Mobilization of Dendritic Cell Precursors Into the Circulation by Administration of MIP-1α in Mice

Yanyun Zhang, Hiroyuki Yoneyama, Yong Wang, Sho Ishikawa, Shin-ichi Hashimoto, Ji-Liang Gao, Philip Murphy, Kouji Matsushima

Background: Dendritic cells (DCs) play a central role in immune responses and may be useful adjuvants for tumor vaccine therapy. We previously reported that F4/80+ B220– MHC Class II+ DC precursors expressing the CC chemokine receptors CCR1 and CCR5 are mobilized rapidly into the circulation in mice injected with Propionibacterium acnes and are recruited into inflammatory tissue by macrophage inflammatory protein 1α (MIP-1α), which binds to CCR1 and CCR5. Here we investigate the mechanisms of DC precursor mobilization and the antitumor effect of these cells in mice.

Methods: Numbers of DC precursors in peripheral blood were determined in P. acnes-treated mice (groups of 10 C57BL/6 B6 mice, CCR1−/− mice, CCR5−/− mice, and B6 mice treated with antibody to MIP-1α or control antibody) and in B6 mice injected with recombinant MIP-1α. MIP-1α–mobilized DC precursors matured by treatment with granulocyte–macrophage colony-stimulating factor, interleukin 4, and tumor necrosis factor-α and pulsed with B16 melanoma lysates were assayed for their ability to confer protective immunity against tumor challenge in vivo and to induce cytotoxic T lymphocytes against B16 tumor cells in vitro. Results: The recruitment of DC precursors into the circulation by P. acnes administration was higher in B6 mice (12.6%, 95% confidence interval [CI] = 9.1% to 16.1%) than in CCR1−/− (9.0%, 95% CI = 7.5% to 10.5%), CCR5−/− (6.3%, 95% CI = 5.2% to 7.3%), or anti-MIP-1α antibody–treated (6.6%, 95% CI = 5.7% to 7.5%) mice. Injection of MIP-1α also mobilized DC precursors into the circulation (13.1%, 95% CI = 10.8% to 15.6%). Matured MIP-1α–mobilized DC precursors pulsed with B16 tumor lysates elicited B16-specific antitumor immunity in vitro and in vivo. Conclusions: MIP-1α and its receptors are important in recruiting DC precursors into the circulation. DC precursors mobilized rapidly by MIP-1α may provide sufficient useful DC precursors for DC-based vaccination in cancer treatment.

Dendritic cells (DCs) are central operators in immune responses, especially in initiating the primary immune response, by presenting antigens to naive T cells (1). DCs are generated from hematopoietic progenitor cells through several differentiation stages, proceeding from DC progenitor cells, to DC precursor cells, to immature DCs, and, finally, to mature DCs (2–8). Immature DCs are distributed throughout tissues and organs. In response to various stimuli (e.g., tumor necrosis factor-α [TNF-α], CD40L, and lipopolysaccharide), immature DCs differentiate into mature DCs and migrate into the T-cell areas in the draining lymph nodes, where they initiate T-cell responses (9–12). Because of their role as the master cells in immune responses, DCs have been used as vaccination adjuvants for infections, tumors, and autoimmune diseases. Several strategies have been considered to improve vaccine efficacy; one of these is to increase the numbers of DCs in the circulation as a source of endogenous DCs for tumor vaccination.

We recently reported (12) that DC precursors lacking expression of the pan-macrophage marker F4/80, the early B-cell marker B220, and the class II major histocompatibility complex (MHC) protein but expressing the alphaX integrin chain (i.e., F4/80+ B220− CD11c+ MHC II−) appear rapidly in the circulation and migrate into the liver of wild-type C57BL/6 (B6) mice that have been injected intravenously with P. acnes. Intravenous administration of P. acnes into mice rapidly increases the levels of inflammatory cytokines in the circulation (12,13) and dramatically changes the profile of genes expressed in the liver (14). Among the genes induced in the liver is the gene for the cytokine macrophage inflammatory protein 1α (MIP-1α) (14).

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See “Notes” following “References.”

DOI: 10.1093/jnci/djh024

Journal of the National Cancer Institute, Vol. 96, No. 3, © Oxford University Press 2004, all rights reserved.
Because the blood DC precursors induced by P. acnes injection specifically express the CC chemokine receptors CCR1 and CCR5 and because MIP-1α, a ligand for CCR1 and CCR5, recruits these cells to the liver to form granulomas (12), we hypothesized that MIP-1α binding to CCR1 and CCR5 on DC precursors mediates the effect of the rapid mobilization of DC precursors into circulation caused by P. acnes administration. To investigate this possibility, we analyzed DC responses in mice treated with an antibody against this chemokine and in mice deficient for its receptors. Furthermore, we examined whether recombinant MIP-1α induces DC precursor mobilization into the circulation. Finally, we studied whether P. acnes- and MIP-1α-mobilized DC precursors differentiate into mature DCs that can induce antitumor protective immunity in a B16 melanoma model.

METHODS

Animals

Female B6 and BALB/c mice (8–10 weeks old) were purchased from CLEA (Tokyo, Japan). CCR1 gene-deficient (CCR1−/−) mice (15) and CCR5−/− mice (16) were generated as described. Both knockout mice strains were backcrossed with B6 mice for eight generations and were kept under pathogen-free conditions in the Animal Facility of the Department of Molecular Preventive Medicine, School of Medicine, the University of Tokyo, Tokyo, Japan. Mice were kept in a sterile environment in microisolators at all times and were provided with autoclaved drinking water and autoclaved food ad libitum.

All animal experiments complied with the standards set out in the Guidelines for Care and Use of Laboratory Animals of The University of Tokyo, Japan.

Cytokines and Antibodies

Recombinant murine stem cell factor and anti-c-kit antibody (ACK-2) were provided by Dr. Tetsuo Sudo, Basic Research Institute of Toray (Kanagawa, Japan), and murine granulocyte–macrophage colony-stimulating factor (GM-CSF) was provided by Kirin Brewery (Tokyo, Japan). Murine TNF-α (mTNF-α) was purchased from R&D Systems (Minneapolis, MN). The interleukins IL-2, IL-4, and IL-7 and goat anti-mouse MIP-1α antibody were purchased from Genzyme-Technich (Cambridge, MA). DEC-205 (NLDC145), a rat monoclonal antibody (MAb) to murine DCs, was purchased from CLEA (Tokyo, Japan). CCR1 gene-deficient (CCR1−/−) mice were provided by Dr. Shiro Kanegasaki (Effector Cell Institute, Tokyo, Japan). Other MAbS and reagents used for immunostaining were obtained from Pharmingen (San Diego, CA) unless otherwise indicated.

Cell Lines

The B16 melanoma cell line and the EL4 thymoma cell line were provided by Dr. Kazuyoshi Takeda (Juntendo University, Tokyo, Japan). These cell lines were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Gibco, Rockville, MD) containing 10% fetal calf serum (FCS), penicillin G (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified incubator supplemented with 5% CO2.

Preparation of Peripheral Blood Mononuclear Cells and Their Derived DC Precursors

B6, CCR1−/−, and CCR5−/− mice (groups of 10 mice) were separately injected, via the tail vein, with 1 mg of heat-killed P. acnes (11828; American Type Culture Collection, Manassas, VA) in 100 μL of phosphate-buffered saline (PBS) or 20 μg of human recombinant MIP-1α in 100 μL of PBS. For blocking experiments, goat anti-mouse MIP-1α antibody (100 μg per mouse in 100 μL of PBS) or control goat immunoglobulin G (IgG) (100 μg per mouse in 100 μL of PBS; Sigma-Aldrich, St. Louis, MO) was intravenously injected 8 hours before the P. acnes injection. Peripheral blood (0.8 mL per mouse) was obtained by cardiac puncture from anesthetized mice at the indicated time intervals after P. acnes injection or MIP-1α administration. Peripheral blood mononuclear cells (PBMCs) were prepared from peripheral blood by density separation with Nycodil (Nynomed Pharma, Oslo, Norway). F4/80 B220−CD11c+ cells were sorted from these PBMCs as previously described (12). In brief, PBMCs were stained with biotinylated anti-F4/80 MAb followed by Cy-chrome–conjugated streptavidin, phycoerythrin (PE)–labeled anti-B220 MAb (clone RA3–6B2), and fluorescein isothiocyanate (FITC)–labeled anti-CD11c MAb (clone HL3) for flow cytometry analysis and sorting of F4/80 B220+CD11c+ DC precursors by using a cell sorter (EPICS ELITE; Coulter Electronics, Hialeah, FL). Re-analysis by flow cytometry showed that the purity of these sorted F4/80+ B220+CD11c+ DC precursors was greater than 99%. In some experiments, PBMCs were stained with biotinylated anti-CD11c MAb, Cy-chrome–conjugated streptavidin, and PE–FITC-labeled anti-B220 MAb for flow cytometry analysis and sorting.

DC Maturation and Culture Conditions

Mature DCs were generated from PBMC–derived B220−CD11c+ DC precursors and bone marrow–derived hematopoietic progenitor cells as previously described (12). In brief, purified peripheral blood–derived B220+CD11c+ DC precursor cells from groups of five mice injected with P. acnes or with recombinant MIP-1α were cultured at a concentration of 3 × 105 cells/mL in IMDM containing 10% FCS, GM-CSF (4 ng/mL), and IL-4 (10 ng/mL) for 4–5 days to induce their differentiation into immature DCs. To generate bone marrow–derived immature DCs, B6 mouse bone marrow cells were stained sequentially with biotinylated anti-c-kit MAb and Streptavidin MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). c-kit+ hematopoietic progenitor cells were magnetically isolated with a MiniMACS separator (Miltenyi Biotec) from the bone marrow of femurs and tibiae of B6 mice and were then cultured in IMDM containing 10% FCS, murine stem cell factor (5 ng/mL), GM-CSF (4 ng/mL), and IL-4 (10 ng/mL) for 9 days to generate immature DCs. Immature DCs were further cultured in fresh IMDM containing 10% FCS, GM-CSF (4 ng/mL), and mTNF-α (5 ng/mL) for 3 days to induce their maturation (5,8,12).

Immunofluorescence Staining

Immunofluorescence analysis of DCs was performed as previously described (5,6,8,12). In brief, B220+CD11c+ DC precursors and mature DC cells prepared and isolated from groups of five mice as described above (2 × 105 to 4 × 105
cells) were incubated with rat anti-DEC-205 MAb followed by FITC-labeled goat anti-rat IgG (Fab')2 antibodies (Caltag, Camarillo, CA) or with FITC-labeled MAb against CD40, F4/80, CD11b, or CD80 and PE-labeled MAb against Ia, CD8α, or CD86 followed by flow cytometry analysis using a cell sorter.

Mixed Leukocyte Reaction Assay

The mixed leukocyte reaction (MLR) assay was performed as previously described (7,8,12). In brief, DCs derived from B220<sup>–</sup>CD11c<sup>+</sup> DC precursors and bone marrow were prepared as described above from groups of five mice. Macrophages were harvested from the peritoneal cavities of B6 mice (n = 3) by injecting the cavities with 10 mL of PBS containing 1 mM EDTA (Sigma-Aldrich). DCs and macrophages (3 × 10<sup>5</sup> cells/mL) were incubated in IMDM containing 10% FCS and mitomycin C (MMC; 15 µg/mL) in six-well plates (Nunc A/S, Roskilde, Denmark) at 37 °C for 3 hours to arrest their proliferation. After several washes with PBS, these B6 mouse–derived stimulator cells were suspended in IMDM containing 10% FCS at concentrations ranging from 1 × 10<sup>3</sup> to 3 × 10<sup>4</sup> cells/mL, as indicated. One hundred microliters of the above stimulator cell suspension was added to each well of 96-well plates (Nunc A/S) that contained allogeneic CD4<sup>+</sup> T cells (3 × 10<sup>5</sup> cells/100 µL per well) that had been magnetically isolated from BALB/c mice using CD4 Microbeads (Miltenyi Biotec). Five days later, T-cell proliferation was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) as previously described (5,7,8,12). In brief, 15 µL of MTT (5 µg/mL in PBS) was added to each well, and the plates were incubated at 37 °C for an additional 4 hours. The resultant absorbance at 550 nm was read by a microplate immunoreader. Medium alone was used as the negative control. Results are expressed as the mean of three wells from three individual experiments.

Pulsing DCs With Tumor Cell Lysates

B16 tumor cells were rapidly frozen and thawed three times to produce tumor cell lysates. Immature DCs (2 × 10<sup>5</sup> cells/mL) derived from B220<sup>–</sup>CD11c<sup>+</sup> DC precursors of P. acnes– or MIP-1α–treated mice and from bone marrow c-kit<sup>+</sup> cells were incubated in six-well plates in the presence of B16 tumor cell lysates (6 × 10<sup>5</sup> cells equivalent/mL) in IMDM containing 10% FCS, GM-CSF (4 ng/mL), and mTNF-α (5 ng/mL) in a humidified incubator at 37 °C and 5% CO<sub>2</sub>. After 3 days, these tumor cell lysate–pulsed DCs were used in vaccination.

Tumor Model and DC-Based Vaccination

Naive B6 mice (groups of 20) were injected subcutaneously in their abdominal wells on days 0, 7, and 14 with 1 × 10<sup>6</sup> DCs of the following types: B16 tumor cell lysate– or PBS–pulsed DCs derived from MIP-1α–mobilized B220<sup>–</sup>CD11c<sup>+</sup> cells or bone marrow–derived c-kit<sup>+</sup> cells at a stimulator to responder cell ratio of 1:20. PBS-pulsed DCs derived from MIP-1α–mobilized B220<sup>–</sup>CD11c<sup>+</sup> cells and tumor lysate alone were used as controls. Fresh medium containing IL-2 and IL-7 was exchanged every 4 days. At day 21, cytolytic activity of the primed T cells as effector cells was determined with the Cytotoxicity Detection Kit as mentioned above.

Statistical Analyses

Differences among groups of mice in the number of mobilized DC precursors and in the ability of T cells to produce IFN-γ in vitro to kill B16 tumor cells and to produce IFN-γ were evaluated using one-way analysis of variance and the multiple comparison method of Scheffe using StatView version 5.0 (SAS Institute, Cary, NC). Differences in survival among groups of mice were evaluated with a log-rank test of the Kaplan–Meier survival curves. Statistical tests were two-sided. P values less than .05 were considered to be statistically significant.

Assays for Cytotoxic T Lymphocyte Activity and Interferon Gamma Secretion

To confirm that tumor-specific cytotoxic T lymphocytes (CTLs) had been generated in the immunized mice, splenic CD3<sup>+</sup> T cells from tumor-free mice that survived (n = 8 in each group of surviving mice) were restimulated ex vivo by culturing in IMDM containing 10% FCS and MMC-treated B16 tumor cells (1 × 10<sup>5</sup>). Five days later, the MMC-treated B16 tumor cells had been lost in the cultures and the T cells were collected for measuring CTL activity and interferon gamma (IFN-γ) secretion. In brief, these restimulated effector T cells (2 × 10<sup>5</sup> in 100 µL per well) were added to the wells containing target B16 or EL4 tumor cells (5 × 10<sup>3</sup> in 100 µL per well) in 96-well plates. After 20 hours, supernatant from each well was collected for measuring cytolytic activity against target B16 and EL4 cells with a Cytotoxicity Detection Kit (LDH; Boehringer Mannheim, Mannheim, Germany) and for measuring IFN-γ production with the mouse IFN-γ enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Woburn, MA). In some experiments, the target B16 tumor cells (1.5 × 10<sup>5</sup> cells/mL) were incubated with a MAb to MHC class I molecules (anti-H2Db/ H2Kb; 50 µg/mL) or with control antibody (anti-H2Dd; 50 µg/mL) at 37 °C for 30 minutes before the addition of restimulated effector T cells to evaluate the specificity of CTL activity.

In addition, splenic CD3<sup>+</sup> T cells (1.0 × 10<sup>6</sup> cells/mL) isolated from naive B6 mice were cultured in the presence of IL-2 and IL-7 (5 ng/mL each) and primed at days 0, 7, and 14 with B16 tumor cell lysate–pulsed DCs derived from MIP-1α–mobilized B220<sup>–</sup>CD11c<sup>+</sup> cells or bone marrow–derived c-kit<sup>+</sup> cells at a stimulator to responder cell ratio of 1:20. PBS-pulsed DCs derived from MIP-1α–mobilized B220<sup>–</sup>CD11c<sup>+</sup> cells and tumor lysate alone were used as controls. Fresh medium containing IL-2 and IL-7 was exchanged every 4 days. At day 21, cytolytic activity of the primed T cells as effector cells was determined with the Cytotoxicity Detection Kit as mentioned above.
RESULTS

Involvement of CCR1 and CCR5 in the Mobilization of F4/80$^+$ B220$^+$ CD11c$^+$ DC Precursors Into the Circulation by P. acnes and MIP-1α

In our previous study (12), we demonstrated that the F4/80$^-$ B220$^-$ CD11c$^+$ DC precursors mobilized into the peripheral blood by P. acnes injection express CCR1 and CCR5 at high levels. To examine whether these two chemokine receptors are responsible for the mobilization of F4/80$^-$ B220$^-$ CD11c$^+$ DC precursors into the circulation, wild-type, CCR1$^{-/-}$, and CCR5$^{-/-}$ B6 mice were injected intravenously with P. acnes. In agreement with our previous observation, F4/80$^-$ B220$^-$ CD11c$^+$ cells were mobilized into the circulation (constituting 12.6%, 95% confidence interval [CI] = 9.1% to 16.1%, of PBMCs) in wild-type B6 mice 24 hours after P. acnes injection (Fig. 1, A). By contrast, administration of P. acnes to CCR1$^{-/-}$ and CCR5$^{-/-}$ mice resulted in statistically significantly less mobilization of F4/80$^-$ B220$^-$ CD11c$^+$ cells into the circulation (6.3% [95% CI = 5.2% to 7.3%] in CCR5$^{-/-}$ mice and 9.0% [95% CI = 7.5% to 10.5%] in CCR1$^{-/-}$ mice; both P<.001; Fig. 1, A), suggesting that CCR5 and, to a lesser extent, CCR1 play important roles in mobilizing F4/80$^-$ B220$^-$ CD11c$^+$ cells into the circulation of P. acnes–treated mice.

Because MIP-1α is involved in recruiting F4/80$^+$ B220$^+$ CD11c$^+$ DC precursors to the livers of mice injected with P. acnes (12) and is a ligand for CCR1 and CCR5 (17), we next examined the effect of anti-MIP-1α antibody on P. acnes–induced mobilization of F4/80$^+$ B220$^+$ CD11c$^+$ cells into the circulation. Intravenous administration of B6 mice with anti-MIP-1α antibody 8 hours before injection of P. acnes resulted in a statistically significant reduction in the mobilization of F4/80$^-$ B220$^-$ CD11c$^+$ cells into the circulation compared with mice treated with control IgG (6.6%, 95% CI = 5.7% to 7.5%, and 12.8%, 95% CI = 11.5% to 14.1%, respectively; P<.001) (Fig. 1, B). These results indicate that binding of MIP-1α to its receptors CCR1 and CCR5 is necessary for the mobilization of F4/80$^+$ B220$^+$ CD11c$^+$ cells into the circulation that is associated with P. acnes injection.

Rapid Mobilization of B220$^+$ CD11c$^+$ Cells Into the Circulation by Single Intravenous Administration of Recombinant MIP-1α

To further assess whether MIP-1α could directly mobilize B220$^+$ CD11c$^+$ cells into the circulation, B6 mice were injected intravenously with doses of recombinant MIP-1α ranging from 0 to 2000 μg. As shown in Fig. 2, A, a single administration of MIP-1α could mobilize B220$^+$ CD11c$^+$ cells into peripheral blood, with a plateau at 20 μg (12.1%, 95% CI = 11.6% to 12.7%). Single injections of MIP-1α mobilized not only B220$^-$ CD11c$^+$ cells but also B220$^+$ CD11c$^+$ cells, although the percentage of B220$^+$ CD11c$^+$ cells (12.1%, 95% CI = 12.0% to 12.1%) always exceeded that of B220$^-$ CD11c$^+$ cells (3.6%, 95% CI = 3.5% to 3.6%; Fig. 2, B). Percentages of both cell types reached a peak between 8 and 24 hours and declined by 72 hours after MIP-1α injection (Fig. 2, B). The mobilizing effect of MIP-1α on B220$^+$ CD11c$^+$ cells was lower in CCR5$^{-/-}$ and CCR1$^{-/-}$ mice (6.5%, 95% CI = 6.3% to 6.8%, and 9.2%, 95% CI = 8.8% to 9.7%, respectively) than in wild-type mice (13.1%, 95% CI = 10.8% to 15.5%; both P<.001), suggesting that both CCR1 and CCR5 are involved in MIP-1α–mediated mobilization of B220$^+$ CD11c$^+$ cells (Fig. 2, C).

Differentiation of MIP-1α–Mobilized Circulating B220$^+$ CD11c$^+$ Cells Into Mature DCs Ex Vivo

We next examined whether the B220$^+$ CD11c$^+$ cells mobilized by MIP-1α can differentiate into mature DCs. Flow cytometric analysis demonstrated that the B220$^+$ CD11c$^+$ cells that were mobilized by MIP-1α injection expressed high levels of the
Cruited by P. acnes

Peripheral blood was obtained by cardiac puncture, and peripheral blood mononuclear cells were isolated. Some cells were matured by culturing them with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) for 5 days and then with GM-CSF and murine tumor necrosis factor-α (TNF-α) for 3–4 days (see “Methods” section). The phenotypes (i.e., immunofluorescence intensity) of both dendritic cell (DC) precursors (dotted lines) and mature DCs (solid lines) were analyzed by immunostaining with antibodies to CD205, CD11c, CD40, F4/80, CD11b, CD80, and CD86 (see “Methods” section). Data are representative of three independent experiments, all with similar results.

Immunization of Mice With MIP-1α–Mobilized DCs

We next examined whether these MIP-1α–mobilized DC precursors could be used in DC-based vaccination for tumor prevention. For these analyses, we used the B16 melanoma tumor model, which has been widely used for studying vaccination-based tumor biotherapy (18). All mice vaccinated with PBS-pulsed DCs derived from MIP-1α–mobilized DC precursors or immunized with tumor lysates alone or with PBS died within 25 days after B16 tumor cell challenge (Fig. 5, A). By contrast, approximately 50% of B6 mice that were first vaccinated with P. acnes–mobilized, MIP-1α–mobilized, or bone marrow–derived DCs that had been pulsed with B16 tumor lysates and were subsequently challenged with B16 tumor cells survived and remained tumor-free for more than 60 days following the primary B16 tumor inoculation (Fig. 5, A). This period included a rechallenge with B16 tumor cells 30 days after the first challenge.

To determine whether the response was tumor specific, we isolated splenic CD3+ T cells obtained from mice still alive 60 days after the primary B16 challenge and assayed levels of IFNγ, a reflection of immune activation, and cytotoxic activity against B16 and EL4 tumor cells. The splenic CD3+ T cells produced high levels of IFNγ in vitro when stimulated with B16...
tumor cells but not when stimulated with EL4 cells (Fig. 5, B) and showed cytotoxic activity against B16 tumor cells but not EL4 tumor cells (Fig. 5, C). Furthermore, antibodies to MHC class I molecules abrogated the cytolytic activity against B16 tumor cells (Fig. 5, C). Together, these results suggest that mice vaccinated with B16 tumor cell lysate—pulsed DCs when cultured in vitro—primed B16 tumor cells had generated MHC class I molecules abrogated the cytolytic activity against B16 tumor cells. Furthermore, antibodies to MHC class I molecules abrogated the cytolytic activity against B16 tumor cells but not EL4 tumor cells.

Finally, we examined whether naive splenic T cells that had been primed in vitro with B16 tumor cell lysate—pulsed DCs in the presence of IL-7 and IL-2 had tumor-specific cytotoxic activity. As Fig. 6 shows, splenic T cells primed with either MIP-1α—mobilized or bone marrow—derived DCs that had been pulsed with B16 tumor lysates had cytotoxic activity against B16 tumor cells but not EL4 tumor cells.

**Discussion**

In this study, we found that mobilization of inflammation-associated DC precursors into the circulation is mediated by the interaction of MIP-1α and its receptors, CCR1 and CCR5. A single administration of recombinant MIP-1α could rapidly mobilize B220+CD11c+ DC precursors into the peripheral blood. Moreover, these DC precursors could differentiate into mature DCs when cultured in vitro in the presence of GM-CSF and TNF-α, and they acquired the capacity to generate CTLs against tumor cells, leading to tumor rejection. Thus, mobilization of DC precursors by injecting MIP-1α may provide a novel way to collect a large number of appropriate DC precursors for DC-based immunotherapy.

Chemokines direct the trafficking of subsets of leukocytes into various tissues in homeostasis as well as in inflammatory states in vivo (19). Previous studies demonstrated that the migration of immature and mature DCs may be regulated by distinct chemokines (7,12,19–21). Bone marrow—derived immature human DCs, which express high levels of CCR1, CCR5, and CCR6, are directed to migrate in response to MIP-1α, MIP-1β, monocyte chemotactic protein-3, and MIP-3α (22,23).

In mature human DCs, by contrast, expression of these chemokine receptors is reduced and that of CCR7 is markedly in-

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*Peripheral blood mononuclear cells were obtained from wild-type (WT) mice (C57BL/6) and knockout mice lacking the CC chemokine receptors CCR1 (CCR1−/−) or CCR5 (CCR5−/−) that had been injected with P. acnes and subjected to cardiac puncture 24 hours later. Cell surface antigens were analyzed by immunofluorescence staining. Percentage of positive cells was determined by fluorescence-activated cell sorting. Data shown are representative of three experiments, all with similar results.
compared with a log-rank test of Kaplan–Meier curves. Mice (groups of 20) injected with phosphate-buffered saline and medium alone were used as controls. A further step was added for mice (groups of 20) injected with phosphate-buffered saline–pulsed MIP-1α–mobilized DCs (i.e., unstimulated DCs), B16 tumor lysate alone, and medium alone were used as controls. A) Survival was observed over time after the first challenge with tumor cells (i.e., day 0 on graph). Survival rate was compared with a log-rank test of Kaplan–Meier curves. B and C) Splenic T cells isolated from the surviving immunized mice (n = 8 per group) at day 60 after tumor challenge were restimulated in vitro with mitomycin C–treated B16 tumor cells for 5 days. The restimulated T cells as effector cells were incubated with target B16 or EL4 tumor cells for 20 hours (see “Methods” section). The supernatant was assayed for interferon γ (B) and for cytolytic activity (C). Results are presented as the mean ± 95% confidence intervals of eight mice in each treatment group. *P < .001.

creased, resulting in a vigorous response to stimulation with secondary lymphoid–organ chemokine (SLC) and EB11-ligand chemokine (ELC) (13, 22, 23). Our previous study (20) demonstrated that CCR6 is not detectable in murine DCs and that immature murine DCs, which express CCR1, CCR5, and CCR7, migrate in response to MIP-1α and SLC. By contrast, mature murine DCs express high levels of CCR7 but not of CCR1 and CCR5, resulting in skewed migration toward SLC (7, 20). However, it remains unclear how DC precursor migration is directed in response to various stimuli. DC migration in vivo involves three steps: mobilization into the blood, recruitment from blood to peripheral tissues, and remobilization from peripheral to lymphoid tissues, where the immature DCs finally differentiate into fully mature DCs to promote immune responses. Although the first step has not received much attention, it is important to understand how this step is regulated for understanding the pathologic role of DCs in various inflammatory diseases and in tumor development.

We previously demonstrated that P. acnes infection of mice leads to the recruitment of F4/80+B220-CD11c+ DC precursors into peripheral blood during the development of hepatic granulomas (12). We have found that rapid mobilization of DC precursors into the circulation can be caused by other stimuli in addition to P. acnes administration, because infection with live herpes simplex virus and acute graft-versus-host reaction also induce similar mobilization of DC precursors in mice (Yoneyama H, Matsuno K, Zhang Y, Nishiwaki T, Kitabatake M, Ueha S, et al.: unpublished observations). Taking advantage of chemokine receptor gene–deficient mice, we found that the number of F4/80+B220-CD11c+ DC precursors in peripheral

**Fig. 5.** Induction of tumor-specific cytotoxic T cells and tumor immunity by vaccination with macrophage inflammatory protein 1α (MIP-1α)–mobilized dendritic cell (DC) precursors. Immature MIP-1α–mobilized DCs, P. acnes–recruited DCs, and bone marrow (BM)–derived DCs were pulsed with lysates from B16 melanoma tumor cells. Groups of 20 C57BL/6 mice were immunized by subcutaneous injection of 1 × 106 B16 tumor cell lysate–stimulated DCs on days 0, 7, and 14. On day 21, the immunized mice were then challenged by subcutaneous injection with 2 × 105 viable B16 tumor cells. Mice (groups of 20) injected with phosphate-buffered saline–pulsed MIP-1α–mobilized DCs (i.e., unstimulated DCs), B16 tumor lysate alone, and medium alone were used as controls. A) Survival was observed over time after the first challenge with tumor cells (i.e., day 0 on graph). Survival rate was compared with a log-rank test of Kaplan–Meier curves. B and C) Splenic T cells isolated from the surviving immunized mice (n = 8 per group) at day 60 after tumor challenge were restimulated in vitro with mitomycin C–treated B16 tumor cells for 5 days. The restimulated T cells as effector cells were incubated with target B16 or EL4 tumor cells for 20 hours (see “Methods” section). The supernatant was assayed for interferon γ (B) and for cytolytic activity (C). Results are presented as the mean ± 95% confidence intervals of eight mice in each treatment group. *P < .001.

**Fig. 6.** Generation of tumor-specific cytotoxic T cells in vitro by macrophage inflammatory protein 1α (MIP-1α)–mobilized circulating dendritic cells (DCs). Isolated splenic T cells from naive C57BL/6 mice were primed with B16 tumor cell lysate–pulsed MIP-1α–mobilized or bone marrow (BM)–derived DCs (see “Methods” section). Unpulsed DCs and tumor lysate alone were used as controls. The primed T cells (i.e., cytotoxic T cells) were titrated by serial dilution, and their lytic activity against B16 and EL4 target cells was assayed. The data represent mean value ± 95% confidence intervals of specific lysis from five independent experiments.

Journal of the National Cancer Institute, Vol. 96, No. 3, February 4, 2004 ARTICLES 207
blood of CCR5−/− and, to a lesser extent, CCR1−/− mice was statistically significantly reduced compared with that in wild-type mice in response to P. acnes injection. Moreover, in the present work, systemic administration of anti-MIP-1α antibodies markedly reduced the mobilizing effect of P. acnes infection on F4/80−/−B220 CD11c+ DC precursors in vitro. Thus, it is likely that the interaction between MIP-1α and its receptors, CCR1 and CCR5, regulates the migration of F4/80−/−B220 CD11c+ DC precursors in vivo. The similarity between the phenotypes of DC precursors obtained from P. acnes–primed wild-type, CCR1−/−, and CCR5−/− mice (Table 1) and from MIP-1α−/−treated mice (Fig. 3) suggests that signaling through CCR1 and CCR5 induced by MIP-1α probably does not discriminate subpopulations of DC precursors.

DC precursor mobilization was not completely abrogated in either CCR1−/− or CCR5−/− mice, suggesting that, although both CCR1 and CCR5 are involved in the mobilization of DC precursors by P. acnes injection, neither is absolutely required for this process. DC precursor mobilization was inhibited more strongly in CCR5−/− mice than in CCR1−/− mice, suggesting that the interaction of MIP-1α with CCR5 may be more important than that with CCR1. In this context, it is interesting to note that MIP-1α has been reported to bind more strongly to CCR5 (Kd = 8.9 nM) than to CCR1 (Kd = 14 nM) in competition binding assays using CCR1- or CCR5-transfected Chinese hamster ovary cells (24,25). The difference between CCR1 and CCR5 effects may also be related to cell trafficking. It was recently reported (26) that both CCR1 and CCR5 are internalized after interaction with their ligands but that only CCR5 can be recycled. In this respect, both CCR1 and CCR5 are involved in leukocyte arrest on endothelial cells, whereas CCR5 may further be involved in subsequent spreading and extravasation, even after internalization, by interacting with the endothelium (26).

Although distinct chemokine receptor usage is possible when immature DCs in the peripheral blood in a simple way immature DCs in the clinic should be simple and widely available. Such manipulations of patients’ cells, protocols to use DCs in the clinic should be simple and widely available. Such protocols include increasing the recovery of DC precursors or immature DCs in the peripheral blood in a simple way (28). Although administration of Flt3-ligand has been reported to increase tissue-resident DC numbers by approximately 10-fold (28), this study is, to our knowledge, the first to describe a way to increase the number of blood DC precursors in a short time. The recruitment into blood is important because blood samples are easier to obtain than tissue samples. Moreover, the blood DC precursors we identified induce a stronger immune response of T helper type 1 cells than tissue-resident DCs (12,21). We also found that MIP-1α–mobilized DC precursors could differentiate into mature DCs and that vaccination of mice with DCs that had been stimulated with B16 tumor cell lysates could induce both tumor resistance in vivo and vigorous CTL responses specific to B16 tumor cells in vitro. Because single injections of MIP-1α did not induce any detectable inflammatory response or liver injury in vivo (data not shown), we believe it is possible that MIP-1α can be used to mobilize DC precursors for the purpose of DC-based cancer therapy. However, it is not yet known whether similar numbers of DC precursors can be obtained by administering MIP-1α to humans or how useful MIP-1α–mobilized DCs will be in the treatment of cancer patients.

We found that intravenous injection of MIP-1α resulted in substantial increases in the peripheral blood of not only B220−/−CD11c+ cells but also B220−/−CD11c− cells. It has recently been demonstrated that murine B220−/CD11c+ DCs in secondary lymphoid organs produce high levels of type 1 interferon and that the development of murine B220−/CD11c+ DCs and B220−/CD11c− myeloid DCs from bone marrow is differentially regulated by Flt3-ligand and GM-CSF in vitro (29,31). It has also been reported that B220−/CD11c+ DC precursors in normal peripheral blood have the capacity to generate all the DC subpopulations present in mouse lymphoid organs (30). However, we have not been able to induce the differentiation of these MIP-1α–mobilized B220−/CD11c+ cells into mature DCs on ex vivo culture in the presence of either Flt3-ligand or GM-CSF plus IL4 plus TNF-α; consequently, the splenic T cells from vaccinated mice had no activity in CTL generation against tumors on ex vivo culture (Yoneyama H, Zhang Y, Ishikawa S, Matsushima K: unpublished observations). The discrepancy between the results with B220−/CD11c+ cells and those with B220−/CD11c+ cells may be due to differences in culture conditions (31). Alternatively, the B220−/CD11c+ cells we observed could be newly induced DC precursor populations in the circulation in response to inflammatory stimuli. Thus, it is also possible that these cells have distinct origins from the previously reported B220−/CD11c+ cells that normally exist in the blood (30). However, further characterization of normal blood B220−/CD11c+ cells is needed because these cells could be the recently described plasmacytoid DC precursors and not common DC precursors (31).

In summary, we have demonstrated that the interaction of MIP-1α and its receptors plays a role in DC precursor mobilization in response to P. acnes or MIP-1α administration. It is not yet known whether this rapid mobilization is due to the release of DC precursors that are anchored on the extracellular matrix of bone marrow as hematopoietic progenitor cells, nor is it known whether MIP-1α acts directly or indirectly to mobilize DC precursors. In addition, we have shown that ex vivo culture of MIP-1α–mobilized DC precursors led to their differentiation into mature DCs with the capacity to elicit CTL responses to tumor cells leading to tumor rejection by vaccinated mice. These data suggest that MIP-1α directs the migration of DC precursors in vitro and raises the possibility that MIP-1α injection may be useful for collecting large number of DC precursors for DC-based immunotherapy in humans.
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NOTES

We thank Dr. Yi Zhang (Division of Hematology/Oncology, University of Pennsylvania School of Medicine, Philadelphia) for his review of the manuscript. We also thank Drs. Yahui Wang, Makoto Haino, Shigenori Nagai, Satoshi Ueha, Masahiro Kitabatake, Nobuyuki Onai, and Aya Onai for their assistance. Manuscript received April 4, 2003; revised November 25, 2003; accepted December 8, 2003.