

Effects of rhM-CSF expressed in silkworm on cytokine productions and membrane molecule expressions of human monocytes¹

Ji Xiao-Hui², SUN Lu-Hong³, QIN Jun-Chuan⁴, YAO Kun³, DING Ru-Ning³, LI Huan-Di³, ZHU De-Xi⁴

(Institute of Clinical Pharmacology, ³Department of Immunology, Nanjing Medical University, Nanjing 210029;

⁴Department of Biochemistry, Nanjing University, Nanjing 210008, China)

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ABSTRACT

AIM: To study the effects of recombinant human macrophage colony-stimulating factor (rhM-CSF) expressed in silkworm on cytokine productions and membrane molecule expressions of monocytes.

METHODS: The rhM-CSF was added to the human peripheral blood monocyte cultures and 3 d later, the culture supernatants and cells were collected, respectively. TNF- α , IL-6, IL-8, and IFN- α levels in the supernatants were detected by biological activity test or ELISA and expressions of CD11b, CD16, HLA I, and HLA II on the cellular surface were examined by the method of alkaline phosphatase anti-alkaline phosphatase complex (APAAP).

RESULTS: The rhM-CSF promoted TNF- α , IL-6, and IL-8 inductions of monocytes and increased the percentages of CD11b, CD16, HLA I, and HLA II molecule expression on monocytes.

CONCLUSION: The rhM-CSF plays a role in monocyte function up-regulation and has a certain practical value in immunological therapy.

INTRODUCTION

A recombinant human macrophage colony-stimulating factor (rhM-CSF) expressed in silkworm has been prepared^(1,2) and it has been observed to have an enhancing effect on hematopoietic recovery of irradiation-injured mice and raise monocyte and granulocyte counts, showing a good practical prospect in clinical therapies of leukocytopenic diseases⁽³⁾. In this paper, we demonstrate the

effect of the rhM-CSF expressed in silkworm on cytokine productions and membrane molecule expressions of monocytes to provide experimental evidence for the application of the rhM-CSF in immunological therapy.

MATERIALS AND METHODS

rhM-CSF The preparation of the rhM-CSF has been previously described^(1,2). In brief, human M-CSF cDNA containing native signal sequence and amino acid residues 1 - 149 of hM-CSF was inserted into BmNPV transfer vector pBE284 and a recombinant transfer vector pBE284-M-CSF was formed. BmN cell was cotransfected with wild type viral DNA and pBE284-M-CSF DNA. The recombinant baculovirus Bm284-M-CSF, which has a high expression activity of hM-CSF, was obtained by homologous recombination *in vivo*. The rhM-CSF was separated from hemolymph of silkworm larvae infected with recombinant virus Bm284-M-CSF and was purified. The specific activity was 1.2×10^9 CFU/g.

Monoclonal antibodies The monoclonal antibodies to CD11b, CD16, HLA I antigen, and HLA II antigen, respectively, were all from Hematology Institute of Chinese Academy of Medical Sciences (Tianjin, China).

Cytokine detecting kits Detecting kits of TNF- α , IL-6, and IL-8 were purchased from Genzyme Co (USA).

Other agents LPS and PMA were purchased from Sigma Co. rhIFN- α (1×10^9 U/L) and rhTNF- α (5×10^9 U/L), which were used in the experiment as positive controls, were provided by Shanghai Institute of Biological Products, Ministry of Public Health, China and Biochemistry Department, Nanjing University, respectively. rhINF- γ was provided by Immunology Department, the Second Military Medical University (Shanghai, China). Alkaline phosphatase anti-alkaline phosphatase complex (APAAP) kit was purchased from Neomarker Co (USA). Viruses of VSV and NDV-4F

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² Correspondence to Ji Xiao-Hui.

Phn 86-25-666-2874. Fax 86-25-650-8960.

E-mail immune@njmu.edu.cn

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strains were propagated by our laboratory. L929 and WISH cell-lines were cultured in RPMI-1640 medium containing 10 % fetal calf serum (FCS).

Peripheral blood monocytes After centrifugation with Ficoll-Hypaque lymphocyte separating medium peripheral blood mononuclear cells (PBMC) were separated from heparin-anticoagulated and Hanks' Sol-diluted peripheral blood of healthy adults. After washed thrice, the cells were adjusted to 1×10^6 /L with RPMI-1640 medium supplemented with 10 % FCS. The resuspended cells were seeded into 96-well plates, 200 μ L/well. After a 2-h adherence step at 37 °C, the wells were washed twice to remove any nonadherent cells. The adherent cells left in the wells were monocytes.

Inductions and detections of cytokines The monocytes were stimulated with rhM-CSF 1×10^6 U/L or 5×10^6 U/L for some days so as to induce cytokines. In IL-6 and IL-8 groups, rhM-CSF was the only stimulator. In TNF- α group, the cells were stimulated with rhM-CSF for 3 d and some of them were simultaneously treated with LPS 10 mg/L and PMA 20 mg/L for 2 d from the second day to the third day. In IFN group, the cells were also stimulated with rhM-CSF for 3 d and the next day till the beginning of rhM-CSF treatment, some wells were added with IFN- γ 1×10^6 U/L and then with NDV (1:1280) on the third day. The supernatants were harvested by centrifugation, at 600 \times g for 15 min for cytokine detection. The supernatants of cultured cells treated with IFN- γ were heated at 56 °C for 30 min in order to inactivate the activity of IFN- γ in case it disturbed IFN- γ detection. TNF- α , IL-6, and IL-8 were determined by double-antibodies sandwich ELISA and the assays were performed according to the kit protocol. TNF- α was simultaneously detected by cytotoxic assay with L929 cells and rhTNF- α was used as the standard. IFN- α activity was examined by the routine procedure of microcytopathy inhibition assay with WISH cells in 96-well plate and rhIFN- α was used as the standard.

Test for membrane molecules The monocytes stimulated with rhM-CSF 1×10^6 U/L or IFN- γ 1×10^6 U/L for some days were resuspended with the medium and then smeared. Using monoclonal antibodies to CD11b, CD16, HLA I, and HLA II molecules, the expressions of CD11b, CD16, HLA I, and HLA II were tested by the method of APAAP staining following the kit protocol. The stained smears were observed for positivity for every antigen expression of the cells by counting 200 cells.

Statistical analysis Data were presented as

$\bar{x} \pm s$. The statistical analysis was carried out using paired *t*-test. $P < 0.05$ was taken to be significant.

RESULTS

Effect of rhM-CSF on TNF- α production in monocytes rhM-CSF stimulation enhanced TNF- α production in human peripheral blood monocytes. The monocytes treated with rhM-CSF secreted more TNF- α when induced by LPS + PMA, indicating that there was a synergetic effect of M-CSF combined with LPS + PMA on inducing TNF- α . The effect of rhM-CSF was dose-dependent. Data showed in Tab 1.

Tab 1. rhM-CSF induced induction of TNF- α in monocytes. $\bar{x} \pm s$, $n = 6$ samples for each group. $^b P < 0.05$ vs the corresponding control (0).

10 ⁻⁶ \times rhM-CSF (U/L)	Treatment		TNF- α	
	LPS	PMA	Cytotoxicity (KU/L)	ELISA (ng/L)
0	-	-	4 \pm 2	160 \pm 42
1	-	-	10 \pm 5 ^b	239 \pm 43 ^b
5	-	-	13 \pm 4 ^b	259 \pm 54 ^b
0	+	+	41 \pm 9	423 \pm 65
1	+	+	145 \pm 33 ^b	691 \pm 89 ^b
5	+	+	212 \pm 51 ^b	1058 \pm 116 ^b

Effect of rhM-CSF on IL-6 production in monocytes One-, two-, three-, and four-day after rhM-CSF stimulation, the concentration of IL-6 (ng/L) increased by 63.4 %, 39.5 %, 60.9 %, and 98.8 %, respectively in the supernatants of PBMC stimulated with rhM-CSF 1×10^6 U/L, and by 69.4 %, 49.1 %, 96.2 %, and 199.4 % respectively in the supernatants of PBMC stimulated with rhM-CSF 5×10^6 U/L, compared with control on the corresponding day. The kinetics curve in IL-6 induction in monocytes was shown in Fig 1, which indicated that rhM-CSF stimulation of monocytes enhanced IL-6 production and this increase was maximum 4 d after stimulation.

Effect of rhM-CSF on IL-8 production in monocytes One-, two-, three-, and four-day after stimulation, the concentration of IL-8 (ng/L) increased by 32.2 %, 29.7 %, 32.9 %, and 57.4 %, respectively in the supernatants of PBMC treated with rhM-CSF 1×10^6 U/L, and by 46.1 %, 42.3 %, 54.6 %, and 95.8 %, respectively in the supernatants of PBMC treated with rhM-CSF 5×10^6 U/L, compared with control

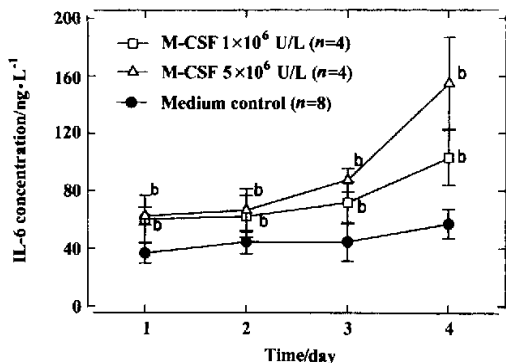


Fig 1. Kinetics curves for induction of IL-6 in human peripheral blood monocytes by sandwich ELISA. $P < 0.05$ vs control.

on the corresponding day. In Fig 2, the dynamic characteristic of IL-8 induction in monocytes is given. The data indicates that rhM-CSF promoted IL-8 induction in monocytes and with the passage of M-CSF stimulation time, the concentration of IL-8 increased.

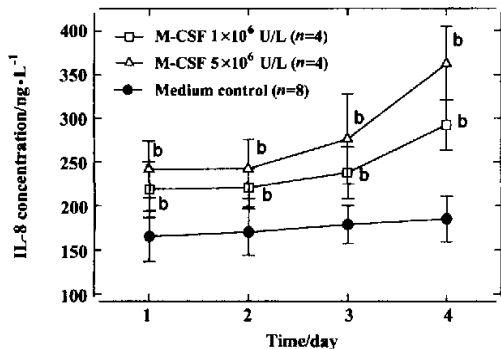


Fig 2. Kinetics curves for induction of IL-8 in human peripheral blood monocytes by sandwich ELISA. $P < 0.05$ vs control.

Effect of rhM-CSF on IFN- α secretion in monocytes Antiviral activity of IFN was not detected in culture supernatants of monocytes stimulated with rhM-CSF alone. A low level of antiviral activity of IFN could be detected in culture supernatants of monocytes stimulated with IFN- γ or NDV alone. A high level of IFN could be found in cultured monocytes treated with IFN- γ + NDV. Although rhM-CSF slightly promoted IFN- α secretion induced by IFN- γ , NDV, or IFN- γ + NDV, the significance was not marked (Data not shown).

Effect of rhM-CSF on membrane antigen molecules of monocytes The data from the assays for testing membrane antigen expressions of monocytes treated with rhM-CSF are presented in Tab 2.

rhM-CSF increased the percentage of CD11b, CD16, HLA I, and HLA II molecules, respectively, just as IFN- γ did. But the enhancing effect of rhM-CSF was not as strong as that of IFN- γ in inducing CD11b, HLA I, and HLA II expressions, while it might be stronger than IFN- γ in inducing CD16 expression. With increase in stimulation time, the enhancing effects became more and more obvious.

DISCUSSION

Macrophage colony-stimulating factor (M-CSF) is considered to be an activator and up-regulator of monocyte-macrophage function⁽⁴⁾. In this paper, it was shown that the rhM-CSF expressed in silkworm could activate monocytes and increase the production of cytokines such as TNF- α , IL-6, and IL-8 and the expression of cellular surface molecule such as CD11b, CD16, HLA I, and HLA II.

IL-6 and IL-8 are important immune factors which play a significant role in immunoregulation and inflammation. IL-8 is a chemotactic factor for neutrophils and lymphocytes and can also activate these cells. It had been proved that M-CSF stimulated monocyte production of IL-1 or TNF- α ^(5,6). Recently IL-8 was found to be induced in human peripheral blood monocytes by M-CSF⁽⁷⁾. But there were different results from the studies on the effect of M-CSF on IL-6 induction⁽⁸⁻¹⁰⁾. In this paper, we demonstrated that stimulation of human peripheral blood monocytes with M-CSF induced IL-6 and IL-8 secretion in a concentration-dependent manner within 4 d, and the concentration of IL-6 and IL-8 in the culture supernatants increased with the passage of M-CSF stimulation time. In addition, we demonstrated that M-CSF enhanced TNF- α induction and had a synergetic effect with LPS and PMA on the induction. It is inferred that M-CSF may enhance immune reaction and inflammation by promoting secretion of cytokines such as TNF- α , IL-6, and IL-8.

As for IFN- α induction by M-CSF, there are contradictory studies. In our experiment, the enhancing effect of rhM-CSF on IFN- α production in human peripheral blood monocytes was not significant.

CD16 molecule is a Fc receptor. HLA I (MHC I molecule), HLA II (MHC II

Tab 2. Percentage (%) of CD11b, CD16, HLA I, and HLA II positive cells in peripheral blood monocytes under different treatments. $\bar{x} \pm s$. $n=3$ samples for each group. $^bP < 0.05$ vs medium control.

Treatment	Stimulating for 3 d				Stimulating for 7 d			
	CD11b	CD16	HLA I	HLA II	CD11b	CD16	HLA I	HLA II
rhM-CSF	31 ± 8 ^b	61 ± 11 ^b	55 ± 9 ^b	57 ± 11 ^b	52 ± 13 ^b	81 ± 2 ^b	65 ± 7 ^b	71 ± 15 ^b
IFN-γ	45 ± 8 ^b	37 ± 8 ^b	64 ± 19 ^b	66 ± 8 ^b	64 ± 7 ^b	54 ± 4 ^b	85 ± 12 ^b	84 ± 14 ^b
Medium control	14 ± 3	4 ± 1	18 ± 7	19 ± 3	20 ± 3	12 ± 3	22 ± 4	26 ± 8

molecule), and CD11b (Mac-1) are all adhesion molecules. Our experiments show that the treatment of monocytes with M-CSF could promote CD16, HLA I, HLA II, and CD11b expressions. The expression enhancement of these membrane molecules could promote immune response and inflammatory reaction. Held *et al* have published a similar study, which shows that the expressions of Ia antigen (MHC II molecule in mice) and Mac-1 molecule increased in spleen macrophages of the mice administered with M-CSF⁽¹⁰⁾. According to the results of the present study, in which rhM-CSF augmented TNF-α, IL-6, and IL-8 secretion and increased CD11b, CD16, HLA I, and HLA II expressions, we consider that M-CSF plays an important role as a modulator of inflammatory reaction and may be of significance in treating infections related to immunodeficiency, especially monocyte-macrophage deficiency.

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家蚕表达的 rhM-CSF 对人单核细胞的细胞因子 诱生和膜分子表达的影响¹

季晓辉², 孙路虹³, 秦浚川⁴, 姚 堃³, 丁如宁³,
李焕娣³, 朱德熙⁴ (南京医科大学临床药理学
研究所,³免疫学研究室, 南京 210029; ⁴南京大学
生物化学系, 南京 210008, 中国)

关键词 巨噬细胞集落刺激因子; 单核细胞;
细胞因子类; 免疫调节

目的: 研究家蚕表达的重组人巨噬细胞集落刺激因

子(rhM-CSF)对单核细胞细胞因子诱生和膜分子表达的影响. **方法:** 将该 rhM-CSF 加入人外周血单核细胞培养液中, 数天后分离培养上清和细胞. 以生物活性法或/和 ELISA 法检测上清中的 TNF- α , IL-6, IL-8 和 IFN- α ; 以 APAAP 法检测细胞表面的 CD11b, CD16, HLAI 和 HLAII 分子. **结果:** rhM-CSF 促进单核细胞 TNF- α , IL-6 和 IL-8 的诱生, 提高表达 CD11b, CD16, HLAI 和 HLAII 分子的细胞的百分率. **结论:** 该 rhM-CS 对单核细胞功能可起上调作用, 在免疫学治疗方面具有很好的应用前景.

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