cytometry.

**Manual cell count**

Cells were harvested after incubation with 10 mM EDTA for 10 min. Cells were centrifuged and resuspended in PBS. The number of viable cells was assessed by 0.4% trypan blue (Sigma-Aldrich) staining and counted using a hemocytometer (Neubauer).

**Proliferation assay**

Cell proliferation was determined by [3H]thymidine incorporation. Cells were pulsed with 0.5 μCi of [3H]thymidine (PerkinElmer) for the last 24 h of the culture period. The cells were then harvested onto a Packard Unifilter Plate using a MicroMate 196 Cell Harvester and counted using a TopCount Microplate Scintillation Counter (Packard Instrument).

**Colonies-forming assay**

Freshly isolated bone marrow cells at density of 5 × 10^4 cells/ml or Lin−, CD117+ cells at density of 10^5 cells/ml were cultured in 35-mm dishes in triplicates in 1 ml of semisolid methylcellulose-based medium (MethoCult SF-3HTM3236; StemCell Technologies) supplemented with 10% bovine serum. Dishes were cultured at 37°C in a humidified atmosphere containing 5% CO₂. Colonies were counted under the microscope at day 14 for bone marrow cells and day 7 for Lin−, CD117+ cells. The number of colonies consisting of more than 50 cells was counted, and the average number of colonies and SDs were calculated from triplicate plates. The types of colonies were identified based on their morphological characteristics (26, 27).

**Immunocytochemistry**

A total of 2–5 × 10^5 cells were smeared onto the TBS-coated glass slides. The air-dried slides were fixed with ice-cold absolute methanol. After blocking with 3% skim milk for 30 min and 3% H₂O₂ for 10 min, the slides were incubated with 1 μg/ml biotinylated primary Abs for 1 h and subsequently with 1 μg/ml streptavidin-peroxidase polymer (Sigma-Aldrich) for 30 min. After three washes, the slides were incubated with liquid diamobenzidine-positive substrate (DakoCytomation) for 5–10 min in the dark. Then the slides were counterstained with hematoxylin (Sigma-Aldrich), and mounted in DPX mounting medium (Sigma-Aldrich). All incubations were done at room temperature.

**Allogeneic MLR**

Bone marrow cells were isolated from femurs of 12-wk-old BALB/c mice. A total of 2 × 10^4 bone marrow cells or 5 × 10^5 Lin−, CD117+ cells/well were cultured in plates that had been precoated with 10 μg/ml Fc or CD137-Fc protein. In parallel, cells were treated with 100 ng/ml GM-CSF plus 25 ng/ml IL-4 (Peprotech) to generate immature DC. Maturation of DC was induced on day 6 by addition of 1 μg/ml LPS (Sigma-Aldrich). Cells were treated with 50 μg/ml mitomycin C at 37°C for 2 h to disable the proliferation on day 7. Subsequently, cells were harvested by incubation with 10 mM EDTA at room temperature for 10 min, washed twice with PBS, and served as stimulator cells. CD4+ T cells were isolated from splenocytes of 8-wk-old C57BL/6 mice by magnetic selection using the mouse CD4 MicroBead kit (Miltenyi Biotec) to a purity of >99%, as analyzed by flow cytometry for CD4. A total of 10^5 CD4+ T cells were cocultured with stimulator cells at a 1:10 ratio in 96-well round-bottom plates for 3 days and pulsed with 0.5 μCi of [3H]thymidine per well for the last 18 h. The rate of T cell proliferation was determined with a scintillation counter (Packard Instrument).

**ELISA**

The concentrations of IL-10 and IL-12p70 in cell supernatants were determined by mouse IL-10 and IL-12 (p70) ELISA MAX sets (BioLegend) according to the manufacturer’s instructions. All measurements were done in triplicate.

**Photographs**

Morphological changes of cells were documented by using a Zeiss Axiovert 40 inverted microscope and Canon PowerShot G6 digital camera.

**Statistics**

Statistical significance was determined by the two-tailed unpaired Student’s t test.

**Results**

**CD137 ligand signaling regulates survival and proliferation of murine bone marrow cells**

Because CD137 agonists (recombinant CD137 protein and anti-CD137 ligand Abs) have been shown to induce proliferation and endomitosis in peripheral monocytes, a cell population that was believed not to be able to proliferate (19, 22), we hypothesized that they may also be able to induce proliferation in the precursor of monocytes, the hematopoietic progenitor cells.

Bone marrow cells were isolated from the femur bones of BALB/c mice. The cells were cultured on tissue culture plates that had been coated with a fusion protein consisting of the extracellular domain of human CD137-Fc and the constant domain of human IgG1 (Fc). Fc protein was used as a negative control. Coating
was performed overnight at 4°C, and plates were washed with PBS afterward. Cell numbers were determined by counting using a hemocytometer and viability was determined by trypan blue staining twice a week. A total of $2 \times 10^6$ bone marrow cells were plated per condition, and ~90% of them died during the first 3 days. But from day 3 onward, the number of live cells started to increase in the wells that had been coated with CD137-Fc protein until day 10, after which the live cell number declined again. By that time, all cells in the other conditions had died. This result and the decline of cell numbers in the CD137-Fc-treated wells after day 10 may be due to exhaustion of nutrients.

But more live cells were present in the CD137-Fc-coated wells compared with Fc-coated wells or untreated wells (PBS) throughout the experiment. Even at day 21 when the experiment

**FIGURE 1.** CD137 ligand cross-linking induces survival and proliferation of bone marrow cells. Murine bone marrow cells at a density of $10^6$ cells/ml were cultured on plates coated with indicated concentrations of CD137-Fc or Fc protein or on uncoated plates (PBS). A, Time course for cells cultured on tissue culture plates that had been coated with a fusion protein consisting of the extracellular domain of human CD137-Fc and the constant domain of human IgG1 (Fc). Fc was used as a negative control. A total of $2 \times 10^6$ cells were plated, and the number of live cells was determined by trypan blue staining and counting at indicated time points. PBS (squares), Fc (10 µg/ml) (ovals), CD137-Fc (10 µg/ml) (diamonds), and G-CSF (0.1 ng/ml daily) (triangles) are shown. Results shown are mean ± SD of four countings. B, Dose response for experimental conditions shown in A, but the number of live cells was determined at day 12. C and D, $[^3]$H]thymidine incorporation of $10^6$ bone marrow cells (C) or $5 \times 10^5$/well Lin$^-$/CD117$^+$ cells (D) were cultured for 7 days and labeled for the last 24 h with 0.5 µCi of $[^3]$H]thymidine. The rate of proliferation was determined with a scintillation counter (Packard Instrument). Results shown are mean ± SD of triplicate measurements. E, Tracking of cell division. Freshly isolated bone marrow cells were either not treated or stained with 5 µM CFSE and were immediately (day 0) analyzed by flow cytometry. CFSE-stained bone marrow cells were incubated for 14 days on immobilized Fc or CD137-Fc protein, or were treated daily for 14 days with 0.1 ng/ml G-CSF and analyzed by flow cytometry for CFSE fluorescence. Histograms depict the percentage of CFSE-negative cells gated in M1 region. *, p < 0.01. All experiments were performed at least three times with comparable results.
was terminated, CD137-treated conditions contained >10^5 live cells. G-CSF that had been added daily to the cells until day 21 resulted in a less rapid decline in cell numbers during the first 7 days, but had no effect afterward (Fig. 1A). Enhancement of cell numbers by CD137 protein was dose-dependent. Saturation was reached at a coating concentration of 20 g/ml CD137-Fc, but a significant enhancement in live cell numbers could already be observed at 1.2 g/ml (Fig. 1B).

The increase in the number of living cells by CD137-Fc could be due to survival, proliferation, or a combination of both. We determined therefore the rate of CD137-induced proliferation, by measuring DNA synthesis and cell division, using [3H]thymidine and CFSE labeling, respectively. Concentrations of Fc and CD137-Fc protein ranging from 0.15 to 40 μg/ml were used to coat plates before 10^5 bone marrow cells were added for 7 days. CD137-Fc induced a significant proliferation at concentrations as low as 1.2 μg/ml and reached its maximum activity at 20 μg/ml. Proliferation of cells in the Fc-coated wells was not different from that of cells in uncoated wells (PBS) (Fig. 1C). Both, the number of live cells and the [3H]thymidine incorporation rate exhibited an identical dose-dependency. CD137-Fc protein needs to be immobilized onto the tissue culture plates to induce proliferation and survival of bone marrow cells. CD137-Fc in its soluble form is not active. When CD137-Fc was added to the cells as a soluble protein and its immobilization to the tissue culture plates was prevented by precoating the plates with serum, no increase in cell numbers was observed (Fig. 1C).

CD137-Fc protein needs to be immobilized onto the tissue culture plates to induce proliferation and survival of bone marrow cells. CD137-Fc in its soluble form is not active. When CD137-Fc was added to the cells as a soluble protein and its immobilization to the tissue culture plates was prevented by precoating the plates with serum, no increase in cell numbers was observed (Fig. 1C). This demonstrates that CD137-Fc works by cross-linking CD137 ligand on bone marrow cells or a subset of these cells, and excludes that the observed effects are due to potential contaminants in the protein preparation.
The subset of bone marrow cells that is considered to be most enriched for hematopoietic progenitor cells is negative for lineage markers and positive for CD117 cells (c-kit), the receptor for stem cell factor. Lin\(^-\), CD117\(^+\) cells constitute only 0.5–1% of bone marrow cells. CD137-Fc increased proliferation of Lin\(^-\), CD117\(^+\) cells to the same extent (about 5-fold) found in total bone marrow cells, and soluble CD137-Fc was inactive (Fig. 1D). But total cell proliferation was \(\sim\)10-fold higher in Lin\(^-\), CD117\(^+\) cells than in unfractionated bone marrow cells.

A total of 36 \(\times\) 10\(^6\) bone marrow cells were labeled with 5 \(\mu\)M CFSE. Labeling efficacy was verified by flow cytometry and the area of CFSE-negative cells was defined as in the M1 region (Fig. 1E). Only 0.9% of the CFSE-labeled cells were in M1. The CFSE-labeled cells were cultured for 14 days on immobilized CD137-Fc or Fc protein, or treated daily with 0.1 ng/ml G-CSF. Fluorescence of CFSE-labeled bone marrow cells that were cultured in the presence of Fc control protein had diminished by day 14, indicating some cell division had occurred but only 0.9% of the cells were in

![Figure 3](image-url)
M1, which was the same percentage as for freshly labeled cells (Fig. 1E). In contrast, CD137-Fc induced strong proliferation causing 44% of the cells to shift into the M1 region. G-CSF also induced cell division, and 9.1% of the G-CSF-treated cells were in M1 (Fig. 1E).

CD137 ligand signaling induces colony formation in hematopoietic progenitor cells

Proliferation induced by CD137 ligand stimulation was also confirmed by colony assays. Bone marrow cells were cultured for 14 days in methylcellulose medium containing 10% bovine calf serum on tissue culture plates with immobilized CD137-Fc or Fc protein. There were on average only 3 ± 1 colonies in the Fc-coated dishes compared with 40 ± 10 colonies in the CD137-Fc-coated dishes. In addition, colonies on Fc protein contained fewer than 100 cells, whereas colonies on CD137-Fc protein were composed of up to 5000 cells (Fig. 2, A and B).

The Lin\(^{−}\), CD117\(^{+}\) cells formed colonies at a much higher rate than bone marrow cells. Because Lin\(^{−}\), CD117\(^{+}\) cell colonies had grown to confluency by day 14, they had to be assessed already at day 7 (Fig. 2, C–F).

CD137 induced similar looking colonies in total bone marrow cells as in Lin\(^{−}\), CD117\(^{+}\) cells, implying that it was the Lin\(^{−}\), CD117\(^{+}\) cells among the bone marrow cells that responded to CD137. Most colonies (80.8 ± 1.9%) were colorless with dark, dense cores or consisted of granular or foamy cells. Based on these morphological characteristics, these colonies were identified as CFU-GM, (26, 27). CFU-macrophages (CFU-M), recognizable by their larger and individually identifiable cells, were also induced by CD137 and constituted 16.9 ± 1.4% of the colonies. Very few (2.3 ± 0.5%) CFU granulocytes, smaller and more tightly packed cells, could be identified (Fig. 2, D–F). No additional growth factors besides CD137 were present in this assay to support colony formation.

Because CD137 ligand signaling induced formation of myeloid colonies and of cells with myeloid morphology, it was possible that CD137 protein exerted its activities through induction of GM-CSF. However, a neutralizing anti-GM-CSF Ab had no influence on the appearance, proliferation rate (Fig. 2G), nor on the morphology (data not shown) or number and size of colonies of Lin\(^{−}\), CD117\(^{+}\) cells (Fig. 2H).

CD137 ligand signaling changes the morphology of murine bone marrow cells

Concomitant with survival and proliferation CD137-Fc protein induced adherence and morphological changes. These morphological changes were of the same type in the subpopulation of whole bone marrow cells that survived until day 7 (Fig. 3A) as in the Lin\(^{−}\), CD117\(^{+}\) cells (Fig. 3B). However, the magnitude of the response was greater in Lin\(^{−}\), CD117\(^{+}\) cells, as a higher percentage of the Lin\(^{−}\), CD117\(^{+}\) cells responded to the CD137 ligand cross-linking. Many more cells attached to CD137-Fc-coated plates than to the Fc-coated or untreated control plates (Fig. 3B). During the first 3 days, CD137-Fc induced spreading in the attached cells, and some grew a tail-like extension on one end and lamellopodia on the opposite end. By day 7, the tails had grown longer, sometimes reaching five times the length of the cell body, whereas the lamellopodia were changing to filopodia. Some cells had several branches and displayed star-like shapes. From days 10–17, some cells adopted round and flat ovoid shapes. Several morphological characteristics described were coexisted in the culture. From day 21 onward, filopodia disappeared and tails shortened, the cells adopted spindle-like shapes (Fig. 3B). The appearance of the tail-like extensions and the lamellopodia and filopodia coincided with the phase of cell growth. They disappeared after day 21 when the cells stopped proliferating and the cell numbers.
started to decline. Soluble CD137-Fc protein did not induce such morphological changes (Fig. 3B).

**Expression of CD137 and CD137 ligand on bone marrow cells**

The described proliferative response of bone marrow cells to CD137 ligand agonists implied CD137 ligand expression by bone marrow cells. Furthermore, if the CD137 ligand induced proliferation were to occur in the bone marrow, then CD137 should also be expressed there.

Indeed, CD137 ligand is expressed constitutively by a subset of murine bone marrow cells. By immunocytochemical staining with a polyclonal or a monoclonal anti-CD137 ligand Ab, 8.9 \( \pm \) 3.8%
or 6.8 ± 0.7% of freshly isolated and untreated bone marrow cells, respectively, were found to express CD137 ligand (Fig. 4A). Also, the expression of CD137 on a few (4.2 ± 0.4%) but distinct number of cells in the murine bone marrow was detected immunocytochemically (Fig. 4B). Expression of CD137 and CD137 ligand mRNAs could also be verified by RT-PCR (data not shown).

**CD137 ligand signaling induces differentiation to monocytic cells**

Induction of colony formation, especially of the CFU-GM type had already implied that CD137 ligand signaling induces differentiation of bone marrow cells as well as of Lin−, CD117<sup>+</sup> cells to the myeloid lineage. We confirmed this by analyzing cell marker expression by flow cytometry. The Lin−, CD117<sup>+</sup> cells were entirely depleted of mature cells assuring that any mature cells present after CD137 ligand stimulation would necessarily have to have differentiated from hematopoietic progenitor cells (Fig. 5A). Lin−, CD117<sup>+</sup> cells cultured for 7 days in the presence of immobilized CD137-Fc protein contained around 30 – 40% of CD11b<sup>+</sup>, CD14<sup>+</sup>, F4/80<sup>+</sup>, or CD11c<sup>+</sup> cells, whereas expression of these markers in the Fc controls was negligible (Fig. 5B). The number of live cells expressing these myeloid markers was enhanced accordingly by CD137 ligand signaling (Fig. 5C).

Total bone marrow cells cultured on immobilized CD137-Fc protein also had an increase in CD11b<sup>+</sup>, CD14<sup>+</sup>, F4/80<sup>+</sup>, or CD11c<sup>+</sup> cells, although mature myeloid cells such as monocytes and macrophages, among total bone marrow cells that are known to respond to CD137 ligand signals with survival and proliferation (19, 20, 22), may have contributed in that case (data not shown).

The identical nature of CD137 effects on total bone marrow cells and Lin−, CD117<sup>+</sup> cells, and the fact that the Lin−, CD117<sup>+</sup> cells respond more strongly than the total bone marrow cells suggest that the small subpopulation in the bone marrow cells that responded to CD137-Fc protein is largely contained within Lin−, CD117<sup>+</sup> cells.

**CD137 ligand signaling induces macrophage differentiation**

The comparable number of cells expressing macrophage markers (CD14, F4/80) and DC markers (CD11c) that CD137 ligand signal had induced in Lin−, CD117<sup>+</sup> cells was compatible with macrophage and DC differentiation. To clarify the nature of cells that had been derived from hematopoietic progenitor cells after differentiation with CD137 protein, we assessed their biological activities. Lin−, CD117<sup>+</sup> cells that were exposed for 7 days to CD137-Fc exhibited an enhanced phagocytosis compared with Fc-treated cells (28.1% vs 57.9%) (Fig. 6A). Cells that had been treated with M-CSF as a positive control for inducing macrophage differentiation displayed a higher phagocytic activity than Fc-treated control cells (34.2% vs 28.1%), but surprisingly were not as active as CD137-Fc-treated cells (34.2% vs 57.9%). This increased phagocytic activity suggested a macrophage differentiation.

Because only ~60% of the Lin−, CD117<sup>+</sup> cells that had been differentiated with CD137-Fc were phagocytically active, and because CD137-Fc treatment had induced expression of CD11c, it
was possible that some of the CD137-Fc-treated Lin−, CD117+ cells had differentiated to DC. We tested this possibility by using CD137-Fc-treated cells as stimulator cells in an allogeneic MLR. CD137-Fc-treated cells did not enhance T cell proliferation and neither did the Fc control protein nor M-CSF (Fig. 6b). However, cells that had been treated with GM-CSF plus IL-4, standard conditions for DC generation, induced significant T cell proliferation (Fig. 6b).

DC also differ from macrophages in their ability to secrete IL-12 (28). Fc-, CD137-Fc-, or M-CSF-treated Lin−, CD117+ cells secreted large amounts of IL-10 upon LPS stimulation but little IL-12 (Fig. 6c). GM-CSF plus IL-4-treated cells had the reverse pattern with high levels of IL-12 and low levels of IL-10. CD137 ligand signals enhanced IL-10 and reduced IL-12 slightly but significantly (Fig. 6c). High levels of IL-10 and low IL-12 secretion of CD137-Fc-treated Lin−, CD117+ cells is in agreement with the enhancement of phagocytosis and the inability to induce T cell proliferation in an allogeneic MLR, and demonstrate that CD137 ligand signaling induces differentiation to macrophages but not to DC.

**Discussion**

The CD137 receptor/ligand system is involved in a number of different immune activities (8, 9, 29), and reverse signaling by CD137 ligand has been documented in monocytes, DC, B cells, and T cells where it has been shown to regulate activation, survival proliferation, and apoptosis (12). Our current study adds murine hematopoietic progenitor cells to this list, and demonstrates that CD137 ligand signals also regulate hematopoiesis.

CD137 protein promoted survival, colony formation, and proliferation of hematopoietic progenitor cells, and was more potent than G-CSF. The activity of CD137 lasted for more than 3 wk after a single dose, whereas the short half-life of G-CSF required it to be added daily. CD137-induced proliferation of bone marrow cells and Lin−, CD117+ cells resulted in an increase in cell number and in colony formation. The colonies formed in response to CD137 ligand signaling were CFU-GM and CFU-M, i.e., myeloid precursor cells, indicating that CD137-induced proliferation was accompanied by a differentiation of bone marrow cells toward the myeloid lineage. Myeloid differentiation was supported by the increase in CD11b expression.

In Lin−, CD117+ cells, equal amounts of macrophage (CD14, F4/80) and DC (CD11c) markers were induced by CD137 protein. But despite induction of CD11c expression, CD137 ligand signals did not direct differentiation toward DC, as shown by the inability of CD137-exposed cells to induce T cell proliferation in a MLR, and to secrete IL-12. Instead, high levels of IL-10 and increased phagocytic activity show that CD137 ligand signaling induces macrophage differentiation in hematopoietic progenitor cells (28). The spindle-like morphology of the cells is also more consistent with a macrophage than a DC differentiation. The differentiative influence of CD137 ligand signaling toward macrophages was specific because it did not enhance the number of lymphoid, megakaryocytic, or erythroid cells.

CD137 ligand cross-linking has been reported previously to support DC maturation. In these studies, however, DC were derived from human umbilical cord blood CD34+ cells or peripheral monocytes via culture with cytokine mixtures for 1–7 days before exposure to CD137 protein (14–16). Our findings are not contradictory to these reports, but rather they demonstrate wider biological activities of CD137 in myelopoiesis. Although CD137 enhances maturation of immature DC derived from hematopoietic progenitor cells, it will induce macrophage differentiation in hematopoietic progenitor cells when given as a first and sole signal.

A role of the CD137 receptor/ligand system in hematopoiesis is also evident from gene modified mice and mice treated with anti-CD137 Abs. Mice injected with anti-CD137 Abs have a 10-fold increased the number of bone marrow cells with a HSC phenotype (30). Similarly, CD137-deficient mice have an increased number of myeloid precursor cells in bone marrow, spleen, and blood (31). It is tempting to speculate that this increase in precursor cells is caused by a missing differentiation signal due to the absence of CD137 or its blocking by the Ab. Interestingly, transgenic mice overexpressing CD137 ligand on APC have a 3-fold elevated number of macrophages (32). Anti-CD137-treated mice display additional hematopoietic defects such as thrombocytopenia, anemia, and lymphopenia (30). The underlying mechanisms for these effects are currently unknown.

Our data are partly in agreement with earlier studies that demonstrated that CD137 protein in combination with M-CSF increases proliferation of bone marrow-derived osteoclast progenitors, but inhibits M-CSF plus receptor activator of NF-κB ligand (RANKL)-induced osteoclast differentiation (33, 34). Some of the osteoclast progenitor cells that proliferated in response to CD137 plus M-CSF in the study by Saito et al. (33) may also have been contained within the Lin−, CD117+ cell population of our study. What is surprising, however, is that CD137 protein inhibited osteoclast differentiation as it promoted macrophage differentiation because both cell types are closely related if not identical. This difference could be due to the different starting cell populations or the presence of M-CSF.

The Lin−, CD117+ cell population contains progenitor cells of different stages in development ranging from multipotent long-term HSC to already committed progenitor cells (35–37). Long-term HSCs are defined by their ability to give rise to the lymphoid and myeloerythroid lineages for life after transplantation into lethally irradiated recipients, whereas short-term HSCs and committed CFU have more limited self-renewal capacity and are capable to respond to stimuli within 6–14 days (37, 38). The fact that CD137 protein induced proliferation, colony formation and myeloid differentiation of Lin−, CD117+ cells before day 14, suggests that the responding cells were short-term HSCs and myeloid progenitor cells, including CFU-GM and CFU-M. Whether more primitive progenitor cells such as long-term HSCs can also be activated by CD137 will need to be determined in future studies.

The induction of proliferation, colony formation, increase in cell number, and differentiation of bone marrow cells, in particular of the Lin−, CD117+ subpopulation, identifies novel biological activities for CD137 and its ligand by demonstrating a role for this receptor/ligand system in hematopoiesis. This study extends previous knowledge that signals through CD137 ligand induce activation, proliferation, and survival in monocytes and enhance maturation of DC, thereby showing that CD137 and CD137 ligand regulate multiple aspects of myeloid cell biology.

**Disclosures**

The authors have no financial conflict of interest.

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