ELSEVIER

FEBS Letters 339 (1994) 239–242

FEBS 13667

FEBS LETTERS

# Monoclonal non-specific suppressor factor (MNSF) inhibits the IL4 secretion by bone marrow-derived mast cell (BMMC)

Morihiko Nakamura*, Ricardo M. Xavier, Yoshinori Tanigawa

*Department of Biochemistry, Shimane Medical University, Izumo 693, Japan*

Received 7 December 1993

**Abstract**

We investigated whether monoclonal non-specific suppressor factor (MNSF) has an effect on the IL4 secretion by BMMC sensitized with anti-DNP IgE monoclonal antibody. The sensitized BMMC showed a maximum increase of IL4 secretion at 10 ng/ml antigen activation. When MNSF (10 U) was added, it remarkably suppressed the IL4 secretion (82 ± 3%). MNSF also suppressed the IL4 secretion by the mast cell line, CFTL-15, but not CFTL-12. Northern blot analysis showed that, when the same amounts of MNSF were added, IL4 mRNA expression was decreased, suggesting that the suppression results from the inhibition of a transcript.

*Key words:* Non-specific cytokine; Suppressor factor; Interleukin-4; Mast cell

## 1. Introduction

Monoclonal non-specific suppressor factor (MNSF) is a lymphokine that is a product of the concanavalin A (Con A)-activated murine T cell hybridoma and inhibits the generation of lipopolysaccharide (LPS)-induced immunoglobulin secreting cells in an antigen non-specific manner [1]. MNSF fractionated on Sephadex G-100 in phosphate buffered saline (PBS, pH 7.3) appeared as an aggregate with a $M_w$ of 70,000, but when fractionated in 0.5 M pyridine-acetic acid buffer (pH 4.0), it appeared as two species of 24,000 and 16,000 Da which showed pI peaks of 5.3 and 5.7, respectively, on isoelectric focusing [1]. Further purified MNSF using reverse-phase HPLC showed a single band with a $M_w$ of 12,000 on SDS-PAGE. The N-terminal amino acid sequence of the protein shows no strong homology to any of the sequences of known biologically active proteins [2].

Previous studies on the mode of action of MNSF have demonstrated that the target cells involved are both LPS-activated B cells and Con A-activated T cells. In addition to activated lymphocytes, the function of various tumor cell lines of mouse origin is altered by a specific cell surface membrane receptor protein [3,4]. More recently, we have isolated the human non-specific suppressor factor (hNSF) from ascitic fluid of a patient with systemic lupus erythematosus (probably the human counterpart of MNSF) and observed that an anti-pan T cell receptor α chain (TCR-α) monoclonal antibody could cross-react with it (Xavier, R. et al. (1994) J. Immunol; in press).

It is evident that transformed or IL3-dependent murine mast cell lines [5–8] and murine bone marrow-derived mast cells (BMMC) [9,10] can produce a number of cytokines, like IL1, IL3, IL4, IL5, IL6, IFN-γ, GM-CSF, TNF-α, in response to cross-linking of the high affinity receptor for IgE (Fcε R) or to ionomycin. IL3 has been clearly shown to be a mast cell growth factor [11]; IL4 is critical for IgE production [12]; IL5 is a potent eosinophil differentiation and activation factor [13].

Helper T cells are another cell type known to release various lymphokines. However, the activation of these lymphokines seems to be largely restricted to the cells with which they interact. By contrast, stimulated mast cells function by releasing a number of cytokines into the extracellular fluid and, therefore, have a wider variety of target cells. In this report, we tested the possibility that MNSF could be related to the mode of action of BMMC from the viewpoint of IL4 secretion.

## 2. Materials and Methods

### 2.1. Materials

MNSF was obtained from the ascites of F1 mice (AKR/J/Balb/c) after injection of MNSF-producing E17 hybridoma into the peritoneal cavity. Purification with the use of anti-MNSF monoclonal antibody (MO6) was done as described previously [14]. The MNSF was further purified by combination of ODS-120T and phenyl 5PW reverse-phase HPLC column (Tosoh Corp., Tokyo Japan). 1 U of MNSF activity was defined as the amount that causes 50% suppression of LPS-induced

*Corresponding author. Fax: (81) (853) 22-4963.

*Abbreviations:* MNSF, monoclonal non-specific suppressor factor; BMMC, bone marrow-derived mast cells; IL, interleukin.


*SSDI* 0014-5793(94)00051-V

antibody responses by mouse splenocytes when added at culture initiation [1].

*2.2. Cell culture*

Mouse BMMC were obtained by culturing bone marrow cells of female (10–12 week old) Balb/c mice (Clear, Japan) for 3 weeks in 25% WEHI-3 conditioned medium and 75% enriched medium (RPMI1640 containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol). BMMC contained >95% mast cells according to Neutral red staining [15]. CFTL-12 and CFTL-15 were obtained by culturing fetal liver from NFS/N mice in IL3 [16].

*2.3. Sensitization and challenge of BMMC and mast cell lines*

BMMC, CFTL-12 or CFTL-15 ($1 \times 10^5$ cells/ml) were sensitized with 1 μg/ml of anti-dinitrophenyl (DNP) IgE monoclonal antibody for 3 h in 25% WEHI-3-conditioned medium/75% enriched medium described above (BMMC) or the enriched medium only (cell lines) and were then washed three times with Hank's balanced salt solution (HBSS). To measure IL4 secretion in the culture medium, the cells were resuspended in 25% WEHI-3 conditioned/75% enriched medium at a concentration of $1 \times 10^5$/ml and challenged with $DNP_{23}$–BSA antigen (10 ng/ml) for 6 h.

*2.4. Enzyme-linked immunosorbent assay (ELISA) for IL4*

To measure mouse IL4, a sandwich ELISA was developed by using two distinct rat monoclonal antibodies to IL4. Briefly, the first antibody (Pharmingen; #18031) was coated onto ELISA plates (Nunc, Denmark; 0.2 μg/well) and then samples obtained from BMMC or mast cell line cultures were added. Subsequently, the biotinylated rat anti-mouse IL4 monoclonal antibody (Pharmingen; #18024) at 0.1 μg/well was added and then streptavidin–alkaline phosphate at 80 ng/well. The plates were washed with PBS/0.05% Tween 20 at each step of this procedure. The alkaline phosphatase substrate was in 0.1 M 2-aminoethanol (Sigma), 1 mM $MgCl_2$, pH 9.8. Sensitivity was 1 U/ml. The unitage was defined by the CT-4S proliferative assay as the concentration required for half-maximal stimulation [17].

*2.5. Analysis of IL4 stored in granules of BMMC*

Cytoplasmic granules were prepared by the method of Roussel and Greenberg [18]. In brief, cells were washed in HBSS and resuspended in disruption buffer (0.25 M sucrose, 0.01 M HEPES, 4 mM EGTA, pH 7.4). They were lysed by decompression at 0°C after equilibrating at 450 psi nitrogen for 20 min. After the addition of $MgCl_2$ to 5 mM, the homogenate was digested with DNase I (from bovine pancreas, type IV, Sigma) at 1,000 U/ml, 22°C, 30 min. Nuclei were removed by filtration through Nucleopore filters (Nucleopore) and the resulting homogenate was cooled to 0°C. 5 ml aliquots were layered on 20 ml of 48% Percoll and ultracentrifuged ($60,000 \times g$) for 10 min. The resulting gradient was fractionated from the bottom by insertion of a stainless- steel tube from the top, and the first 5 ml high-density Percoll fraction was removed via attached polyethlene tubing. Granules have been shown to the present in the high-density Percoll fractions on the basis of granule markers such as β-glucuronidase and cytolysin. To eliminate the Percoll, the granule fractions were ultracentrifuged ($85,000 \times g$) for 2 h, and then the granules were removed and diluted with sterile water and then stored at −70°C. The granules were quickly thawed at 37°C and then solubilized in 3 M NaCl for 1 h on ice. The solubilized granules were then dialized against PBS and tested for IL4 by ELISA.

*2.6. Northern blot analysis*

Either antigen-activated BMMC ($2 \times 10^7$ cells) or mast cell lines (CFTL-12, $5 \times 10^7$; CFTL-15, $2 \times 10^7$ cells) were cultured in the presence or absence of MNSF (10 U), harvested and the total cellular RNA was isolated by the methods of Sambrook et al. [19]. 20 μg of RNA was fractioned on 1% agarose formaldehyde gels, and transferred to ECL-Hybond. Hybridization, labeling of cDNA probe and detection were done according to the ECL-Gene Detection System (Amersham). The mouse IL4 cDNA probe was a 424 bp *Eco*RI–*Hin*dIII fragment which includes the 373 bp *Rsa*I fragment of mouse IL4 cDNA and 51 bp of pGEM-3 [11].

Table 1
Pre-formed IL4 in BMMC

| | Total amounts detected (U/ml)* | |
|---|---|---|
| | Experiment 1 | Experiment 2 |
| Unsensitized BMMC | 8 ± 3 | 5 ± 1 |
| Sensitized BMMC | 24 ± 5 | 15 ± 3 |

*Purification of BMMC ($1 \times 10^6$ cells) cytoplasmic granules and detection of the amount of IL4 were done as described in section 2. The amount of IL4 obtained from the same number of sensitized and antigen-stimulated BMMC was 728 ± 66 U/ml.

## 3. Results and Discussion

While studying possible effects of MNSF on mast cells, we observed that it greatly influenced the production of IL4 by mast cells, and this is the subject of the present report. In preliminary experiments, we had observed that the maximum increase in IL4 secretion by activated BMMC was detected after 6 h of antigen stimulation. To obtain an optimal condition of antigen concentration to activate BMMC, anti-DNP IgE-sensitized BMMC were tested for the maximum increase of IL4 secretion (Fig. 1). A concentration of 10 ng/ml of DNP–BSA resulted in a maximum increase, and hence was used for studies of the following inhibition tests.

MNSF was added to BMMC cultures at the time of antigen challenge. The secretion of IL4 was inhibited, dose dependently, by the addition of MNSF (Fig. 2A). However, complete suppression of IL4 secretion was not observed, suggesting that some of the secreted IL4 could have been preformed and stored in granules. We tested this possibility by disrupting BMMC and detecting the amounts of stored IL4 as described in section 2. Table 1 shows that little IL4 could be detected after disrupting BMMC, indicating that IL4 was released from newly

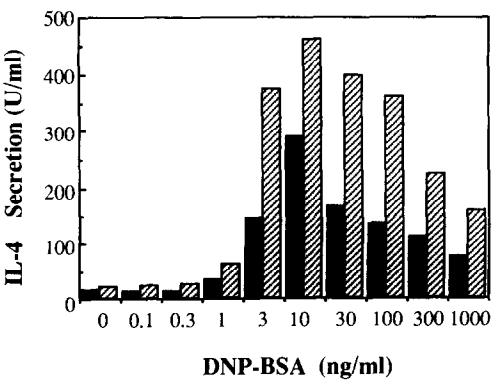


Fig. 1. Effect of various concentrations in DNP-BSA antigen on the IL4 secretion by BMMC. BMMC ($2 \times 10^4$, ■; $1 \times 10^5$ cells, □) were sensitized with anti-DNP–BSA IgE monoclonal antibody (1 μg/ml) and then challenged with DNP–BSA antigen (10 ng/ml). IL4 secreted into the BMMC culture supernatant was detected by ELISA as described in section 2. Experimental values are means of triplicate determinations.

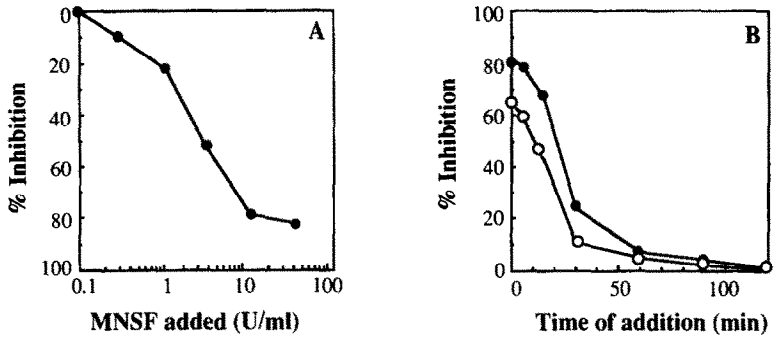


Fig. 2. (A) Effect of MNSF on IL4 secretion by BMMC. BMMC ($1 \times 10^5$ cells) were sensitized and challenged with antigen as described in the legend to Fig. 1. Various concentrations of MNSF were added at the initiation of the cultures. For controls, PBS was added in place of MNSF. Secreted IL4 was detected and percentage suppression was calculated by comparison with the control response of 462 U/ml. (B) Kinetics of suppression of MNSF. MNSF was added to the BMMC cultures at indicated times; ●, 10 U MNSF added; ○, 5 U. Percentage suppression was estimated as described in the legend to Fig. 2.

synthesized pools and not stored in granules after sensitization. We speculate that the incomplete effect might be due to the following reasons. First, an alternative pathway not influenced by MNSF might exist. Second, the presence of an inhibitor, for instance, soluble MNSF receptor, may control the function.

The kinetics of suppression of MNSF were examined to determine whether MNSF would also suppress IL4 secretion during various periods of culture. As can be seen in Fig. 2B, maximum suppression was observed when MNSF was added at the time of antigen challenge, whereas no significant effect could be detected when it was added during the later culture period, thereby indicating that MNSF acts on BMMC at an early stage. In contrast, MNSF inhibits the antibody response in all culture periods [3].

We investigated whether suppression of IL4 secretion could also be observed in murine mast cell lines. MNSF inhibited the secretion of IL4 in CFTL-15, whereas no significant suppression was observed in CFTL-12 (Fig. 3). CFTL-15 would be a good model to investigate the functional mechanism of MNSF. It is conceivable that MNSF can not act on CFTL-12 due to lack of MNSF receptors on the cell surface. It is unclear at present whether MNSF acts directly on BMMC. Binding assays of $^{125}$I-labelled MNSF to BMMC are under way to clarify receptor expression on the cells. We reported previously that IFN-$\gamma$-activated murine spleen cells produce MNSF, and that IFN-$\gamma$ enhances the activity of MNSF on target cells by an increment related to the number of MNSF receptors [20,21]. Further studies are in progress to determine whether IFN-$\gamma$ is involved in the functional mechanism of BMMC.

To determine whether the suppression of IL4 secretion resulted from a decrease in the normal stimulated accumulation of IL4 mRNA, Northern blot analyses were carried out. As can be seen in Fig. 4A, the expression of

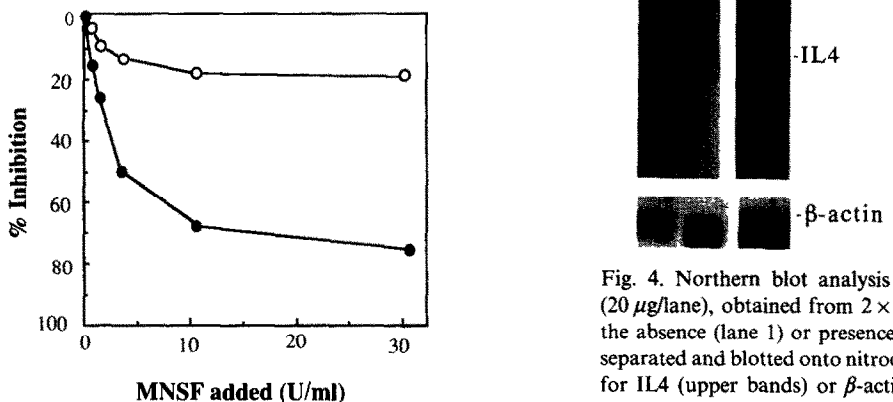


Fig. 3. Effect of MNSF on the IL4 secretion by mouse mast cell lines. CFTL-12, ○; and CFTL-15, ●; were sensitized and challenged as described in section 2. Control and stimulated levels of IL4 production were 18 and 231 U/ml for the CFTL-12, and 61 and 558 U/ml for the CFTL-15 cell line.


Fig. 4. Northern blot analysis of IL4 mRNA. (A) Poly(A$^+$) RNA (20 $\mu$g/lane), obtained from $2 \times 10^7$ BMMC activated with antigen in the absence (lane 1) or presence (lane 2) of MNSF (10 U), were size-separated and blotted onto nitrocellulose and hybridized with the probe for IL4 (upper bands) or $\beta$-actin (bottom bands). Lane 3 shows the basal level of IL4 mRNA. The migrated positions of 18 S ribosomal RNA are indicated in the RNA blots. (B) Poly(A$^+$) RNA (20 $\mu$g/lane), obtained from mast cell lines activated with antigen in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of MNSF (10 U), were probed with IL4 cDNA; lanes 1 and 2, CFTL-15 ($2 \times 10^7$ cells); lanes 3 and 4, CFTL-12 ($5 \times 10^7$ cells).

IL4 mRNA was inhibited in the presence of MNSF in the BMMC culture. The incomplete suppression of IL4 mRNA expression might be comparable to that of IL4 secretion. No IL4 mRNA was expressed in BMMC in the absence of antigen. In CFTL-15 cells, MNSF inhibited the IL4 mRNA expression to the same extent as in BMMC, while this effect was not observed in CFTL-12 (Fig. 4B). In the absence of antigen, the basal levels of IL4 mRNA in both CFTL-12 and −15 cells were nil (data not shown). It remains to be clarified whether or not the suppression is a consequence of a decreased rate of transcription and/or increase in specific RNase that degrades the transcripts.

Further studies are currently in progress to clarify the specific mode of action of MNSF in BMMC. We are now attempting to isolate cDNA for MNSF and hNSF by the use of synthetic oligonucleotide primers corresponding to the N-terminal amino acid sequences of MNSF.

*Acknowledgements:* We thank Y. Nishikouri and K. Ueno for technical assistance. This work was supported in part by a Grant-in-aid for Scientific Research No. 05770316 from the Ministry of Education, Science and Culture, Japan.

## References

[1] Nakamura, M., Ogawa, H. and Tsunematsu, T. (1986) J. Immunol. 136, 2904–2909.
[2] Nakamura, M., Ogawa, H. and Tsunematsu, T. (1992) Cell. Immunol. 139, 139–143.
[3] Nakamura, M., Ogawa, H. and Tsunematsu, T. (1988) Cell. Immunol. 116, 230–239.
[4] Tsunematsu, T., Ogawa, H., and Nakamura, M. (1987) in: New-Horizons in Animal Models for Autoimmune Disease (M Kyogoku, and H. Wigzell, eds.) pp. 323–329, Academic Press, Tokyo.
[5] Nable, G., Galli, S.J., Dvorak, A.M. and Cantor, H. (1981) Nature 291, 332–334.
[6] Pierce, J.H., Difiore, P.P., Aaronson, S.A., Potter, M., Pumphery, J., Scott, A. and Ihle, J.N. (1985) Cell 41, 685–693.
[7] Wodnar-Filipowicz, A., Heusser, C.H. and Moroni, C. (1989) Nature 339, 150–152.
[8] Plaut, M., Pierce, J.H., Watson, C.J., Hanley-Hyde, J., Nordan, R.P. and Paul, W.E. (1989) Nature 339, 64–67.
[9] Burd, P.R., Rogers, H.W., Gordon, J.R., Martin, C.A., Jayaraman, S., Wilson, S.D., Dvorak, A.M., Galli, S.J. and Martin, M.E. (1989) J. Exp. Med. 170, 245–257.
[10] Gurish, M.F., Ghildyal, N., Arm, J., Austin, K.F., Avraham, S., Reynolds, D. and Stevens, R.L. (1991) J. Immunol. 146, 1527–1533.
[11] Fung, M.C., Hapel, A.J., Ymer, S., Cohen, D.R., Johnson, R.M., Campbell, H.D. and Young, I.G. (1984) Nature 307, 233–237.
[12] Mosmann, T.R., Bond, M.W., Coffman, R.L., Ohara, J. and Paul, W.E. (1986) Proc. Natl. Acad. Sci. USA 83, 5654–5658.
[13] Sanderson, S.J., Campbell, H.D. and Young, I.G. (1988) Immunol. Rev. 102, 29–32.
[14] Nakamura, M., Ogawa, H. and Tsunematu, T. (1987) J. Immunol. 138, 1799–1803.
[15] Gordon, J.R. and Galli, S.J. (1990) Nature 346, 274–277.
[16] Brown, M.A., J. Pierce, C.J., Watoson, J.F. Ihle and Paul, W.E. (1987) Cell 50, 809–818.
[17] Hu-Li, J., Ohara, J., Watson, C., Tsang, W. and Paul, W.E. (1989) J. Immunol. 142, 800–807.
[18] Roussel, E. and Greenberg, A.H. (1989) J. Immunol. 142, 543–549.
[19] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Lab. Press. New York.
[20] Nakamura, M., Ogawa, H. and Tsunematsu, T. (1990) Cell. Immunol. 130, 281–290.
[21] Nakamura, M., Ogawa, H. and Tsunematsu, T. (1992) Cell. Immunol. 39, 131–138.