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Quick method of multimeric protein production for biologically active substances such as human GM-CSF (hGM-CSF)

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ABSTRACT

The C-terminal fragment of C4b-binding protein (C4BP)-based multimerizing system was applied to hGM-CSF to induce dendritic cells (DCs) from peripheral blood monocytes (PBMCs), to see whether the C4BP could stimulate immature DCs, since DCs, equipped with pattern recognition receptors such as toll-like receptors (TLRs), are hypersensitive to various immunologically active molecules like LPS. *hGM-CSF* gene was merged to the 3′-terminal region of the *C4BPα-chain* gene, and the transfected human 293FT cells produced sufficient amount of octameric hGM-CSF, which resulted in iDCs with the same phenotype and the same response to a TRL4 ligand, LPS and a TLR3 ligand, poly I:C, as those induced with authentic monomeric hGM-CSF. These results suggest that the C4BP-based multimerizing system could facilitate the design of self-associating multimeric recombinant proteins without stimulating iDCs, which might be seen with the other multimerizing systems such as that using Fc fragment of IgM.

Introduction

Recombinant proteins are generally produced as single chain molecules. In contrast, natural biological ligand–receptor systems are often multimeric, assembling different molecules into complexes and bringing together different functions. Moreover, it is frequently observed that monomeric ligand–receptor interactions at the cell surface are not able to trigger signal transduction or cellular activation. There have only been a few attempts to produce recombinant multimeric molecules mimicking natural biological system, which were usually unsatisfactory mainly due to the undesirable effects of these multimerizing systems such as the immunoglobulin Fc fragment-based multimerizing system that interacts with cell surface receptors and activates complement [1,2]. There are also multimerizing systems based on leucine zippers [3], chemical polyethylene glycol linkage [4], diabodies [5], streptavidin [6], and protein A [7], which use intracellular or foreign proteins. Therefore, they are likely being immunogenic. Chemical linkages using polyethylene glycol are not stable enough *in vivo* and do not mimic natural biological complexes [4].

C4b-binding protein (C4BP) is a spider-like molecule [8,9] involved in the regulation of the complement cluster family and consists of short consensus repeat units (Fig. 1). Five to seven α-chains, that bind C4b and one β chain, which binds protein S, or eight α-chains only are covalently associated together at their C-terminal portion [10]. A multimerizing system was designed based on the C-terminal portion of the *C4BPα chain gene* [11], and the *hGM-CSF* gene was selected as an example of a multimeric recombinant protein to see whether iDCs, which are among the most sensitive cells to immunological stimulation, could be induced from peripheral blood mononuclear cells to confirm that the C4BPα-multimerizing system does not show immunostimulation.

Materials and methods

Cells and medium. 293FT cells (Invitrogen) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich) supplemented with 10% FCS (Moregate, Queensland, Australia), penicillin (50 U/ml), and streptomycin (50 U/ml) (Invitrogen). HepG2 cells were obtained from ATCC (Manassas, VA, USA).

Antibodies for cell staining. The mouse mAbs HI149 (anti-human CD1a), M-T101 (anti-human CD1b), FITC conjugated mouse anti-human mAbs, G46-2.6 (anti-HLA-abc) and G46-6 (anti-HLA-DR), and phycoerythrin (PE)-conjugated mouse mAb HB15e (anti-CD83) and IT2.2 (anti-CD86) were all purchased from BD Pharmingen (San Diego, CA, USA). PE-conjugated goat F(ab′)$_2$ antibody to mouse IgG (IM0855) was from Beckman Coulter (Fullerton, CA, USA).

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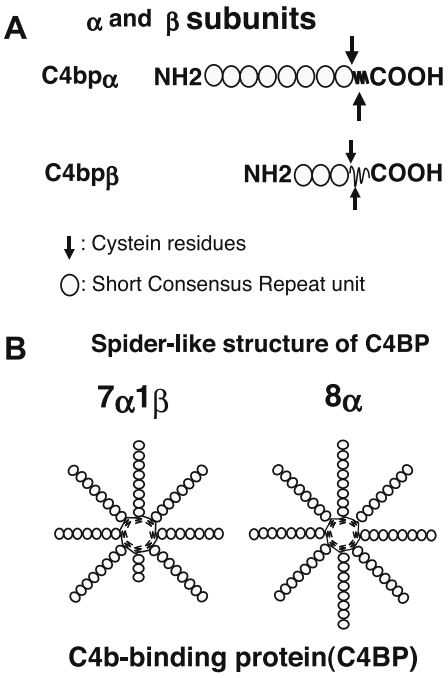

Fig. 1. Spider-like structure of the C4b-binding protein (C4BP). (A) There are two subunits of C4BP: C4BPα consists of eight short consensus repeat (SCR) units and a C-terminal sequence. C4BPβ consists of three SCR units and a C-terminal sequence. (B) C4BP is a high molecular mass (570 kDa) glycoprotein in plasma. The major form is usually composed of seven C4Bpα subunits and one C4BPβ subunit (7α1β) or eight C4Bpα subunits (8α) linked by disulfide bridges. In electron microscope-derived images, C4BP appears as a spider-like structure [9].

DNA constructs. The human *GM-CSF* gene was amplified from the plasmid pCM-hGM (RIKEN DNA Bank, Japan) [12] by PCR using the following primers:

5′-CGCGGATCCATGTGGCTGCAGAGCCTGCT-3′
5′-GGAATTCCTCCTGGACTGGCTCCCAGC-3′

Amplified DNA was digested with BamHI and EcoRI. The digested DNA was subcloned between BamHI and EcoRI sites of plasmid pEF-1a/Myc/6× His/A (Invitrogen) and designated as hGM-CSF/EF1/Myc/His.

Total RNA was extracted from human HepG2 cells using an RNeasy kit (Qiagen, Hilden Germany) and the C-terminal region of the human *C4BPα* gene was cloned by RT-PCR methods using the following primers:

A-1: 5′-CGGAATTCTTCGGGACAGGTCCTGC-3′
A-2: 5′-GCTCTAGATAGTTCTTTATCCAAAG-3′

The amplified C-terminal region of the human *C4BPα* gene was digested with EcoRI and XbaI, subcloned 3′ to the *hGM-CSF* gene in hGM-CSF/EF1/Myc/His between the EcoRI and XbaI sites, and designated as hGM-CSF-C4BPαEF1/Myc/His.

Production of recombinant multimeric GM-CSF. Transfection of 293FT cells with hGM-CSF/EF1/Myc/His or hGM-CSF-C4BPα/EF1/Myc/His was performed using polyethyleneimine as described previously [13,14], and G418-resistant clones were selected. The conditioned culture media of the selected clones were collected, sterilized with a 0.2 μm filter, and quantified using hGM-CSF ELISA kit (R&D Systems, Minneapolis, MN, USA) for the further experiments. As a reference, recombinant hGM-CSF purchased from Peprotech EC (London, Great Britain) was used (see Fig. 2).

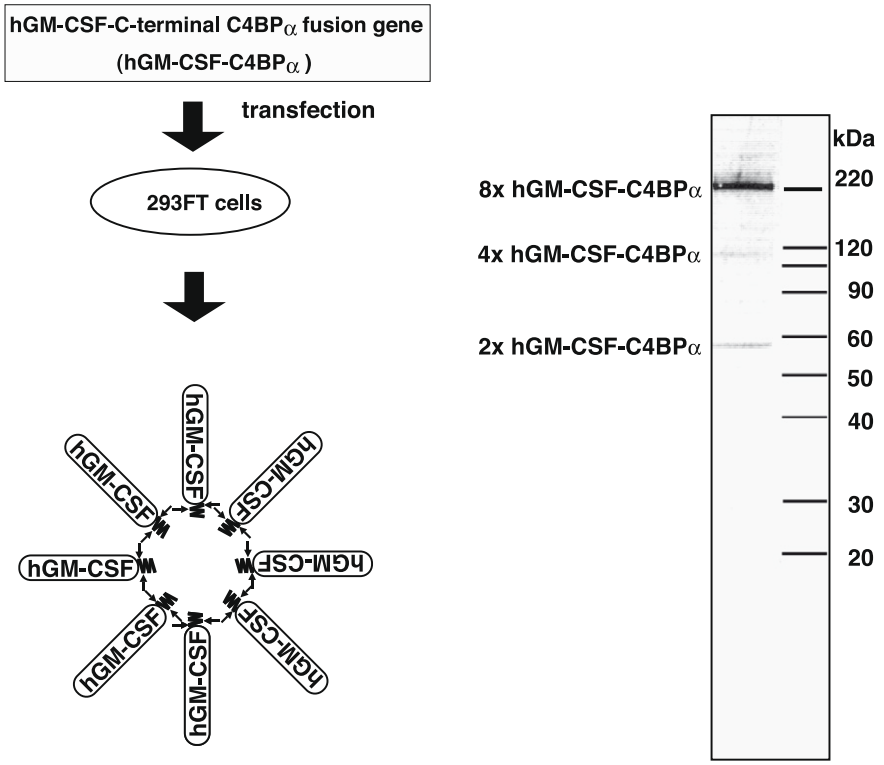

Fig. 2. Production of recombinant octameric hGM-CSF. The hGM-CSF-C4BPα fusion gene was transfected into 293T cells. An immunoblot analysis with anti-myc-epitope antibody revealed a molecular mass of 224 kDa, indicating an octamer of hGM-CSF, as well as small amounts of 112 kDa (tetramer) and 56 kDa (dimer) proteins.

PBMC-derived immature DCs. Immature DCs were obtained from PBMCs as described previously [13]. Briefly, PBMCs were freshly isolated with Ficoll-paque (Amersham-Pharmacia, Uppsala, Sweden) from the peripheral blood of healthy volunteers, and $CD14^+$ monocytes were separated immediately by magnetic depletion using a monocyte isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) containing hapten-conjugated antibodies to CD3, CD7, CD19, CD45RA, CD56, and anti-IgE Abs, and a magnetic cell separator (MACS, Miltenyi Biotec) in accordance with the manufacturer's instructions, routinely resulting in >90% purity of $CD14^+$ cells. The cells were cultured in 24-well culture plates for 6–7 days in complete medium supplemented with 20 ng/ml IL-4 (Biosource Intl., Camarillo, CA, USA) and 50 ng/ml of hGM-CSF obtained from PeproTech EC, or from the conditioned culture medium of 293FT cells transfected with *hGM-CSF* gene or *hGM-CSF-C4BPα* gene, in order to obtain iDCs. After 4–6 days of incubation, fluorescent activated cell sorter (FACS) analysis was performed to analyze the phenotype of the cells.

Immunoblotting. The cells were lysed in triple-detergent lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.1% SDS, 100 g/ml phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1% Nonidet P-40, and 0.5% sodium deoxycholate). The obtained samples were run on a 4–12% NuPAGE Bis–Tris gel (Invitrogen) using MES (morpholine ethanesulfonic acid), SDS (sodium dodecyl sulfate) buffer (1000 mM MES, 1000 mM Tris, 70 mM SDS, 20 mM EDTA) under non-reducing conditions and transferred to a PVDF (polyvinylidene difluoride) membrane (ATTO, Tokyo, Japan). The membrane was incubated with rabbit anti-myc serum (Invitrogen) and immunoblotting was carried out using horseradish peroxidase conjugated-goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) and a 3,3′,5,5′-tetramethylbenzidine (TMB) substrate kit for peroxidase (VECTOR Laboratory, Burlingame, CA).

Electron microscopy. The conditioned culture medium containing hGM-CSF-C4BPα gene-derived multimeric hGM-CSF was dialyzed against 0.1 M NH_4OAc/0.05 M NH_4HCO_3, pH 7.35, were adsorbed to thin carbon films and were negatively stained with 4.0% uranyl acetate. The photographs were taken at a primary magnification of 40,000 in a Hitachi H-7500 transmission electron microscope, operating at 80 kV.

Results

hGM-CSF produced by 293FT cells could induce iDCs

The first question was whether 293FT cells could produce enough amount of hGM-CSF to prepare iDCs from PBMCs. Human embryonic 293FT cells were transfected with the *hGM-CSF* gene and the hGM-CSF concentration was analyzed by ELISA. As a result, the hGM-CSF concentration in the conditioned medium was always high enough for dendritic cell preparation; between 150 and 200 ng/ml after transient transfection, or with cloned 293FT cells producing hGM-CSF.

The second question was whether the conditioned medium of the *hGM-CSF*-transfected 293FT cells could induce iDCs together with IL-4, because dendritic cells are equipped with pattern recognition receptors such as toll-like receptors that enable DCs to respond to very scarce amounts of stimulants such as LPS or nucleotides. Flow cytometric analysis of DCs prepared with the conditioned culture medium of *hGM-CSF*-transfected 293FT and commercial IL-4 showed the typical phenotype of iDCs derived from PBMCs, suggesting that the conditioned medium of 293FT did not contain any stimulants that induced the maturation of DCs (Fig. 3, right panels).

Production of multimeric hGM-CSF

Next, it was attempted to produce multimeric hGM-CSF using the *hGM-CSF-C4BPα* gene to see whether it could induce iDCs from PBMCs as well as authentic monomeric hGM-CSF. A chimeric gene of *hGM-CSF* fused to *C4BPα* was transfected into 293FT cells and the conditioned culture medium was analyzed by ELISA for hGM-CSF. Immunoblotting was also performed to analyze the *hGM-CSF-C4BPα* chimeric gene product under non-reducing conditions, which was observed as a band with a molecular mass of 220 kDa as well as faint bands with molecular masses of 56 and 112 kDa (Fig. 3). The predicted molecular weight of the monomeric *hGM-CSF-C4BPα* gene product is 28 kDa (Table 1), indicating that the major product of the *hGM-CSF-C4BPα* gene was an octameric protein, but dimer and tetramer proteins were also produced.

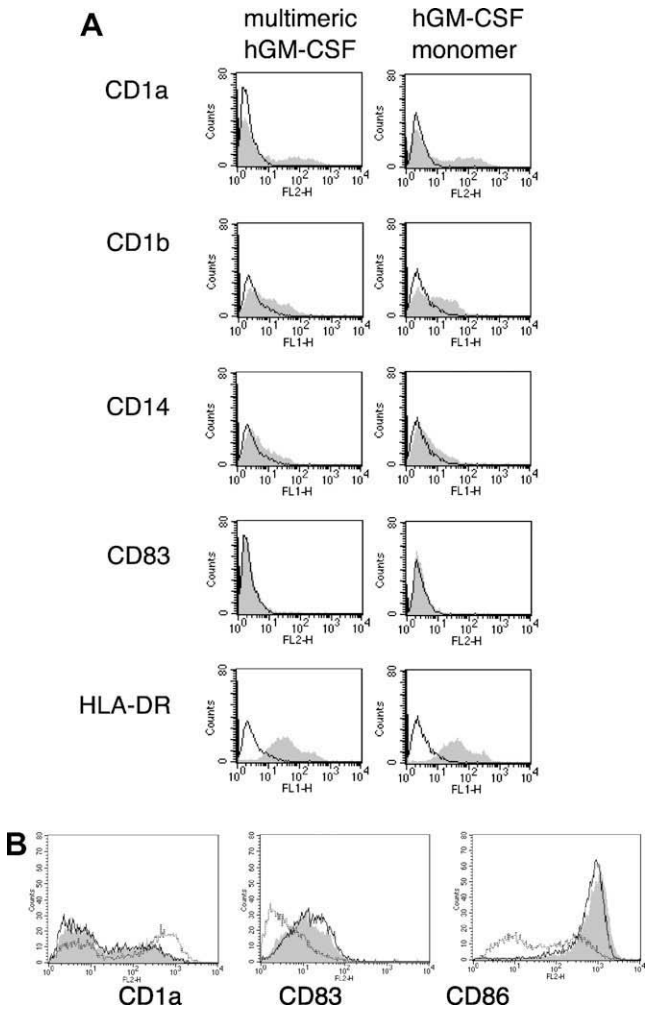

Fig. 3. FACS analysis of the immature dendritic cells. (A) The hGM-CSF gene or the hGM-CSF-C4BPα fusion gene was transfected into 293FT cells and conditioned medium containing hGM-CSF or multimeric GM-CSF was used in combination with IL-4 to induce dendritic cells from peripheral blood monocytes. The resultant DCs either with hGM-CSF (right panels) or multimeric GM-CSF (left panels) showed an identical phenotype each other. The results from the hGM-CSF gene and the hGM-CSF-C4BPα fusion gene are shown as grey lines and negative control data are shown as black lines. (B) The immature DCs induced with multimeric hGM-CSF showed the same response to LPS and polyI:C as that of the iDCs induced with hGM-CSF. Multimeric hGM-CSF-induced iDCs were incubated with LPS (200 ng/ml) (solid line) or poly I:C (100 μg/ml) (filled line) for 48 h and FACS analysis was performed. The iDCs without LPS or poly I:C is shown as dotted line.

Visualization of multimeric hGM-CSF

The purified multimeric hGM-CSF was examined in the electron microscope and a typical field view is shown in Fig. 4A. Several hGM-CSF-C4BPα molecules were examined and the molecules

Table 1
Characteristics of hGM-CSF-C4Bpα monomer with myc and 6× histidine tag.

	Value*
Length	245 amino acids
Molecular weight	27961.0 Da
Isoelectric point	5.07
Net charge at pH 7	−12.3

* The values were calculated using Gene Inspector 1.6 software (Textco BioSoftware, West Lebanon, NH, USA).

exhibited a morphology that resembles the aggregation of globular molecules (Fig. 4B). Human GM-CSF is a flattened globular molecule that is about 4.0 and 2.4 nm thick [15], the size of which corresponds with the electron micrographs of multimeric hGM-CSF.

Multimeric hGM-CSF produced by 293FT cells could induce iDCs

Finally, the induction of iDCs from PBMC using recombinant IL4 and multimeric hGM-CSF was attempted. The conditioned culture medium of 293FT cells transfected with *hGM-CSF-C4BPα* gene and recombinant IL4 were used to make iDCs from PBMC. FACS analysis showed the typical phenotype of iDCs (Fig. 3, left panels). The obtained iDCs were also incubated with either LPS (200 ng/ml for 48 h) or poly I:C (100 μg/ml for 48 h) and the typical phenotypic responses of iDCs to LPS and poly I:C were observed, such as the down-regulation of CD1a surface expression and up-regulation of CD83 and CD86 surface expression (Fig. 3B).

Discussion

For the protein expression, there are several methods such as those using *Escherichia coli*, insect cells, or mammalian cells, but each has their own advantages and disadvantages [16]. First, *E. coli* expression techniques are probably the most popular. The techniques necessary to express sufficient amounts of protein are relatively simple and the amount of time necessary to generate an over-expressing strain is very short. Therefore, *E. coli* is widely used for the expression of commercially important proteins. However, protein expression in *E. coli* does have some disadvantages. Recombinant eukaryotic proteins produced in *E. coli* are not properly modified and often precipitate into insoluble aggregates called "inclusion bodies." The recombinant protein could only be recovered in an active form by solubilization in denaturing agents followed by careful renaturation. Moreover, it is relatively difficult

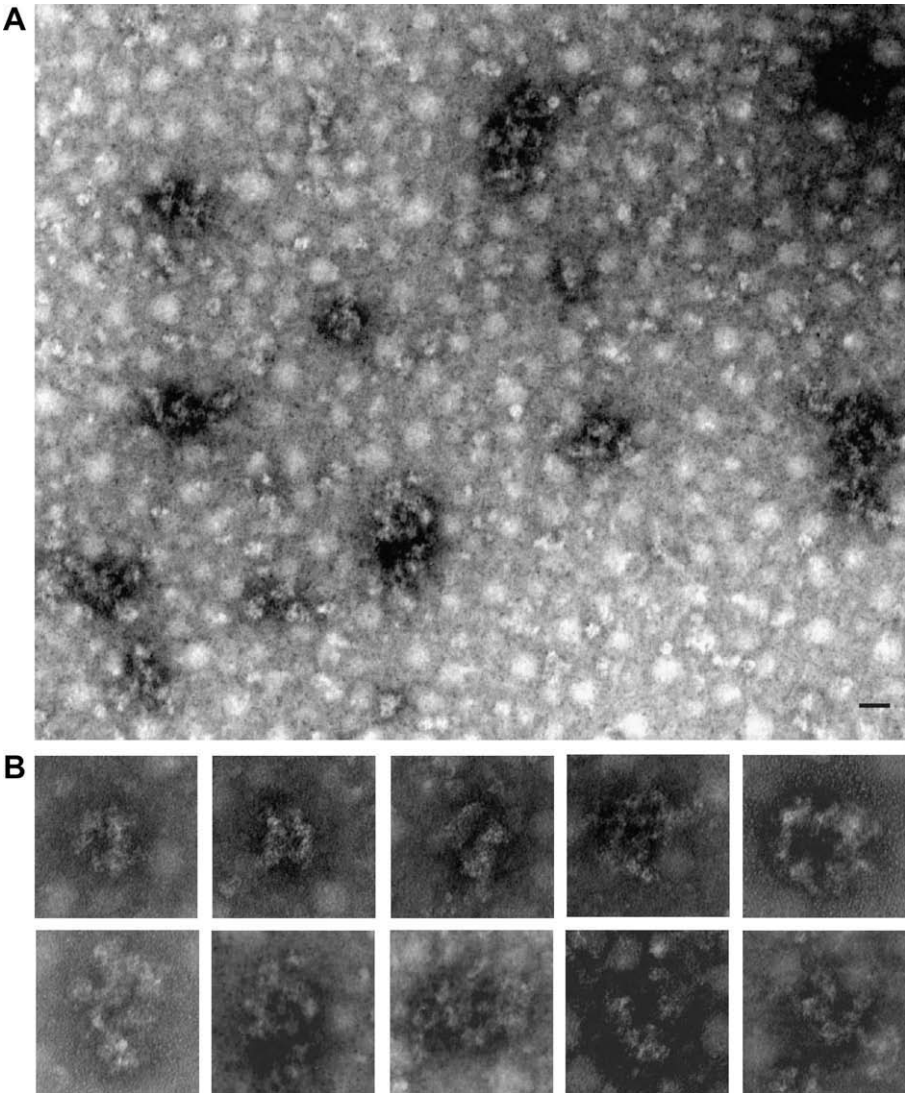

Fig. 4. Electron micrographs of multimeric hGM-CSF. (A) Field view of multimeric hGM-CSF. (B) Selected images demonstrating the aggregation of globular molecules of hGM-CSF. The scale bar in (A) represents 60 nm.

to arrange the secretion of the expressed proteins from *E. coli* if the amount is large. In addition, if the desired recombinant protein was hGM-CSF for DC preparation, the hGM-CSF produced should absolutely be without any trace of LPS, but it is very difficult to obtain recombinant proteins expressed in *E. coli* that are free of LPS. The baculoviral expression system using insect cells also has a number of advantages. With this system, proteins can be expressed at high levels and usually in the proper cellular compartment. For example, membrane proteins are usually localized to the membrane and nuclear proteins to the nucleus in insect cells as well as in mammalian cells, although the proteins expressed in insect cells are not always properly modified. Compared to the above two systems, mammalian expression techniques have certain advantages, especially for the expression of higher eukaryotic proteins. The expressed proteins are usually properly modified and they accumulate in the correct cellular compartment, but it is difficult to perform large-scale expression experiments. To obtain large amounts of recombinant protein using eukaryotic cells, CHO cells are often used because of the high amounts of the desired protein that can be obtained via dihydrofolate reductase (DHFR) [17] based amplification of recombinant genes using increasing concentrations of methotrexate (MTX) [18]. However, the DHFR amplification process is lengthy and may require several months to isolate and characterize a stable, amplified line of CHO cells. Moreover, even after the long amplification period, the amount of product is not always enough for some experimental uses.

This study utilized 293FT cells instead of CHO cells, which were transfected with the *hGM-CSF* gene to see if they could produce sufficient amounts of hGM-CSF to induce iDCs in combination with IL-4 in a laboratory setting. All the procedures took no more than one month from the cloning of the gene, which included subcloning into the vector plasmid, transfection into 293FT cells, production of sufficient amounts of hGM-CSF, and analysis of the quality of the hGM-CSF obtained for the preparation the iDCs. The resultant iDCs showed the same phenotype as iDCs incubated with commercial hGM-CSF and IL-4 as well as the same response to a TLR-4 ligand, LPS, and to a TLR-3 ligand, poly I:C.

Recently, many biologically active substances, such as cytokines and chemokines, have been identified, some of which are often needed for use in further experiments immediately. Therefore, there is a clear need for methods to obtain newly identified biologically substances immediately and without difficulty. The eukaryotic expression system using 293FT cells is a promising candidate because this system can obtain biologically active substances of good quality within a few weeks, and the amount of these substances is usually enough for further experiments because these substances have very high biological activity.

Furthermore, using a C-terminal fragment of the *C4BPα* gene fused to the 3′ end of the *hGM-CSF* gene, the recombinant multimeric hGM-CSF was obtained, which was successfully secreted into the culture medium of the cells despite of its high M_W of 200 kDa, and it was shown that this recombinant hGM-CSF could be used for iDCs preparation from PBMCs, whose phenotype was exactly the same as those iDCs incubated with authentic monomeric hGM-CSF and IL-4. These iDCs, prepared with multimeric hGM-CSF and IL-4, responded to LPS and poly I:C in exactly the same manner as those prepared with authentic monomeric hGM-CSF, which supported the notion that the C4BPα-multimerizing system can be used without the potential risk of immunological stimulation, because iDCs are among the cells that are most sensitive to immunological stimulation. Furthermore, it took no more than one month to obtain multimeric hGM-CSF with the C4BPα-multimerizing system using 293FT cells, which was enough to obtain iDCs from PBMCs.

In conclusion, the present C4BP-based multimerizing system combined with 293FT cells was shown to be the promising quick method to produce sufficient amounts of recombinant multimeric protein with biological activity without any untoward stimulation of the immune system.

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