Generation of CD1+RelB+ Dendritic Cells and Tartrate-Resistant Acid Phosphatase–Positive Osteoclast-Like Multinucleated Giant Cells From Human Monocytes

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We previously showed that granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) stimulate the differentiation of human monocytes into two phenotypically distinct types of macrophages. However, in vivo, not only CSF but also many other cytokines are produced under various conditions. Those cytokines may modulate the differentiation of monocytes by CSFs. In the present study, we showed that CD14+ adherent human monocytes can differentiate into CD1+relB+ dendritic cells (DC) by the combination of GM-CSF plus interleukin-4 (IL-4) and that they differentiate into tartrate-resistant acid phosphatase (TRAP)-positive osteoclast-like multinucleated giant cells (MGC) by the combination of M-CSF plus IL-4. However, the monocyte-derived DC were not terminally differentiated cells; they could still convert to macrophages in response to M-CSF. Tumor necrosis factor-α (TNF-α) stimulated the terminal differentiation of the DC by downregulating the expression of the M-CSF receptor, c-fms mRNA, and aborting the potential to convert to macrophages. In contrast to IL-4, interferon-γ (IFN-γ) had no demonstrable effect on the differentiation of monocytes. Rather, IFN-γ antagonized the effect of IL-4 and suppressed the DC and MGC formation induced by GM-CSF + IL-4 and M-CSF + IL-4, respectively. Taken together, these results provide a new aspect to our knowledge of monocyte differentiation and provide evidence that human monocytes are flexible in their differentiation potential and are precursors not only of macrophages but also of CD1+relB+DC and TRAP-positive MGC. Such a diverse pathway of monocyte differentiation may constitute one of the basic mechanisms of immune regulation.

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convert to macrophages in response to M-CSF was aborted. These results provide evidence that human monocytes are flexible in their differentiation potential and are precursors not only of macrophages but also of DC and osteoclast-like MGC.

MATERIALS AND METHODS

Medium. RPMI 1640 medium (Nissui Pharmaceutical Co, Ltd, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS; Bocknek Laboratories Inc, Toronto, Ontario, Canada), 2 mmol/L L-glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin. FCS was shown to contain 0.003 ng of lipopolysaccharide per milliliter by the Limulus amebocyte lysate test.

Cytokines. Recombinant human (rh) GM-CSF (1 × 10^6 U/mg) was kindly provided by Kirin Brewery Co, Ltd (Maebashi, Japan) and Schering-Plough Japan (Osaka, Japan). GM-CSF from the two companies did not differ in activity and showed the same results. rhM-CSF (1 × 10^6 U/mg) was kindly supplied by Morigana Milk Industry Co, Ltd (Tokyo, Japan). rhTNF-α (1 × 10^6 U/mg) was supplied by Dainippon Pharmaceutical Co, Ltd (Osaka, Japan) and recombinant human interferon-γ (rhIFN-γ; 1 × 10^6 U/mg) was supplied by Toray Industries, Inc (Tokyo, Japan). rhIL-4 (1 × 10^6 U/mg) was obtained from Genzyme Corp (Boston, MA).

Preparation and culture of human monocytes. PBMC were obtained from buffy coats of normal healthy volunteers by centrifugation on a Ficoll-Metrizoate density gradient (Lymphoprep; Nycomed, Oslo, Norway) and were suspended in medium. PBMC were placed into monocyte-isolating plates (MSP plate; Japan Immunoresearch Laboratories, Co, Ltd, Takasaki, Japan) and incubated for 2 hours at 37°C in a humidified 5% CO2 atmosphere. Thereafter, the nonadherent cells were removed by repeated washing and the adherent cells were collected by vigorous pipetting. More than 97% of the recovered adherent cells were judged to be monocytes by morphology, nonspecific esterase staining (cells were stained using a kit for α-naphthyl butyrate esterase), CD14 positivity, and phagocytic ability of latex particles. Monocytes plated in flat-bottomed 96-well tissue culture plates (1 × 10^5/24-well plate; Falcon 3072) were incubated with 200 μL of culture medium with or without various concentrations of M-CSF or GM-CSF for 6 days at 37°C in 5% CO2. The cells were pulsed with 0.5 μCi (18.5Bq) of [3H]Tdr (New England Nuclear, Boston, MA) per well during the last 16 hours of culture. At the end of the culture period, the cells were harvested on glass fiber filters (Ready Filter; Beckman Instruments, Fullerton, CA). To block nonspecific FcR-mediated binding of MoAbs, cells were fixed with 0.2% paraformaldehyde (Sigma) and analyzed by cytofluorography.

Determination of monocyte DNA synthesis. Monocytes (1 × 10^5 cells) in flat-bottomed 96-well tissue culture plates (Falcon 3072) were incubated with 200 μL of culture medium with or without various concentrations of M-CSF or GM-CSF for 6 days at 37°C in 5% CO2. The cells were pulsed with 0.5 μCi (18.5Bq) of [3H]Tdr (New England Nuclear, Boston, MA) per well during the last 16 hours of culture. At the end of the culture period, the cells were harvested on glass fiber filters (Ready Filter; Beckman Instruments, Inc, Fullerton, CA) on a semiautomated sample harvester, and harvested on glass fiber filters (Ready Filter; Beckman Instruments, Fullerton, CA). To block nonspecific FcR-mediated binding of MoAbs, cells were fixed with 0.2% paraformaldehyde (Sigma) and analyzed by cytofluorography.

Determination of the number of adherent cells. The number of adherent monocytes or monocyte-derived macrophages was determined by the method described previously by Nakagawara and Nathan. Briefly, cultures were depleted of medium by gentle aspiration and replenished with 50 to 200 μL of 1% (wt/vol) cetfytrimethyl ammonium bromide (Cetavlon; Wako Pure Chemical Industries, Ltd, Osaka, Japan) in 0.1 mol/L citric acid with 0.05% (wt/vol) naphthol blue black (Sigma Chemical Co, St Louis, MO) at room temperature for 3 minutes. This treatment readily lysed the adherent cells and liberated stained intact nuclei, which were then counted by using a TATA1 hemocytometer (Kayagaki Irikakogyo, Tokyo, Japan). The results are expressed as the mean and standard deviations of triplicate wells.

Uptake of acetylated LDL (Dih-Ac-LDL). Cells were added with Dih-Ac-LDL (10 μg/mL) culture medium, acetylated low-density lipoprotein labeled with 1, 1'-diocadecyl-1-1-3, 3, 3'-tetramethyl-indo-carboxyanine perchlorate; Biomedical Technologies Inc, Stoughton, MA) and incubated for 4 hours at 37°C. After being washed, the percentage of cells labeled with Dih-Ac-LDL was determined by counting at least 300 cells using a fluorescence microscope; the results are expressed as the mean and standard deviations of triplicate experiments.

Phagocytosis assay. The phagocytosis of latex particles or EA (sheep erythrocytes sensitized with IgG of rabbit antisherp erythrocyte antiserum) was determined as described previously. Briefly, cells were washed once and incubated with 0.2% latex particles (average diameter, 1 μm; Dow Chemical Co, Indianapolis, IN) for 4 hours or with 0.4% EA for 1 hours at 37°C. The cells were washed vigorously three times with warm medium to remove noninternalized beads or EA, and then noninternalized EA were lysed with lysing buffer. The percentage of cells ingesting more than 3 latex particles or one EA was determined by counting at least 300 cells; the results are expressed as the mean and standard deviations of triplicate experiments.

Phenotyping with monoclonal antibodies (MoAbs). The expression of leucocyte cell surface markers or cytoplasmic antigens was assessed using the following MoAbs: anti-CD14 (Leu M3), anti-CD11b (Leu-15), anti-CD11c (Leu M5), anti-CD15 (Leu-M1), anti-CD14-DR (L243), anti-CD14-DQ (Leu-10), anti-CD3 (Leu-4), anti-CD4 (Leu-3a), and anti-CD19 (Leu-12) from Becton Dickinson; anti-CD1a (OKT-6) and anti-CD7 (OKT-9) from Ortho Diagnostic Systems (Raritan, NJ); anti-CD11a (SPV-L7), anti-CD11b (NU-T2), and anti-CD45 (MEM-85) from Nichirei Corp (Tokyo, Japan); and antiCD1c (L-161) provided by Dr M. Furue (Yamanashi Medical School, Japan); anti-CD64 (anti-FcγRI, 32.2), anti-CD32 (anti-FcγR II, IV, 3), anti-CD16 (FcγRIII, 3G8) from Medarex (West Lebanon, NH); anti-CD54 (ICAM-1, 5C2) and 710F (antibody reactive to monocytes but not to T cells, B cells, and granulocytes) provided by Dr Y. Koyama (Tokyo Institute for Immunopharmacology, Tokyo, Japan); anti-CD80 (anti-B7, L307), anti-CD81 (anti-B70, IT2.1), and anti-CD40 (B-B20) from Serotec (Oxford, UK); and Lag (antibody specifically reactive to Birbeck granules of human LC) and rel B (rabbit polyclonal antibody reacts with rel B) from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Some of the MoAbs were already conjugated to either fluorescein isothiocyanate (FITC; anti-CD3 and anti-CD4) or phycoerythrin (PE; anti-CD19, anti-CD80, and anti-CD86); otherwise, FITC-conjugated goat F(ab')2, antimouse IgG + IgM (Cappel, West Chester, PA) was used as a second-step antibody for indirect staining. The binding of murine CTLA4-human Ig fusion protein (CTLA4-Ig; provided by Dr P. Lane, Basel Institute, Basel, Switzerland) was also assessed by staining with FITC-conjugated goat antihuman IgG (CalTag, Burlingame, CA). To block nonspecific FeR-mediated binding of MoAbs, cells were incubated for 60 minutes at 0°C with normal goat serum (Cedarlane Laboratories Ltd, Hornby, Ontario, Canada) before staining. For all experiments, irrelevant control MoAbs of the same IgG isotype and second-step controls were included. Stained cells were fixed with 1% paraformaldehyde (Sigma) and analyzed by cytofluorography on a FACSScan (Becton Dickinson Immunocytometry System) equipped with Lysis 2 software. For the staining of adherent macrophages, the cells were dislodged in the presence of 0.2% lidocaine, washed, and then spotted on the coverslips. The resultant adherent cells on the coverslips were then stained with various antibodies. Staining was evaluated with a fluorescence microscope, as described...
Previously,22 to assess the reactivity of anti-HLA-DR, Lag, or rel B, immunohistochemical immunoperoxidase staining of cytospin preparations (fixed in acetone) were also made by a highly sensitive streptavidin-biotin technique (Histofine; Nichirei Corp).

**Immunoblot analysis.** Cell lysates were prepared in a lysis buffer containing 1% Triton X-100, 20 mmol/L Tris-HCl (pH 8.0), 137 mmol/L NaCl, 10% glycerol, 1 mmol/L NaVO₄, 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 50 μg/mL antipain, 20 μg/mL leupeptin, 50 μg/mL pepstatin A, and 20 μg/mL aprotinin. Lysates were cleared by centrifugation at 15,000 rpm for 15 minutes at 4°C. The protein content of cell lysates was determined by BCA protein assay kit (Pierce Corp, Rockford, IL). Cell lysates (10 μg protein/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 10% gel concentration, and the separated proteins were then transferred to an Immobilon P membrane (Millipore, Bedford, MA) using a semidyrect electrobolization system (Sartorbiot II-S; Sartorius Inc, Gottingen, Germany). The membrane was blocked with Tris-buffered saline (TBS; 10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl) containing 1% nonfat milk, 1% bovine serum albumin, 0.05% Tween 20 for 5 hours and then incubated for 1 hour with 2 μg/mL of specific anti-relB antibody (Santa Cruz Biotechnology, Inc) or normal rabbit IgG at room temperature. After four washes in TBS, 0.05% Tween 20, the membrane was incubated at room temperature for 1 hour with horseradish peroxidase-conjugated goat antirabbit IgG (Santa Cruz Biotechnology, Inc). After three washes, the membrane was incubated in Amersham ECL Reagent and bands were visualized on Polaroid Type T667 film using an ECL Mini-camera (Amersham International plc, Buckinghamshire, UK).

**Isolation of RNA and Northern blot analysis.** The isolation of total RNA and Northern blot analysis were performed as described previously.23 Briefly, cells were washed once with phosphate-buffered saline and then lysed with denaturing solution containing 4 mol/L guanidium thiocyanate, 25 mmol/L sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 mol/L 2-mercaptoethanol (2-ME). After transfer to a polypropylene tube, total RNA was extracted by sequential addition of 2 mol/L sodium acetate, pH 4.0, water-saturated phenol, and chloroform-isoamylalcohol (49:1), followed by centrifugation at 10,000g for 20 minutes at 4°C and isopropanol precipitation. Total RNA was size-fractionated by electrophoresis after denaturation with glyoxal and dimethyl sulfoxide and then transferred to a nylon membrane (Pall BioSupport, East Hills, NY). After transfer, the membrane was baked under vacuum at 80°C, boiled in 80 mmol/L Tris-HCL, pH 8.0, for 5 minutes, and prehybridized by using prehybridization buffer (5 Prim-3Prim, Inc, West Chester, PA) and formamide at 42°C for 3 hours. Hybridization for c-fms RNA was performed with human c-fms cDNA probe kindly provided by Dr P. Robbins (Boston, MA). Blots were washed at 45°C for 30 minutes in 0.5% sarcosyl, and 0.1 mol/L 2-mercaptoethanol (2-ME). After washing with 0.1% sodium dodecyl sulfate and then analyzed using a Fuji BAS 2000 Bio-image Analyzer (Fuji Photo Film Co, Ltd, Tokyo, Japan).

**Mixed leukocyte reactions (MLRs).** Nylon wool-passed resting autologous or allogeneic T lymphocytes (2 × 10⁵) were cultured with graded doses of stimulator cells (DC or macrophages) in 96-well flat-bottomed microplates ( Falcon 3072) in 0.2 mL of medium per well. The proliferative response was measured after 6 days by adding 0.5 μCi (1 Ci = 37 GBq) of [³H]Tdr to each well and harvesting the cells 16 hours later. [³H]Tdr incorporation by cellular DNA was counted in a liquid scintillation counter. The results are expressed as the mean cpm and standard deviations of triplicate wells.

**Assay for the formation of MGC.** Monocytes (2.5 × 10⁶ cells/well in 24-well tissue culture plates) were cultured with M-CSF (10⁴ U/mL) in the presence or absence of various concentrations of IL-4 or IFN-γ for 14 days. After culture, the cells were fixed and stained with Diff-Quik (Kokusai Syaku Corp, Kobe, Japan) and counted on an inverted microscope. Cells with 3 or more nuclei per cell were defined as MGC. The results are expressed as the mean MGC counts and standard deviations of triplicate wells.

**TRAP staining.** Cells were fixed and stained for TRAP activity using a commercial kit (Sigma Diagnostics, St Louis, MO) according to the manufacturer’s instructions. TRAP-positive cells appeared dark red.

**Electron microscopy.** Monocytes cultured with GM-CSF and IL-4 for 7 days were centrifuged to make pellets. They were fixed in 2.5% glutaraldehyde at 4°C for 2 hours. After washing with 0.1 mol/L cacodylate buffer (pH 7.4), they were postfixed in 1% osmium tetroxide at 4°C for 2 hours, dehydrated in a graded series of ethanol, and embedded in Epok 812. Ultrathin sections were cut by an Ultratome Nova (LKB, Uppsala, Sweden) and observed in a Hitachi H-800 electron microscope (Hitachi, Tokyo, Japan) after staining with uranyl acetate and lead citrate.

**RESULTS**

**IL-4 but not IFN-γ suppressed the generation of macrophages from monocytes incubated with GM-CSF and induced the generation of cells with DC morphology.** We have previously shown that GM-CSF stimulates the differentiation of human monocytes into CD14⁺ macrophages.16 However, in the present study, the differentiation into macrophages of monocytes brought about by GM-CSF was inhibited by the addition of IL-4. Instead, culture with GM-CSF + IL-4 resulted in the generation of nonadherent cells that displayed a dendritic morphology with delicate membrane projections that were veil-like or sheet-like processes and were best shown when the cells were stained with anti-HLA-DR antibodies (Fig 1A through D). IL-4 alone was not sufficient to maintain the survival of monocytes. The effect of IL-4 was dose-dependent, and almost all of the cells recovered at 100 U/mL of IL-4 were nonadherent DC (Fig 2A). Electron microscopy analysis showed that most of the cells extended long cytoplasmic processes, showing a dendritic appearance (Fig 1G). They often had an indented nucleus and ample cytoplasm containing well-developed Golgi apparatus, rough endoplasmic reticulum, a few small lysosomes, and a microfilamentous structure. In contrast to macrophages, the cells did not ingest latex beads (>97.5% negative). Neither the phagocytosis of E. coli (9.8%) nor the rosette formation with EA (98.8%) was observed. Moreover, the cells did not express Ac-LDL receptor (Ac-LDLR; >97.1% negative) nor the rosette formation with EA (98.8% negative) nor the rosette formation with EA (98.8% negative), both of which are also typical markers of macrophages. These morphologic and functional properties of the cells indicated that they were so-called DC. The DC nature of the cells was further suggested by the finding that the cells expressed the relB subunit of the NF-κB complex (Fig 1E), because recent studies have shown that the expression of relB correlates with the differentiation of DC but not with that of monocyte/macrophages.24,25 In our study, the exis-
Fig 1. Morphology and cell-associated antigen expression of cells generated from human monocytes in response to GM-CSF plus IL-4. (A and B) Phase contrast micrographs of DC (A) and macrophages (B) generated from human monocytes by culture for 7 days with GM-CSF [500 U/mL] + IL-4 [100 U/mL] and GM-CSF [500 U/mL] alone, respectively (original magnification × 200). (C through F) Immunoperoxidase staining of DC (8 days of culture) for HLA-DR (C), Rel B (E), or the control antibodies control mouse IgG (D) and normal rabbit IgG (F) (original magnification × 400). (G) Electron micrograph of DC (original magnification × 6,000).

tence of rel B in monocyte-derived DC but not in fresh monocytes or monocyte-derived macrophages was confirmed by immunoblot analysis (Fig 3). A specific signal that was calculated at 67 to 68 kD, which is matched to the

known molecular weight of rel B, was detected in monocyte-derived DC by anti-rel B antibody, but fresh monocytes or monocytes-derived macrophages did not express this band (Fig 3).

The generation of DC from monocytes cultured with GM-CSF + IL-4 did not require cell proliferation, because the DNA synthesis in monocytes induced by GM-CSF was completely inhibited by the addition of IL-4 (Fig 2B). IFN-γ also inhibited the DNA synthesis induced by GM-CSF, but it had no demonstrable effect on the differentiation of monocytes (Fig 2B). Rather, IFN-γ suppressed the DC generation induced by GM-CSF + IL-4 in a dose-dependent manner (Fig 2C).

T-cell stimulating activity and cell surface antigen expression of the DC generated from monocytes by GM-CSF + IL-4. The most important characteristic of DC is their strong T-cell stimulating activity; DC, but not monocytes/macrophages, stimulate both autologous and allogeneic T cells in MLR. We therefore investigated monocyte-derived DC in terms of their ability to stimulate the proliferation of resting allogeneic or autologous T cells. Monocyte-derived DC, but not monocyte-derived macrophages induced by GM-CSF alone, strongly stimulated T-cell response in allogeneic MLR (Fig 4A). Even when the cell numbers were increased to 1 × 10⁵ (DC:T-cell ratio = 1:2), the macrophages did not trigger the proliferation of T cells, and fresh monocytes or M-CSF-induced macrophages (0.05 to 1 × 10⁵/well) similarly failed to stimulate allogeneic T cells (data not shown; but DC were at least 20 times more potent than monocyte-derived macrophages or fresh monocytes). Consistent with their strong T-cell stimulating activity, monocyte-derived DC, but not macrophages, could form clusters or rosettes with resting T cells within 24 hours (data not shown). Monocyte-derived DC were also a more powerful T-cell stimulator than were macrophages in autologous MLR, although the level of T-cell response in autologous MLR was lower than that in allogeneic MLR (Fig 4B).

In agreement with their strong T-cell stimulating activity, monocyte-derived DC expressed high levels of major histocompatibility complex (MHC) class II antigens (not only HLA-DR but also HLA-DQ) and other antigens, including
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A

![Graph showing the effects of IL-4 on adherent macrophages](image)

B

![Graph showing the effects of IL-4 and IFN-γ on DNA synthesis](image)

C

![Graph showing the effects of IFN-γ on adherent macrophages](image)

Fig 2. IL-4 but not IFN-γ suppresses the GM-CSF-induced monocyte differentiation into adherent macrophages and stimulates the generation of nonadherent DC. (A) Effects of IL-4 on the recovery of adherent macrophages in the culture of monocytes incubated with GM-CSF. Monocytes (2 x 10^5 cells/well) were cultured with GM-CSF (500 U/mL) in the presence or absence of various doses of IL-4 for 7 days. The numbers of adherent macrophages were counted as described in the Materials and Methods. Data are expressed as the mean ± SD of triplicate wells from one representative experiment of five performed. (B) Effects of IL-4 and IFN-γ on the GM-CSF-induced DNA synthesis of monocytes. Monocytes (1 x 10^5 cells/well) were incubated with GM-CSF (500 U/mL) in the presence or absence of IL-4 or IFN-γ for 6 days, and DNA synthesis was measured as described in the Materials and Methods. The level of DNA synthesis is expressed as mean ± SD cpm values of ³HdTdr incorporation of triplicate wells from one representative experiment of three performed. (C) IFN-γ restored the suppressive effect of IL-4 on the recovery of adherent macrophages in the culture of monocytes incubated with GM-CSF. Monocytes (1.9 x 10^5 cells/well) were cultured with GM-CSF (500 U/mL) plus IL-4 (100 U/mL) in the presence or absence of various doses of IFN-γ for 7 days. The numbers of adherent macrophages and nonadherent DC were counted as described in the Materials and Methods. Data are expressed as the mean ± SD of triplicate wells from one representative experiment of three performed.

Fig 3. Immunoblot analysis of relB in monocyte-derived DC. Lysates (10 µg protein/lane) from nonadherent PBMC (lane 1), fresh monocytes (lane 2), DC generated from monocytes by the cooperation of GM-CSF (500 U/mL) + IL-4 (100 U/mL) (lane 3), GM-CSF-induced (500 U/mL) monocyte-derived macrophages (lane 4), and M-CSF-induced (100 U/mL) monocyte-derived macrophages (lane 5) were immunoblotted as described in the Materials and Methods using anti-relB polyclonal antibody. Specific band correspondence to relB was detected only in the lysate from monocyte-derived DC.

adhesion molecules and costimulatory molecules that are likely to be critical in the capacity of DC to present antigen to resting T cells (CD40, CD80, CD86, CD54, CD11a, CD44, etc; Fig 5). However, the monocyte-derived DC did not express monocyte/macrophage markers (CD14, CD71, 710F, FcγRI, CD16, and FcγRIII) or lymphocyte markers (CD3 and CD19), but a low level of CD32 (FcγRII) or CD4 expression was detected (Fig 5). They also expressed CD11c (data not shown). The cells expressed CD11b and a high level of CD1a, CD1b, and CD1c, i.e., the MHC class I-like differentiation antigens that are expressed by epidermal LC (Fig 5). However, the proportion of cells that expressed Lag antigen (Birbeck granule-associated antigen) was low (<4% of the cells reacted with Lag; data not shown).

TNF-α downregulated the expression of c-fms and the potential to convert to macrophages of the DC generated from monocytes by GM-CSF + IL-4. It has been reported that TNF-α + GM-CSF is required for the generation of DC from CD34+ precursor cells or CD33+CD14dim cells in the blood. However, we found that a combination of GM-CSF (500 U/mL) + TNF-α (10 to 100 U/mL) did not stimulate the generation of DC from monocytes. However, we did find TNF-α influenced the capacity to convert to macrophages of DC generated from monocytes by GM-CSF + IL-4. DC generated from human monocytes by incubation with GM-CSF (500 U/mL) + IL-4 (100 U/mL) for 7 days were washed once and then incubated further with GM-CSF + IL-4 in the presence or absence of TNF-α (50 U/mL) for 2 days. When TNF-α–nontreated or TNF-α–treated monocyte-derived DC were cultured with medium alone, both cells died (Fig 6A and B). However when both TNF-α–nontreated or TNF-α–treated monocyte-derived DC were cultured with GM-CSF alone, almost all of the cells were alive and the cells recovered maintained DC nature, similar to findings in the cells cultured with GM-CSF + IL-4 (Fig 6A and B). However, we noted that TNF-α–nontreated and TNF-α–treated monocyte-derived DC showed a different
A T-cell stimulating activity of the monocyte-derived DC. DC and macrophages were obtained from human monocytes by incubation with GM-CSF (500 U/mL) + IL-4 (100 U/mL) and GM-CSF (500 U/mL) alone, respectively, for 8 days. Nylon wool passed resting allogeneic (A) or autologous (B) T lymphocytes (2 x 10^5) were cultured with graded doses of stimulator cells (DC or macrophages), and a proliferative response was measured after 6 days as described in the Materials and Methods. Note the strong stimulatory capacity of DC relative to macrophages. Data are expressed as the mean ± SD of triplicate wells from one representative experiment of three performed.

response to M-CSF. When TNF-α–nontreated DC were cultured with M-CSF, about 90% of the recovered cells were adherent macrophages and phagocytized latex particles (Fig 6A). In contrast, TNF-α–treated DC did not respond to M-CSF and died as in culture with medium alone (Fig 6B). When monocyte-derived DC were incubated with 1, 10, 50, and 100 U/mL of TNF-α for 48 hours, 85%, 44%, 9.5%, and 7.5% of them converted to macrophages after incubation with M-CSF, respectively. Kinetic study showed that 100%, 85%, 68.8%, and 7.5% of monocyte-derived DC incubated with 50 U/mL of TNF-α for 6, 12, 24, and 48 hours converted to macrophages after incubation with M-CSF, respectively. These results indicate that monocyte-derived DC require incubation with 50 U/mL of TNF-α for 48 hours to lose the response to M-CSF.

Northern blot analysis showed that the mRNA of the Ac-LDLR, a typical macrophage marker, and the mRNA of the M-CSF receptor c-fms were positive in both M-CSF– and GM-CSF–induced monocyte-derived macrophages, although the amount of the mRNA of c-fms was much less in GM-CSF–induced macrophages compared with M-CSF–induced macrophages (Fig 7, lanes 3 and 4). As stated above, monocyte-derived DC did not express Ac-LDLR. In agreement with this result, neither TNF-α–treated nor TNF-α–

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Fig 5. A representative FACS analysis of DC recovered from monocytes cultured with GM-CSF plus IL-4. Monocytes were cultured with GM-CSF (500 U/mL) + IL-4 (100 U/mL) for 10 days. Media were changed at 7 days. After culture, the cells were washed and then stained with various antibodies as described in the Materials and Methods. The upper left panel shows the forward and side scatter properties of monocyte-derived DC. Gated cells were 98% of total nonadherent cells and show uniform high forward and side scatter properties. The data are shown as histograms depicting the number of cells exhibiting various fluorescence intensities. The left lines are controls and were stained with a mixture of control IgG1 and IgG2a. Data are from one representative experiment of four performed.
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Fig 6. Effect of TNF-α on the potential to convert to macrophages of DC generated from monocytes by GM-CSF and IL-4. DC obtained from monocytes cultured with GM-CSF (500 U/mL) + IL-4 (100 U/mL) for 7 days were washed, divided into two portions, and then incubated further with GM-CSF (500 U/mL) + IL-4 (100 U/mL) in the presence or absence of TNF-α (50 U/mL) for 2 days. After culture, the cells were washed and then cultured with M-CSF (10^4 U/mL), GM-CSF (500 U/mL), GM-CSF + IL-4 (100 U/mL), or medium alone for 6 days. After culture, the number of adherent macrophages and DC were counted. (A) TNF-α–nontreated monocyte-derived DC. (B) TNF-α–treated monocyte-derived DC. Data are expressed as the mean ± SD of triplicate wells from one representative experiment of three performed.

nontreated monocyte-derived DC expressed the mRNA of Ac-LDLR (Fig 7, lanes 1 and 2). However, the expression of the mRNA of c-fms was distinct between these two DC; TNF-α–nontreated DC expressed the mRNA of c-fms and the level was the same as that in M-CSF–induced monocyte-derived macrophages, whereas TNF-α–treated DC did not express c-fms mRNA (Fig 7, lanes 1 and 2). The amounts of the mRNA of actin and the ribosomal RNA were not significantly different between these cells.

These results indicated that the DC generated from monocytes by the combination of GM-CSF + IL-4 were not the terminally differentiated cells and that they could convert to macrophages by M-CSF, because they expressed c-fms. However, the DC cannot convert to macrophages by GM-CSF, whereas GM-CSF is necessary to maintain their survival. The results further suggest that TNF-α stimulates the downregulation of c-fms expression in the DC and aborts the potential to convert to macrophages provided by M-CSF.

TNF-α–treated DC relative to nontreated DC were extremely potent stimulators of T-cell proliferation in the allogeneic MLR (data not shown). The development of strong T-cell stimulatory function in the TNF-α–treated DC is probably due to the upregulation of DR antigen, adhesion molecule (CD54), and costimulatory molecules (CD80, CD86, and CD40; data not shown). However, TNF-α had no demonstrable effect on the expression of CD1a or LFA-1 of the DC (data not shown).

Generation of TRAP-positive MGC from human monocytes by the combination of M-CSF and IL-4. We next examined the effects of IL-4 and IFN-γ on the differentiation of monocytes stimulated with M-CSF. When monocytes were cultured by a combination of M-CSF + IL-4 for 14 days, MGC containing 3 or more nuclei were recovered, whereas no MGC were detected in monocytes cultured with
M-CSF alone (Fig 8A). MGC formation was dependent on the dose of IL-4; MGC formation was already detected at 10 U/mL of IL-4 and reached a maximum level at a dose of 250 U/mL (Fig 8A). MGC appeared at about 10 days of culture and reached a maximum level at about 14 days (data not shown). However, IFN-γ (1 to 500 U/mL) had no effect on MGC formation (Fig 8A). Rather, IFN-γ antagonized the effect of IL-4 and suppressed the MGC formation induced by IL-4 and M-CSF (Fig 8B). The MGC possessed TRAP activity, which is known as a marker of osteoclasts (Fig 9).

To determine whether the potential to form MGC is restricted to monocytes, we next examined the effect of M-CSF + IL-4 on monocyte-derived macrophages. MGC formation occurred even when M-CSF–induced monocyte-derived macrophages (monocytes were incubated with M-CSF alone for 8 days to obtain macrophages) were further incubated with M-CSF + IL-4 for 14 days (Fig 8C). However, only a small number of MGC was formed when GM-CSF–induced monocyte-derived macrophages (monocytes were incubated with GM-CSF for 8 days) were further incubated with M-CSF + IL-4 (Fig 8C). These results suggest that, even after the commitment to M-CSF–induced macrophages, monocytes have the capacity to form MGC, whereas commitment to GM-CSF–induced macrophages abrogates this capacity. One reason for the ineffectiveness of GM-CSF–induced macrophages may be the low expression of the M-CSF receptor c-fms, because the level of expression of c-fms mRNA in the GM-CSF–induced macrophages was markedly lower than that in the M-CSF–induced macrophages, as shown in Fig 7.

**DISCUSSION**

We previously showed that two phenotypically distinct types of macrophages can be generated from human monocytes by stimulation with GM-CSF and M-CSF. The present study showed that CD14+relB+ DC and TRAP+ MGC can also be generated from human monocytes by the cooperation of GM-CSF with IL-4 and M-CSF with IL-4, respectively, although the terminal differentiation into mature DC required further stimulation with TNF-α. Our findings add a new aspect to our knowledge of monocyte/macrophage differentiation and provide evidence that monocytes are flexible in their differentiation potential and are precursors not only of macrophages but also of CD14+relB+ DC and TRAP+ osteoclast-like MGC.

Using the same combination of cytokines (GM-CSF + IL-4), the generation of DC from an adherent fraction of PBMC and the functional maturation of the DC followed by TNF-α were also reported by Sallusto and Lanzavecchia, but they did not clarify the precursor and the potential to convert to macrophages was not described. Although DC and monocyte/macrophages share a common precursor, until now, there was no clear-cut evidence to show that DC can also be generated from monocytes. Our present findings of the generation of DC from human monocytes by GM-CSF plus IL-4 suggest that monocytes are at least a precursor of some DC. Human peripheral blood preparations do not yield 100% pure monocytes. Therefore, it could be argued that there may have been an overgrowth of contaminating blood DC rather than monocyte conversion to DC. However, the monocytes used were greater than 97% pure (they were adherent, showed strong expression of CD14, and showed nonspecific esterase activity and phagocytic activity) and the generation of DC from monocytes cultured with GM-CSF + IL-4 did not require cell proliferation, because the DNA synthesis in monocytes induced by GM-CSF was completely
inhibited by the addition of IL-4, providing strong evidence for monocyte conversion to DC. In contrast to blood DC, DC generated from monocytes cultured with GM-CSF + IL-4 expressed a high level of CD1, which is an MHC class I-like differentiation antigen and is expressed by epidermal LC.\textsuperscript{29} CD1a is also expressed on DC derived from CD34\textsuperscript{+} hematopoietic precursors cultured with GM-CSF + TNF-\textalpha.\textsuperscript{32} Therefore, monocyte-derived DC may belong to a subset of DC including epidermal LC or DC from CD34\textsuperscript{+} hematopoietic precursors.

The results of the present study showed that DC generated from monocytes with GM-CSF + IL-4 cannot convert to macrophages in response to GM-CSF, although GM-CSF is necessary to maintain their survival. However, we showed that the DC still express c-fms and possess the ability to convert to macrophages in response to M-CSF. After treatment with TNF-\textalpha, the DC lost both the expression of c-fms mRNA and the capacity to revert to macrophages in response to M-CSF. This finding is in agreement with recent studies that show a lack of the M-CSF receptor c-fms in murine DC\textsuperscript{30} and an important role of GM-CSF for the survival of DC.\textsuperscript{31} Thus, our findings strongly indicate that the differentiation of monocytes to DC by GM-CSF + IL-4 is true differentiation and that the monocyte-derived DC are not interconvertible with macrophages, although TNF-\textalpha is required to complete the terminal differentiation.

Sallusto and Lanzavecchia\textsuperscript{10} showed that TNF-\textalpha upregulates the T-cell stimulating capacity in MLR of DC generated from adherent PBMC by the combination of GM-CSF + IL-4. We also found that TNF-\textalpha resulted in the upregulation of the T-cell stimulating activity of monocyte-derived DC in MLR (data not shown). These results suggest that terminal differentiation of monocyte-derived DC by TNF-\textalpha accompanies the functional maturation.

CD34\textsuperscript{+} hematopoietic precursor cells\textsuperscript{5,7} and a subset of CD33\textsuperscript{+}CD14\textsuperscript{+} circulating blood cells were recently deduced as precursors of functional human DC.\textsuperscript{11} A combination of GM-CSF and TNF-\textalpha was required for the generation of DC from CD34\textsuperscript{+} precursor cells.\textsuperscript{5,7} However, TNF-\textalpha is not enough to generate DC from monocytes in combination with GM-CSF; the combination of GM-CSF and IL-4 is required, although TNF-\textalpha is necessary for the terminal differentiation of monocyte-derived DC. In contrast to monocytes, cytokines such as GM-CSF and IL-4 do not appear to be necessary for the differentiation of CD33\textsuperscript{+}CD14\textsuperscript{+} DC precursors into mature DC, although their maturation is enhanced by TNF-\textalpha and GM-CSF.\textsuperscript{11} Taken together, these results strongly imply that subpopulations of DC precursors may exist in adult peripheral blood, possibly at different stages of differentiation (CD14\textsuperscript{+} monocytes, CD33\textsuperscript{+}CD14\textsuperscript{+} cells, or CD34\textsuperscript{+} precursors), with distinct requirements for cytokines for their functional maturation.

Another interesting finding of our study is that IL-4 stimulated the formation of MGC from human monocytes by cooperating with M-CSF. The important role of IL-4 in MGC formation from monocyte/macrophages was reported previously in mouse bone marrow cells and rat microglia.\textsuperscript{32,33} In the case of rat microglia, IL-4 alone is enough to induce the MGC formation.\textsuperscript{35} However, in our study, IL-4 alone was ineffective for MGC formation and required the presence of M-CSF. Thus, IL-4 can elicit the production of MGC, but only when used as a costimulant with growth factors that efficiently sustain the viability, growth, and differentiation of monocytes. Our results are consistent with the data from mouse bone marrow cells. In that study, MGC were formed in cultures stimulated with IL-4 in combination with IL-3, M-CSF, or GM-CSF.\textsuperscript{32} However, in the present study, the combination of IL-4 and GM-CSF induced DC instead of MGC. The discrepancy might be attributable to the difference in cell source. In contrast to our study, a suppressive effect of IL-4 on MGC formation by human monocytes was reported by others, but the system was different from ours and they used Con A as a stimulus of MGC formation.\textsuperscript{32}

IFN-\gamma has been shown to induce human monocytes,\textsuperscript{34,36} human alveolar macrophages,\textsuperscript{37} and rat microglia\textsuperscript{33} MGC formation. However, in our study, IFN-\gamma alone and a combination of IFN-\gamma and M-CSF did not induce MGC. We found that IFN-\gamma antagonized the effect of IL-4 and suppressed the MGC formation induced by IL-4 and M-CSF. The discrepancy in effect may be due to the differences of cell source, serum supplemented, and/or costimulatory molecules, ie, human monocytes versus human alveolar macrophages\textsuperscript{37} or rat microglia,\textsuperscript{36} fetal bovine serum versus human serum,\textsuperscript{35} and M-CSF versus Con A.\textsuperscript{34,36}

Our results showed that MGC are formed not only from fresh monocytes but also from macrophages induced by M-CSF. Thus, the capacity to form MGC remains after the differentiation of monocytes to macrophages. However, GM-CSF–induced macrophages were unable to form MGC. The expression of c-fms mRNA in the GM-CSF–induced macrophages was markedly lower than that in the M-CSF–induced macrophages; this is a possible reason for the inability of GM-CSF–induced macrophages to form MGC.

In disease-free individuals, MGC are found as osteoclasts, cells that are thought to be important in the physiologic resorption of bone. Osteoclasts are considered to be a member of the monocyte/macrophage family, and an important role of M-CSF in osteoclast formation has been shown in the osteopetrotic (op/op) mouse, which is a naturally occurring knockout of M-CSF.\textsuperscript{38,39} MGC of mononuclear phagocytic origin are also observed in various pathologic entities, including granulomas formed in response to tuberculosis, foreign bodies, and other infectious agents, including HIV, and in response to neoplasms and idiopathic conditions such as sarcoidosis and Crohn’s disease.\textsuperscript{40,44} However, because of the difficulty of isolation and characterization, the functional significance and precise mechanism of MGC formation in granulomas are still controversial. The relationship between osteoclasts and MGC in granuloma is also not known. In the present study, MGC generated from monocytes by IL-4 + M-CSF were ‘TRAP’, a typical characteristic of osteoclasts. The production of TRAP \textsuperscript{+} MGC in large numbers from human monocytes in vitro should help to achieve a better understanding of the physiology of MGC in granulomas and osteoclasts in bone and of the relationship between these cells.

Based on our experimental results described here and on the results of our previous study\textsuperscript{16} summarized in Fig 10, it
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Generation of CD1+RelB+ dendritic cells and tartrate-resistant acid phosphatase-positive osteoclast-like multinucleated giant cells from human monocytes

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