# Anti-tumor effect of combining CC chemokine 22 and an anti-CD25 antibody on myeloma cells implanted subcutaneously into mice

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Received November 27, 2008; Accepted May 7, 2009

DOI: 10.3892/mmr_00000171

**Abstract.** Chemokines are known to have anti-tumor effects due to their chemoattractant properties, which stimulate the accumulation of infiltrating immune cells in tumors. CCL22 (macrophage-derived chemokine, MDC) attracts killer T cells, helper T cells and antigen-presenting cells expressing the CCL22 receptor, CCR4. Thus, CCL22 gene expression results in the accumulation of these cells in tumors, and has been shown to suppress lung and colon cancer growth in mice. In the present study, early-stage subcutaneous tumor growth in a mouse multiple myeloma cell line stably expressing CCL22 (MPC-CCL22) was decreased compared to tumor growth in control cells (MPC-mock). However, the final extent of tumor growth in these cell lines was almost equivalent. Regulatory T cells, which express CD25, CD4 and CCR4, are known to cause immune disruption. We therefore investigated the association of regulatory T cells with the progressive decrease in CCL22 anti-tumor effect observed in late-stage experimental multiple myelomas. Tumor growth in MPC-CCL22 cells was observed to drastically decrease, to the point of complete tumor regression, when CD4 or CD25 T cells were depleted. Here, we document the drastic anti-tumor effect of a combination of CCL22 and anti-CD25 antibody on multiple myeloma cells.

## Introduction

Chemokines are a family of small cytokines that primarily induce the directed migration of hematopoietic cells when bound to their seven-transmembrane, G protein-coupled receptors (1,2). Because they function as chemoattractants for several immune effector cell types, chemokines are attractive candidates for immune cell-based approaches to cancer gene therapy. Several studies describe the anti-tumor effects induced by infiltrating immune cells upon their chemokine-mediated accumulation within the tumor mass. The regression of XCL1 (lymphotactin)-expressing engineered myeloma was mediated by increased infiltration of T cells, neutrophils and macrophages, leading to a reduction in tumorigenicity (3,4). CCL22 (macrophage-derived chemokine, MDC) is a CC chemokine that appears to be specifically secreted by a macrophage. CCL22 is a potent chemoattractant for CD8 and CD4 T cells, as well as for dendritic cells expressing the CCL22 receptor CCR4 (5,6), and is involved in chronic inflammation mediated by the continuous homing of dendritic cells and lymphocytes (7,8). Solid tumor growth in mice was suppressed by the recruitment of these cell types into tumors induced to express CCL22 using an adenoviral vector (9). Due to its tendency to function as a chemoattractant for killer T cells, helper T cells and antigen-presenting cells, CCL22 is considered to be an attractive candidate gene for immune cell-based cancer therapy. However, one drawback for such CCL22-based therapy is the immunosuppressive condition caused by the fact that CCL22 also recruits regulatory T cells into the tumor mass. Here, we demonstrate that a drastic anti-tumor CCL22 effect can be sustained in multiple myeloma tumors by reducing the recruitment of regulatory T cells into tumors using an anti-CD25 antibody.

## Materials and methods

*Cell culture and reagents.* Derivatives of the murine MPC-1 multiple myeloma cell line, one stably expressing CCL22 (MPC-CCL22) and the other a control vector (MPC-mock), as well as the stably expressing CCR4 L1.2 cell line (L-CCR4) were kindly provided by Dr Osamu Yoshie (Department of Microbiology, Kinki University, Osaka, Japan). MPC-CCL22 and MPC-mock were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine, 50 $\mu$M 2-mercaptoethanol, 100 U/ml penicillin and 100 mg/ml streptomycin. The mouse L1.2 pre-B-cell line was kindly provided by Dr E. Butcher (Stanford University

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*Key words:* chemokine, CCL22, gene therapy, regulatory T cells

School of Medicine). Mouse CD4 T cell lines (OT4H.1D5) were kindly provided by Dr Shinsaku Nakagawa (Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan) and were maintained in RPMI-1640 medium supplemented with FBS, 2 mmol/l L-glutamine, 50 $\mu$M 2-mercaptoethanol, 100 U/ml penicillin, and 100 mg/ml streptomycin. The cultures were maintained at 37˚C in a humidified atmosphere containing 5% $CO_2$ and 95% air. Recombinant mouse CCL22 was purchased from R&D Systems (Minneapolis, MN, USA).

*Reverse transcription-polymerase chain reaction*. RT-PCR was performed as previously described (10). Briefly, total RNA was extracted using an RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. First-strand cDNA was prepared from an RNA template (2 $\mu$g) using an oligo(dT)18 primer and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed at 42˚C for 50 min and then at 70˚C for 15 min. PCR amplification was performed using the Takara Ex Taq HS PCR kit (Takara Bio, Shiga, Japan) under following conditions: 26 or 30 cycles of denaturation at 94˚C for 60 sec, annealing at 60˚C for 60 sec, and extension at 72˚C for 60 sec. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and CCL22, 26 and 30 cycles were performed, respectively. All primers were verified to yield the expected products under the indicated conditions. PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. The sequences of the primers were as follows: GAPDH sense 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' and antisense 5'-CATGTGGGCCATGAGGTCCACCAC-3', CCL22 sense 5'-GGTCCCTATGGTGCCAATG-3' and antisense 5'-TTATCAAAACAACGCCAGGC-3'.

*Chemotaxis assay*. MPC-CCL22 and MPC-mock cells were cultured in serum-free AIM-V medium (Gibco BRL, Grand Island, NY) for 48 h, and the conditioned media were collected. L-CCR4 cells were seeded in the upper wells of Transwell chambers with a 5-$\mu$m pore size (Kurabo, Osaka, Japan) in 100 $\mu$l of assay buffer (0.1% bovine serum albumin in RPMI-1640). Aliquots of the conditioned media were pre-treated with an anti-mouse CCL22 antibody (R&D Systems) or a control IgG for 30 min at 37˚C before being applied to the lower chambers at a final volume of 600 $\mu$l. After incubation for 6 h at 37˚C, the number of cells that had migrated into the lower chambers was counted using light microscopy (magnification x40). All assays were performed in triplicate.

*Animals*. Inbred 6-week-old female BALB/c mice were purchased from Sankyo Labo Service (Hamamatsu, Japan). The study was conducted in accordance with the standards established in the Guidelines for the Care and Use of Laboratory Animals of Toyama University. These guidelines stipulate that mice harboring tumors ≤20 mm in diameter should, prior to tumor measurment using calipers, be sacrificed and counted as having a cancer-associated death.

*Implantation of tumor cells into syngeneic mice*. Mice were subcutaneously inoculated with MPC-1 cells (1x10$^6$ cells in 50 $\mu$l PBS) in the dorsal region on day 0. After hypodermic implantation, the longest and shortest tumor diameter was measured every 3 days. Tumor volume was approximated using the formula: tumor volume (mm$^3$) = long diameter x short diameter$^2$ x 1/2.

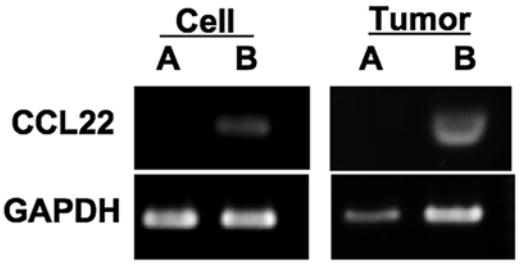


Figure 1. Expression of CCL22 in MPC-mock (A) and MPC-CCL22 (B) cells and subcutaneous tumors as assessed by reverse transcription polymerase chain reaction (RT-PCR). CCL22 expression was higher in MPC-CCL22 cells than in MPC-mock control cells. Expression of CCL22 was also detected in subcutaneous tumors. GAPDH served as a loading control.

*In vivo depletion analysis*. Hybridomas producing i) the PC61 5.3 CD25 T cell specific antibody, ii) the GK1.5 CD4 T cell specific antibody, and iii) the 53-6.72 CD8 T cell specific antibody were purchased from American Type Culture Collection (VA, USA). To deplete the CD8, CD4 and CD25 T cells *in vivo*, mice were intraperitoneally injected with 200 $\mu$l PC61 5.3, GK1.5 and 53-6.72 mAb fluid twice a week until the time of sacrifice.

*Statistical analysis*. The mean and SD were calculated for all variables. Statistical significance was calculated using the unpaired Student's t-test when appropriate. $P<0.05$ was considered statistically significant.

## Results

*CCL22-mediated anti-tumor and survival effect*. CCL22 expression was initially compared in MPC-mock and MPC-CCL22 cell lines by RT-PCR (Fig. 1). MPC-CCL22 cells expressed greater levels of CCL22 mRNA compared to MPC-1-mock cells. BALB/c mice were subcutaneously implanted with 1x10$^6$ MPC-mock (n=7) or MPC-CCL22 (n=8) cells in the dorsal region. Following implantation, the size of the tumors was measured every 3 days. On day 15, the group of mice implanted with MPC-CCL22 cells had a significantly smaller tumor volume than the MPC-mock group (Fig. 2A). All mice in the MPC-mock group survived until day 27. In contrast, 7 of 8 mice in the MPC-CCL22 group survived until day 42. Complete tumor regression was observed on day 27 in one of the MPC-CCL22 tumor-harboring mice (Fig. 2B). CCL22 expression in the tumors was compared using RT-PCR (Fig. 1), and was found to be much higher in the subcutaneous tumors of the MPC-CCL22 group than in those of the MPC-mock group.

*Chemotaxis of CD4 T cells to MPC-CCL22 cells*. We next examined whether soluble CCL22 produced by MPC-CCL22 cells was chemotactic for mouse L1.2 cells stably expressing

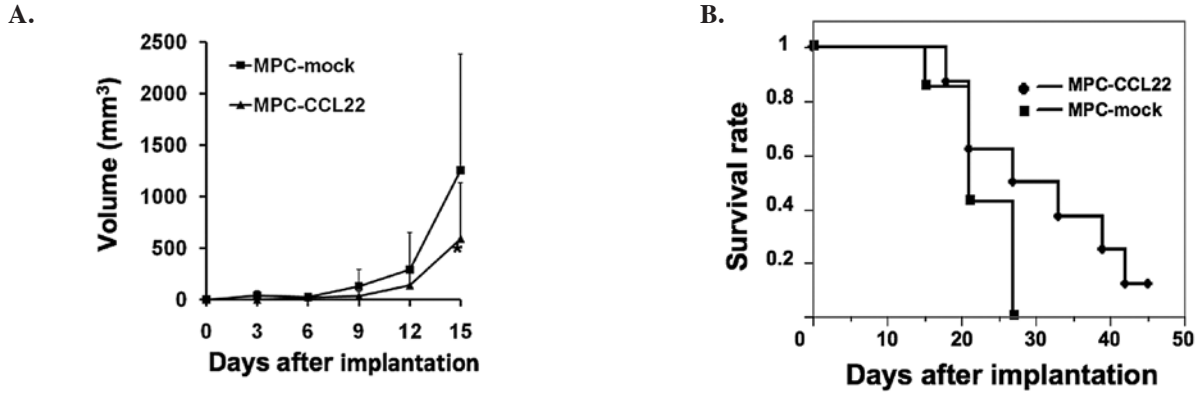


Figure 2. (A) Volume of subcutaneous tumors 15 days after the subcutaneous transplantation of MPC-mock and MPC-CCL22 cells (MPC-mock, n=7; MPC-CCL22, n=8). Tumor volume was observed to be less in MPC-CCL22 cells compared to MPC-mock cells. Error bars indicate the SD (p=0.0008). (B) Survival curve of the subcutaneaously implanted mice by Kaplan-Meier plotting shows increased survival for the MPC-CCL22 group (p=0.09, log-rank test).

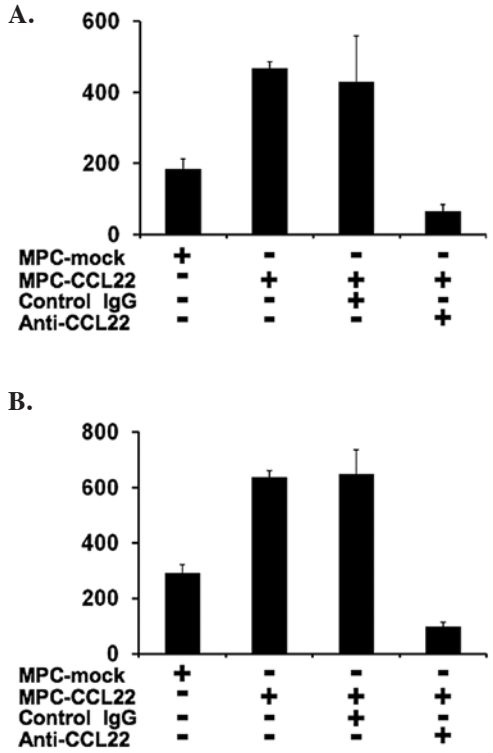


Figure 3. Chemotactic activity of CCL22 secreted by MPC-CCL22 and MPC-mock cells. MPC-CCL22 and MPC-mock supernatants were added to the lower wells with or without neutralizing anti-CCL22 Ab. After 6 h, the number of cells that had migrated into the lower wells of Transwell chambers was counted using light microscopy. Conditioned media from MPC-CCL22 and MPC-mock cells induced L-CCR4 and OT4H.1D5 cell chemotaxis. Anti-CCL22 Ab completely abrogated this chemotactic activity.

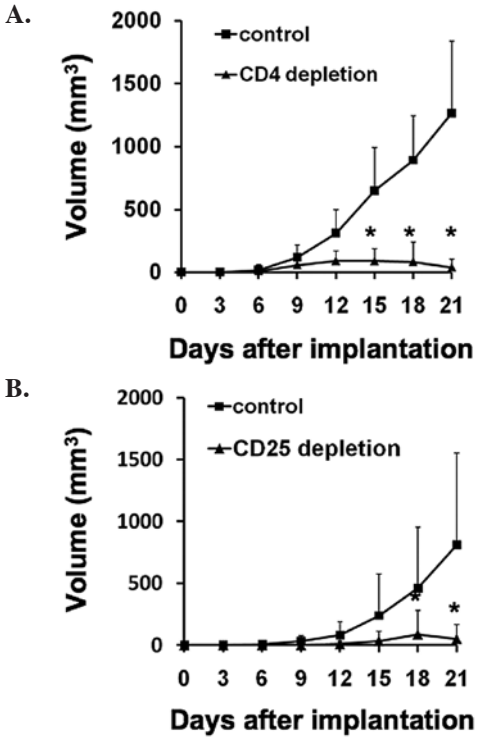


Figure 4. Growth of MPC-CCL22 tumors with or without the depletion of CD4 T (A) and CD25 T (B) cells. Tumor size in the CD4+ T cell-depleted group was smaller than that observed in the control group (p=0.0033). Similarly, tumor size was smaller in the CD25 T cell-depleted group compared to the control group (p=0.0252).

human CCR4. Cultured supernatant from MPC-CCL22 cells significantly induced the migration of L-CCR4 cells. This effect was completely neutralized by an anti-CCL22 antibody, but not by a control IgG (Fig. 3A). The culture media also significantly induced the migration of OT4H.1D5 cells. Again, anti-CCL22 antibody, but not control a IgG, completely neutralized the chemotactic activity of the MPC-CCL22 cultured supernatant (Fig. 3B). We confirmed that CD4+ T cells responded to the chemotactic stimulus induced by CCL22, and considered this recruitment to indicate the progress of tumor growth in the MPC-CCL22 group.

*Effect of the depletion of CD4 and CD25 T cells on tumor growth*. BALB/c mice were subcutaneously implanted with $1x10^6$ MPC-CCL22 cells in the dorsal region with or without the depletion of CD4 T cells (n=8 for each group). Following implantation, the size of the tumor was measured every 3 days. Mice depleted of CD4 T cells had a significantly

smaller tumor size compared to the controls (Fig. 4A). All mice in the depletion group survived, while, by day 39 in the control group, 7 of 8 mice had been sacrificed due to large tumor size. One of the 8 mice in the depletion group developed no tumors, while 5 of 8 mice in the depletion group developed a tumor that later regressed.

## Discussion

Previous studies have shown that CD4 and CD25 T cells prevent tumor growth. We hypothesized that CD4 and CD25 T cells that chemotactically respond to CCL22 are responsible for this effect. To examine this hypothesis, we subcutaneously injected BALB/c mice with $1x10^6$ MPC-CCL22 cells in the dorsal region with or without the depletion of CD25 T cells (n=5 for each group). Following transplantation, tumor size was measured every 3 days. Mice depleted of CD25 T cells had a significantly smaller tumor size (Fig. 4B).

This is compelling evidence that the combination of CCL22 chemokine and anti-CD25 antibody can be effective as an anti-myeloma therapy. Chemokines are attractive molecules for cancer gene therapy. Treatment using this technique is categorized as cell-based cancer immunotherapy due to the involvement of chemokine-mediated immune cell chemotaxis, as well as the angiostatic activity of some chemokines (11,12). CCL22 functions as a chemotactic agent for cells expressing the CCL22 receptor, CCR4 (6,13). Cell types responding to CCL22 include CD8 T cells, CD4 T cells and dendritic cells (14-16). Guo *et al* were the first to report that the intratumoral injection of a recombinant CCL22-encoding adenovirus resulted in tumor regression in a murine lung cancer model. This antitumor response was demonstrated to be CD8 and CD4 T cell dependent (9). As shown in Fig. 2, we also observed tumor regression in stably expressing CCL22 murine multiple myeloma tumors (MPC-CCL22) compared to control cell tumors (MPC-mock).

Notably, tumor regression was not observed 18 days after tumor implantation. Our findings indicate that tumor regression was independent of CD8 T cells (data not shown) and that CD4 T cells suppressed this regression (Fig. 4A). It is widely known that the normal immune system harbors a regulatory T cells population specializing in immune suppression, and that the autoimmune-suppressive CD4 T cell population constitutively expresses CD25. Given the immunosuppressive activity of regulatory T cells, we observed tumor growth in the presence of an anti-CD25 antibody (Fig. 4B). The anti-CD25 antibody drastically inhibited MPC-CCL22 tumor growth in our model. CCL22 and CCL17 (thymus activation-regulated chemokine, TARC) share the CCR4 receptor (6). In experimental lung and colon tumor models (9,17), both chemokines had similarly potent anti-tumor effects, resulting from the chemoattraction of CD8 T, CD4 T and dendritic cells to the tumor site. Cancer gene therapy strategies using CCL22 and/or CCL17 are thought to be promising therapeutic approaches because killer T cells, helper T cells and antigen-presenting cells are critical to the antitumor immunoresponse. Although positive results have been obtained for experimental cancer gene therapy, the interaction of cancer cell-derived chemokines with immune cells has yet to be investigated in a clinical setting (18,19). CCL5 (regulated on activation, normal T-cell expressed and secreted, RANTES) and CCL2 (monocyte chemotactic protein-1, MCP-1) are the most commonly observed chemokines in cancer (20). In breast cancer, decreased CCL2 expression is correlated with longer relapse-free survival and decreased tumor-associated macrophage (TAM) (21). Higher levels of CCL5 expression were associated with an increase in TAM and lymph node metastasis (22). CCL17 and CCL22, in particular, are significantly increased in the serum of the vast majority of Hodgkin lymphoma patients (23). The expression of these chemokines by cancer cells is related to increased populations of regulatory T cells in the gastric cancer mass (24). Phase II clinical trials using the humanized monoclonal anti-CD25 antibody daclizumab in lymphoma patients are taking place at NCI (http://clinicaltrials.gov/ct/show/NCT00001941?order=2). In addition, a phase I clinical trial using an anti-CCR4 mAb (KM2760) in patients with CCR4-positive T cell leukemia/lymphoma is currently in progress in Japan. Ishida *et al* reported that mAb could not only eliminate CCR4 positive tumor cells, but also that it could overcome the immunosuppressive effect of regulatory T cells (25). Because regulatory T cells have an inhibitory effect on the surrounding effector T cells, a combination strategy supporting the accumulation of various effector immune cells by CCL22 and the elimination of regulatory T cells using daclizumab and/or KM2760 might be an effective therapeutic approach to cancer.

## Acknowledgements

We thank Drs Takashi Nakayama and Osamu Yoshie, (Department of Microbiology and SORST, Kinki University School of Medicine, Osaka, Japan) for helpful advice and expert technical support.

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