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Full Length Research Paper

Heterologous expression of biologically active chicken granulocyte-macrophage colony stimulating factor in *Pichia pastoris*

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Granulocyte-macrophage colony stimulating factor (GM-CSF) is an effective nucleic acid adjuvant, which can enhance the body immunity, especially in the immune response to the DNA vaccine. In this study, we investigated the function of recombinant chicken GM-CSF (rchGM-CSF). Chicken GM-CSF (chGM-CSF) gene sequence was cloned, the signal peptide in the N terminal sequence was cut, and 3' end of the stop codon was changed. The rchGM-CSF was labeled with a C-terminal c-myc and His antigen. The recombinant *Pichia pastoris* expression vector pPICZαA-rchGM-CSF was constructed by inserting the reformed cytokines genes in pPICZαA. The pPICZαA-rchGM-CSF was expressed in GS115 cells. After being screened by yeast peptone dextrose (YPD) containing high concentrations of Zeocin and direct PCR, the positive clone was cultured in flask with buffered minimal methanol (BMMY) and expression induced by methanol. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis suggested that rchGM-CSF was expressed and secreted into BMMY and the secreted peak was generally at 72 h in BMMY medium. After large-scale induced expression, the supernatant was collected by centrifuge, concentrated by centrifugal ultrafiltration and purified by His Bind Ni-NTA chromatography. The MTT (3-(4, 5-dimethylthiazolyl-2) -2, 5-diphenyltetrazoliumbromide) assay results showed that rchGM-CSF could stimulate lymphocyte proliferation. The experiment laid the foundation for further study of immune adjuvant.

Key words: Granulocyte-macrophage colony stimulating factor (GM-CSF), *Pichia pastoris*, secretory expression, biological activity.

INTRODUCTION

Granulocyte-macrophage colony stimulating factor (GM-CSF) plays an important role in immune response. As a crucial immune cytokine, the GM-CSF can stimulate leucocyte to express major histocompatibility complex (MHC), promote mature and migration of dendritic cells and macrophages (Papatriantafyllou, 2011). Based on the network system of hematopoietic cells factor, the GM-CSF can regulate the body immune system function in the whole, activate the dendritic cells and macrophages in regional application, stimulate antigen presenting cells (APCs) activation in local application, enhance immunity activity by fusion protein and activate antigen expression cells *in vitro*. GM-CSF can enhance dendritic cells' ability of antigen presentation and the creation of IL-2, activating CD4+ T cells to enhance the ability of secreting antibody and also enhance the function of CD8+ T cells (Papatriantafyllou, 2011; Tovey and Lallemand, 2010). GM-CSF also is a key regulator of IL-1beta production. Furthermore, It was reported that GM-CSF play a key role in the activation of Th1 and Th17 cells(McGeachy, 2011; Wei et al., 2011).

Most of the reports on yeast-expressed GM-CSF focus on humans and mice (Shim et al., 2011; Williams and

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Galipeau, 2011). The recombinant human GM-CSF as an important therapeutic intervention has been used in clinic (Gupta and Emens, 2010; Papatriantafyllou, 2011; Waterer, 2011). GM-CSF confers resistance to influenza by enhancing innate immune mechanisms that depend on alveolar macrophages. Pulmonary delivery of this cytokine has the potential to reduce the morbidity and mortality due to influenza virus (Huang et al., 2011; Sever-Chroneos et al., 2011). GM-CSF also increases mucosal and systemic immunogenicity of an H1N1 influenza DNA vaccine administered into the epidermis of non-human primates (Loudon et al., 2010). There were few reports about recombinant chicken GM-CSF (rchGM-CSF) function (Kaiser et al., 2005). RchGM-CSF was reported to improve the protective effect of subunit nucleic acid vaccine of the chicken infectious bronchial virus. (Tan et al., 2009). The purpose of this study was to express rchGM-CSF in *Pichia pastoris* yeast and investigate the biological activity.

MATERIALS AND METHODS

Construction of the vector

According to the gene sequence of the chGM-CSF, which was cloned in our lab, the signal peptide of the sequence was predicted by the SignalP3.0 database. A pair of primers were designed according to the coding sequence (CDS) coding region of which signal peptide was removed. The forward and reverse primers were 5'- CCGAATTCTACTCCTGCTGCTACAAAGT -3' and 5'-GCGGCCGCTTGGATGCAGTCTTTCTC -3' containing EcoRI and NotI site. The DNA (383 bp) fragment coding for chGM-CSF was amplified by RT-PCR from the total RNA that was previously isolated from chicken cecal tonsil. The PCR product was inserted into the expression vector pPICZaA (Invitrogen, USA). The recombinant plasmid pPICZaA-rchGM-CSF was confirmed by restriction analysis and sequencing.

Transformation and expression of rchGM-CSF

The recombinant pPICZaA-chGM-CSF was transformed into *P* Gs115 by electroporation according to the method as described in the *P. pastoris* expression manual (Invitrogen, USA). The transformed strain was analyzed by colony PCR to verify the integration of the recombinant gene. Positive transformants were tested for their ability to secrete rchGM-CSF into the cell culture supernatants as follows: Cells were inoculated into 5 ml of buffered minimal glycerol (BMGY) medium and allowed to grow for 24 h, 1% (v/v) methanol was added to the culture at intervals of 12 h during an incubation period of 120 h. The supernatants were collected and analyzed by 12% SDS-PAGE.

Expression, purification and of western blot analysis of rchGM-CSF

Positive transformants were picked up from the plates and inoculated into a flask containing 10 ml BMGY medium. After culturing at 30°C, 200 rpm for 36 h, the culture was centrifuged at 4°C, 3,000 rpm for 5 min. The collected cells were cultured in 30 ml buffered minimal methanol (BMMY) at 30°C for 96 h again, and the methanol was added every 24 h. To obtain the highest yield of protein, different culture parameters including pH and concentration of methanol added daily were evaluated. Scale-up expression was performed under the optimized cultivation conditions. The culture was centrifuged at 5,000 rpm for 15 min and the supernatant was concentrated by ultrafiltration. The concentrated sample was loaded onto chromatography column (Novagene Ltd., USA) and eluted with linear gradient of 0 to 2.0 mol/L NaCl in phosphate buffered saline (PBS). The eluted fractions were analyzed by 12% SDS-PAGE. Protein samples were separated by 12% SDS-PAGE and electroblotted onto a millpore polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, USA). The membrane was blocked with 5% (w/v) milk in 10 mM Tris-HCl with 150 mM NaCl (pH 8.0) and 0.1% (v/v) Tween-20 (TBST) for 2 h at room temperature (RT). The membrane was then incubated for 2 h at RT with anti-His monoclonal antibody (Novagene Ltd., USA). After washing three times, the membrane was incubated for 1 h with peroxidase-conjugated goat anti-mouse IgG (Novagene Ltd., USA). The membrane was washed with TBST and the specific protein bands were visualized by enhanced luminol-based chemiluminescent (ECL) detection kit (Amersham Pharmacia Biotech, Canada).

MTT proliferation assay

The growth-promoting effects of rchGM-CSF on chicken lymphocytes (CHLC) were determined using 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazoliumbromide (MTT) cell proliferation assay. CHLC were cultured in RPMI1640 (Gibco, USA) with 10% (v/v) fetal bovine serum (FBS) and kept in an incubator with 5% CO_2 at 37°C. Cells were adjusted to a concentration of 1×10^5 cells/ml and seeded in 96-well flat-bottom plates. Then, different concentrations (5^{-1} to 5^{-4}) of rchGM-CSF was added to each well and cultured for 48 h at 37°C. 10 µl of 0.5 mg/ml MTT (Sigma, USA) were added to each well and incubated at 37°C for 4 h. The formazan crystals in viable cells were then dissolved in 100 µl/well of dimethyl sulfoxide (DMSO, Sigma, USA). The absorbance of the color in each well was quantified as absorbance units (AU) at 450 nm wavelength using a Bio-Rad model 550 microplate reader (Bio-Rad Molecular Bioscience Group, USA). All data were analyzed using SPSS 12.0 for windows. All values are expressed as means ± standard error (SE). The difference was considered significant if $p<0.05$.

RESULTS

Transformation and selection of rchGM-CSF expression strain

The predicted sequence of the signal peptide by SignalP3.0 is 72 bp of nucleotides at the 5' end. In redesigning the primer, the chGM-CSF sequence's length was 383 bp (Figure 1). The expression vector pPICZαA-chGM-CSF was constructed. The recombinant expression vector and empty vector were transformated into competence GS115 yeast respectively. The yeasts were inoculated in yeast peptone dextrose (YPD) (including 100 µg/ml Zeocin).

The positive clones were selected and cultured in BMGY and induced expression of rchGM-CSF. The supernatant of BMMY culture medium were collected each at 24, 48, 72, and 96 h. SDS-PAGE analysis and silver staining coloration were done after the trichloroacetic acid (TCA) precipitation. There was a strap

GAATTCTACTCCTGCTGCTACAAAGTGTACACCATCCTGGAAGAAATAACGAGTC
ACTTGGAGAGCACAGCGGCCACAGCAGGTCTGTCCTCGGTACCCATGGACATC
AGGGATAAAACCTGTCTGCGTAACAACCTGAAAACATTCATAGAGTCCTTGAAAA
CAAATGGGACAGAGGAAGAAAGCGGAATCGCCTTTCAGCTGAACAGAGTTCAC
GAGTGTGAACGCCTCTTCTCGAACATAACTCCCACCCCCCAGGTTCCTGATAAG
GAATGTAGAACTGCACAAGTATCGAGGGAAAAATTCAAAGAGGCATTAAAAACTT
TCTTTATTTACCTCTCTGATGTGCTCCCAGAGGAGAAAGACTGCATCCAAGCGG
CCGC

Figure 1. chGM-CSF sequence. The underlined sequences are the upstream and downstream sequence primers. chGM-CSF, Chicken granulocyte-macrophage colony stimulating factor.

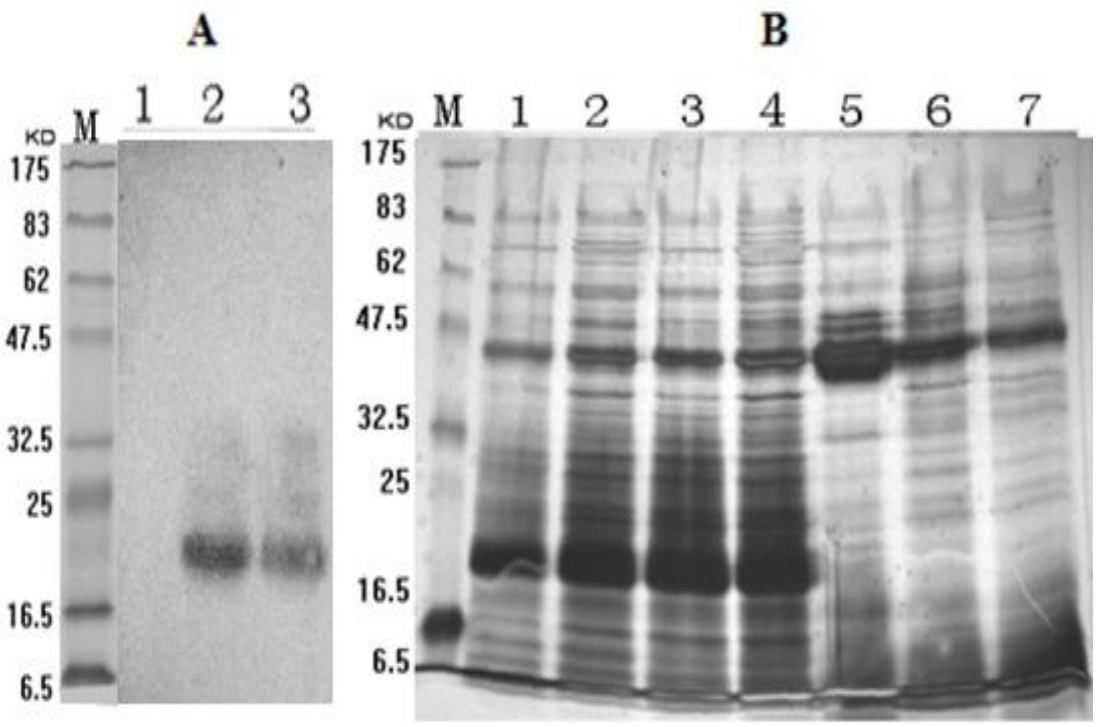

Figure 2. Identification of culture medium supernatant. A, Western blot analysis; M, marker; 1, the 36th hour BMGY; 2 and 3, the third day BMMY culture medium supernatant. B:M, marker, 1-4:1-4 days BMMY supernatant; 5, the 36th hour BMGY; 6, empty vector transformation GS115 BMMY supernatant; 7, the untransformational GS115 supernatant. BMMY, Buffered minimal methanol; BMGY, buffered minimal glycerol.

at the position of 20 kDa (Figure 2). It is illustrated that the rchGM-CSF was secreted by the positive yeast induced by methanol. Grey scale analysis indicated the expression was at the peak (at 72 h), but there was no obvious difference compared with the other time. Western blot analysis showed the specific immune reaction at 20 kDa (Figure 2). The results of the SDS-PAGE were concordant to western blot. The expression dose of the rchGM-CSF was 10 mg/L in culture.

Purification of the rchGM-CSF

The BMMY culture supernatant was collected by centrifugation and condensed into 5 ml after superfiltration and salt elimination. The rchGM-CSF was subsequently

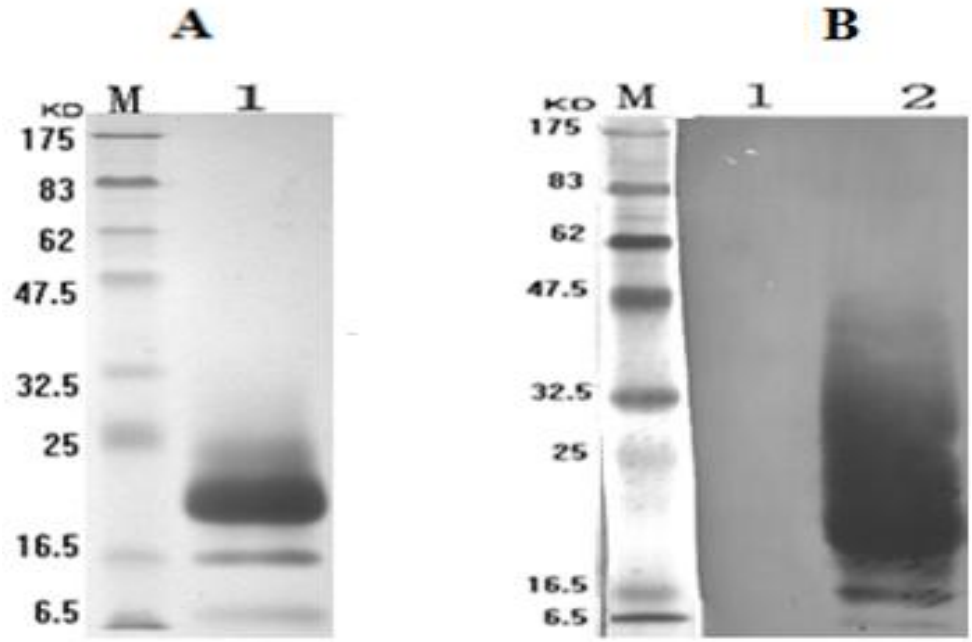

Figure 3. Analysis of purified rchGM-CSF. A, The result of SDS-PAGE; M, marker; 1, the second column long eluent. B, The result of western blot; M, marker; 1, eluent passed no column; 2, the second column long eluent. rchGM-CSF, Recombinant chicken granulocyte-macrophage colony stimulating factor.

purified. SDS-PAGE and western blot analysis indicated that the rchGM-CSF was purified (Figure 3).

Activity assay of rchGM-CSF

The biological activity of rchGM-CSF was detected by the MTT method. The rchGM-CSF was diluted with the 1640 culture medium containing 10% fetal bovine serum and taken into the 96 hole cell plate. The final concentration was 20 μg/ml. Five times multiproportion was diluted to four gradients. The results show that the 1:5 rchGM-CSF dilution could stimulate the lymphocytic proliferation conspicuously. It was indicated that the purified rchGM-CSF had good biological activity (Figure 4).

DISCUSSION

GM-CSF is known to stimulate macrophage differentiation and proliferation, and to activate APCs. GM-CSF also is an attractive adjuvant for a DNA vaccine on account of its ability to recruit APCs to the site of antigen synthesis, as well as its ability to stimulate the maturation of dendritic cells (DCs). As a DNA vaccine immune adjuvant, the GM-CSF is used to control the specific immune response (Gupta and Emens, 2010). There are lots of reports which indicate that the GM-CSF can enhance the DNA vaccine immune effect. It was reported that GM-CSF gene adjuvant enhanced the immunogenicity of DNA replicon vaccine of *Clostridium botulinum* neurotoxin serotype A (Li et al., 2011). Consistently, GM-CSF co-expressing DNA modified vaccinia Ankara simian immunodeficiency virus vaccine and prevented infection (Lai et al., 2011). In addition, GM-CSF increased mucosal and systemic immunogenicity of an H1N1 influenza DNA vaccine administered into the epidermis of non-human primates (Loudon et al., 2010). Furthermore, GM-CSF and IL-2 as adjuvant, enhance the immune effect of protein vaccine against foot-and-mouth disease (Zhang et al., 2011).

In this study, we choose MTT assay to determine the biological activity of the rchGM-CSF being purified. The MTT assay is applied to assess the viability and the proliferation of lymphocyte. It can also be used to determine the biological activity of cytokines, since those cytokines would stimulate or inhibit lymphocyte viability and growth (Bernas and Dobrucki, 2002). Among different dilution of rchGM-CSF, the 1:5 dilution was most effective and economic. In this dilution, proliferation of lymphocyte is more active conspicuously than other dilution. It was also indicated that the purified rchGM-CSF had good biological activity. It can be use as an immune adjuvant for DNA vaccine in clinic in the future.

The GM-CSF of humans or rats expressed by the *P. pastoris* was glycosylated differently, so the molecular weight is larger than prediction of weight. It was predicted that the recombinant chGM-CSF has potential glycosyla-

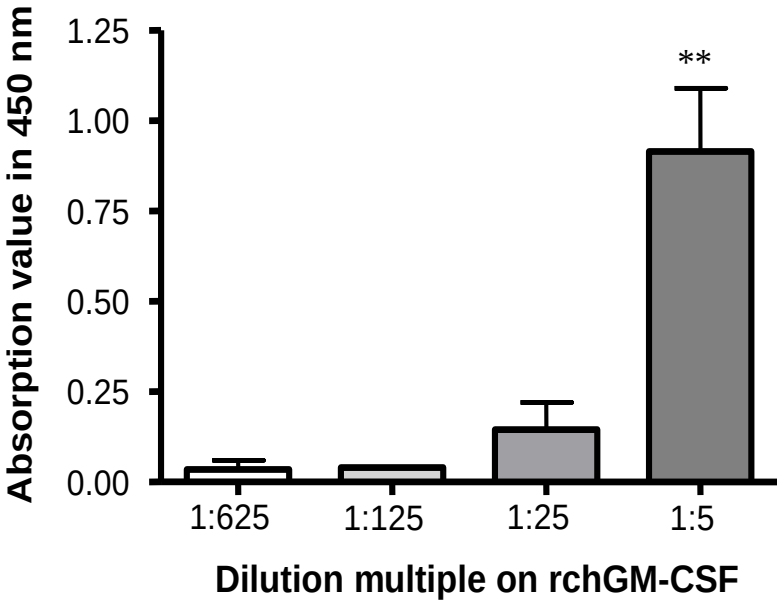

Figure 4. The lymphocyte proliferation assay. Stimulation of cell growth in cultured chicken lymphocytes (CHLC) by rchGM-CSF. CHLC (1×10^5 cells/ml) were seeded in 96-well plates, followed by incubation in different concentrations (5^{-1} – 5^{-4}) of rchGM-CSF. The number of viable cells in cultures was measured by MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazoliumbromide) cell proliferation assay. Asterisks indicate P values of less than 0.05 (*) and less than 0.01 (**). rchGM-CSF, Recombinant chicken granulocyte-macrophage colony stimulating factor.

tion site. The results of SDS-PAGE and western blot show that the practical location of the strap was larger than prediction of theory about 3 to 5 kDa (Bhatacharya et al., 2007; Srinivasa Babu et al., 2008). Our results show that most rchGM-CSF was glycosylated, the molecular weight increased, but small amount of protein was not glycosylated. The two purified main straps have specific immune reactions with the anti-His antibody. The molecular weight of the small one is 17 kDa according to the theory (Figure 3). In the supernatant analysis of small scale induction expression, there was no 17 kDa strap in the result of western blot. The possible reason was that the dose of the protein, which was glycosylated, was too low to be detected by western blot. After purification, the concentration of the protein increased, so it can be detected.

In conclusion, we expressed the rchGM-CSF in *P. pastoris* and proved its determinate biologic activity. The experiment laid the foundation for the further study of immune adjuvant.

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REFERENCES

Bernas T, Dobrucki J (2002). Mitochondrial and nonmitochondrial reduction of MTT: interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes. Cytometry, 47: 236-242.

Bhatacharya P, Pandey G, Mukherjee KJ (2007). Production and purification of recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF) from high cell density cultures of *Pichia pastoris*. Bioprocess Biosyst. Eng. 30: 305-312.

Gupta R, Emens LA (2010). GM-CSF-secreting vaccines for solid tumors: moving forward. Discov. Med. 10: 52-60.

Huang FF, Barnes PF, Feng Y, Donis R, Chroneos ZC, Idell S, Allen T, Perez DR, Whitsett JA, Dunussi-Joannopoulos K, Shams H (2011). GM-CSF in the lung protects against lethal influenza infection. Am. J. Respir. Crit. Care Med. 184: 259-268.

Kaiser P, Poh TY, Rothwell L, Avery S, Balu S, Pathania US, Hughes S, Goodchild M, Morrell S, Watson M, Bumstead N, Kaufman J, Young JR (2005). A genomic analysis of chicken cytokines and chemokines. J Interferon Cytokine Res. 25: 467-484.

Lai L, Kwa S, Kozlowski PA, Montefiori DC, Ferrari G, Johnson WE, Hirsch V, Villinger F, Chennareddi L, Earl PL, Moss B, Amara RR, Robinson HL (2011). Prevention of infection by a granulocyte-macrophage colony-stimulating factor co-expressing DNA/modified vaccinia Ankara simian immunodeficiency virus vaccine. J. Infect. Dis. 204: 164-173.

Li N, Yu YZ, Yu WY, Sun ZW (2011). Enhancement of the immunogenicity of DNA replicon vaccine of Clostridium botulinum neurotoxin serotype A by GM-CSF gene adjuvant. Immunopharmacol. Immunotoxicol. 33: 211-219.

Loudon PT, Yager EJ, Lynch DT, Narendran A, Stagnar C, Franchini AM, Fuller JT, White PA, Nyuandi J, Wiley CA, Murphey-Corb M, Fuller DH (2010). GM-CSF increases mucosal and systemic immunogenicity of an H1N1 influenza DNA vaccine administered into the epidermis of non-human primates. PLoS One, 5: e11021.

McGeachy MJ (2011). GM-CSF: the secret weapon in the T(H)17 arsenal. Nat. Immunol. 12: 521-522.
Papatriantafyllou M (2011). Cytokines: GM-CSF in focus. Nat. Rev. Immunol. 11: 370-371.
Sever-Chroneos Z, Murthy A, Davis J, Florence JM, Kurdowska A, Krupa A, Tichelaar JW, White MR, Hartshorn KL, Kobzik L, Whitsett JA, Chroneos ZC (2011). GM-CSF modulates pulmonary resistance to influenza A infection. Antiviral Res. 92(2): 319-328.
Shim DS, Schilter HC, Knott ML, Almeida RA, Short RP, Mackay CR, Dent LA, Sewell WA (2011). Protection against Nippostrongylus brasiliensis infection in mice is independent of GM-CSF. Immunol. Cell Biol. doi: 10.1038/icb.2011.69
Srinivasa Babu K, Antony A, Muthukumaran T, Meenakshisundaram S (2008). Construction of intein-mediated hGMCSF expression vector and its purification in Pichia pastoris. Protein Expr. Purif. 57: 201-205.
Tan B, Wang H, Shang L, Yang T (2009). Coadministration of chicken GM-CSF with a DNA vaccine expressing infectious bronchitis virus (IBV) S1 glycoprotein enhances the specific immune response and protects against IBV infection. Arch. Virol. 154: 1117-1124.
Tovey MG, Lallemand C (2010). Adjuvant activity of cytokines. Methods Mol. Biol. 626: 287-309.
Waterer G (2011). GM-CSF and influenza: will saving mice help humans? Am. J. Respir. Crit. Care Med. 184: 157-158.
Wei WC, Su YH, Chen SS, Sheu JH, Yang NS (2011). GM-CSF plays a key role in zymosan-stimulated human dendritic cells for activation of Th1 and Th17 cells. Cytokine, 55: 79-89.
Williams P, Galipeau J (2011). GM-CSF-based fusion cytokines as ligands for immune modulation. J. Immunol. 186: 5527-5532.
Zhang C, Wang B, Wang M (2011). GM-CSF and IL-2 as adjuvant enhance the immune effect of protein vaccine against foot-and-mouth disease. Virol. J. 8: p. 7.