A Pivotal Role for CC Chemokine Receptor 5 in T-Cell Migration to Tumor Sites Induced by Interleukin 12 Treatment in Tumor-bearing Mice

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ABSTRACT

Interleukin (IL) 12 treatment in the CSA1M and OV-HM, but not in Meth A tumor models, induces tumor regression that is associated with T-cell migration to tumor sites. Here, we investigated the role of the CC chemokine receptor (CCR)5 in T-cell migration induced after IL-12 treatment. In the two IL-12-responsive tumor models (CSA1M and OV-HM), IL-12 treatment up-regulated the mRNA expression of CCR5 in splenic T cells as well as ligands for CCR5, such as macrophage inflammatory protein (MIP) 1α and MIP-1β in tumor masses. In contrast, the expression of CCR5 in spleens and MIP-1α/MIP-1β in tumor masses was marginally induced before and even after IL-12 treatment in the Meth A model in which T-cell migration is not observed. T cells infiltrating tumor masses in the former two IL-12-responsive models expressed CCR5. Administration of a synthetic CCR5 antagonist, TAK-779 to tumor-bearing mice during IL-12 immunotherapy prevented T-cell migration and tumor regression. Furthermore, anti-CCR5 antibody was found to inhibit T-cell migration in the lymphoid cell migration assay. Namely, although spleenic T cells prepared from IL-12-treated CSA1M or OV-HM-bearing mice migrated into the corresponding tumor masses in recipient mice, the migration was inhibited when donor T cells were treated with anti-CCR5 antibody before the injection. These results indicate a critical role for CCR5 in the induction of T-cell migration to tumor sites after IL-12 treatment.

INTRODUCTION

The migration of leukocytes into sites of inflammation is a multistep process mediated by a series of cellular/molecular interactions (1). Whereas the requirement for adhesion molecules in this process has been well appreciated (2, 3), recent studies have shown that an additional class of molecules, the chemoattractant cytokines (chemokines), and their receptors also play a fundamental role in leukocyte migration (4, 5). Namely, several chemokines have been shown to up-regulate lymphocyte adhesion to endothelial cells or isolated endothelial ligands (6–8). In the T cell, it has been proposed that chemokines serve to activate T-cell integrins to mediate firm binding to the vascular adhesion molecules such as VCAM-14 and ICAM-1 before migration into inflammatory tissues. IL-12 has pleiotropic effects on T cells and natural killer cells (9, 10), including the stimulation of lymphokine expression, especially of IFN-γ (11, 12). This cytokine has also been shown to exhibit potent antitumor activity in a number of mouse tumor models (13–15). Although IFN-γ is a critical requirement for the IL-12-mediated antitumor effect (14, 15), IFN-γ production alone is not sufficient to induce significant therapeutic effects (16). Instead, we have observed that tumor regression is associated with a massive T-cell infiltration into tumor masses and in situ IFN-γ production (15, 17, 18). Our preceding study also revealed that such an infiltration takes place through very late antigen-4/lymphocyte function-associated antigen-1 adhesion pathways because the infiltration was almost completely inhibited by either set of anti-very late antigen-4 plus anti-VCAM-1 or anti-lymphocyte function-associated antigen-1 plus anti-ICAM-1 mAbs (19). In contrast to strong evidence for the role of adhesive mechanisms, it remains unclear whether the chemoattractant system is involved in IL-12-induced T-cell migration to tumor sites. There are only a few studies that reported the involvement of chemokines in tumor immunity (20–22).

A number of T-cell chemokines (23–27) and chemokine receptors (28, 29) has thus far been identified. Recent studies have revealed the role of a Th1 cytokine receptor CCR5 and the corresponding chemokines in the recruitment of Th1 cells to inflammatory sites such as the synovial lesion in rheumatoid arthritis (30, 31). Moreover, we have most recently shown that IL-12 is the cytokine that can induce the expression of CCR5 on TCR-stimulated CD4+ and CD8+ T cells (32). In view of such information, this study investigated the role of CCR5 and CCR5-reactive T-cell chemokines (MIP-1α and MIP-1β) in the IL-12-induced T-cell migration in two IL-12-responsive and one IL-12-unresponsive tumor (as control) models. Exclusively in IL-12-responsive models, IL-12 treatment up-regulated the expression of CCR5 and the relevant chemokines (MIP-1α and MIP-1β) in splenic T cells and tumor masses, respectively. A large portion of T cells that have migrated to tumor masses expressed CCR5. Importantly, a CCR5 antagonist or anti-CCR5 Ab inhibited the migration of IL-12-stimulated T cells to tumor masses and IL-12 induction of tumor regression. These results indicate that CCR5 plays a critical role in IL-12-mediated T-cell migration to tumor sites that is responsible for the induction of tumor regression.

MATERIALS AND METHODS

Tumor Cell Lines. CSA1M fibrosarcoma (BALB/c origin; 33), OV-HM ovarian carcinoma ([C57BL/6 × C3H/He]F1 [B6C3F1] origin) (34), and Meth A fibrosarcoma (BALB/c) were used.

Mice. Male BALB/c and female (C57BL/6 × C3H/He)F1 (B6C3F1) mice were obtained from Shizuoka Experimental Animal Center (Hamamatsu, Japan) and used at 6–9 weeks of age.

Reagents. Mouse mIL-12 and mouse recombinant human interferon-γ were provided from Genetics Institute, Inc. (Cambridge, MA) and Shionogi Research Laboratories (Osaka, Japan), respectively. The CCR5 antagonist, TAK-779 [N,N-dimethyl-N-4-[[2-(4-methylphenyl)-6,7-dihydro-5H-benzocyclohepten-8-yl]carbon-yl]amino]benzyl]-tetrahydro-2H-pyran-4-3751

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4 The abbreviations used are: VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; IFN-γ, interferon-γ; IL, interleukin; IL-12R, IL-12 receptor; RIL, recombinant interleukin; CCR, CC chemokine receptor; Ab, antibody; mAb, monoclonal antibody; MIP, macrophage inflammatory protein; TCR, T-cell receptor; RANTES, regulated on activation, normal T cell expressed and secreted; β2M, β2 microglobulin; PCR, polymerase chain reaction; TIL, tumor-infiltrating lymphocyte.
amine chloride; M, 531.13] was synthesized in one of our laboratories (S. O., T. I.) based on the structure shown in Ref. 35. Rabbit antiserum CCR5 Ab was prepared as described previously (36). Briefly, rabbits were immunized with glutathione S-transferase protein with the NH2-terminal portion of CCR5, and sera were fractionated into IgG using a protein A-agarose column. Control rabbit IgG was obtained from Jackson ImmunoResearch (Philadelphia, PA).

Preparation of Tumor-bearing Mice. Mice were inoculated s.c. with CSA1M (1 × 105/mouse), OV-HM (5 × 105/mouse), or Meth A (5 × 105/mouse) tumor cells.

IL-12 Treatment. Mice received i.p. injections of rIL-12 in a dose of 0.5 µg/mouse three or four times every other day.

Treatment with CCR5 Antagonist. TAK-779 was dissolved in 5% man-nitol solution. Mice received injections of 150 µg of TAK-779 in a volume of 100 µl every other day.

Preparation of a T-Cell-enriched Population by Positive Selection. Spleen cells were labeled with superparamagnetic microbeads conjugated to rat antiserum Thy1.2 mAb (Miltenyi Biotec, Inc., Sunnyvale, CA). Labeled cells were separated from unclustered cells by magnetic cell sorting using the MiniMACS (Miltenyi Biotec, Inc.) according to the procedure described in detail (37).

cDNA Probes. MIP-1α, MIP-1β, and RANTES cDNAs were cloned from the mouse macrophage cell line RAW 264. RNA was isolated from RAW 264 cells that were treated for 12 h with 100 µg/ml lipopolysaccharide (Escherichia coli 0127:B8; Difco Laboratories). CCR1 and CCR5 cDNAs were cloned from unfractionated spleen cells. RNA was isolated from unfractionated spleen cells that were treated for 48 h with 5 µg/ml concanavalin A, harvested and then restimulated for 48 h with 100 units/ml mouse rIL-2 (Shionogi Pharmaceutical Co. Ltd.). These RNAs were used as templates for first-strand cDNA synthesis. The complete mouse MIP-1α, MIP-1β, RANTES, CCR1, and CCR5 coding sequences were cloned from these cDNAs by use of Taq DNA polymerase, standard PCR conditions, and the following primers: (α) MIP-1α, a 5′ sense oligonucleotide CTCACCCCTGTCACCTGCTCAA and a 3′ antisense oligonucleotide GGCTCAAGCCCTGTCTCTACAC based on sequences 44–66 and 395–416, respectively, from the sequence of MIP-1α (38); (b) MIP-1β, a 5′ sense oligonucleotide CAGCCTGTGCAAACCTAACC and a 3′ antisense oligonucleotide TCAGTTCAACTCAAGGTAC based on sequences 30–49 and 322–341, respectively, from the sequence of MIP-1β (39); (c) RANTES, a 5′ sense oligonucleotide TCTCCCAACGCCCCTGCC and a 3′ antisense oligonucleotide GATGCGGATTTTCCAGAG based on sequences 13–30 and 425–443, respectively, from the sequence of RANTES (40); (d) CCR1, a 5′ sense oligonucleotide ATGGAGATTTGATTTTACAG and a 3′ antisense oligonucleotide TCAGAAGCCAGCAGAG based on sequences 1–22 and 1051–1068, respectively, from the sequence of CCR1 (41); and (e) CCR5, a 5′ sense oligonucleotide GTCCCTCTCAAGAGCTTG and a 3′ antisense oligonucleotide AAAAGTGACCCAGTCTAA based on sequences 1–23 and 1093–1112, respectively, from the sequence of CCR5 (42). The PCR products were purified by agarose gel electrophoresis and ligated to the T-vector as described previously (43). Briefly, Bluescript (Stratagene, La Jolla, CA) plasmid was digested with EcoRV and incubated with Taq polymerase with the use of standard buffer conditions in the presence of 2 mm dTTP for 2 h at 70°C. After phenol extraction and precipitation, the T-vector was ready for cloning. PCR products were then ligated to the vector. βM was kindly provided by Dr. Takeshi Tokuhisa (Chiba University Medical School, Chiba, Japan).

Measurement of mRNA Expression. Total cellular RNA was isolated by the acid guanidinium thiocyanate-phenol chloroform method, and mRNA levels were determined using the RNase protection assay according to the procedure described previously (44). Briefly, 10 µg of total cellular RNA was hybridized in solution to a 32P-labeled antisense riboprobe for overnight at 55°C in 80% formamide. The plasmid was linearized with HindIII (MIP-1α and CCR1), XhoI (MIP-1β), NotI (RANTES), or psr (CCR5), and an in vitro transcription was performed in the presence of [α-32P]UTP. The protected fragment (205 bp for MIP-1α, 312 bp for MIP-1β, 331 bp for RANTES, 182 bp for CCR1, and 260 bp for CCR5) was separated on a denaturing sequencing gel. As an internal control for the amount of RNA loaded on the gel, RNA was simultaneously hybridized to an antisense 32P-labeled probe for the βM gene (127 bp).

A Lymphoid Cell Migration Assay. The assay system was essentially the same as described previously (19). Briefly, splenic T cells suspended to a concentration of 5 × 107/ml in 1 ml of diluent were allowed to react with 5 × 105/mкл PKH-26 dissolved in 1 ml of diluent for 5 min at 37°C. Labelling was stopped by adding 2 ml of FCS, and cells were washed five times with RPMI 1640 containing 10% FCS. Mice with similar tumor sizes (~7 mm in diameter) were used as recipients for this assay. PKH-26-labeled T cells (3 × 105 cells) were injected i.v. into recipient (IL-12-unreated, homolo-gous tumor-bearing) mice, and 24 h later, cryostat sections of tumor masses were prepared. The entry of fluorescence-labeled donor T cells was quantified under fluorescence microscopy and expressed as the mean cell number ± SE/section.

In Situ Hybridization. MIP-1α and MIP-1β probes prepared for the RNase protection assay were used to prepare RNA probes for in situ hybridization. The DNAs cloned into pBluescript plasmids were linearized and used for in vitro transcription. In the case of MIP-1α, the plasmid was either linearized with HindIII and then transcribed with T3 polymerase to yield the antisense probe or linearized with BamHI and transcribed with T7 polymerase to yield the sense (control) probes; whereas for MIP-1β, the plasmid was linearized with BamHI and transcribed with T7 polymerase to generate the antisense probe or linearized with HindIII and transcribed with T3 polymerase to generate the sense probe. Transcription reactions were carried out using a Digoxigenin RNA Labeling kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer’s instructions.

Samples were dehydrated with a graded series of ethanol and embedded in paraffin. Serial sections (4-µm thick) were cut and mounted on slides coated with 3-(triethoxysilyl)-propylamine (Merck-Schuchardt, Munchen, Germany). They were stored at 4°C until use. Three sections were used as follows: (a) the first section was stained with H&E; (b) the second section was hybridized with MIP-1α or MIP-1β antisense probes; and (c) the third section was hybridized with sense probes as control. The procedure of in situ hybridization was described previously in detail (45).

Staining Procedure of Immunohistochemical Examination. The following reagents were purchased to perform immunohistochemical examination: (a) biotinylated goat antirabbit IgG (Jackson Immuno Research, West Grove, PA); (b) biotinylated rat antirat CD4 and antirat CD8 (PharMingen, San Diego, CA); (c) biotinylated rat IgG (Jackson Immuno Research); and (d) Histofine SA-PO kit and Histofine DAB kit (Nichirei Co. Ltd., Tokyo, Japan). Staining of CD4 and CD8 was performed according to the procedure described previously (46).

Preparation of TIL from Tumor Masses. Tumors were disaggregated into single cell suspensions by a modification of the enzyme digestion technique originally described by Russell et al. (47). Briefly, tumors from tumor-bearing mice 3–4 weeks after tumor cell inoculation (unless other-wise indicated) were removed and minced with scissors and surgical blade into ~1-mm³ pieces. The minced tumor fragments were then digested in RPMI 1640 containing 20% FCS and 200 units/ml collagenase (Worthington Co., Lakewood, NJ) at 37°C in a humidified atmosphere with 5% CO2 for 4 h. The supernatant was filtered through nylon mesh to remove clumps and washed immediately in cold RPMI 1640. Cell fractionation was carried out by sedimentation over Ficoll solution. Briefly, Ficoll (Pharmacia Bio-technology, Uppsala, Sweden) was dissolved in PBS, and 20% v/v of Ficoll solution was prepared in a plastic 15-ml centrifugation tube (Corning Glass Works, Corning, NY). A single cell suspension consisting of a viable tumor and lymphoid cells (5–10 × 106) plus dead cells in a 5-ml volume was loaded over 3 ml of 20% Ficoll solution, and centrifuged for 20 min at 2500 rpm. Cells banding between medium and Ficoll solution were collected. This fraction contained viable lymphoid cells as well as viable tumor cells but was depleted of dead cells and cell debris. This fraction was used as a TIL population.

Detection of CCR5 on TIL. A TIL population (a mixture of viable TIL and tumor cells) was first incubated with anti-FcγRII/III/m Ab (2,4G2) to prevent the staining mAbs from binding with the Fc receptors. These treated cells were then stained with phycoerythrin-conjugated anti-CCR5 mAb (PharMingen, San Diego, CA) and a mixture of allophycocyanin-conjugated anti-CD4 and anti-CD8 mAbs (PharMingen). The stained cells were analyzed by FACSCalibur (Becton Dickinson, Mountain View, CA). CCR5 expression was detected by gating on CD4+ and CD8+ cells.
RESULTS

Enhanced Expression of CCR5 Chemokine Receptor mRNA by Splenic T Cells after IL-12 Treatment in CSA1M and OV-HM but not in Meth A Tumor Models. Our earlier studies have shown that rIL-12 administration induces complete regression of CSA1M and OV-HM tumors (15, 48) but not of Meth A tumors (19). We have also found that tumor regression in the former two IL-12-responsive models was associated with enhanced T-cell infiltration (15, 17, 19). Recent studies (30, 31) demonstrated a crucial role of chemokine receptors, particularly of CCR5 in the migration of Th1 cells to inflammatory sites. To investigate the role of CCR5 in the migration of T cells to tumor sites, we examined whether mRNA for CCR5 is expressed by splenic T cells from tumor-bearing mice and whether such an expression is up-regulated after IL-12 treatment. Fig. 1A shows that IL-12 treatment induces enhanced expression of CCR5 mRNA by unfractionated spleen cells from CSA1M- and OV-HM-bearing mice. In contrast, such an up-regulation was not induced in the IL-12-unresponsive Meth A model. CCR5 expression in the CSA1M and OV-HM models was mediated mainly by splenic T cells because purified T cells exhibited higher levels of CCR5 mRNA (Fig. 1B). CCR5 expression was observed in spleen cells from CSA1M- and OV-HM-bearing mice before and after IL-12 treatment. However, high levels of CCR1 expression were observed in spleen cells from CSA1M- and OV-HM-bearing mice (data not shown). This is compatible with the observations that IL-12R expression is higher in T cells from CSA1M- or OV-HM-bearing mice (data not shown). Thus, it is an expression is up-regulated after IL-12 treatment.

mRNA Expression of CCR5-reactive Chemokines in Tumor Masses from IL-12-untreated and -treated Mice. We next examined whether CCR5-reactive chemokines are induced in tumor masses before and after IL-12 treatment in two IL-12-responsive tumor models (CSA1M and OV-HM) models in comparison with an IL-12-unresponsive tumor (Meth A) model. The results for mRNA expression of three CCR5-reactive chemokines (MIP-1α, MIP-1β, and RANTES) are summarized in Fig. 2. In two IL-12-responsive tumor models, mRNAs for MIP-1α and MIP-1β were detected in tumor masses even before IL-12 treatment, and IL-12 treatment up-regulated mRNA expression of these chemokines. RANTES mRNA expression was slightly and appreciably up-regulated in CSA1M and OV-HM tumor masses, respectively, after IL-12 treatment. In contrast, mRNA levels of MIP-1α and MIP-1β were weak or negligible before and even after IL-12 treatment in the Meth A model. However, high levels of RANTES mRNA expression were observed in Meth A tumor masses irrespective of IL-12 treatment. Chemokines are known to be expressed not only by lymphoid cells (macrophages and T cells) but also by tumor cells and tumor-associated fibroblasts/endothelial cells (4). We examined whether tumor cells used here express any of three T-cell chemokines constitutively or in response to stimulation with inflammatory cytokines such as IFN-γ. mRNAs for MIP-1α and MIP-1β were not detected by either type of tumor cells irrespective of IFN-γ stimulation, whereas Meth A tumor cells expressed RANTES mRNAs even before exposure to IFN-γ (data not shown). Thus, it is likely that the induction of RANTES, particularly in the Meth A tumor mass, is because of the capacity of tumor cells to express this chemokine constitutively.

By in situ hybridization, we further examined the localization of cells expressing MIP-1α and MIP-1β mRNA around and inside CSA1M tumor masses. Whereas CSA1M tumors from IL-12-untreated mice had few MIP-1α or MIP-1β-expressing cells (data not shown), increased numbers of cells expressing these chemokines mRNAs were observed in tumors from IL-12-treated mice (Fig. 3).

These cells were localized massively and scattered inside and around a tumor mass, respectively, but did not exist in skin and s.c. tissues adjacent to a tumor mass (data not shown). Considering the
failure of CSA1M cells to express these chemokines and an increase in lymphoid cell migration after IL-12 treatment, tumor-infiltrating inflammatory cells appear to be responsible for a large part of MIP-1\(\alpha\)/MIP-1\(\beta\) mRNA expression. Similar observations were made for OV-HM tumors, although the frequency of MIP-1\(\alpha\)/MIP-1\(\beta\) mRNA-expressing cells was less intense (data not shown).

**Accumulation of CCR5\(^+\) Cells in CSA1M and OV-HM Tumor Masses after IL-12 Treatment.** Our previous studies have also demonstrated that lymphoid cells infiltrating CSA1M and OV-HM tumors include large numbers of CD4\(^+\) and CD8\(^+\) T cells (15, 17). The CD4\(^+\) T-cell subset was dominant in CSA1M, whereas the CD8 subpopulation was dominant in OV-HM (19). The representative CD4\(^+\) and CD8\(^+\) T-cell infiltration is shown for CSA1M and OV-HM tumor masses, respectively (Fig. 4). We examined whether these tumor-infiltrating T cells express CCR5. Strikingly elevated levels of CCR5 mRNA accumulation were seen in CSA1M and OV-HM tumor masses after IL-12 treatment (Fig. 5), suggesting the migration of CCR5\(^+\) cells to tumor masses.

CCR1 expression was also up-regulated after IL-12 treatment (Fig. 5). However, moderate levels of CCR1 expression were already observed before IL-12 treatment. Considering the fact of CCR1 expression by non-T cells (Fig. 1), this suggests that CCR1\(^+\) cells such as macrophages exist in CSA1M and OV-HM tumor masses and that the accumulation of these cells is also enhanced by IL-12 treatment along with the migration of CCR5\(^+\) cells. In contrast, the accumulation of both CCR5 and CCR1 is weak in the IL-12-unresponsive Meth A model in which lymphoid cell migration is not induced.

**Tumor-infiltrating T Cells Express CCR5.** We next aimed to directly demonstrate whether tumor-infiltrating T cells express CCR5. TILs were isolated from CSA1M and OV-HM tumor masses 1 day after the third IL-12 injections. Because TILs were contaminated by a considerable number of viable tumor cells, they were stained with anti-CCR5 as well as a mixture of anti-CD4 plus anti-CD8. T-cell migration is shown on the section from CSA1M or OV-HM tumor, respectively. ×400.
Inhibition of IL-12-induced Tumor Regression and Intratumoral T-Cell Infiltration by Administration of the CCR5 Antagonist TAK-779. Recently, a CCR5-specific inhibitor (antagonist) was developed (35). Using this reagent, we examined whether administration of the inhibitor to tumor-bearing mice affects tumor regression that is otherwise induced by IL-12 treatment. IL-12 treatment (0.5 μg/mouse/time, four times every other day) induced regression of all growing OV-HM tumors. The administration of the CCR5 inhibitor was started 1 day before IL-12 treatment, and seven additional injections were given every other day. As shown in Fig. 7, IL-12-mediated tumor regression was prevented by administration of the inhibitor except for one mouse. Furthermore, we determined whether inhibition of tumor regression by TAK-779 is based on the down-regulation of intratumoral T-cell infiltration. Fig. 8 shows that the IL-12-induced migration of both CD8\(^+\) (the major subset) and CD4\(^+\) T cells to OV-HM tumor masses is inhibited by administration of TAK-779. Similar observations to those in Figs. 7 and 8 were made in the CSA1M model (data not shown).

Prevention of IL-12-induced T-Cell Migration to Tumor Masses by anti-CCR5 Ab. To more accurately examine the function of CCR5 in intratumoral T-cell migration, anti-CCR5 Ab was used instead of the CCR5 antagonist, and to more directly evaluate the effect of the CCR5 blockade, a previously described lymphoid cell migration assay (19) was used. In this assay, spleen cells from IL-12-untreated and IL-12-treated tumor-bearing mice were transferred i.v. into IL-12-untreated, homologous tumor-bearing recipient mice. Our previous study (19) demonstrated that donor cells only from IL-12-treated tumor-bearing mice migrate to tumor masses and that tumor-infiltrating cells in this migration assay are mostly T cells. IL-12-induced enhancement of T-cell migration occurred in CSA1M and OV-HM but not in Meth A tumor models (19). Therefore, donor T cells were prepared from CSA1M or OV-HM tumor-bearing mice that had been untreated or treated with IL-12. These cells were labeled with a fluorescein dye (PKH-26) and transferred i.v. into IL-12-untreated, corresponding tumor-bearing mice. As shown in Fig. 9, T-cell migration to recipients’ tumor masses did not differ greatly between donor T cells from normal and IL-12-untreated tumor-bearing mice. In contrast, donor T cells from IL-12-treated tumor-bearing mice exhibited enhanced migration. Such an enhanced T-cell migration was inhibited when donor cells were treated with anti-CCR5 Ab before the transfer. These results indicate that CCR5 plays an important role in the induction of T-cell migration to tumor sites.

DISCUSSION

This study shows that whereas splenic T cells in tumor-bearing mice express CCR5 mRNA and tumor masses express MIP-1α/MIP-1β mRNA, both of these mRNA expressions are up-regulated...
after IL-12 treatment in two IL-12-responsive tumor models. CD4⁺ and CD8⁺ T cells that have migrated to tumor sites after IL-12 treatment express CCR5. More importantly, their migration and tumor regression were blocked by a synthetic CCR5 antagonist or anti-CCR5 Ab. In contrast, the expression levels of MIP-1α/MIP-1β and CCR5 were marginal or weak even after IL-12 treatment in an IL-12-unresponsive tumor model in which T-cell migration was not induced after IL-12 treatment. Thus, these results indicate that the CCR5 chemokine system plays an important role in T-cell migration to tumor sites after IL-12 treatment.

CCR1 and CCR5 have been described to represent the receptors for CC chemokines such as MIP-1α, MIP-1β, and RANTES (28, 29). Recent studies have implied that CCR5 plays a critical role in the migration of Th1 cells to inflammatory sites such as the rheumatoid synovium (30, 31). Whereas these observations support the requirement for CCR5 in their recruitment to inflammatory sites, CCR5 expression may not necessarily demonstrate the requirement for this chemokine receptor in the process of recruitment. In this context, the present study showed not only enhanced expression of CCR5 on splenic T cells in CSA1M- and OV-HM-bearing mice after IL-12 treatment, but also CCR5 expression on T cells that migrated into CSA1M and OV-HM tumor masses after the treatment. More importantly, the blockade of CCR5 using a CCR5 antagonist or anti-CCR5 Ab inhibited IL-12-induced tumor regression/intratumoral T-cell migration. Thus, these results provided the direct demonstration for CCR5 function in T-cell migration to tumor sites (see below).

Whereas CCR5 expression in splenic T cells was up-regulated after in vivo IL-12 treatment, these observations are indirect evidence for the effect of IL-12 on CCR5 induction/up-regulation. Regarding this, our recent studies (32, 50) demonstrated the capacity of IL-12 to induce CCR5 expression. Resting mouse T cells express neither CCR5 nor IL-12R, and they express IL-12R upon TCR triggering (37). Stimulation of TCR-triggered T cells with IL-12 resulted in CCR5 induction (32). This was observed for both CD4⁺ and CD8⁺ T cells. We have also shown that most human T cells are allowed to express CCR5 by IL-12 stimulation if their TCR is triggered (50). Thus, it is obvious that IL-12 is capable of inducing CCR5 expression on both mouse and human T cells; thus far they are sensitized with antigen and express IL-12R.

Our previous studies showed that spleen cells from CSA1M-bearing mice contain allophycocyanin-presenting processed tumor antigens and T cells already sensitized to tumor antigens (51, 52). Furthermore, splenic T cells from CSA1M-bearing mice exhibited enhanced levels of IL-12R β1 and β2 mRNA expression compared with normal T cells (49). This was also observed in splenic T cells from OV-HM-bearing mice but not from Meth A-bearing mice. Consistent with this, splenic T cells from CSA1M- or OV-HM-bearing...
mice but not from Meth A-bearing mice displayed enhanced IL-12 responsiveness (49). These observations are compatible with the present results that IL-12-induced CCR5 expression is observed for splenic T cells from CSA1M- or OV-HM-bearing mice but not from Meth A-bearing mice. Together, the results support the possibility that splenic T cells that were rendered CCR5+ after IL-12 treatment contain tumor-sensitized T cells.

For leukocyte binding to blood vessel endothelium (1–8), chemokines have been proposed as critical of the signaling elements for integrin activation. Therefore, it is also important to examine whether CCR5-reactive chemokines are induced in tumor masses. In the CSA1M and OV-HM models, MIP-1α and MIP-1β mRNA expression was detected before IL-12 treatment and up-regulated after the treatment. Because both CSA1M and OV-HM tumor cells themselves failed to express these chemokines, its expression is considered to be attributable to a few inflammatory cells infiltrating tumor masses before IL-12 treatment. Consistently, our previous studies showed that before IL-12 treatment, a significant albeit slight level of cellular infiltration is observed (53). Moreover, detectable levels of VCAM-1 and ICAM-1 were also observed to be expressed on tumor vasculature (46). In a lymphoid migration assay, donor cells could migrate to tumor masses in IL-12-untreated tumor-bearing recipient mice; thus, far donor cells were from IL-12-treated tumor-bearing mice (this study and Ref. 19). The capacity of IL-12-unexposed tumor masses to accept IL-12-treated donor T cells is assumed to rely on detectable levels of the expression of VCAM-1/ICAM-1 as well as MIP-1α/ MIP-1β. IL-12 treatment up-regulated the expression of both VCAM-1/ICAM-1 (46) and MIP-1α/MIP-1β/RANTES (this study). Thus, the up-regulation of these chemokines correlates with enhanced T-cell migration in the CSA1M and OV-HM models. In contrast, the expression levels of MIP-1α/MIP-1β were very low even after IL-12 treatment in the Meth A model.

Evidence for the participation of the chemokine system in T-cell migration in tumor cell has been provided in the studies using chemokine antagonists (54–56). Met-RANTES is one of chemokine analogs that act as RANTES antagonists (57). The ability of this antagonist to reduce inflammation was observed in models of arthritis (54), renal inflammation (55), and colitis (56). In this study, TAK-779 that has recently been shown to block the function of Th1 inflammation-associated chemokines. TAK-779 antagonists (57). The ability of this antagonist to reduce inflammation was observed in models of arthritis (54), renal inflammation (55), and colitis (56). In this study, TAK-779 that has recently been shown to block the function of Th1 inflammation-associated chemokines. TAK-779 antagonists (57). The ability of this antagonist to reduce inflammation was observed in models of arthritis (54), renal inflammation (55), and colitis (56). In this study, TAK-779 that has recently been shown to

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