

Augmented Anti-tumor Effect of Dendritic Cells Genetically Engineered by Interleukin-12 Plasmid DNA

Masataka Yoshida, Jun-Ichiro Jo and Yasuhiko Tabata*

Department of Biomaterials, Institute for Frontier Medical Sciences, Kyoto University,
53 Kawara-cho Shogoin, Sakyo-ku Kyoto 606-8507, Japan

Received 26 January 2009; accepted 12 March 2009

Abstract

The objective of this study was to genetically engineer dendritic cells (DC) for biological activation and evaluate their anti-tumor activity in a tumor-bearing mouse model. Mouse DC were incubated on the surface of culture dishes which had been coated with the complexes of a cationized dextran and luciferase plasmid DNA complexes plus a cell adhesion protein, Pronectin[®], for gene transfection (reverse transfection). When compared with the conventional transfection where DC were transfected in the medium containing the complexes, the level of gene expression by the reverse method was significantly higher and the time period of gene expression was prolonged. Following the reverse transfection of DC by a plasmid DNA of mouse interleukin-12 (mIL-12) complexed with the cationized dextran, the mIL-12 protein was secreted at higher amounts for a longer time period. When injected intratumorally into mice carrying a mass of B16 tumor cells, the DC genetically activated showed significant anti-tumor activity.

© Koninklijke Brill NV, Leiden, 2010

Keywords

Dendritic cells, immune response, anti-tumor effect, cationized dextran, interleukin-12

1. Introduction

Dendritic cells (DC) are antigen-presenting cells, which possess the capacity to generate primary immune responses [1]. After the antigen uptake, DC process these antigens into small peptides and move into secondary lymphoid organs to present the antigenic peptides to lymphocytes for their immunological activation [2–4]. Generally, DC are activated to biologically mature and subsequently obtain immunological responsibility. The mature DC express high levels of cell-surface major histocompatibility complexes, antigen complexes and co-stimulatory molecules. Lymphocytes, such as naïve T-cells, helper CD4⁺ T-cells, cytolytic CD8⁺ T-cells, naïve B-cells, memory B-cells, natural killer cells and natural killer T-cells,

* To whom correspondence should be addressed. Tel.: (81-75) 751-4121; Fax: (81-75) 751-4646; e-mail: yasuhiko@frontier.kyoto-u.ac.jp

are critical elements of immunological responses. DC play a central role in the natural immunization. Therefore, because of the function of DC, DC generated *ex vivo* are highly expected to be promising cells, suitable for vaccination in cancer and infectious diseases [5]. However, the immune response of DC is not strong enough for vaccination in vigorous tumors. It is necessary for successful DC-based tumor vaccination to activate the immunological function for anti-tumor response.

Interleukin-12 (IL-12) is a heterodimeric cytokine produced by DC, macrophages, polymorphonuclear leukocytes and keratinocytes [6]. The anti-tumor effect of DC genetically modified with an IL-12 gene was examined in mouse tumor models [7]. IL-12 enhances the anti-tumor effect of natural killer cells and cytotoxic T lymphocytes (CTL) activities, plays a key role in the induction of Th1-type immune responses including interferon-gamma (IFN- γ) production, and has IFN- γ /IFN-inducible protein-10-dependent anti-angiogenic effects [8]. It has been reported that following intratumoral injection, bone-marrow-derived DC, genetically modified with an IL-12 gene, showed enhanced uptake of tumor antigens at the tumor site and moved into secondary lymphoid organs to induce an immune response against the tumor [9]. Intratumoral injection of IL-12-engineered DC suppressed the *in vivo* growth of tumors established and induced a strong anti-tumor T-cell response [9]. However, in the studies reported, retrovirus or adenovirus vectors have been used for genetic engineering of DC. This is because virus vectors generally have high transfection efficiency. Adenoviruses and retroviruses have several clinical limitations, such as mutagenesis, carcinogenesis, and induction of immune responses. From the clinical viewpoint, it is desirable to develop an efficient and safe non-viral carrier to genetically engineer DC for tumor vaccination. Cationized polysaccharides of spermine-introduced dextran and pullulan have been explored as a non-viral carrier for gene transfection [10–13]. Since polysaccharides have reactive OH groups, the chemical modification is easy to give them chemical, physical and biological functions. In addition, polysaccharides are composed of different sugars which can be recognized by some sugar-recognizable cell receptors. Complexation with the cationized polysaccharide allows plasmid DNA to prepare a size-condensed structure with a positive charge, which efficiently allows plasmid DNA to internalize into cells *via* receptor-mediated endocytosis.

As the conventional procedure of gene transfection, a plasmid DNA is complexed with the non-viral carrier, and then added to the culture medium of cells for gene transfection. In this case, serum cannot be generally added to the culture medium, although it is essential to give cells biological conditions good for culture. This is because the plasmid DNA-carrier complexes often interact with serum components, leading to the suppressed ability of gene transfection. To overcome the technical problem, a new transfection system has been introduced [14]. Cells are cultured on the surface of substrate which was coated with the plasmid DNA-cationized dextran complexes and gene transfected in the presence of serum (reverse transfection). The

transfection method enhanced the level of gene transfection to a significantly higher extent compared with the conventional one.

The objective of this study is to investigate the gene transfection of DC for the genetic engineering by the spermine–dextran of non-viral carrier and compared with that of other transfection reagents commercially available. Moreover, gene transfection by the reverse transfection method with the spermine–dextran/plasmid DNA complexes was carried out to enhance gene transfection efficiency [14]. DC genetically engineered by the complexes of mouse interleukin 12 (mIL-12) plasmid DNA and spermine–dextran were intratumorally injected to tumor-bearing mice to evaluate the *in vivo* anti-tumor effect. We examined the IFN- γ production and anti-tumor activity of spleen cells isolated from tumor-bearing mice injected with engineered DC.

2. Materials and Methods

2.1. Materials

Dextran with a weight-average molecular weight of 74 000 and spermine were purchased from Sigma (St. Louis, MO, USA). Pronectin[®] (Lot No. R1-7K002-A) was kindly supplied by Sanyo Chemical Industries (Kyoto, Japan). Other chemicals were obtained from Nacalai Tesque (Kyoto, Japan) and used without further purification.

2.2. Preparation of Cationized Dextran Derivatives

Spermine was introduced to the hydroxyl groups of dextran by a N,N'-carbonyldiimidazole (CDI) activation method [15]. Spermine (1870 mg) and CDI (225 mg) were added to 50 ml dehydrated dimethyl sulfoxide (DMSO) containing 50 mg dextran. Following agitation at 35°C for 20 h, the reaction mixture was dialyzed against ultra-pure double-distilled water (DDW) for 2 days with a dialysis membrane (molecular mass cut-off 12–14 kDa, Viskase, Willowbrook, IL, USA). Then, the solution was freeze-dried to obtain the spermine-introduced dextran (spermine–dextran). When determined by conventional elemental analysis, the molar percentage of spermine introduced to the hydroxyl groups of dextran was 12.7 mol%.

2.3. Preparation of Plasmid DNA

The plasmid DNAs used were the pGL3 plasmid coding for a firefly luciferase gene (Luciferase Reporter Vectors-pGL3, Promega, Madison, WI, USA), the pEGFP-N1 plasmid coding enhanced green fluorescent protein (Takara Bio, Shiga, Japan) and the pGEG. mIL-12 plasmid coding for a bicistronic expression cassette for murine IL-12 p35 and p40 genes [16], which was kindly supplied by Dr. Mazda, Kyoto Prefectural University of Medicine (Kyoto, Japan). The pGEG is composed of CAG (cytomegalovirus immediate-early enhancer-chicken beta-actin hybrid) promoter, SV40 (Simian vacuolating virus 40) polyA additional signal, EBV oriP (Epstein–Barr virus origin of plasmid replication), EBV EBNA1 (EBV-encoded nuclear

antigen-1) gene and another CAG promoter. The plasmid DNA was propagated in an *Escherichia coli* strain (DH5 α) and purified by the Qiagen plasmid Mega kit (Qiagen K.K., Tokyo, Japan) according to the manufacturers' instructions. Both the yield and purity of plasmid DNA were evaluated by UV spectroscopy (Ultrospec 2000, Pharmacia Biotech, Cambridge, UK). The absorbance ratio at wavelengths of 260 to 280 nm for plasmid DNA solution was measured to be between 1.8 and 2.0.

2.4. Preparation of Polyion Complexes

Polyion complexes were prepared by mixing an aqueous solution of spermine–dextran with that of plasmid DNA. Briefly, various amounts of spermine–dextran were dissolved in 50 μ l DDW and mixed with 50 μ l phosphate-buffered saline (PBS, 10 mM, pH 7.4) solution containing 2.5 μ g plasmid DNA, followed by leaving for 15 min at room temperature to obtain the polyion complexes of spermine–dextran and plasmid DNA. The polyion complexes composition was calculated on the basis of the nitrogen number of spermine–dextran (N) per the phosphorus number of plasmid DNA (P) and expressed as the N/P ratio. To complex the plasmid DNA with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), 50 μ l Opti-MEM (Invitrogen) containing 5.0 μ g Lipofectamine was mixed with 50 μ l Opti-MEM containing 2.5 μ g plasmid DNA, followed by leaving for 15 min at room temperature.

2.5. Preparation and Culture of DC

Mouse immature DC were obtained from bone marrow cells (BMC) through the differentiation induction according to the conventional procedure previously reported [17]. Briefly, mouse BMC were harvested from the femur and tibia of 4-week-old female ICR mice or 6-week-old female C57BL/6 mice (body weight 20 g, Shimizu Laboratory Supplies, Kyoto, Japan). Erythrocytes contaminated in BMC were lysed with ammonium chloride solution and cells were seeded in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with penicillin (100 U/ml, Sigma-Aldrich), streptomycin (100 μ g/ml, Sigma-Aldrich), 10 vol% heat-inactivated FCS (Hyclone Laboratories, Logan, UT, USA) and 200 U/ml recombinant mouse-derived granulocyte macrophage colony-stimulating factor (rmGM-CSF, 1×10^7 U/mg, Peprotech/Tebu, Frankfurt, Germany) on day 0. The medium was changed every 2 days thereafter. The cells cultured for 9–14 days were used for all experiments as mouse immature DC.

2.6. Conventional Gene Transfection

DC were seeded in each well of a 12-well multi-dish culture plate (Corning, Corning, NY, USA) at a density of 2.5×10^5 cells/well and cultured in 1 ml of RPMI-1640 medium with 10 vol% FCS and 50 U/ml rmGM-CSF for 24 h. Immediately after the medium was exchanged by FCS and rmGM-CSF-free RPMI-1640 medium, 100 μ l polyion complexes solution containing 2.5 μ g plasmid DNA at different N/P ratios was added, and transfection culture was performed for 3 h. After

the medium was changed to RPMI-1640 with 10 vol% FCS and rmGM-CSF, cells were incubated further for 1, 4 and 7 days.

2.7. Reverse Gene Transfection

In vitro gene transfection by the reverse method was performed according to the method described previously [14]. Briefly, succinic anhydride (90.1 mg) was added to 20 ml of 100 mg/ml gelatin solution in DMSO, followed by agitation at room temperature for 18 h to allow to introduce the carboxyl groups to the amino groups of gelatin for anionization. When determined as the extent of amino groups decreased by the trinitrobenzene sulfonic acid method [18], the molar amount of carboxylic groups introduced was 100 mol%. The aqueous solution of anionized gelatin (100 µg/ml) and Pronectin (200 µg/ml) was placed into each well of 12-well multi-dish culture plate and left at 37°C for 1 h for coating. After PBS washing, the well was coated with the polyion complexes solution containing 2.5 µg plasmid DNA at an N/P ratio of 3. After 30 min incubation, the wells were washed with PBS. Then, DC (2.5×10^5 cells/well) were seeded on the complexes-coated well, followed by cell culture in the RPMI-1640 medium with 10 vol% FCS and 50 U/ml rmGM-CSF for 2, 5 and 8 days.

2.8. Gene Expression Assay

To assay the level of luciferase expression, transfected cells were washed with PBS once, lysed in 100 µl cell-culture lysis reagent (Promega, Madison, WI, USA), transferred into a micro reaction tube, and the cell debris was separated by centrifugation (14×10^3 rpm, 20 min). Then, 100 µl luciferase assay reagent (Promega) was added to 20 µl supernatant while the relative light unit (RLU) of the sample was determined by a luminometer (MicroLumatPlus LB 96V, Berthold, Tokyo, Japan). The total protein of each well was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturers' instructions. The RLU was divided by the protein amount to normalize the influence of number variance of cells on the luciferase activity. Each experimental group was carried out three times independently.

To assay GFP expression efficacy, cells transfected were washed with PBS twice and detached by trypsinization. After centrifugation (1000 rpm, 5 min), the cells were resuspended and washed with PBS twice. Cells (1×10^4) were measured by fluorescence activated cell sorter (FACS) Calibur with Cell Quest Pro Software (Becton Dickinson Biosciences, San Jose, CA, USA). The percentage of positive cells was calculated by setting the background population as 98% negative when analyzing control cells [19].

After the reverse transfection culture with the spermine–dextran/mIL-12 plasmid DNA complexes for different time periods, the amount of mIL-12 produced was measured by the mIL-12 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturers' instructions.

2.9. Evaluation of Plasmid DNA Internalization into Cells

Plasmid DNA was labeled with Cy5 using the Label IT[®] Cy5 Labeling Kit (Mirus Bio, Madison, WI, USA). The Cy5-labeled plasmid DNA was complexed with spermine–dextran. The cells were treated in cold acetate buffer containing 0.3 vol% acetic acid, 0.085 M NaCl and 5.0 mM KCl for 1 min, washed with cold PBS to remove the complexes bound to the cell surface. The cells were collected by trypsinization, then 1×10^4 cells were measured by FACSCalibur flow cytometry. The percentage of Cy5-positive cells was calculated by setting the background population as 98% negative when analyzing control cells.

2.10. Microscopic Observation of Intracellular Localization

After the conventional and reverse transfection of cultures for 1 day with the complexes of Cy5-labeled plasmid DNA and the spermine–dextran, cells were washed and fixed with 0.25 wt% glutaraldehyde solution in PBS, followed by staining nucleus with Hoechst 33342 (Molecular Probes, Eugene, OR, USA). Imaging data of cells were collected on an Olympus Fluoview FV300 confocal laser scanning microscope (Olympus) and processed with Adobe Photoshop 6.0 software (Adobe Systems, San Jose, CA, USA).

2.11. Cell Viability

Cytotoxicity after gene transfection was assayed using a cell counting kit (Nacalai Tesque). The medium was changed to RPMI-1640 with FCS and rmGM-CSF, 10 μ l 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) solution was added and the cells incubated for another 4 h. The absorbance of samples was measured at 450 nm using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA). The percentage cell viability was expressed as 100% for non-transfected, control cells.

2.12. Evaluation of Anti-tumor Effect

B16 melanoma cells (1×10^5 cells/mouse) suspended in 200 μ l of PBS were subcutaneously inoculated into the right flank of C57BL/6 mice on day 0. DC were genetically engineered by the reverse transfection with the complexes of spermine–dextran/mIL-12 plasmid DNA as described previously. On day 9, when the tumor size reached a approx. 0.10–0.05 cm³, tumor therapy with DC genetically engineered was started. DC genetically engineered were taken off with a rubber policeman, washed twice and resuspended in PBS. Then, 100 μ l DC (1×10^6 cells) suspended in PBS was intratumorally injected. At different time periods after intratumoral injection, the longest and widest diameter of tumor mass were measured with a caliper and the tumor volume was calculated by using the formula of (length) \times (width)² \times 1/2 [20]. As controls, PBS, non-transfected, original DC or DC transfected with spermine–dextran/GFP–plasmid DNA complexes by the reverse method were injected.

2.13. In Vitro Cytokine Release Assays

Splenocytes were obtained from the spleen harvested from tumor-bearing mice 7 days after intratumoral injection with PBS, original DC or those transfected with the complexes of spermine–dextran and GFP or mIL-12 plasmid DNA by the reverse method. Briefly, the spleen taken was pressed through a sterile gauze, rinsed twice. These cells (1×10^6 cells) were co-cultured in a 100-mm dish (Corning) with mytomycin C (Sigma)-treated B16 cells (1×10^6 cells) in culture medium containing 25 U/ml recombinant mouse IL-2 (2.5×10^6 U/mg; Prospec, Rehovot, Israel) for 6 days. Then, the culture medium was collected and the amount of interferon- γ (IFN- γ) was measured by mouse IFN- γ (R&D Systems) ELISA kits according to the manufacturers' instructions.

2.14. Measurement of Cytotoxic T-Lymphocyte (CTL) Activity

Splenocytes harvested as described above (1×10^6 cells) were stimulated in a 100-mm dish with mytomycin C-treated B16 cells (1×10^6 cells) in culture medium containing 25 U/ml recombinant mouse IL-2 for 2 days. Then, the cells stimulated were used as effectors for measurement of CTL activity. B16 (target) cells (5×10^4 cells each) were cultured in a 96-well multi-dish culture plate (Corning) for 24 h. After washing twice, various number of effector cells were added to the plate. After 4 h co-culture, the supernatant (50 μ l) was collected, and the lactate dehydrogenase (LDH) activity was measured using the LDH-Cytotoxic Test (Wako, Osaka, Japan). The CTL activity was expressed as the number percentage of target cells injured by splenocytes to those completely injured by adding 0.2 vol% Tween-20.

2.15. Evaluation of In Vivo Tumor Immunity

B16 cells (1×10^5 cells/mouse) suspended in 200 μ l PBS were subcutaneously inoculated into the both flanks of C57BL/6 mice on day 0. DC were genetically engineered by reverse transfection with the complexes of spermine–dextran/mIL-12 plasmid DNA as described previously. On day 9, 100 μ l DC (1×10^6 cells) suspended in PBS was injected into the tumor mass grown in the right flank of the mice. At 29 days after intratumoral injection, each tumor volume in the both flanks was calculated as described above. As controls, PBS, non-transfected, original DC or those transfected with spermine–dextran/GFP–plasmid DNA complexes by the reverse method was injected similarly.

2.16. Statistical Analysis

Numerical values were expressed as mean \pm SEM unless otherwise indicated. Comparisons of parameters between the 4 groups were made using one-way analysis of variance, followed by the Newman–Keuls multiple comparison test. $P < 0.05$ was considered to be significant.

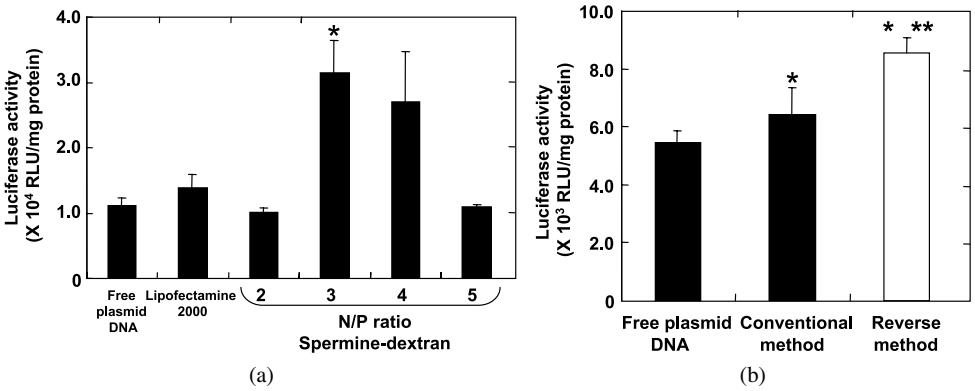


Figure 1. (a) The level of luciferase expression in DC 1 day after transfection of luciferase plasmid DNA complexed with the spermine–dextran at different N/P ratios by the conventional method. As controls, free plasmid DNA and plasmid DNA complexes with Lipofectamine 2000 are used. The amount of plasmid DNA used for transfection is 2.5 μ g. (b) The level of luciferase expression in DC 1 day after transfection of luciferase plasmid DNA complexed with the spermine–dextran at a N/P ratio of 3.0 by the conventional (filled column) and reverse methods (open columns). As control, free plasmid DNA is used. The amount of plasmid used for transfection is 2.5 μ g. * P < 0.05 versus the expression level of DC transfected with free plasmid DNA. ** P < 0.05 versus the expression level of DC transfected with the spermine–dextran/plasmid DNA complexes by the conventional method.

3. Results

3.1. Gene Expression by Spermine–Dextran/Plasmid DNA Complexes

Figure 1 shows the level of luciferase expression for DC 1 day after transfection of luciferase plasmid DNA complexed with the spermine–dextran at different N/P ratios by the conventional and reverse method. In the conventional method, the level of gene expression by the spermine–dextran/plasmid DNA complexes at a N/P ratio of 3.0 was significantly higher than those of free plasmid DNA and the plasmid DNA complexes of Lipofectamine 2000 or the spermine–dextrans at other N/P ratios (Fig. 1a). On the other hand, the expression level by the reverse transfection method was significantly higher than that of the conventional one (Fig. 1b).

Figure 2 shows the time-course of the percent internalized of GFP–plasmid DNA and GFP expression for DC transfected with free plasmid DNA and spermine–dextran/plasmid DNA complexes by the conventional and reverse methods. The plasmid DNA in the free form could be hardly internalized while little gene expression was observed. For the plasmid DNA in the complexes form, there was no difference in the time-course of plasmid DNA internalized into DC different between the conventional and reverse methods, while the percentage of plasmid DNA internalized was significantly higher for the reverse method than for the conventional one (Fig. 2a). The level of GFP expressed by the conventional method decreased with culture time, whereas it was retained at a high value for the reverse method (Fig. 2b).

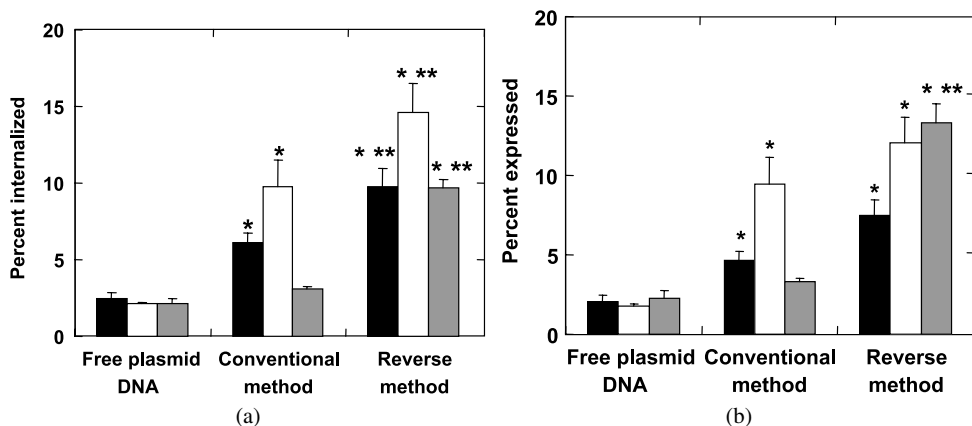


Figure 2. Time-course of percent GFP-plasmid DNA internalized (a) and GFP expression for DC (b) transfected with free plasmid DNA and spermine-dextran/plasmid DNA complexes by the conventional and reverse methods. The cells were cultured for 1 (black columns), 4 (open columns) and 7 days (gray columns). The amount of plasmid DNA used for transfection is 2.5 μ g. The N/P ratio of spermine-dextran/plasmid DNA complexes is 3.0. * $P < 0.05$ versus the percent GFP-plasmid DNA internalized or GFP expression for DC transfected with free plasmid DNA at the corresponding time. ** $P < 0.05$ versus the percent GFP-plasmid DNA internalized or GFP expression for DC transfected with the spermine-dextran/plasmid DNA complexes by the conventional method at the corresponding time.

Figure 3 shows the confocal laser microscopic images of DC 1 day after transfection with free plasmid DNA and spermine-dextran/plasmid DNA complexes by the conventional and reverse methods. The plasmid DNA in the free form was hardly internalized into DC. The number of internalized-plasmid DNA for the reverse method tended to be larger than that of the conventional one.

3.2. Viability of DC Transfected by the Conventional and Reverse Methods

Figure 4 shows the viability of DC 1 day after transfection with free plasmid DNA, plasmid DNA-Lipofectamine 2000 complexes and spermine-dextran/plasmid DNA complexes by the conventional and reverse methods. The cell viability decreased significantly by the conventional transfection method with the spermine-dextran and Lipofectamine 2000 complexes with plasmid DNA. In contrast, the viability after the reverse transfection culture was similar to that of non-transfected, original cells.

IL-12 expression of DC transfected by spermine-dextran/mIL-12 plasmid DNA complexes. Figure 5 shows the time-course of mIL-12 production of DC transfected with PBS, complexes of spermine-dextran and GFP or mIL-12 plasmid DNAs by the reverse method. The amount of mIL-12 produced by DC transfected with the spermine-dextran/mIL-12 plasmid DNA complexes was significantly higher than that of non-transfected, original DC until at least 14 days after transfection. The amount of mIL-12 produced by DC transfected with spermine-

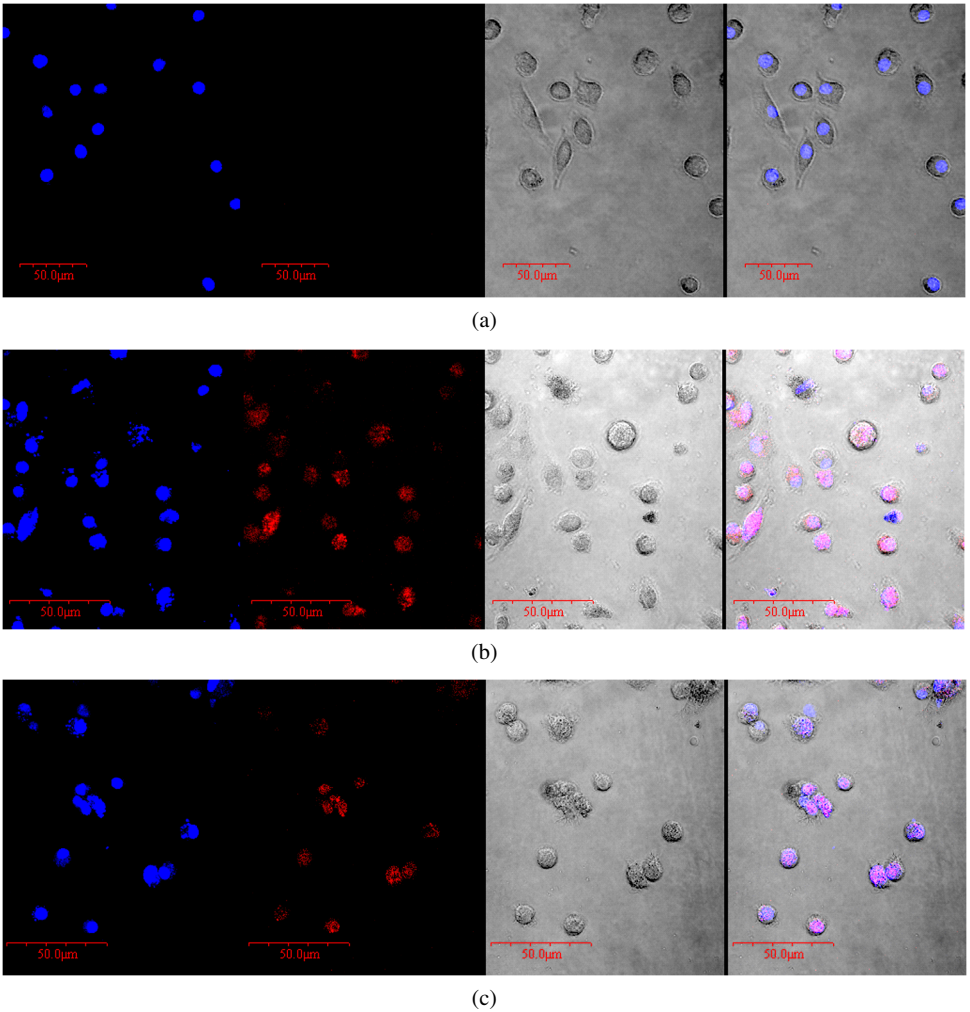


Figure 3. Confocal laser microscopic images of DC 1 day after transfection with free plasmid DNA (a) and spermine–dextran/plasmid DNA complexes by the conventional (b) and reverse methods (c). The plasmid DNA was labeled with Cy5. The amount of plasmid DNA used for transfection is 2.5 μg . The N/P ratio of spermine–dextran/plasmid DNA complexes is 3.0. The red and blue points indicate the plasmid DNA and cell nucleus, respectively. This figure is published in the online edition of this journal, that can be accessed *via* <http://www.brill.nl/jbs>

dextran/GFP–plasmid DNA complexes increased with culture time and reached plateau at 11 days.

3.3. In Vivo Anti-tumor Effect of DC Genetically Engineered with Spermine–Dextran/mIL-12 Plasmid DNA by the Reverse Transfection Method

Figure 6 shows the time-course of tumor volume change after the intratumoral injection of PBS, non-transfected, original DC and DC transfected with complexes

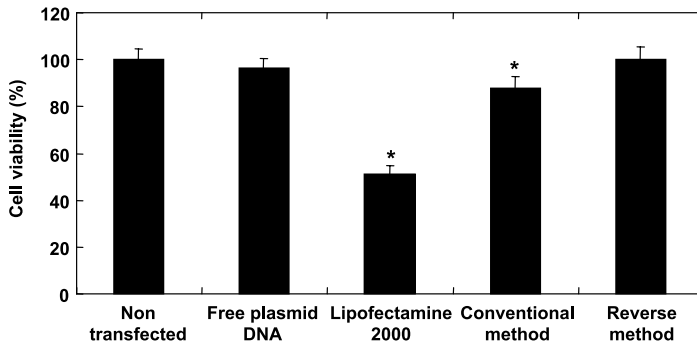


Figure 4. Viability of DC 1 day after transfection with free plasmid DNA, plasmid DNA–Lipofectamine 2000 complexes and spermine–dextran/plasmid DNA complexes by the conventional and reverse methods. The amount of plasmid DNA used for transfection is 2.5 μ g. The N/P ratio of spermine–dextran/plasmid DNA complexes is 3.0. * $P < 0.05$ versus the viability of DC without transfection.

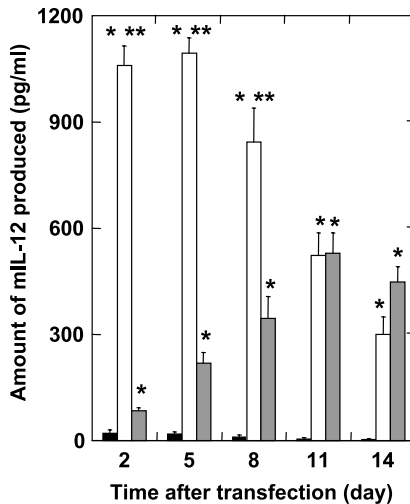


Figure 5. Time-course of mIL-12 production in DC transfected with PBS (closed bars), complexes of spermine–dextran and GFP (gray bars) or mIL-12 plasmid DNAs (open bars) by the reverse method. The amount of plasmid DNA used for transfection is 2.5 μ g. The N/P ratio of spermine–dextran/plasmid DNA complexes is 3.0. * $P < 0.05$ versus the amount of mIL-12 produced by DC transfected with PBS at the corresponding time. ** $P < 0.05$ versus the amount of IL-12 produced of DC transfected with spermine–dextran/GFP–plasmid DNA complexes at the corresponding time.

of spermine–dextran and GFP or mIL-12 plasmid DNAs by the reverse method. The intratumoral injection of DC transfected with the spermine–dextran/mIL-12 plasmid DNA complexes by the reverse method tended to suppress the *in vivo* growth of tumor cells even at the early time period and exhibited significantly higher anti-tumor effect *in vivo* than that of PBS, original DC, and DC transfected with spermine–dextran/GFP–plasmid DNA complexes.

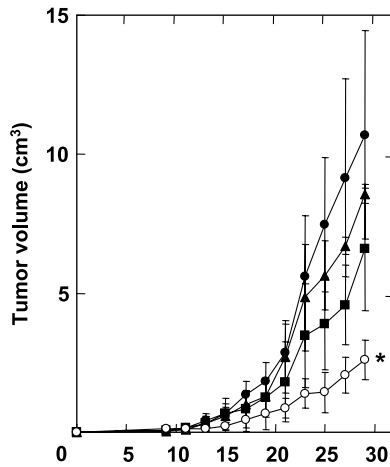


Figure 6. Time-course of tumor volume change after intratumoral injection of PBS (●), non-transfected, original DC (▲) and DC transfected with complexes of spermine–dextran and GFP (■) or mIL-12 plasmid DNAs (○) by the reverse method. * $P < 0.05$ versus the tumor volume of other groups at the corresponding time.

3.4. IFN Production and CTL Activity of DC Genetically Engineered with Spermine–Dextran/mIL-12 Plasmid DNA by the Reverse Transfection Method

Figure 7 shows the IFN- γ secretion and CTL activity by the splenocytes of tumor-bearing mice 7 days after intratumoral injection of PBS, non-transfected, original DC, and DC transfected with complexes of spermine–dextran and GFP or mIL-12 plasmid DNAs by the reverse transfection method. Intratumoral injection of DC genetically engineered with the spermine–dextran/mIL-12 plasmid DNA complexes by the reverse method enhanced IFN- γ production of splenocytes to a significantly greater extent compared with that of original DC or those genetically engineered with spermine–dextran/GFP–plasmid DNA complexes by the reverse transfection method (Fig. 7a). For DC transfected with the spermine–dextran/mIL-12 plasmid DNA complexes, as the effector/target ratio increased, the CTL activity of splenocytes harvested became high to a significantly greater extent compared with that of original DC or those genetically engineered with spermine–dextran/GFP–plasmid DNA complexes by the reverse transfection method (Fig. 7b).

3.5. Evaluation of In Vivo Tumor Immunity

To confirm the induction of systemic and therapeutic immunity, the growth of contralateral non-treated tumor, which is distant from the tumor mass injected with DC genetically engineered by the spermine–dextran/mIL-12 plasmid DNA complexes, was evaluated (Table 1). Intratumoral injection of DC genetically engineered significantly suppressed the growth of the tumor mass, irrespective of the direct injection.

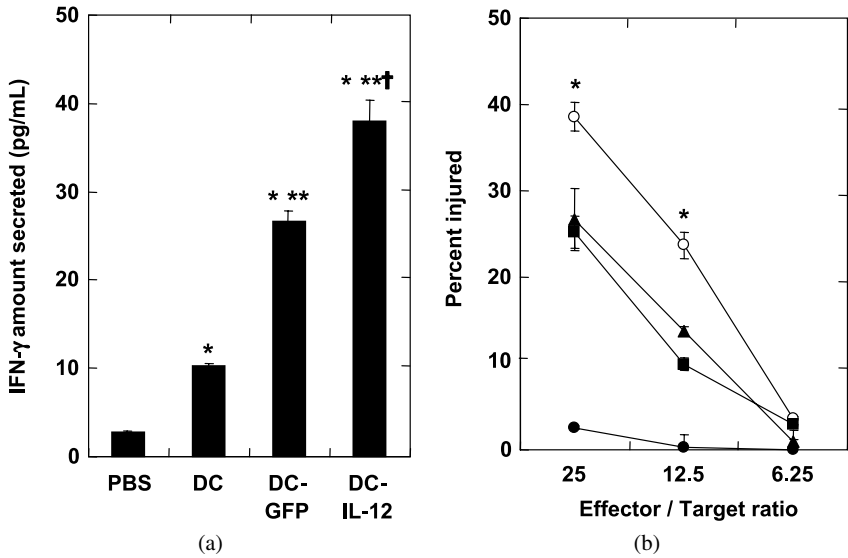


Figure 7. (a) IFN- γ secretion by splenocytes of tumor-bearing mice 7 days after intratumoral injection of PBS, non-transfected, original DC (DC) and DC transfected with complexes of spermine–dextran and GFP (DC–GFP) or mL-12 plasmid DNAs (DC–IL-12) by the reverse method. * $P < 0.05$ versus the amount of IFN- γ secreted for the PBS group. ** $P < 0.05$ versus the amount of IFN- γ secreted for the DC group. † $P < 0.05$ versus the amount of IFN- γ secreted for the DC–GFP group. (b) CTL activity as a function of effector/target ratio. CTL activity was expressed as the percentage of B16 (target) cells injured by co-culture with splenocytes (effector) harvested from tumor-bearing mice 7 days after the intratumoral injection of PBS (●), non-transfected, original DC (▲) and DC transfected with complexes of spermine–dextran and GFP (■) or mL-12 plasmid DNAs (○) by the reverse method. * $P < 0.05$ versus the percentage of B16 cells injured by other groups at the corresponding effector/target ratio.

Table 1.

Tumor volume in both flanks of tumor-bearing mice with or without intratumoral of genetically engineered DC

	Tumor volume (cm ³)	
	Injected	Not injected
PBS	9.9 ± 1.4	9.6 ± 1.9
DC	8.0 ± 0.4	7.8 ± 0.9
DC–GFP	7.3 ± 0.0	8.5 ± 0.0
DC–IL-12	5.6 ± 0.9*	5.9 ± 0.1*

Tumor volume was measured 29 days after inoculation.

* $P < 0.05$ versus the tumor volume of other groups.

4. Discussion

The present study clearly demonstrated that DC genetically engineered by IL-12 plasmid DNA complexed with the spermine–dextran enhanced the anti-tumor effect

through the immune surveillance potential. The *in vivo* growth of tumor cells was significantly suppressed by injection of engineered DC (Fig. 6). The splenocytes isolated from tumor-bearing mice following the injection of engineered DC showed the IFN production and CTL activity (Fig. 7). The tumor growth suppression was observed even for the tumor mass without the direct DC injection (Table 1).

To genetically engineer cells, it is necessary to develop the technology and methodology of gene transfection. Viral vectors have been mainly used for this purpose because of the high transfection efficiency. However, there are scientific and ethical barriers to use the viral transfection for clinical applications. As one trial to tackle this issue, various non-viral carriers have been investigated [21–24]. To enhance the level of gene expression, in addition to the research and development of gene carriers themselves, it is indispensable to improve the culture technology and methodology of cells for gene transfection. It is highly expected that the culture conditions always affect the biological state of cells which strongly contribute to the level of gene transfection.

In this study, spermine–dextran was used for gene transfection. Complexation with the spermine–dextran of a non-viral vector enabled the plasmid DNA to decrease the apparent size of spermine–dextran plasmid DNA complexes small enough to allow their cellular internalization and to have a positive surface charge which can interact with the cell surface of negative charge. Moreover, it has been reported that many types of sugar-recognizable receptor are expressed on DC [25]. Taken together, it is highly possible that the spermine–dextran/plasmid DNA complexes were internalized into the DC *via* receptor-mediated endocytosis. It is highly conceivable that the features promoted the transfection of plasmid DNA for DC, enhancing the level of gene expression.

Dextran is a water-soluble, naturally occurring polysaccharide with multiple hydroxyl groups applicable to chemical modification. It also has low immunogenicity and a long history for the clinical use as a plasma expander [26]. The level of gene expression of DC by the spermine–dextran/plasmid DNA complexes at a N/P ratio of 3.0 was significantly higher than that by Lipofectamine 2000 of a commercially available transfection reagent (Fig. 1a). It has been previously reported that the N/P ratio affects not only the physicochemical properties of the complexes, but also the gene transfection efficiency [11, 13]. At low N/P ratios, the complexes formed are unstable and have too large molecular size to be internalized into cells, resulting in low gene transfection efficiency. On the other hand, at high N/P ratios, the complexes are too stable to dissociate and have high cytotoxicity due to the excessive amount of cationic spermine–dextran, resulting in low gene transfection efficiency. Therefore, it is possible that the complexes at a N/P ratio of 3.0 have a condition suitable for gene transfection of DC. Considering these points, the spermine–dextran is a promising non-viral vector to enhance the gene expression of DC through the enhanced receptor-mediated endocytosis.

We have demonstrated that the reverse transfection method gives cells good culture conditions, resulting in enhanced and prolonged gene expression with less

cytotoxicity [14]. The efficacy of reverse transfection in gene expression was also confirmed in this study for the luciferase (Fig. 1b), GFP (Fig. 2) and mIL-12 plasmid DNAs (Fig. 5). The higher efficiency of gene transfection can be explained in terms of the unique feature of the reverse method. DC are always cultured on complexes of plasmid DNA and spermine–dextran of a non-viral carrier. It is likely that the localization of plasmid DNA close to cells enhances the possibility of cellular internalization of plasmid DNA, resulting in increased efficiency of gene transfection. In addition, the transfection culture can be performed in the presence of serum, which is a condition better for cell culture. It is apparent from Fig. 3 that the plasmid DNA was localized in the nucleus of cells at higher amounts. This may be also due to the efficient and continuous supply of plasmid DNA to cells (Fig. 2). Different behaviors between the time-course of percent internalized of GFP–plasmid DNA (Fig. 2a) and that of GFP expression (Fig. 2b) for DC transfected with spermine–dextran/plasmid DNA complexes by the reverse method were observed. It will take some time to express protein after gene transfection. Thus, the time between the plasmid DNA internalization and gene expression would be observed. When cells internalize the complexes too much, it is conceivable that their capacity to take up decreases. Therefore, the percent internalized would decrease at day 7.

Although less cytotoxicity was observed (Fig. 4), transfection of the spermine–dextran/GFP–plasmid DNA complexes affected not only the amount of mIL-12 produced in DC (Fig. 5), but also that of IFN- γ secreted by splenocytes (Fig. 7a). It is well known that DC themselves tend to produce IL-12 by various stimuli [27, 28]. Therefore, it is possible that only the stimulus of plasmid DNA transfection allows DC to produce IL-12. Enhancement of IFN production by spermine–dextran/GFP–plasmid DNA complexes may be explained in terms of interaction of DC and IL-12. It has been reported that IFN production from DC is influenced by IL-12 action [29, 30]. The roles of DC and T-cells in the tumor immunity are as follows [1]. DC phagocytoses a tumor antigen in the tumor tissue. Then, DC process these antigens into small peptides and move into the lymphoid organs, such as the spleen. During these steps, DC are biologically matured and obtain immunological responsibility, which express high levels of cell-surface major histocompatibility complexes antigen complexes and co-stimulatory molecules. Receptors of helper CD4⁺ T-cells (Th0) in the lymphoid organs interact with the MHC class II of the DC, followed by the differentiation into the T-helper 1 (Th1) or 2 (Th2) cells and IFN production. On the other hand, it has been reported that IL-12 promotes the differentiation of Th0 cells into Th1 cells [31–33], enhances CTL activity by CD8⁺ T-cells [34], and infiltration of T-cells into the tumor tissue [35]. In the present study, the intratumoral injection of DC genetically engineered with spermine–dextran/mIL-12 plasmid DNA complexes by the reverse method suppressed tumor growth (Fig. 6) and induced a strong anti-tumor T-cell response (Fig. 7). These results suggest that mIL-12 over-expression at the tumor site or, alternatively, in the secondary lymphoid sites could be important for the anti-tumor response. Chemokine production induced by mIL-12/IFN- γ elaboration at the tumor site would be responsible for

these effects, at least in part, through enhanced recruitment of cytolytic effector cells into tumors as well as possibly anti-angiogenic effects. It is apparent from Fig. 6 that the tumor growth was gradually suppressed by the intratumoral injection of DC genetically engineered with spermine–dextran/mL-12 plasmid DNA complexes by the reverse transfection method (Fig. 6). It may be due to that there are many steps in tumor immunity as described above; consequently, it takes some time to express the tumor suppression. Systemic immune responses, as demonstrated by IFN- γ production and CTL activity (Fig. 7), were also significantly higher and tumor specific when injected DC genetically engineered. These findings well correspond to those of research where DC genetically engineered with IL-12 gene by retrovirus were intratumorally injected [9]. These results indicate that high cytotoxic activity in association with a Th1-type response could indeed contribute to the profound *in vivo* anti-tumor effects. Experiments involving a subsequent rechallenge with additional tumors (Table 1) further confirmed systemic anti-tumor immunity after treatment intratumoral injection of DC with genetically engineered.

In conclusion, we developed a novel efficient gene delivery into DC with spermine–dextran of a non-viral vector. Intratumoral injection of DC genetically engineered is therapeutically feasible, mediates an effective anti-tumor response, and is capable of inducing substantial systemic anti-tumor immunity.

References

1. J. Banchereau and R. M. Steinman, *Nature* **392**, 245 (1998).
2. J. M. Austyn, *J. Exp. Med.* **183**, 1287 (1996).
3. S. Sozzani, P. Allavena, A. Vecchi and A. Mantovani, *J. Leukoc. Biol.* **66**, 1 (1999).
4. M. C. Dieu-Nosjean, A. Vicari, S. Lebecque and C. Caux, *J. Leukoc. Biol.* **66**, 252 (1999).
5. E. Gilboa, *J. Clin. Invest.* **117**, 1195 (2007).
6. A. G. Lamont and L. Adorini, *Immunol. Today* **17**, 214 (1996).
7. A. Ribas, L. H. Butterfield, J. A. Glaspy and J. S. Economou, *Curr. Gene Ther.* **2**, 57 (2002).
8. S. Hiscox and W. G. Jiang, *In Vivo* **11**, 125 (1997).
9. Y. Nishioka, M. Hirao, P. D. Robbins, M. T. Lotze and H. Tahara, *Cancer Res.* **59**, 4035 (1999).
10. J. Jo and Y. Tabata, *Eur. J. Pharm. Biopharm.* **68**, 90 (2008).
11. J. Jo, T. Ikai, A. Okazaki, K. Nagane, M. Yamamoto, Y. Hirano and Y. Tabata, *J. Biomater. Sci. Polymer Edn* **18**, 883 (2007).
12. J. Jo, N. Nagaya, Y. Miyahara, M. Kataoka, M. Harada-Shiba, K. Kangawa and Y. Tabata, *Tissue Eng.* **13**, 313 (2007).
13. J. Jo, T. Ikai, A. Okazaki, M. Yamamoto, Y. Hirano and Y. Tabata, *J. Control. Rel.* **118**, 389 (2007).
14. A. Okazaki, J. Jo and Y. Tabata, *Tissue Eng.* **13**, 245 (2007).
15. G. T. Hermanson, *Bioconjugate Techniques*. Academic Press, San Diego, CA (1996).
16. T. Kishida, H. Asada, E. Satoh, S. Tanaka, M. Shinya, H. Hirai, M. Iwai, H. Tahara, J. Imanishi and O. Mazda, *Gene Ther.* **8**, 1234 (2001).
17. M. B. Lutz, N. Kukutsch, A. L. Ogilvie, S. Rossner, F. Koch, N. Romani and G. Schuler, *J. Immunol. Methods* **223**, 77 (1999).
18. A. F. Habeeb, *Anal. Biochem.* **14**, 328 (1966).
19. F. Lampariello, *Cytometry* **15**, 294 (1994).

20. M. Konishi, Y. Tabata, M. Kariya, H. Hosseinkhani, A. Suzuki, K. Fukuhara, M. Mandai, K. Takakura and S. Fujii, *J. Control. Rel.* **103**, 7 (2005).
21. S. C. De Smedt, J. Demeester and W. E. Hennink, *Pharm. Res.* **17**, 113 (2000).
22. K. Ewert, N. L. Slack, A. Ahmad, H. M. Evans, A. J. Lin, C. E. Samuel and C. R. Safinya, *Curr. Med. Chem.* **11**, 133 (2004).
23. M. C. Pedroso de Lima, S. Neves, A. Filipe, N. Duzgunes and S. Simoes, *Curr. Med. Chem.* **10**, 1221 (2003).
24. A. Hirko, F. Tang and J. A. Hughes, *Curr. Med. Chem.* **10**, 1185 (2003).
25. N. Kanazawa, *J. Dermatol. Sci.* **45**, 77 (2007).
26. U. Klotz and H. Kroemer, *Clin. Pharmacokinet.* **12**, 123 (1987).
27. A. D'Andrea, M. Rengaraju, N. M. Valiante, J. Chehimi, M. Kubin, M. Aste, S. H. Chan, M. Kobayashi, D. Young and E. Nickbarg, *J. Exp. Med.* **176**, 1387 (1992).
28. U. Shu, M. Kiniwa, C. Y. Wu, C. Maliszewski, N. Vezzio, J. Hakimi, M. Gately and G. Delespesse, *Eur. J. Immunol.* **25**, 1125 (1995).
29. U. Grohmann, M. L. Belladonna, R. Bianchi, C. Orabona, E. Ayroldi, M. C. Fioretti and P. Puccetti, *Immunity* **9**, 315 (1998).
30. T. Ohteki, T. Fukao, K. Suzue, C. Maki, M. Ito, M. Nakamura and S. Koyasu, *J. Exp. Med.* **189**, 1981 (1999).
31. C. S. Hsieh, S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra and K. M. Murphy, *Science* **260**, 547 (1993).
32. R. A. Seder, R. Gazzinelli, A. Sher and W. E. Paul, *Proc. Natl. Acad. Sci. USA* **90**, 10188 (1993).
33. R. Manetti, P. Parronchi, M. G. Giudizi, M. P. Piccinini, E. Maggi, G. Trinchieri and S. Romagnani, *J. Exp. Med.* **177**, 1199 (1993).
34. M. Kobayashi, L. Fitz, M. Ryan, R. M. Hewick, S. C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia and G. Trinchieri, *J. Exp. Med.* **170**, 827 (1989).
35. H. Fujiwara, S. C. Clark and T. Hamaoka, *Ann. N.Y. Acad. Sci.* **795**, 294 (1996).