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ORIGINAL ARTICLE

IL-12 gene-modified bone marrow cell therapy suppresses the development of experimental metastatic prostate cancer

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To investigate the immunomodulatory effects of interleukin-12 (IL-12) for treatment of metastatic prostate cancer, we administered adult bone marrow cells (BMC) that were genetically modified by retroviral vector-mediated IL-12 gene transduction in an experimental mouse model of prostate cancer metastasis. This therapy produced significant anti-metastatic effects in bone and lung and prolonged animal survival. Flow cytometric analysis indicated donor BMC could effectively home to bone and lung after treatment. Intensive infiltration of CD4 and CD8T cells in lung metastases and increased systemic natural killer and cytotoxic T lymphocyte activities indicated induction of a significant anti-metastatic immune response after treatment with IL-12 transduced BMC. Our results demonstrate the therapeutic potential of gene-modified BMC gene therapy.
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Introduction

Currently, prostate cancer progression leads to the death of approximately 31 000 men in the United States every year.[1] In many patients, microscopic and clinically evident metastasis has already occurred by the time of diagnosis of the primary tumor.[2] Unfortunately, such metastatic disease is incurable, and there is a critical need to develop new therapies for these patients. Generation of an antitumor immune response through the use of specific tumor-associated antigens and/or immunomodulatory agents is one approach to the development of systemic cancer therapy. To treat prostate cancer, putative tumor-associated antigens including PSA,[3] PSMA,[4] PAP,[5] MUC-1[6] and NY-ESO[7] have been tested or proposed for use as prostate cancer vaccines. However, the antigenic properties of these proteins are not optimal and multiple clinical trials that have tested these proteins as full-length proteins or peptide derivatives as primary vaccines have, in general, failed to show significant clinical responses.[8,9] Tumor RNA[10] or whole-cell vaccines[11] that provide multiple tumor antigen epitopes may have advantages over single protein or peptide vaccines. Specific vaccine approaches that involve gene-modified and irradiated whole tumor have also been considered and have advanced to clinical trials for many malignancies.[12,13]

The delivery of immunostimulatory genes *in situ* into tumor cells through specific gene therapy approaches represents an alternative to gene-modified tumor cell vaccines.[14] For prostate cancer, extensive pre-clinical studies and clinical trials have used adenoviral vector-mediated herpes simplex virus thymidine kinase gene transfer followed by ganciclovir.[15–17] More recent pre-clinical studies using adenoviral vector-mediated delivery of the interleukin-12 (IL-12) gene *in situ* have led to a Phase I/II clinical trial at our institution for men with recurrent prostate cancer.[18] Overexpression of IL-12 in prostate cancer tissues has the potential to generate a localized and systemic antitumor response.[19] Since it is not feasible to inject all metastatic lesions, and systemic delivery of adenoviral vectors does not effectively target metastases,[20] the generation of systemic antitumor responses, indirectly, through gene transfer-stimulated cell-mediated activities, is a promising approach.[19] However, it is important to develop new approaches for delivery of therapeutic genes to disseminated metastases.

Bone marrow cells (BMC) are a reasonable choice for gene-modified cell therapy because hematopoietic stem cells (HSC) have the capacity for self-renewal and introducing genes into multiple hematopoietic lineages. In this study, we developed a retroviral vector-mediated gene-modified cell therapy approach to overexpress IL-12

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in adult BMC. Through this bone marrow transplantation-mediated IL-12 gene therapy approach, we were able to suppress the development of experimental prostate cancer metastasis to lung and bone.

Materials and methods

Animals and metastatic prostate cancer cell line

129/Sv Rosa mice[21] were purchased from The Jackson Laboratory (Bar Harbor, ME) and 8- to 16-week-old mice were used as BMC donors. Four- to five-month-old 129/Sv wild-type mice were maintained at our facility and used as recipients. All mice were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institute of Health (NIH)'s Guide for the Care and Use of Laboratory Animals. Metastatic prostate cancer cell line (178-2 BMA) was generated from 129/Sv mice using the mouse prostate reconstitution (MPR) model system.[22] Cancer cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, and cultures were passaged by trypsinization with 0.025% trypsin. Cell passages 9–11 were used in this study.

Production of infectious retrovirus

Retroviral vectors, DFG-mIL-12 and DFG-eGFP, were kindly provided by Dr Hideaki Tahara (Pittsburgh Cancer Institute, PA). DFG-mIL-12 vector contains cDNAs of p35 and p40 of *mIL-12*. Control retroviral vector DFG-eGFP has the same backbone as DFG-mIL-12 and an eGFP cDNA.[23] Helper-virus-free ecotropic retroviral packaging cell line Phoenix-293 (kindly provided by Dr G Nolan, Stanford, CA) was transfected with plasmid DNA encoding DFG-mIL-12 or DFG-eGFP with lipofectAMINE PLUS reagent (Invitrogen Life Technologies, Carlsbad, CA). The supernatant was filtered through a 0.45-μm filter and concentrated by ultracentrifugation. All vector stocks were titered by transducing NIH3T3 cells using limiting dilutions of the stock with analysis for intracellular mIL-12 or eGFP expression by flow cytometry.

Transduction of BMC with mIL-12 gene

129/Sv Rosa mice were treated with 5-fluorouracil (Calbiochem, San Diego, CA) 150 mg/kg intraperitoneally 6 days before isolating BMC. BMC were flushed from the femoral and tibias and cultured with high glucose DMEM (Sigma, St Louis, MO) containing 10% fetal bovine serum, 2 mM glutamine, 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 100 U/ml penicillin, 100 U/ml streptomycin (Invitrogen Life Technologies). Red blood cells were removed from the bone marrow by ficoll-paque plus (Amersham Biosciences, Piscataway, NJ) centrifugation. After pre-stimulation with 20 ng/ml murine IL-3, 50 ng/ml IL-6, 100 ng/ml stem cell factor, 20 ng/ml granulocyte-colony stimulating factor and 50 ng/ml Flt-3 Ligand (Biosource, Camarillo, CA) for 24 h, BMC were cultured in six-well plate coated with recombination fibronectin CH-296 fragment (25 μg/well, Takara Bio, Japan). BMC were infected once daily at multiplicity of infection (MOI) 2 or 5 for three consecutive days with high titer ($>10^6$/ml) retrovirus. Transduction efficiency of mouse BMC was (15–30%) determined by intracellular murine IL-12 expression or eGFP expression using flow cytometry.

Flow cytometric analysis

After 3 consecutive days of retrovirus infection, BMC were cultured with 2 μM monensin (Sigma) for 24 h to block IL-12 secretion. Following Fc receptor blocking with anti-mouse CD16/CD32, BMC were fixed with 1% formaldehyde and 1% bovine serum albumin (Sigma) in phosphate-buffered saline (PBS) for 15 min at room temperature. Cells were then permeabilized with 0.1% saponin (Sigma) in PBS for 10 min on ice, followed by vortexing. BMC were either stained with phycoerythrin-labeled anti-mouse IL-12 (p70/p40) for intracellular mIL-12 detection or directly analyzed to detect eGFP expression. All antibodies were purchased from BD Biosciences Pharmingen (San Diego, CA). Flow cytometric analysis was performed on a flow cytometer (EPICS XL-MCL Beckman Coulter, Westbook, ME). A total of 25 000 live cells were counted per sample.

Lac-Z gene expression

After treatment, blood and bone marrow of recipient mice were examined for donor 129/Sv Rosa mice BMC homing at different time points. C_{12}FDG lac-Z gene expression kit (Molecular Probes, Eugene, OR) was used for detection of β-galactosidase in peripheral blood (PB) and BMC following the manufacture's instruction using flow cytometry. Expression of Lac-Z gene in frozen tissues was detected by enzyme histochemistry. Frozen sections were fixed with 4% paraformaldehyde for 2 min at 4°C. LacZ staining was performed overnight at 37°C with the following reagents: 35 mM potassium ferrocyanide, 35 mM potassium ferricyanide, 1 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40 (NP-40) and 1 mg/ml X-gal (Life Technologies Inc.).

Metastatic tumor establishment and treatment model

We have established a model system in which highly metastatic mouse 178-2 BMA were injected via tail vein into adult male syngeneic mice, both lung and bone metastases were established within 3 days and ultimately progress to lethality beyond 21 days (data not shown). DFG-mIL-12 or DFG-eGFP transduced BMC (10^6/mouse/100 μl) were injected via tail vein into recipient 129/Sv adult male mice, which received no bone marrow ablation. Control mice were injected either with non-retroviral vector infected BMC (10^6/mouse/100 μl) or with Hank's balanced salt solution (HBSS) medium (Invitrogen Life Technologies). Animals were killed at various time points after treatment. Sera and specific tissues were collected and analyzed at different time points.

Cytokine detection
Serum of treated animals was collected at different time points after treatment and mIL-12 (p70/p40) levels were measured by enzyme-linked immunosorbent assay (ELISA) (Biosource International, Camarillo, CA).

Detection of NK and CTL activities
Splenocytes of recipient mice were isolated at different time points after retroviral vector-transduced BMC treatment and used for *in vitro* cytolytic assays. Natural killer (NK) activity was determined by lysis of ^{51}Cr-labeled YAC cells with splenocytes derived from recipient mice as described previously.[18] The YAC cell line was obtained from American Type Culture Collection. Cytotoxic T lymphocyte (CTL) activity was determined by lysis of target ^{51}Cr-labeled, interferon-γ (IFN-γ)-stimulated 178-2 BMA cells. Effector cells were generated *in vitro* by incubating spleen cells (1.8×10^7 cells/well) with irradiated 178-2 BMA cells (1.2×10^7 cells/well) in 12-well plates for 5 days in the presence of anti-transforming growth factor-β1 antibody (30 μg/ml) and IL-2 (20 units/ml). Target 178-2 BMA cells were incubated with 100 U/ml of IFN-γ for 2 days and then radiolabeled with 100 μCi of ^{51}Cr for 45 min at 37°C. Different E:T cell ratios were incubated together for 4 h. Supernatants were harvested and counted in a gamma counter, and the percentage of specific lysis was calculated as described previously.[24]

Histological assessments of the lung and bone tissues
For quantitation of the lung metastatic deposits, lungs were removed and fixed in Bouin's solution. Metastatic colonies were counted with the aid of a dissecting microscope by two independent observers, and the average of the two counts was reported. For further histological evaluation, additional lung and the femur bone tissues were fixed in 10% neutral formalin, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin stain. These tissues were also frozen in optimal cutting temperature (OCT) compound, cut into 6-μm sections, and fixed with acetone/methanol (1:1, for 10 min) for further immunohistochemical staining. A polyclonal antibody to cytokeratin (Dako, North America, Carpinteria, CA) was used to identify minute cancer cell deposits in the lung or in the bone tissues. Immune cells infiltration in cancer deposits were labeled using monoclonal rat-anti-mouse antibodies for CD4, CD8 and F4/80 (BD-PharMingen, BD Biosciences Pharmingen, San Diego, CA) and were quantified using computer-assisted image analysis following the procedures described previously.[18]

Statistical analysis
Analysis of variance (ANOVA) and Kruskal–Wallis tests were used to make comparisons between the means in spontaneous metastases, densities of immune cell infiltration and cytolytic assays, with pairwise comparison carried out using the *post hoc* multiple comparison procedures and Mann–Whitney tests. Independent Samples *t*-test was used to compare CLT and NK levels, taking into account the results of Levene's Test for Equality of Variances. Changes of IL-12 levels over time between treatment groups were compared using the Repeated Measure ANOVA with Bonferroni *Post Hoc* Tests. Mean levels of IL-12 at two selected time points (6 and 9 days) were compared using ANOVA with Bonferroni *Post Hoc* Tests. Overall animal survival curves were obtained using a Kaplan–Meier analysis. The hazard ratios (HR) and 95% confidence intervals were calculated and evaluated using the Cox proportional hazards regression model. In addition, survival times were censored at 40 days for evaluation of the short-term effect, and analyzed using the same methods. *P*-values of <0.05 were considered statistically significant. All analyses were performed using the SPSS 12.0 software package (SPSS Inc., Chicago, IL).

Results

Anti-metastatic effects of DFG-mIL-12-transduced BMC
To establish a mouse model with bone and lung metastases, 129/Sv male mice were injected with 5000 syngeneic highly metastatic 178-2 BMA cells via tail vein. Preliminary histopathology studies confirmed the presence of tumor nodules in the femur marrow and lung of recipient mice 3 days following injection (data not shown). Based on this information, retroviral vector DFG-mIL-12[23] or control vector DFG-eGFP[23] transduced 129/Sv Rosa[21] BMC (10^6) were injected via the tail vein into recipient 129/Sv male mice without prior bone marrow ablation 3 days following tumor cell injection. The experimental scheme is shown in Figure 1a.

To analyze IL-12 activities in mice, we monitored serum IL-12 levels at 3-day intervals. Sera obtained from mice treated with DFG-mIL-12-infected BMC (MOI = 2) showed a gradual increase in IL-12 level (p70 + p40, ELISA) that was overall significantly higher than in DFG-eGFP transduced BMC and HBSS-treated groups of mice ($P = 0.0026$ and 0.0014, respectively). The increase in DFG-mIL-12 infected BMC-treated mice peaked at days 6–9 after treatment (0.656 ± 0.123 vs -0.013 ± 0.085 ng/ml, $P = 0.0003$ and 0.812 ± 0.176 vs -0.015 ± 0.173 ng/ml, $P = 0.0014$ compared to DFG-eGFP-transduced BMC group at days 6 and 9, respectively). Serum IL-12 was then maintained at a higher level (0.28–0.43 ng/ml) in DFG-mIL-12-transduced BMC-treated mice compared to DFG-eGFP-transduced BMC (0.08–0.13 ng/ml) or HBSS-treated mice (0.03–0.20 ng/ml) (Figure 1b). Recipients receiving BMC transduced at a higher MOI of 5 had correspondingly higher peak serum IL12- levels (2.80 ± 0.40 ng/ml, data not shown).

At day 21 after gene-modified (MOI = 2) BMC therapy, DFG-mIL-12-transduced BMC-treated mice had fewer metastatic lung colonies (mean = 39, s.d. 22, median = 33) compared to mice treated with DFG-eGFP transduced BMC (mean = 74, s.d. 44, median = 55, $P = 0.0197$) or with HBSS (mean = 68, s.d. 38, median = 65, $P = 0.0036$) (Figure 1c). Histochemistry showed that 80% of the mice treated with DFG-eGFP transduced BMC and 83% of the mice treated with HBSS developed

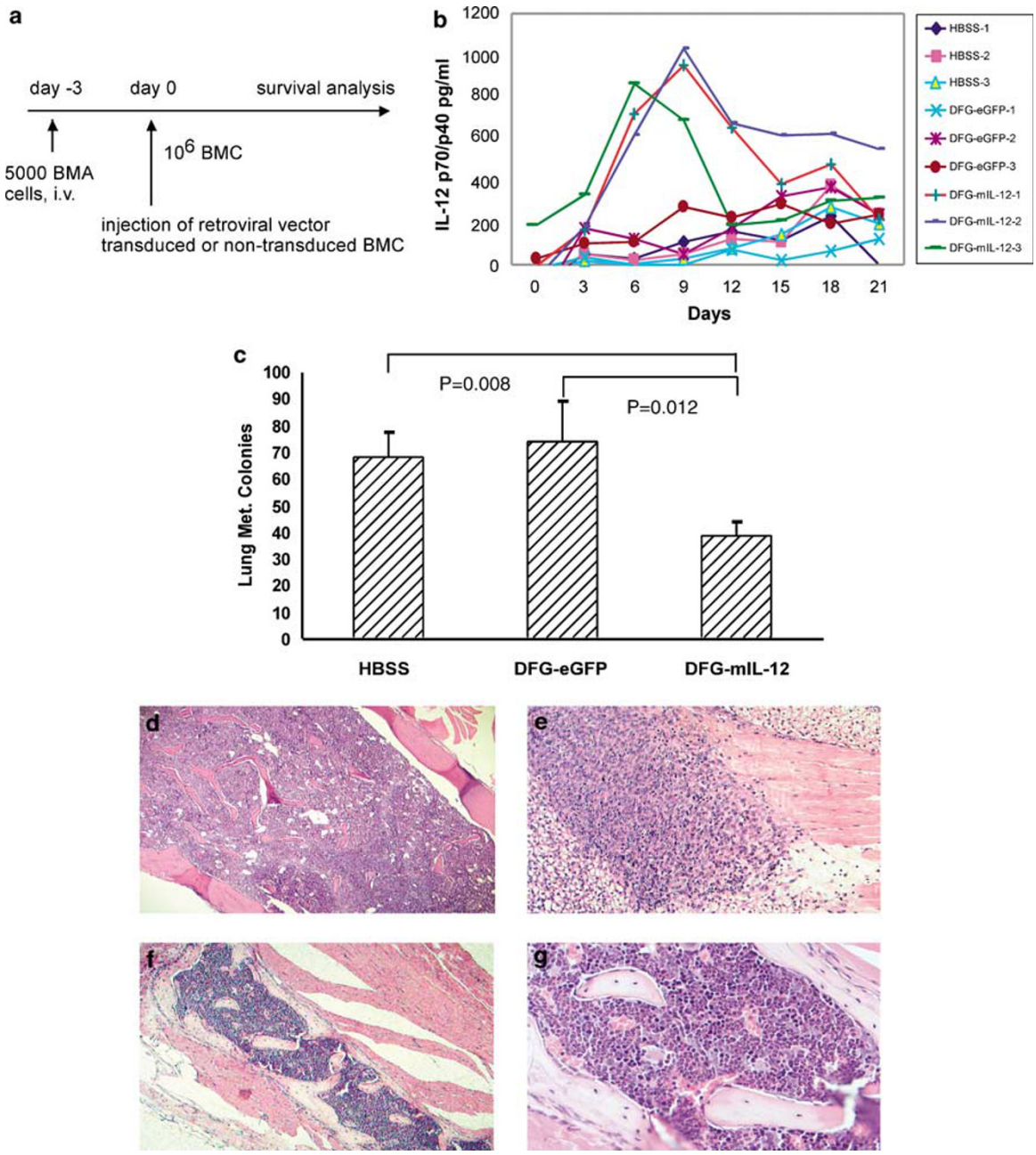

Figure 1 Anti-metastatic effect of DFG-mIL-12-transduced BMC. Retroviral vector-transduced 129/Sv Rosa BMC (10^6) were injected via the tail vein into recipient 129/Sv male mice without prior bone marrow ablation 3 days following tumor cell injection. The experimental scheme is shown in (**a**). Serum IL-12 level was monitored by ELISA (p70/p40) at 3-day intervals following retroviral vector-transduced BMC treatment (MOI = 2) (**b**). At day 21 after treatment, lungs were removed and fixed in Bouin's solution. Formation of lung metastatic colonies was quantified in (**c**). Femurs were fixed and stained with H&E. Formation of bone metastasis at low and high magnification in mice treated with DFG-eGFP-transduced BMC (**d** and **e**, respectively), and in mice treated with DFG-mIL-12-transduced BMC (**f** and **g**, respectively). Data are representative of three mice per group per experiment of two independently performed experiments. H&E, hematoxylin and eosin stain.

bone metastases (data not shown). In mice treated with DFG-eGFP-transduced BMC, the bone metastases were extensive and invaded surrounding muscles (Figures 1d and e at low and high magnification, respectively). In marked contrast, only 17% of the mice treated with DFG-mIL12 transduced BMC had bone metastases, and these tumors were small and less locally invasive (Figures 1f and g at low and high magnification, respectively).

Homing activities of IL-12 transduced BMC

Homing of transplanted BMC to recipient bone marrow is an essential step in engraftment and reconstitution of hematopoiesis following bone marrow transplantation. In this study, the contribution of donor 129/Sv Rosa BMC were analyzed by Lac-Z expression. PB and BMC of tumor bearing mice treated with retroviral vector-transduced BMC (MOI = 5) were collected at days 3

and 21 after treatment and analyzed for Lac-Z expression by flow cytometry. At day 3, up to 18% Lac-Z$^+$ cells were detected in bone marrow (Figure 2a), and 0.16% of Lac-Z$^+$ cells were found in PB of DFG-mIL-12-transduced BMC-treated mice (Figure 2b). However, 21 days after DFG-mIL-12-transduced BMC treatment, 5% of PB cells stained positive for Lac-Z (Figure 2c). Mice treated with control vector DFG-eGFP-transduced BMC demonstrated a similar homing phenomena compared to mice treated with DFG-mIL-12 BMC (data not shown). Control mice injected with HBSS medium were at background level and negative for Lac-Z expression in both BMC and PB at days 3 and 21 (Figures 2a, b and c). Positive Lac-Z expression was also demonstrated in lung by immunohistochemical staining at day 21 after retroviral vector-transduced BMC treatment (Figure 2d).

Systemic antitumor immune response

To analyze the mechanisms responsible for DFG-mIL-12 BMC-mediated anti-metastatic activities, we initially examined immune cell infiltrations in metastatic tumors. Lung metastatic tissues were stained for CD4$^+$ or CD8$^+$ T cells and quantified using computer-assisted image analysis following the procedures described previously.[18] At day 21, there was significantly increased CD4$^+$ cell infiltration in the lung of mice treated with DFG-mIL-12-transduced BMC (mean 98.3 cells/mm^2, s.d. 44.3, median (med.) 87.9 cells/mm^2) compared to that of mice treated with DFG-eGFP-transduced BMC (mean 46.1 cells/mm^2, s.d. 32.9, med. 28.5 cells/mm^2, $P=0.0208$) and compared to HBSS-treated mice (mean 38.9 cells/mm^2, s.d. 24.5, med. 39.5, $P=0.0002$) (Figure 3a). A similar increase was observed in CD8$^+$ T-cell infiltration in mice treated with DFG-mIL-12-transduced BMC (mean 48.3 cells/mm^2, s.d. 23.2, med. 45.7 cells/mm^2) compared to DFG-eGFP-transduced BMC mice (mean 25.4 cells/mm^2, s.d. 12.9, med. 28.9 cells/mm^2, $P=0.0195$) and compared to HBSS-treated mice (mean 21.1 cells/mm^2, s.d. 15.3, med. 17.9 cells/mm^2, $P=0.0011$) (Figure 3b). To determine whether systemic immune responses were associated with the anti-metastatic effects of DFG-mIL-12-transduced BMC therapy, splenocytes from mice treated with DFG-mIL-12 or DFG-eGFP-transduced BMC or HBSS were isolated 7 days after treatment and CTL and NK analyses were performed. Significantly increased CTL (Figure 3d, mean$\pm$s.d.: -7.5 ± 1.54 vs 17.9 ± 17.3, $P=0.0298$ at E:T = 50:1) and NK (Figure 3c, mean$\pm$s.d.: 6.99 ± 5.52 vs 14.78 ± 2.99, $P=0.0475$ at E:T = 50:1) activities were demonstrated in splenocytes from mice treated with DFG-mIL-12-transduced BMC compared to mice treated with DFG-eGFP-transduced BMC. These increased CTL and NK activities paralleled the high serum IL-12 levels in DFG-mIL-12-transduced BMC-treated mice at day 6 following treatment.

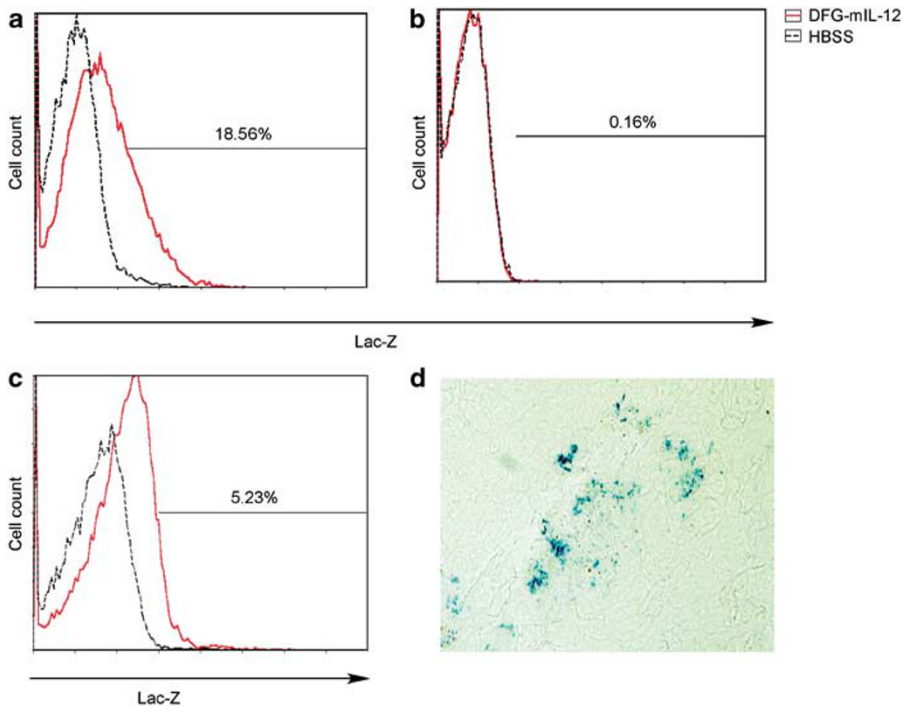

Figure 2 Homing effects of retroviral vector-transduced BMC. Analysis of Lac-Z expression in recipient mice after treatment with retroviral vector-transduced donor 129/Sv Rosa BMC. BM (**a**) and PB (**b**) cells were stained with C_{12}FDG for Lac-Z expression and analyzed by flow cytometry at day 3. At day 21, Lac-Z expression was also analyzed in PB by flow cytometry (**c**) and in lung by immunohistochemical staining (**d**). Black dashed lines indicate background staining of BM and PB from HBSS mice. Data are representative of three mice per group per experiment of two independently performed experiments.

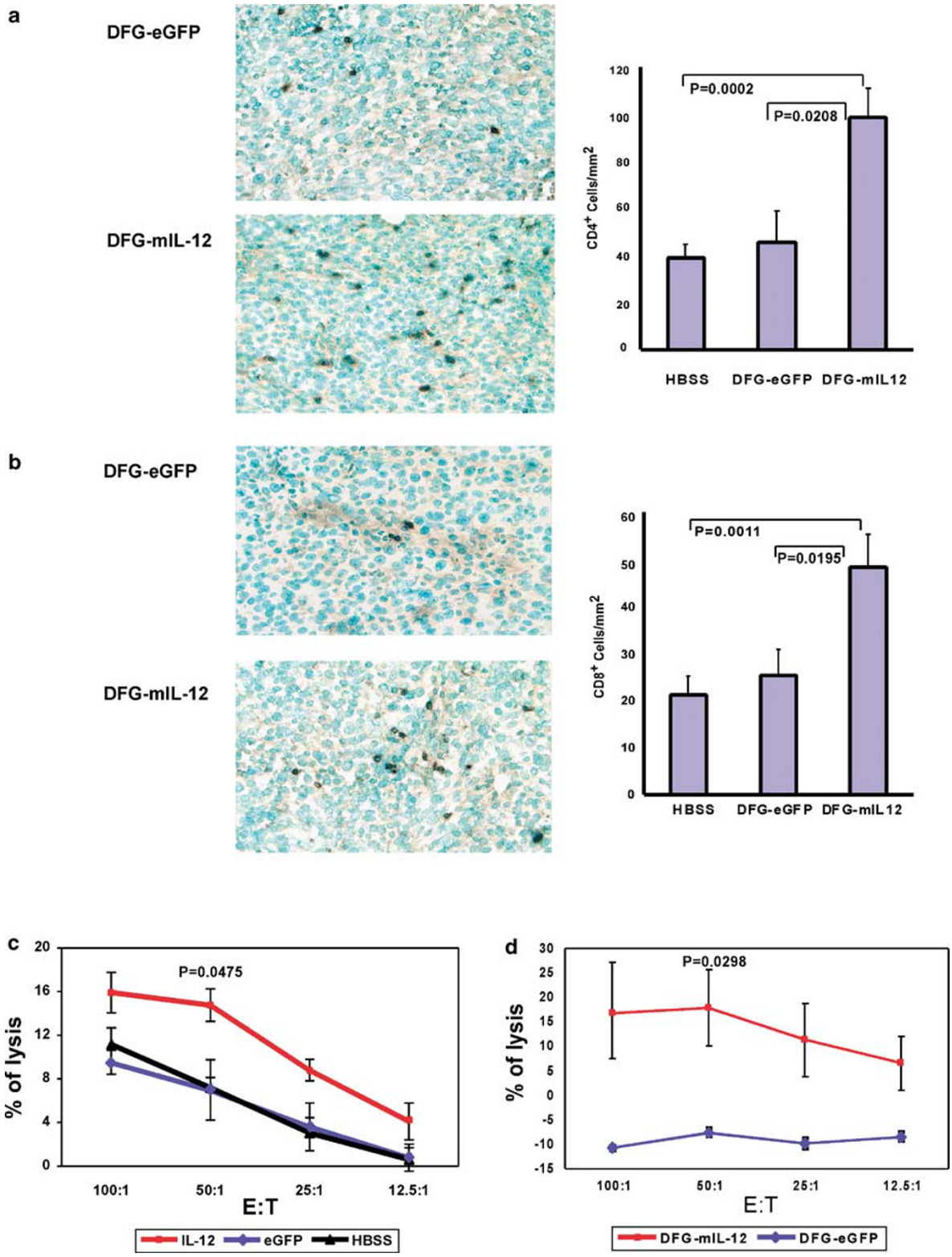

Figure 3 Systemic anti-metastatic immuno responses. At day 21 after treatment, lungs were removed and analyzed by immunohistochemical staining. $CD4^+$ (**a**) and $CD8^+$ (**b**) T-cell infiltrations in lung metastatic tumors were examined in DFG-eGFP-transduced BMC-treated mice (upper left) and in DFG-mIL12-transduced BMC-treated mice (lower left), as well as quantified for both groups as well as HBSS-treated mice (right). Systemic immune responses of NK activities (**c**) and CTL activities (**d**) were analyzed at day 7 after treatment. Data are representative of three mice per group per experiment of three independently performed experiments. *P*-values reported are for the comparison of DFG-mIL12-transduced BMC-treated mice and DFG-eGFP-transduced BMC-treated mice at the selected E:T.

Survival of DFG-mIL-12-transduced BMC-treated mice
The therapeutic activity of gene-transduced BMC for metastatic cancer depends on the stable expression of mIL-12 in BMC that are distributed through blood circulation and likely expanded through progenitor cell differentiation and proliferation. Therefore, we determined if the therapeutic effects of DFG-mIL-12-transduced BMC were sustained.

The study was conducted on 80 mice (four groups, 19–21 per group). Maximum follow-up time recorded was 120 days, with only 9 mice (11.3%) remaining alive at the end of the study. The survival probabilities of mice treated with DFG-mIL-12 transduced BMC were significant higher than ones treated with HBSS (HR = 2.7 confidence intervals (1.4, 5.2), $P = 0.0043$), or non-transduced BMC (HR = 2.8 (1.4, 5.5), $P = 0.0034$) (Figure 4). Only 5.3% of mice in non-transduced BMC-treated group survived till day 120. All mice in HBSS-treated group died within 84 days. The difference in survival between these groups was not significant ($P = 0.8800$). DFG-eGFP-transduced BMC treatment seemed to prolong the survival at first, but the difference was not significant on the final analysis (HR = 1.5 (0.8, 3.0), $P = 0.2306$).

To study this phenomenon further, the data were analyzed 1/3 way through the experiment (at 40 days). Within these 40 days, the mice treated with DFG-mIL-12-transduced BMC already showed a significant survival advantage over all control mice (Figure 4 left part): 66.7% of the DFG-mIL-12-transduced BMC mice vs 10.5% in mice treated with non-transduced BMC (HR = 4.6 (2.0, 10.7), $P = 0.0005$), and vs 10.0% in mice treated with HBSS (HR = 3.7 (1.6, 8.6), $P = 0.0025$). The mice treated with DFG-eGFP-transduced BMC also demonstrated significantly reduced survival probability (35.0%) than mice treated with DFG-mIL-12, and this difference was statistically significant (HR = 2.5 (1.0, 6.0), $P = 0.0443$).

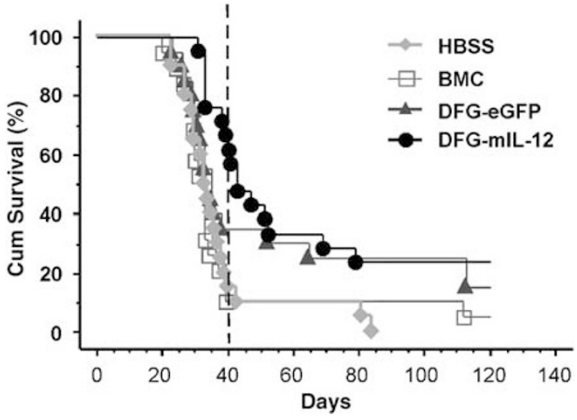

Figure 4 Survival analysis of mice treated with retroviral vector-transduced BMC (Kaplan–Meier plots). Mice harboring metastatic tumors were treated with retroviral vector-transduced BMC, non-transduced BMC or HBSS. Differences between survival probabilities in these groups were evaluated using Cox PH model (HR = 2.7 confidence intervals (1.4, 5.2), $P = 0.0043$) between groups of DFG-mIL-12-transduced BMC and HBSS control, or (HR = 2.8 (1.4, 5.5), $P = 0.0034$) between groups of DFG-mIL-12-transduced BMC and non-transduced BMC ($n = 20$ per group at the initial starting time). The analysis of the data 1/3 way through the experiment (at 40 days) showed a significant increase in survival probabilities for mice treated with DFG-mIL-12-transduced BMC over all control mice and over mice treated with DFG-eGFP-transduced BMC (HR = 2.5 (1.0, 6.0), $P = 0.0443$).

Discussion

In this study, we investigated the therapeutic, immunostimulatory activities of IL-12 to metastatic lesions using gene-modified BMC in a mouse prostate cancer model. The anti-metastatic effects of IL-12 have been shown previously to efficiently control primary tumor growth and systemic metastasis in various pre-clinical models over the short-term.[25–28] Our results show extended anti-metastasis immunity that is mediated by IL-12-transduced BMC.

IL-12 is a heterodimeric pro-inflammatory cytokine that induces the production of IFN-γ, favors the differentiation of helper T cells (Th1) and forms a link between innate resistance and adoptive immunity. *In vitro* studies have also shown that IL-12 can enhance survival and proliferation of early multipotent hematopoietic progenitor cells and linage-committed precursor cells.[29] Systemic administration of recombinant human IL-12 has been hampered by its toxicity for various antitumor and anti-metastatic therapeutic approaches.[30] In constrast, gene therapy with myeloid progenitor cells transduced with IL-12 did not induce hematologic or tissue toxicities commonly associated with systemic IL-12 protein therapy.[31] The retroviral vector, DFG-mIL-12, expresses both IL-12 subunits (p35 and p40) from a polycistronic message utilizing internal ribosome entry site sequences. It has been used to modify the activities of dendritic cells that were introduced into weakly immunogenic tumors using a mouse model system. The results of this study demonstrated the capacity of this approach to induce systemic and therapeutic immunity.[32] In particular, it was clearly demonstrated that IL-12 gene-modified cell therapy allows for the efficient delivery of IL-12 to disseminated metastatic sites, increasing the therapeutic index of this cytokine. In this study, we found that DFG-mIL-12-transduced BMC generated significantly increased levels of serum IL-12 that reached a peak at days 6–9 after treatment. The high levels of IL-12 could promote Th1 immune responses against metastatic tumor cells, and could activate NK cells to induce nonspecific killing of tumor cells. This is indicated by significantly elevated CTL and NK activities in mice treated with DFG-mIL-12-transduced BMC. The significantly increased $CD4^+$ and $CD8^+$ T-cell infiltration in lung metastases suggests a direct antitumor response in the local area. The inverse association between CTL and NK activities and the number of lung metastatic colonies clearly reflect the anti-metastasis effects of the treatment with DFG-mIL-12-transduced BMC.

In our experiments, we transplanted gene-modified BMC in mice with intact hematopoietic systems. Usually, bone marrow transplantation is carried out with recipients that have previously been myeloablated by lethal irradiation to ensure total engraftment of the donor BMC. However, the concept that myeloablation to open space is a prerequisite for marrow stem cell engraftment has been challenged by studies showing high rates of engraftment in non-myeloablated mice.[33,34] Vaccination after non-myeloablative syngeneic stem cell transplantation can achieve stable mixed bone marrow chimerism and generate significantly enhanced tumor-specific immune responses without inducing graft-versus-host disease.[35] Since most prostate cancer patients do not receive high-dose chemotherapy, and therefore have intact, albeit compromised, hematopoietic systems, our model is more realistic and directly translatable to a clinical setting.

Previous bone marrow transplantation studies have shown that donor HSC can effectively home to bone and contribute to short- and long-term hematopoiesis after unconditioned bone marrow transplant.[36] To take advantage of this specific homing effect of BMC, we used DFG-mIL-12-transduced BMC to specifically treat bone and possibly lung metastases. Our results demonstrated that a subpopulation of retroviral vector-transduced LacZ$^+$ BMC homed to host bone marrow within 3 days after treatment and migrated to PB and lung 3 weeks later, which indicate specific homing effects of retroviral vector-transduced BMC in this model (Figure 2). Although specific homing activities were associated with both DFG-mIL-12- and DFG-eGFP-transduced BMC, only DFG-mIL-12-transduced BMC produced significant anti-metastatic activities in lung and bone (Figure 1). These results indicate that IL-12 expression was associated with the anti-metastatic effects, yet, do not specify whether local and/or systemic concentrations of IL-12 protein stimulated the response. Since the localized infiltration of CD4$^+$ and CD8$^+$ T cells was significantly increased within the metastatic lung nodules of DFG-mIL-12-transduced BMC-treated mice compared to DFG-eGFP-transduced BMC-treated mice, it can be argued that local IL-12 concentrations generated by either transplanted BMC homing to the lungs or transplanted BMC-derived immune cells contributed to the anti-metastatic activities.

In our survival study, mice treated with DFG-mIL-12-transduced BMC demonstrated a significant survival advantage compared with DFG-eGFP-transduced BMC or non-transduced BMC or HBSS medium-treated mice within the first third of the follow-up period (Figure 4, left part). This effect may be due to increased immune cell capacity resulting from IL-12-mediated proliferation and colony formation of hematopoietic progenitor cells. However, we did not have direct evidence that DFG-mIL-12-transduced bone marrow stem cells differentiated into T cells and NK cells. Alternatively, the extended survival in mice treated with DFG-mIL-12-transduced BMC may result from direct stimulatory effects of IL-12 on differentiated immune cells and/or anti-angiogenic activities of IL-12 on metastases.[37,38] Interestingly, in long term, both DFG-mIL-12- and DFG-eGFP-transduced BMC-treated mice had extended survival times, and the difference between these groups was no longer significant (Figure 4). It is possible that this difference would have remained at the end of the follow-up period in a larger study, or this could be explained as the effect of longevity, when the mice in both groups surviving beyond a certain point tend to stay alive for equally long time. Alternatively, since a gene-marking study using eGFP-transduced CD34$^+$ cells showed an induction of CTL and antibody responses against eGFP in macaques that received autologous infusions of MLV/eGFP-transduced CD34$^+$ cells,[39] it is possible that transduced eGFP in BMC induced some nonspecific immune responses and extended survival time of mice harboring metastases.

In summary, our study demonstrated that combination of retroviral vector-transduced IL-12 gene therapy with BMC transplantation produced significant anti-metastatic effects associated with local and systemic antitumor immune responses. In our study, we used a metastatic mouse prostate cancer model, yet our results have general significance to treatment of established metastatic disease for various malignancies. Translation of this approach to the clinic will require careful consideration of the specific tumor type, the clinical setting, efficiency of IL-12 gene transduction and, importantly, assessment of the risk of IL-12 integration in BMC.

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