MIP-3α and MIP-1α rapidly mobilize dendritic cell precursors into the peripheral blood

Shan He,* Qi Cao,* Hiroyuki Yoneyama,† Hailiang Ge,* Yi Zhang,‡ and Yanyun Zhang*,[1]

*Institute of Health Sciences and Shanghai Institute of Immunology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and Shanghai Jiao Tong University School of Medicine, Institutes of Medical Sciences, Shanghai, China; †Department of Molecular Preventive Medicine, School of Medicine, The University of Tokyo, Tokyo, Japan; and ‡Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan, USA

Abstract: Acquisition of dendritic cells (DCs) or DC precursors in vitro is critical for DC-based immunotherapy. We reported previously that administration of MIP-1α mobilized a population of F4/80$^-$B220$^-$CD11c$^+$ DC precursors into peripheral blood by the expression of CCR1 and CCR5. In this study, we identified a new subset of CCR6$^+$CCR1$^-$CCR5$^-$B220$^-$CD11c$^+$ cells in MIP-1α-administered mice. When cultured with GM-CSF, IL-4, and TNF-α, these cells differentiated into mature DCs, possessing the typical morphologic characteristics, phenotypes, and antigen-presenting function (termed CCR6$^+$ DC precursors). Although it did not directly drive the CCR6$^+$ DC precursors, MIP-1α could recruit a population of F4/80$^+$CD11c$^-$ monocyte/macrophage-producing MIP-3α in the peripheral blood to mobilize a CCR6$^+$ DC precursor subset of B220$^-$CD11c$^+$ DC precursors. Importantly, exogenous administration of MIP-3α significantly enhanced MIP-1α-induced mobilization of DC precursors. Moreover, these MIP-3α- and MIP-1α-mobilized DC precursors could be prepared for a DC vaccine capable of eliciting CTL responses to tumor cells, leading to tumor rejection in vitro and in vivo. Taken together, this study further demonstrates the mechanism of DC precursor mobilization induced by MIP-1α; that is, besides mobilizing DC precursors with CCR1 and CCR5 expressions, MIP-1α recruited F4/80$^+$CD11c$^-$ monocyte/macrophage-producing MIP-3α, which finally mobilized the CCR6$^+$ DC precursor subset to amplify the B220$^-$CD11c$^+$ DC precursor population. Furthermore, combined administration of MIP-3α and MIP-1α may be an efficient strategy for collecting a large number of DCs appropriate for immunotherapy. *J. Leukoc. Biol.* 84: 1549–1556; 2008.

Key Words: *chemokine · recruited DC · DC-based vaccine*

INTRODUCTION

Dendritic cells (DCs) are professional APCs that play a central role in initiating a primary immune response and are also important to maintain effective memory T cell responses [1–3]. This essential role of DCs in cellular immunity has led to developing feasible and effective DC-based vaccines against tumor antigens to eliminate cancer cells. Approaches that have been tested include pulsing DCs with tumor lysates, tumor antigen peptide, or protein, fusing DCs with tumor cells, and transducing DCs with genes of tumor antigens, cytokines, or chemokines [4–6]. These DC-based vaccine procedures hold promise for the treatment of tumor-bearing patients.

To improve the strategy for DC-based vaccines, it is critical to acquire a large number of appropriate DCs possessing normal function in vitro. DCs can be isolated and cultured from bone marrow, umbilical blood, and peripheral blood. Among these methods, generation of DCs from the peripheral blood is the simplest and most suitable for clinical application. However, DC precursors exist at low numbers (less than 1%) in the PBMC [7, 8], which limits the application of peripheral blood-derived DCs as a cell source for DC-based therapy. In addition, it has been demonstrated that significantly lower percentages of immature and mature DCs are found in the peripheral blood of cancer patients, particularly in those with distant organ metastases, suggesting that the deficiency of DCs may play a part in inducing cancer-related immune suppression [9]. Thus, it is necessary to investigate the mechanisms of DC precursor recruitment and to find new ways to mobilize them into the circulation.

Chemokines and chemokine receptors play an essential role in directing the migration of immune cells [10–12]. The cells expressing certain chemokine receptors can selectively direct their migration into target tissues, where the cognate chemokine(s) are produced [13–15]. We have demonstrated that i.v. administration of *Propionibacterium acnes* or MIP-1α rapidly recruits a group of F4/80$^-$B220$^-$CD11c$^+$ DC precursors into

[1] Correspondence: Institute of Health Sciences and Shanghai Institute of Immunology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and Shanghai Jiao Tong University School of Medicine, Institutes of Medical Sciences, 225 South Chongqing Road, Shanghai 200025, China. E-mail: yyzhang@sibs.ac.cn

Received July 15, 2008; revised August 13, 2008; accepted August 13, 2008.

doi: 10.1189/jlb.0708420

the peripheral blood [16, 17]. These cells expressed high levels of CCR1 and CCR5, and blockade of CCR1 or CCR5 resulted in their reduced mobilization into the peripheral blood. However, this blockade did not completely inhibit their mobilization into the circulation. Thus, these F4/80$^-$B220$^-$CD11c$^+$ DC precursors may contain heterogeneous populations that use multiple chemokines and chemokine receptors for their mobilization and migration.

In the present study, we identified a new subset of CCR6$^+$CCR1$^-$CCR5$^-$B220$^-$CD1 1c$^+$ DC precursors (termed CCR6$^+$ DC precursors) in the peripheral blood of mice administered with MIP-1α, which cannot directly mobilize CCR6$^+$ DC precursors in vivo but in a way, recruit F4/80$^+$CD11c$^-$ monocyte/macrophage-producing MIP-3α, leading to the interaction between MIP-3α and CCR6 expressed on DC precursors, and directing these DC precursors into the circulation. Moreover, in addition to MIP-1α, administration of MIP-3α can serve to increase collection of DC precursors useful as a source of DCs appropriate for immunotherapy.

MATERIALS AND METHODS

Animals

Female C57BL/6 (B6) and BALB/c mice (8–10 weeks) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and were kept under pathogen-free conditions in the animal center of the Shanghai Jiao Tong University School of Medicine (Shanghai, China). CCR1 gene-deficient (CCR1$^{-/-}$) mice and CCR5$^{-/-}$ mice were generated as described previously [18, 19]. Both knockout mouse strains were backcrossed with B6 mice for eight generations. We generated mice deficient in CCR1/CCR5 (CCR1$^{-/-}$CCR5$^{-/-}$) using the following approach: CCR5$^{-/-}$ mice were mated with CCR1$^{-/-}$ mice to generate a F_1 generation. These mice were then backcrossed to CCR5$^{-/-}$ mice, and offspring heterozygous at the CXCR3 locus (–/–) and deficient for a functional CCR5 gene at both loci (–/–) were then selected and interbred. From the subsequent offspring, homozygous, double-deficient mice were selected, but their vitality and fertility were too low for further inbreeding to generate CCR1$^{-/-}$CCR5$^{-/-}$ mice. Assessment of genotypes was performed by PCR. All experiments with knockout mice were performed in the department of Molecular Preventive Medicine, School of Medicine, The University of Tokyo (Tokyo, Japan). Animal care and use were in compliance with institutional guidelines.

Cytokines and antibodies

Human recombinant MIP-1α, which was expressed in *Brevibacillus choshinensis* and purified to homogeneity, was provided by Dr. Shiro Kanegasaki (Effector Cell Institute, Tokyo, Japan). Mouse recombinant GM-CSF, TNF-α, IL-4, IL-2, and IL-7 were purchased from R&D Systems (Minneapolis, MN, USA). Mouse recombinant MIP-3α and neutralizing rabbit anti-mouse CCR1, CCR5, and MIP-3α antibodies were gifts of Professor Xueguang Zhang (School of Medicine, Soochow University, Suzhou, China). All antibodies used for immunofluorescence labeling were obtained from BD PharMingen (San Diego, CA, USA), and microbeads for magnetic sorting were purchased from Miltenyi Biotec (Auburn, CA, USA).

Cell lines

The murine melanoma cell line B16F10 was purchased from the American Type Culture Collection (Manassas, VA, USA). B16-melanoma antigen 1 (MAGE-1) is a subclone of B16F10 cells, stably transduced with a retrovirus encoding the human MAGE-1 gene [20]. Cells were cultured in IMDM (Gibco, Rockville, MD, USA) containing 12.5% FCS, penicillin G (100 U/ml), and streptomycin (100 μg/ml) as complete medium.

Cell preparation

B6, CCR1$^{-/-}$, and CCR5$^{-/-}$ mice were administered separately with MIP-1α (20 μg) and/or MIP-3α (0–100 μg) in 100 μl PBS via the tail vein, with the equivalent dose of PBS as control. For blocking experiments, antibody to CCR1, CCR5, or MIP-3α (100 μg/mouse) or control IgG was i.v.-injected 8 h before administration of MIP-1α. Peripheral blood (0.8 ml per mouse) was obtained by cardiac puncture from anesthetized mice and then separated for PBMC with NycoPrep (Nycomed Pharma, Oslo, Norway). B220$^-$CD11c$^+$ cells were prepared from PBMC by depleting F4/80$^+$ and B220$^+$ cells by magnetic negative selection and further isolating CD11c$^+$ cells by FACS. In some experiments, these B220$^-$CD11c$^+$ cells were separated further into CCR1$^-$CCR5$^-$, CCR1$^-$CCR5$^+$, CCR1$^+$CCR5$^-$, and CCR1$^+$CCR5$^+$ cell subsets by FACS. The purity of sorted cells was consistently more than 98%, as revealed by immunofluorescence analysis.

DC development

DCs were generated as described previously [16, 21, 22]. In brief, purified peripheral blood-derived B220$^-$CD11c$^+$ cells were cultured at a concentration of 3×10^5 cells/ml in complete medium with GM-CSF (4 ng/ml) and IL-4 (10 ng/ml) for 3–5 days to induce immature DCs, which were cultured further in fresh complete medium containing GM-CSF (4 ng/ml) and TNF-α (50 ng/ml) for 2–3 days to induce their maturation.

MIP-3α assay

The peripheral blood was obtained by cardiac puncture from anesthetized mice at 4, 8, 16, 24, 48, and 72 h postadministration of MIP-1α. Serum was collected for measurement of MIP-3α using an ELISA kit (R&D Systems), according to the manufacturer's instructions.

MLR

MLR was performed in accordance with previous methods [23]. DCs derived from B220$^-$CD11c$^+$ or CCR6$^+$ DC precursors were treated with Mitomycin C (MMC; 15 μg/ml, Kyowa Hakko Kogyo Corp., Tokyo, Japan) in complete medium at 37°C for 3 h to arrest their proliferation. After three washes with PBS, these B6 mouse-stimulator cells (10^2–3×10^4) were added to each well of 96-well plates that contained allogeneic CD4$^+$ T cells (3×10^5) that had been isolated magnetically from BALB/c mice using CD4 microbeads. Cells were stimulated ex vivo for 5 days, and 1 μCi/well [H^3]thymidine was added 18 h before the end of culture. The cells were then harvested onto glass fiber filters for measurement of [H^3]thymidine incorporation based on scintillation counting.

Quantitative PCR (Q-PCR)

Total RNA was extracted from the sorted B220$^-$CD11c$^+$ DC precursors and F4/80$^+$D11c$^-$ macrophages using the guanidium thiocyanate-phenol-chloroform method modified for TRIzol (Invitrogen, Carlsbad, CA, USA). cDNA of MIP-3α and tested chemokine receptors were quantified through the Q-PCR technique, all with GAPDH as control. Q-PCR was performed using a SYBR Green PCR mix in ABI Prism 7900HT (Applied Biosystems, Foster City, CA, USA). Thermocycler conditions included an initial holding at 50°C for 2 min and then 95°C for 10 min; this was followed by a two-step PCR program: 95°C for 15 s and 60°C for 60 s for 40 cycles. Data were collected and quantitatively analyzed on an ABI Prism 7900 sequence detection system (Applied Biosystems). The primer sequences were designed using Primer Express Software Version 2.0, provided with the ABI Prism 7900HT (**Table 1**). The GAPDH gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. All values were expressed as fold increase or decrease relative to the expression of GAPDH. The mean value of the replicates for each sample was calculated and expressed as cycle threshold (C_T; cycle number at which each PCR reaches a predetermined fluorescence threshold, set within the linear range of all reactions). The amount of gene expression was then calculated as the difference (ΔC_T) between the C_T value of the sample for the target gene and the mean C_T value of that sample for the endogenous control (GAPDH). Relative expression was calculated as the

TABLE 1. The Oligonucleotides Used for Q-PCR

Gene		Primer (5′ → 3′)
CCR1	Forward	TGCAGGTGACTGAGGTGATTG
	Reverse	AAACAGCTGCCGAAGGTACTTC
CCR5	Forward	GGTGGAGGAGCAGGGACAA
	Reverse	TGTGTCCGTCCCTTTGCAT
CCR6	Forward	ACGAGGAGGACCATGTTGTGA
	Reverse	CAGGCCCAGAACTCCAAGAG
MIP-3α	Forward	GACAGATGGCCGATGAAGCTT
	Reverse	TCACAGCCCTTTTCACCCAGT
GAPDH	Forward	GTGAAGGTCGGAGTCAACG
	Reverse	TGAGGTCAATGAAGGGGTC

difference ($\Delta\Delta C_T$) between the ΔC_T values of the test sample and of the control sample. Relative expression of genes of interest was calculated and expressed as $2^{-\Delta\Delta CT}$.

Chemotaxis assay

The chemotaxis analysis in vitro was performed, as described previously [16], with minor modifications. MIP-1α or MIP-3α (100 ng/ml) was diluted in IMDM without FCS, and 500 μl was added to 24-well tissue-culture plates. Murine type IV collagen-coated transwells (3 μm pore size) were placed in each well, and 120 μl DC suspension (8.0×10^5 cells/well) was added to the top chamber. After incubation at 37°C in 5% CO_2 for 3 h, the cells that had migrated to the bottom chamber were recovered and counted by light microscopy.

Recombinant adenoviral vectors

The recombinant adenovirus (Ad) expressing MAGE-1 gene (termed Ad-MAGE-1) and Ad encoding β-galactosidase (termed Ad-LacZ) were constructed as described previously [24]. In brief, the entire MAGE-1 cDNA and LacZ gene were cloned, respectively, into pAC-CMVpLpA Ad type 5 driven by the cytomegalovirus promoter/enhancer. Ad was prepared by recombination of this plasmid with pJM17, which contains the 35-kb Ad genome, deleted in the El region in 293 cells, which provide the El genes in trans. Recombinant viruses were produced using 293 cells, purified on CsCl density gradient, and titers were determined by plaque assay on 293 cells. The Ad solutions were stored at −80°C.

Transduction of DC with adenoviral constructs

To transduce gene expression in DCs, MIP-3α- and MIP-1α-mobilized DCs were cultured with GM-CSF and TNF-α for 3 days, then incubated with Ad-MAGE-1 or Ad-LacZ at a multiplicity of infection of 100:1 for 2 h at 37°C, and then washed twice with complete medium. The above DCs are referred to as DC-MAGE-1 and DC-LacZ, respectively. Twenty-four hours after gene transduction, Ad-LacZ was collected for 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside staining to evaluate gene transfer efficiency as described previously [24].

DC vaccination and tumor challenge

B6 mice were vaccinated s.c. above the abdominal wall three times at an interval of 7 days with DC-MAGE-1 (1×10^6). Control mice were vaccinated with DC-LacZ or nontransduced DCs alone. These vaccinated mice were challenged s.c. with a lethal dose of 1×10^5 B16-MAGE-1 or with the parental B16F10 tumor cells. Tumor size was evaluated every other day. Forty days after the first challenge, surviving mice were rechallenged s.c. with B16-MAGE-1 cells (2×10^5). Survival differences among groups receiving different vaccinations were monitored following challenge with tumor cells.

Assays for CTL activity and IFN-γ secretion

Splenic $CD3^+$ T cells (1×10^6 cells/ml) isolated from naive B6 mice were stimulated in vitro in the presence of cytokines including IL-2 (5 ng/ml) and IL-7 (5 ng/ml) at Days 0, 7, and 14 with DC-MAGE-1 at a stimulator:responder cell ratio of 1:20. Fresh complete medium containing IL-2 and IL-7 was exchanged every 3 days. At Day 21, the stimulated T cells were collected as effector cells. In some experiments, T cells were isolated from spleens of the survival mice at Day 60 after tumor cell challenge. These T cells (1×10^6 cells/ml) were restimulated in vitro with 1×10^5 MMC-treated B16-MAGE-1 cells. Five days later, the MMC-treated B16-MAGE-1 cells had been lost in the cultures, and the restimulated T cells were collected as effector cells (2×10^5 in 100 μl per well), which were added to 96-well plates containing target B16-MAGE-1 or B16F10 cells (5×10^4 in 100 μl per well). After 20 h, supernatant from each well was collected for measuring IFN-γ secretion (ELISA kit, R&D Systems) and released lactate dehydrogenase using a non-radioactive cytotoxicity detection kit, according to the manufacturer's instructions (Promega, Madison, WI, USA).

Statistical analysis

Differences among groups of mice with respect to the number of mobilized DC precursors and the ability of T cells in vitro to kill tumor cells and to produce IFN-γ were evaluated by one-way ANOVA and the multiple comparison method of Scheffe using StatView Version 5.0 (SAS Institute, Cary, NC, USA). 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 0.05 were considered to be statistically significant.

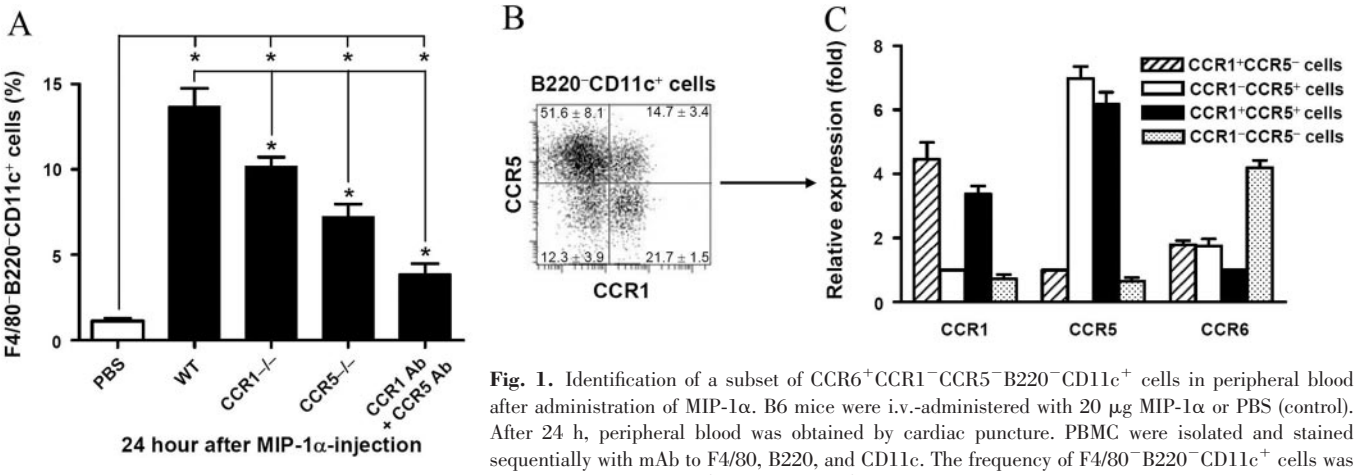

Fig. 1. Identification of a subset of $CCR6^+CCR1^-CCR5^-B220^-CD11c^+$ cells in peripheral blood after administration of MIP-1α. B6 mice were i.v.-administered with 20 μg MIP-1α or PBS (control). After 24 h, peripheral blood was obtained by cardiac puncture. PBMC were isolated and stained sequentially with mAb to F4/80, B220, and CD11c. The frequency of $F4/80^-B220^-CD11c^+$ cells was analyzed using FACS. (A) $F4/80^-B220^-CD11c^+$ cells were mobilized in $CCR1^{-/-}$ mice, $CCR5^{-/-}$ mice, and wild-type (WT) mice, injected with or without antibodies against CCR1 and CCR5, respectively. (B) $B220^-CD11c^+$ cells mobilized in WT mice were analyzed and sorted by FACS according to CCR1 and CCR5 expressions. (C) FACS-sorted $B220^-CD11c^+$ cell subsets were subjected to Q-PCR for expressions of CCR1, CCR5, and CCR6. Data are shown as the means ± SD of 10 mice in each group and are representative of three independent experiments. *, $P < 0.05$.

RESULTS

Identification of MIP-1α-mobilized CCR6$^+$ DC precursors

Using CCR1$^{-/-}$ and CCR5$^{-/-}$ mice, we have demonstrated previously that CCR1 and CCR5 play important roles in mobilizing F4/80$^-$B220$^-$CD11c$^+$ DC precursors into the peripheral blood after i.v. injection of mice with MIP-1α [17]. However, blockade of CCR1 and CCR5 by their neutralizing antibodies did not completely inhibit the mobilization of F4/80$^-$B220$^-$CD11c$^+$ DC precursors into the peripheral blood of mice administered with MIP-1α (**Fig. 1A**). This suggests that some other chemokine receptors, besides CCR5 and CCR1, may also account for mobilizing DC precursors. We examined chemokine receptor expression in freshly isolated blood B220$^-$CD11c$^+$ cells of B6 mice 24 h after i.v. administration of MIP-1α. Most B220$^-$CD11c$^+$ cells expressed CCR1 and/or CCR5 (51.6±8.1% for CCR$^-$CCR5$^+$, 14.7±3.4% for CCR1$^+$CCR5$^+$, and 21.7±1.5% for CCR1$^+$CCR5$^-$), and 12.3 ± 3.9% were CCR1$^-$CCR5$^-$ (Fig. 1B). Interestingly, these CCR1$^-$CCR5$^-$B220$^-$CD11c$^+$ cells predominantly expressed CCR6 (Fig. 1C), suggesting that in addition to CCR1 and CCR5, CCR6 may be responsible for MIP-1α-induced mobilization of this CCR1$^-$CCR5$^-$B220$^-$CD11c$^+$ cell subset.

We next examined whether these CCR6$^+$CCR1$^-$CCR5$^-$B220$^-$CD11c$^+$ cells mobilized by MIP-1α were DC precursors. Freshly isolated CCR6$^+$CCR1$^-$CCR5$^-$ cell subset from B220$^-$CD11c$^+$ cells exhibited monocyte-like morphology (**Fig. 2A**) and expressed high levels of CD11b, moderate levels of CD40, and low levels of Ia, CD80, CD86, and DEC-205, with no expression of F4/80 and CD8α (Fig. 2B). Functionally, these CCR6$^+$CCR1$^-$CCR5$^-$B220$^-$CD11c$^+$ cells lacked the ability to stimulate the proliferation of allogeneic T cells in an in vitro MLR assay (Fig. 2C). However, when cultured with GM-CSF, IL-4, and TNF-α for 5–8 days, these CCR6$^+$CCR1$^-$CCR5$^-$B220$^-$CD11c$^+$ cells exhibited mature DC morphology with eccentric nuclei and polarized lamellipodia (Fig. 2A), expressed high levels of Ia, DEC-205, CD80, and CD86 (Fig. 2B), and gained the potent ability to stimulate the proliferation of allogeneic T cells (Fig. 2C). Thus, CCR6$^+$CCR1$^-$CCR5$^-$B220$^-$CD11c$^+$ cells, which are mobilized into the peripheral blood after MIP-1α administration, in spite of lacking the expressions of CCR1 and CCR5, are DC precursors (termed CCR6$^+$ DC precursors).

MIP-1α enhances the recruitment of F4/80$^+$CD11c$^-$ monocytes/macrophages producing MIP-3α

As CCR6 is not the specific receptor for MIP-1α, the mechanism of MIP-1α-induced mobilization of CCR6$^+$ DC precursors was examined subsequently. Surprisingly, we found that CCR6$^+$ DC precursors did not respond to MIP-1α in vitro in a chemotaxis assay (**Fig. 3A**). This is in sharp contrast to the effect of MIP-1α on mobilization of the CCR6$^+$ DC precursor in vivo (Fig. 1). A previous study showed that the only known ligand for CCR6 is MIP-3α [25, 26]. We then tested whether MIP-1α could mobilize CCR6$^+$ DC precursors indirectly into the circulation via promoting the production of MIP-3α. In an ELISA assay, we found that MIP-3α was increased significantly in serum and peaked by 24 h after administration of MIP-1α (Fig. 3B). Injection of B6 mice with the anti-MIP-3α

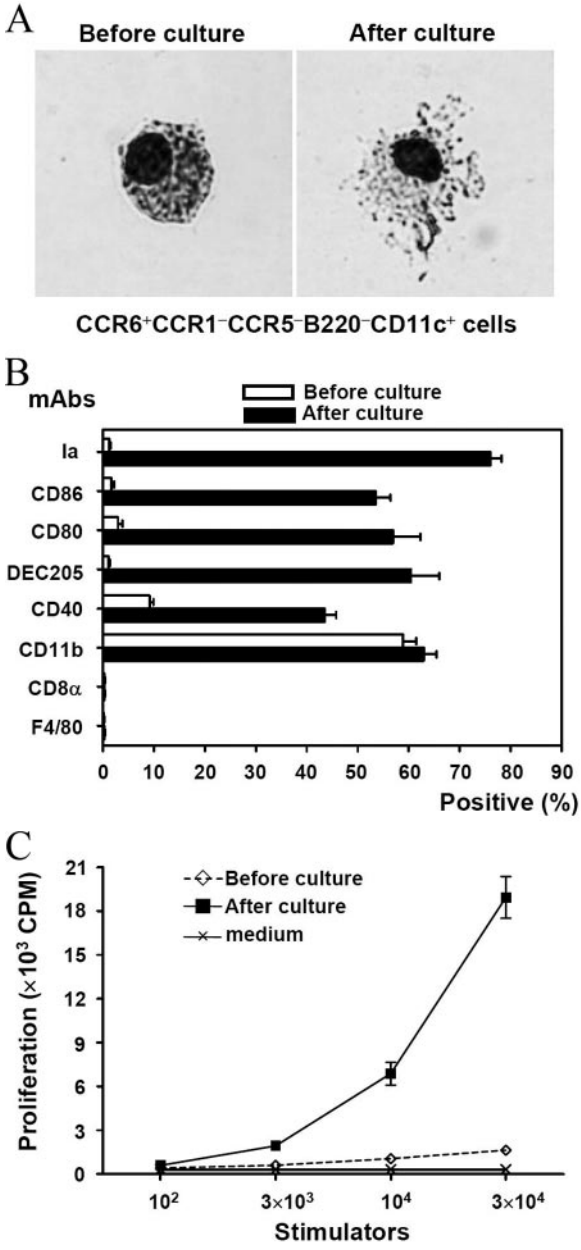

Fig. 2. Characteristics of CCR6$^+$CCR1$^-$CCR5$^-$B220$^-$CD11c$^+$ cells in peripheral blood from mice administered with MIP-1α were sorted and analyzed before and after cultured with GM-CSF and IL-4 for 3–5 days and then with GM-CSF and TNF-α for 2–3 days. (A) Giemsa staining was performed. Original magnification: ×400. (B) The phenotype of these cells was analyzed by immunofluorescence staining with antibodies to DEC205, CD40, F4/80, CD11b, CD80, Ia, CD8α, and CD86. Results are expressed as the means ± SD. (C) Allogeneic MLR was performed using purified splenic T cells (3×10^5 cells/well in a 96-well plate) from BALB/c mice as responder cells. CCR6$^+$CCR1$^-$CCR5$^-$B220$^-$CD11c$^+$ cells before and after culture were treated with MMC to arrest cell proliferation and were used as stimulator cells at the indicated cell numbers. T cell proliferation was measured by incorporation of [^{3}H]thymidine. Results are expressed as the means ± SD of triplicate cultures. All data are representative of three independent experiments.

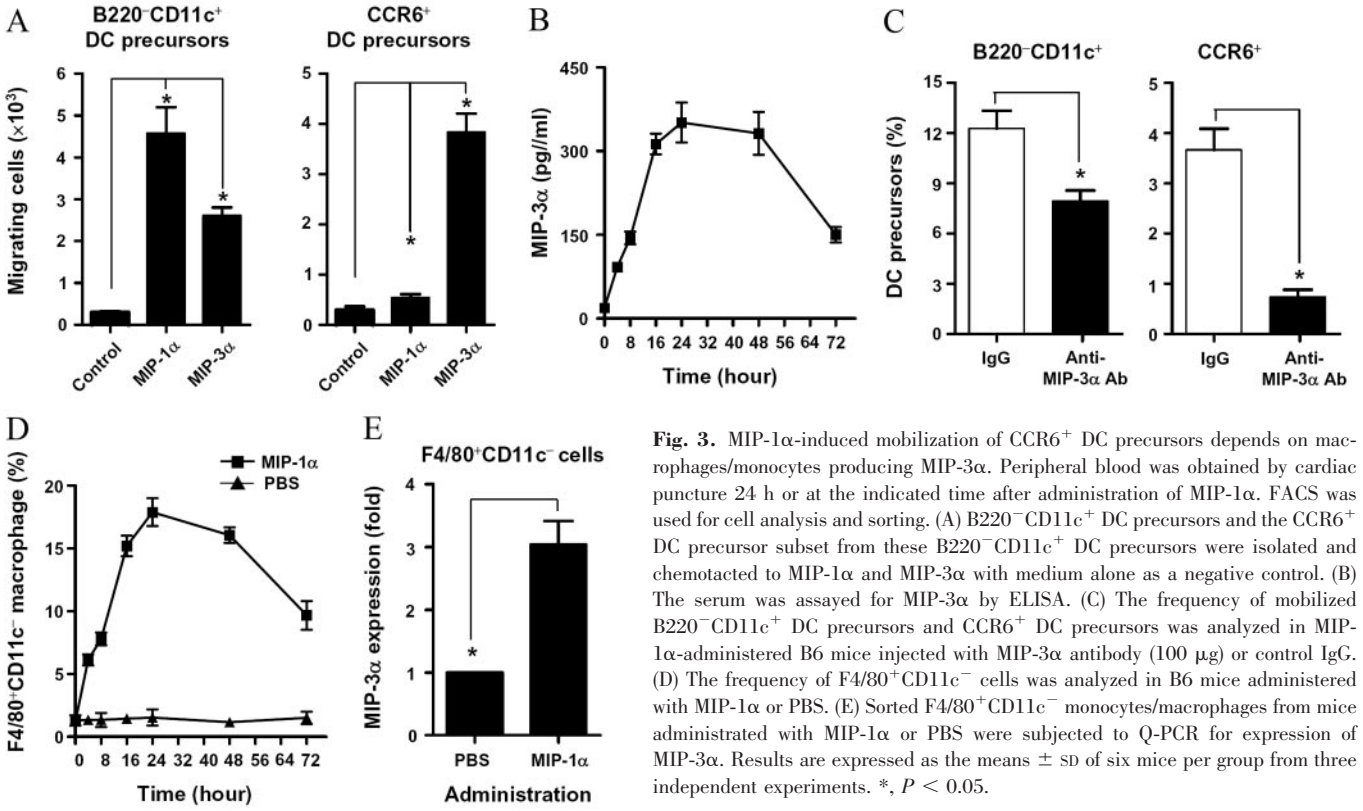

Fig. 3. MIP-1α-induced mobilization of CCR6$^+$ DC precursors depends on macrophages/monocytes producing MIP-3α. Peripheral blood was obtained by cardiac puncture 24 h or at the indicated time after administration of MIP-1α. FACS was used for cell analysis and sorting. (A) B220$^-$CD11c$^+$ DC precursors and the CCR6$^+$ DC precursor subset from these B220$^-$CD11c$^+$ DC precursors were isolated and chemotacted to MIP-1α and MIP-3α with medium alone as a negative control. (B) The serum was assayed for MIP-3α by ELISA. (C) The frequency of mobilized B220$^-$CD11c$^+$ DC precursors and CCR6$^+$ DC precursors was analyzed in MIP-1α-administered B6 mice injected with MIP-3α antibody (100 μg) or control IgG. (D) The frequency of F4/80$^+$CD11c$^-$ cells was analyzed in B6 mice administered with MIP-1α or PBS. (E) Sorted F4/80$^+$CD11c$^-$ monocytes/macrophages from mice administrated with MIP-1α or PBS were subjected to Q-PCR for expression of MIP-3α. Results are expressed as the means ± SD of six mice per group from three independent experiments. *, $P < 0.05$.

antibody 8 h before administration of MIP-1α resulted in a significant reduction of the B220$^-$CD11c$^+$ DC precursor population, herein especially, the CCR6$^+$ DC precursor subset as compared with that in mice treated with control IgG (0.73±0.25% vs. 3.67±0.72%; $P<0.05$; Fig. 3C). This was confirmed further by an in vitro chemotaxis assay that CCR6$^+$ DC precursors responded to the chemoattractant effect of MIP-3α (Fig. 3A). Thus, MIP-3α is responsible for the in vivo mobilization of the CCR6$^+$ DC precursor subset of B220$^-$CD11c$^+$ DC precursors in mice administered with MIP-1α.

It has been demonstrated that MIP-3α is produced predominantly by macrophages and monocytes [27, 28]. We found that i.v. administration of MIP-1α also recruited a population of the F4/80$^+$CD11c$^-$ monocyte/macrophage into the peripheral blood, which peaked by 24 h after MIP-1α administration (Fig. 3D). These MIP-1α-recruited F4/80$^+$CD11c$^-$ monocytes/macrophages produced a significantly higher level of MIP-3α than did the F4/80$^+$CD11c$^-$ monocyte/macrophage derived from mice with PBS treatment (Fig. 3E). Together, these results suggest that administration of MIP-1α recruits a population of F4/80$^+$CD11c$^-$ monocytes/macrophages producing MIP-3α into the circulation, which finally mobilizes the CCR6$^+$ DC precursor subset and amplifies the mobilization of B220$^-$CD11c$^+$ DC precursors.

Administration of MIP-3α and MIP-1α mobilizes DC precursors

We next tested whether administration of MIP-3α plus MIP-1α mobilized more F4/80$^-$B220$^-$CD11c$^+$ DC precursors into the circulation than MIP-1α or MIP-3α alone. As shown in **Figure 4A**, a single dose of MIP-3α significantly increased the percentage of F4/80$^-$B220$^-$CD11c$^+$ cells in the peripheral blood, with a plateau at 10 μg (7.8±0.9%). Although the ability of MIP-3α to mobilize F4/80$^-$B220$^-$CD11c$^+$ cells was less potent than that of MIP-1α, addition of MIP-3α plus MIP-1α could increase the numbers of F4/80$^-$B220$^-$CD11c$^+$ cells significantly as compared with MIP-1α alone (19.5±1.4% vs. 13.0±1.2%; $P<0.05$; Fig. 4B). Thus, combined administration of mice with MIP-3α and MIP-1α can dramatically augment the mobilization of DC precursors into the peripheral blood.

DCs mobilized by MIP-1α and MIP-3α elicit potent anti-tumor activity

To examine whether these DC precursors can be used for eliciting potent anti-tumor activity, we infected the MIP-3α- and MIP-1α-mobilized DC precursor with Ad-MAGE-1 to transduce the expression of MAGE-1 after cultured with GM-CSF and TNF-α for 3 days (termed DC-MAGE-1). Nontransduced DCs and DCs transduced with Ad-LacZ (termed DC-LacZ) were used as controls. Naive T cells derived from normal mice were stimulated with DC-MAGE-1, DC-LacZ, and nontransduced DCs, respectively, in an in vitro culture in the presence of IL-2 and IL-7. An in vitro CTL assay showed that DC-MAGE-1-stimulated T cells exhibited much more potent ability to specifically lyse B16-MAGE-1 cells but not B16F10 cells than did T cells stimulated with DC-LacZ or T cells stimulated with nontransduced DCs (**Fig. 5A**). These data suggest that MAGE-1 gene-tranduced, modified DCs could

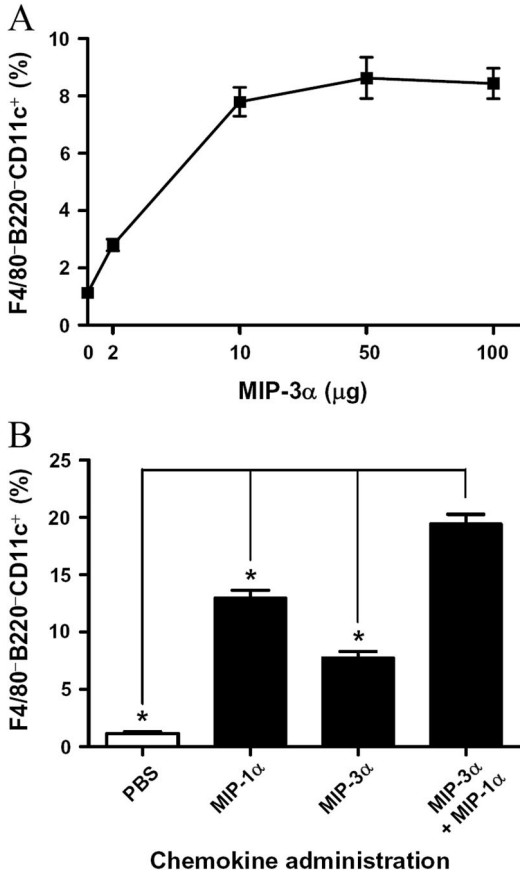

Fig. 4. MIP-3α promotes the mobilization of DC precursors into peripheral blood. B6 mice were administered with the indicated dose of MIP-3α (A) and with 10 μg MIP-3α, 20 μg MIP-1α, or a combination of MIP-3α and MIP-1α (B). After 24 h, PBMC from peripheral blood were isolated from these mice. The frequency of F4/80$^-$B220$^-$CD11c$^+$ DC precursors was analyzed using FACS. Data are expressed as the means ± SD of 10 mice in each group and are representative of three independent experiments. *, $P < 0.05$.

stimulate specific CTL responses to tumor cells bearing MAGE-1 in vitro.

We finally investigated the ability of DC-MAGE-1 to induce the in vivo generation of CTLs specific to tumor antigen MAGE-1. Normal B6 mice were vaccinated s.c. three times at an interval of 7 days with DC-MAGE-1, DC-LacZ, or nontransduced DCs. These vaccinated mice were challenged s.c. with B16-MAGE-1 or the parental B16F10 cells. We found that all mice vaccinated with DC-LacZ or with nontransduced DCs died within 30 days after the B16-MAGE-1 cell challenge. In contrast, 50% of mice receiving the DC-MAGE-1 vaccination survived from B16-MAGE-1 challenge but not from the parental B16F10 cell challenge (Fig. 5B). Furthermore, splenic T cells derived from those mice, survived from B16-MAGE-1 challenge, produced a high level of IFN-γ (Fig. 5C) and showed potent CTL activity against B16-MAGE-1 in vitro but not B16F10 tumor cells (Fig. 5D). Again, all of these mice rechallenged at Day 40 after inoculation of B16-MAGE-1 tumor cells survived without tumor development in the period of over a 90-day observation (Fig. 5B). Together, these results suggest that MIP-3α- and MIP-1α-mobilized DCs can initiate a specific and lasting immunologic protection against tumor growth.

DISCUSSION

Mobilization of DCs and DC precursors into the peripheral blood is of particular interest in the development of DC-based immunotherapy. We reported previously that administration of MIP-1α could mobilize DC precursors by the expression of CCR1 and CCR5 [17]. However, CCR1 and CCR5 are not entirely responsible for the recruitment. The studies herein shed some light on the mechanism of DC precursor mobilization independent of CCR1 and CCR5. After administration of MIP-1α in vivo, we identified a new subset of CCR6$^+$CCR1$^-$CCR5$^-$F4/80$^-$B2 20$^-$CD11c$^+$ DC precursors. These cells did not respond to MIP-1α-mediated migration ex vivo but were mobilized in vivo by MIP-1α-recruited F4/80$^+$CD11c$^-$ monocyte/macrophage-producing MIP-3α. Exogenous administration of MIP-3α mobilized CCR6$^+$ DC precursors and amplified MIP-1α-induced recruitment of B220$^-$CD11c$^+$ DC precursors in the peripheral blood. Thus, combined administration of MIP-3α and MIP-1α may be a novel and more efficient way to collect a large number of DC precursors for further DC-based therapy.

Chemokines and chemokine receptors selectively direct the trafficking of subsets of leukocytes into various tissues in homeostasis as well as in inflammatory states in vivo [12, 29]. The capacity of DCs to migrate to the sites of inflammation, where they capture antigens and subsequently migrate to the local lymph nodes, is regulated by the expression of different chemokines and chemokine receptors [11, 30, 31]. We have reported that the accumulation of the circulating DC precursors is associated with their expression of chemokine receptors CCR1 and CCR5 after MIP-1α administration [17]. However, abrogation of CCR1 and/or CCR5 could not entirely inhibit the mobilization of DC precursors. Thus, it seemed that in addition to CCR1 and CCR5, these DC precursors could use other chemokine receptors for their mobilization and migration. In this study, we identified a new subset of CCR6$^+$CCR1$^-$CCR5$^-$B220$^-$CD11c$^+$ cells. However, although these cells did not express CCR1 or CCR5, after cultured with GM-CSF, IL-4, and TNF-α, they could also differentiate into mature DCs possessing the typical morphologic characteristics, phenotypes, and antigen-presenting function found in allogeneic MLR. Thus, our results demonstrated that the mobilized CCR6$^+$CCR1$^-$CCR5$^-$B220$^-$CD11c$^+$ cells were indeed a subset of DC precursors, and in addition to CCR1 and CCR5, CCR6 may play a role in MIP-1α-induced mobilization of DC precursors into the circulation.

It is somewhat surprising that MIP-1α can mobilize CCR6$^+$ DC precursors in vivo, whereas in vitro, CCR6$^+$ DC precursors did not show a chemotactic response toward MIP-1α. It was hypothesized that MIP-1α did not mobilize CCR6$^+$ DC precursors directly but induced some other cells or chemokines that bound to CCR6 for direction of their migration in vivo. Previous studies have indicated that MIP-3α, a specific and high-affinity ligand for CCR6, is secreted by monocytes/mac-

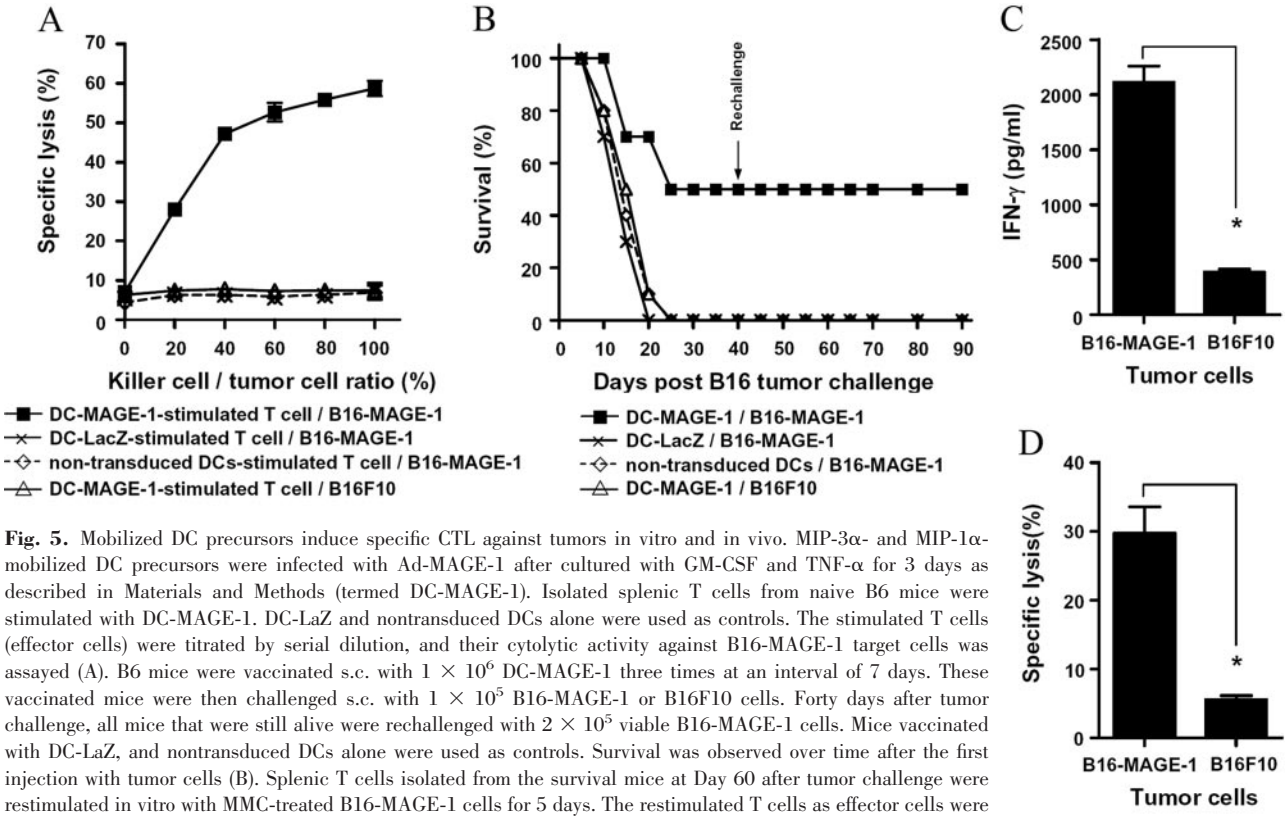

Fig. 5. Mobilized DC precursors induce specific CTL against tumors in vitro and in vivo. MIP-3α- and MIP-1α-mobilized DC precursors were infected with Ad-MAGE-1 after cultured with GM-CSF and TNF-α for 3 days as described in Materials and Methods (termed DC-MAGE-1). Isolated splenic T cells from naive B6 mice were stimulated with DC-MAGE-1. DC-LaZ and nontransduced DCs alone were used as controls. The stimulated T cells (effector cells) were titrated by serial dilution, and their cytolytic activity against B16-MAGE-1 target cells was assayed (A). B6 mice were vaccinated s.c. with 1×10^6 DC-MAGE-1 three times at an interval of 7 days. These vaccinated mice were then challenged s.c. with 1×10^5 B16-MAGE-1 or B16F10 cells. Forty days after tumor challenge, all mice that were still alive were rechallenged with 2×10^5 viable B16-MAGE-1 cells. Mice vaccinated with DC-LaZ, and nontransduced DCs alone were used as controls. Survival was observed over time after the first injection with tumor cells (B). Splenic T cells isolated from the survival mice at Day 60 after tumor challenge were restimulated in vitro with MMC-treated B16-MAGE-1 cells for 5 days. The restimulated T cells as effector cells were incubated with target B16-MAGE-1 or B16F10 cells for 20 h. The supernatant was collected for IFN-γ assay (C), and cytolytic ability was analyzed (D). The data are shown as the means ± SD of 10 mice per group and are representative of three independent experiments. *, $P < 0.05$.

rophages for selective recruitment of CCR6-expressing cells[26, 28]. We found that i.v. administration of MIP-1α resulted in an increase of the F4/80$^+$CD11c$^-$ monocyte/macrophage population besides F4/80$^-$B220$^-$CD11c$^+$ DC precursors into the peripheral blood. Interestingly, these F4/80$^+$CD11c$^-$ monocytes/macrophages produced a high level of MIP-3α. Moreover, after administration of MIP-1α, the F4/80$^+$CD11c$^-$ cell number was increased synchronously with the corresponding MIP-3α level in the blood. Injection of the anti-MIP-3α antibody reduced the MIP-1α-induced mobilization of F4/80$^-$B220$^-$CD11c$^+$ DC precursors significantly, herein especially, the CCR6$^+$ DC precursor subset. Moreover, exogenous administration of MIP-3α promoted DC precursors into the circulation, suggesting that the interaction between the chemokine receptor CCR6 and the related chemokine MIP-3α was involved in the regulation of the mobilization of DC precursors in vivo. Taken together, this study demonstrates further the mechanism of DC precursor mobilization induced by MIP-1α; that is, besides mobilizing DC precursors with CCR1 and CCR5 expressions, MIP-1α recruited F4/80$^+$CD11c$^-$ monocytes/macrophages, producing a high level of MIP-3α, which finally mobilized the CCR6$^+$ DC precursor subset to amplify the B220$^-$CD11c$^+$ DC precursor population.

A pivotal requirement for DC-based immunotherapy against tumors depends on adequate viability and precise capacity of DCs for initiating specific immune responses. The other is to isolate abundant DCs simply, effectively, and practicably. To this end, increasing the recovery of DC precursors or immature DCs in the peripheral blood offers a simple approach [32, 33]. Administration of MIP-1α could increase and mobilize DC precursors rapidly, and these DC precursors could efficiently induce a strong immune response [17]. In this study, we expanded this notion and established a combined protocol, administering MIP-3α and MIP-1α for collection of many more DC precursors. These recruited DC precursors can differentiate into mature DCs and initiate vigorous CTL responses in vitro and in vivo after transduction with the tumor antigen gene. Moreover, T cells primed by those tumor antigen gene-transduced DCs in vivo can elicit protection against the second tumor challenge, which may induce a more persistent immunologic protection and allow their use in a DC-based cancer vaccine in the clinic.

In summary, we demonstrate that CCR6 plays an important role in regulating MIP-1α-induced mobilization of F4/80$^-$B220$^-$CD11c$^+$ DC precursors. In addition to mobilizing DC precursors by their specific receptors CCR5 and CCR1, MIP-1α can recruit F4/80$^+$CD11c$^-$ monocytes/macrophages, producing MIP-3α in the peripheral blood, which substantially mobilizes the CCR6$^+$ DC precursor subset to amplify B220$^-$CD11c$^+$ DC precursors. Exogenous administration of MIP-3α enhances MIP-1α-induced mobilization of DC precursors significantly. Importantly, these MIP-1α- and MIP-3α-mobilized DC precursors can be prepared for DC vaccine with the capacity to elicit CTL responses to tumor cells, leading to tumor rejection in vitro and in vivo. Thus, combined administration of MIP-3α and MIP-1α may be an efficient strategy for collecting a large number of DCs appropriate for immunotherapy.

ACKNOWLEDGMENTS

This work was supported by grants from National Natural Science Foundation of China, 211 Project of Shanghai Jiao Tong University School of Medicine, the Programs of Science and Technology Commission of Shanghai Municipality, and Shanghai Leading Academic Discipline Project. The authors have no conflict of financial interest. We highly appreciate Dr. Sheri M. Skinner (Baylor College of Medicine, Houston, TX, USA) for her critical review of the manuscript and Dr. Hongmei Li (Soochow University, Suzhou, China) for help in statistical analyses. We also thank Drs. Min Jin and Fang Zhao for their kind assistance.

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