Macrophage Colony-Stimulating Factor mediates its Immunosuppressive Activity through the Emerging Myeloid Cells during Tumor Progression

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Macrophage Colony-Stimulating Factor mediates its Immunosuppressive Activity through the Emerging Myeloid Cells during Tumor Progression

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Mechanisms by which tumors evade immune surveillance remain to be addressed. In our previous study, we reported that murine mammary tumor (4T1) cells secrete immunosuppressive soluble factor(s), which was identified to be a 10-100 kDa protein. In the current study, we report that analysis of the proteins in the active fractions revealed the presence of macrophage colony-stimulating factor (M-CSF) as one of the suppressive factors secreted by 4T1 tumor cells. Although previously identified as a cytokine that regulates survival, proliferation, and differentiation of macrophages and monocytes, M-CSF has also been associated with tumor progression and metastasis. To date, the immunosuppressive activity of M-CSF is not well understood. To better understand the immunosuppressive activity of M-CSF, we studied the activity of recombinant murine M-CSF in splenocytes isolated from 4T1 tumor-bearing mice. Reduced levels of interferon-gamma (IFN-γ) by M-CSF were observed in a dose-dependent manner indicating suppressive activities of M-CSF on the T-cell activation. M-CSF reduces the secretion of IFN-γ without affecting its intracellular protein expression. The suppressive activity of M-CSF is dependent on the length of days after tumor inoculation as no activity was observed in splenocytes from one-week tumor-bearing mice. This phenomenon correlates with an increase in the number of myeloid cells in the spleen during tumor progression. The suppressive activity of M-CSF is thus thought to be mediated by the myeloid cells emerging during tumor growth.

Keywords: M-CSF, immunosuppression, splenocyte assay.

1. Introduction

In Japan, cancer is the leading cause of death followed by heart diseases. Although significant progress has been made in the treatment of cancer, current regimen do not benefit every cancer patient. One of the main challenges in the fight against tumor growth is the ability of the tumor cells to evade the immune surveillance. Cancer cells have been shown to evade the immune system through different means including secretion of immunosuppressive factors such as tumor growth factors-β, prostaglandin E2, and granulocyte colony-stimulating factor (G-CSF) among others[1–4]. These factors either directly or through recruitment of suppressive cells enhance not only tumor progression but also metastasis to distance organs[5]. Much effort is currently being focused on the identification of immunosuppressive factors and elucidation of their mechanisms of action.

Macrophage colony-stimulating factor (M-CSF) is a cytokine essential for the differentiation of cells of monocyte origin such as macrophages, osteoclasts, and microglia[6]. In addition to this primary role, M-CSF has also been shown to play a role in tumor angiogenesis where it acts as an angiogenic switch, especially in mammary tumors[7]. Metastasis of tumor cells to other organs such as the lungs and bones has also been shown to be enhanced by M-CSF. For instance, using osteopetrotic (*op/op*) mutant mice, a mouse model that lacks M-CSF signaling, no reduction in tumor growth was observed, but there was a significant delay in tumor invasiveness and metastasis to the lungs[8]. This effect was associated with reduced infiltration of tumor-associated macrophages (TAM) in tumors in the absence of M-CSF. Indeed, inhibition of M-CSF through the use of small interfering RNAs[9] and antibodies[10] or use of clodronate to deplete the macrophages[11] has been shown to suppress tumor growth in mice. Enhanced metastasis by M-CSF through TAM is thought to be due to the released matrix metalloproteases that remodel tumor vasculature leading to leakage of tumor cells into

circulation. However, it has been not well reported that M-CSF has a suppressive effect on T cells in tumor conditions.

Although the spleen is one of the major secondary lymphoid organs, its biological role, especially in pathophysiological conditions, is not well understood. In tumor models and cancer patients, the spleen has been shown to be the major organ to undergo cell expansion resulting in splenomegaly[12,13]. In 4T1 murine tumor models, immature myeloid cells have been reported to be the major cell population that increases significantly in the spleen during tumor development, and G-CSF is one of the factors stimulating this event[2]. Spleen myeloid cells have also been shown to serve as a reservoir for replenishing TAM in tumor areas through a process that requires C-C chemokine receptor type 2 signaling[14]. However, the factors stimulating differentiation of the immature myeloid cells into TAM in the tumor area are not well understood. These observations highlight the importance of spleen cells in tumor biology.

We have developed an evaluation system called splenocyte assay to screen for tumor-derived suppressive factors and elucidate their suppressive mechanisms using splenocytes from tumor-bearing mice. Using this assay system, we previously reported that 4T1 tumor cells secrete soluble factors that suppress interferon gamma (IFN-γ) secretion and the activity was attributed to a protein of between 10-100 kDa[15]. In this study, we report that analysis of the active fractions through chromatography systems and MALDI-TOF-MS indicated the presence of M-CSF as one of the immunosuppressive factors. Recombinant M-CSF showed suppressive activity in splenocytes from tumor-bearing mice in a dose-dependent manner. The activity was not observed in a co-culture of T cells isolated from tumor-bearing mice with T cell-depleted splenocytes from normal mice or in splenocytes from one-week tumor-bearing mice. These results suggest that the suppressive activity of M-CSF in a splenocyte assay is mediated by a myeloid cell population emerging during tumor development.

2. Materials and Methods

2.1 Preparation of Tumor-Bearing Mice and Isolation of Splenocytes

Murine mammary tumor 4T1 cells were purchased from American Type Culture Collection (Manassas, VA, USA), and maintained with RPMI 1640 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum in a 5% CO_2 atmosphere at 37 °C. Balb/c mice were purchased from Kyudo Co. Ltd. (Tosu, Japan) and all animal experiments were carried out according to the guidelines for the proper conduct of animal experiments published by the Science Council of Japan. All experimental protocols were approved by the Ethics Committee and the Animal Care and Use Committee of Kyushu University. Recombinant M-CSF was purchased from Peprotech (Rocky Hill, NJ, USA) and used at the indicated concentrations. Tumor-bearing mice were prepared by inoculating 1 x 10^6 4T1 cells and the tumor was allowed to grow for two weeks or otherwise indicated. Splenocytes were isolated from tumor-bearing mice or normal mice and prepared as described previously[15]. One million splenocytes were cultured in triplicates in the presence or absence of the indicated M-CSF concentrations for 48 hours, and the level of IFN-γ in the conditioned media was determined. T cells were isolated using the Pan T cell Isolation Kit II (Miltenyi Biotec Inc, Auburn, CA) while depletion was done using CD3ε MicroBead Kit (Miltenyi Biotec Inc, Auburn, CA, USA). Successful depletion and isolation of T cells were confirmed by flow cytometry (Guava easyCyte™, Millipore, USA). For the co-culture assay, isolated T cells from tumor-bearing mice were co-cultured with T cell-depleted splenocytes from normal mice, and the activity of M-CSF was tested.

2.2 Fractionation of 4T1 Conditioned Media

Conditioned media (CM) was collected from cultured 4T1 tumor cells. After removing cell debris by centrifugation, the CM was concentrated by ammonium sulfate precipitation at 4°C overnight. The sample was separated by a hydrophobic column (HiTrap™ Butyl HP) using ÄKTAprime plus system (GE Healthcare Life Sciences, USA). The elution was monitored by UV absorbance at 280 nm.

2.3 MALDI/TOF-MS Analysis and Western Blotting

Further several column purification steps were performed, and the active fractions were separated by SDS-PAGE ready for MALDI/TOF-MS analysis or Western blotting. For MALDI/TOF-MS analysis, the gel was stained with Coomassie Brilliant Blue and the protein bands in the active fractions were digested as described before[16]. The mass spectra of the digested samples were obtained using JMS-S3000 (JEOL, Japan). Protein identification was performed from a database search using Mascot algorithms (Matrix Science). For Western blotting, the separated protein bands were transferred to a PVDF membrane, and after being blocked, the membrane was incubated with anti-M-CSF antibody (Peprotech, product No. 315-02) or anti-G-CSF antibody (eBioscience, product No. 16-7353-85). After washing steps, the membrane was incubated with secondary horseradish peroxidase-conjugated antibodies (R&D Systems, Minneapolis, MN, USA) and visualized with a chemiluminescent substrate (ImmunoStar LD; Wako Pure Chemical Industries Ltd., Osaka, Japan).

2.4 Enzyme-linked Immunosorbent Assay (ELISA)

Quantification of IFN-γ was performed by ELISA (R&D Systems), according to the manufacturer's

instructions.

2.5 Statistical Analysis

Differences between experimental groups were tested using either analysis of variance or two-tailed unpaired Student's t-tests. Values are reported as mean ±SD, and typical data are shown. Differences were considered significant if $p < 0.05$.

3. Results and Discussion

3.1 M-CSF is secreted by 4T1 tumor cells and shows strong immunosuppressive activity

To determine the active factors, we fractionated 4T1 conditioned media using hydrophobic columns, and the activity of the resulting fractions was tested in a splenocyte assay. Suppressive activity was observed in fractions 3 and 4 and fractions 9 to 11 (Fig. 1A) indicating the presence of immunosuppressive factors in these fractions.

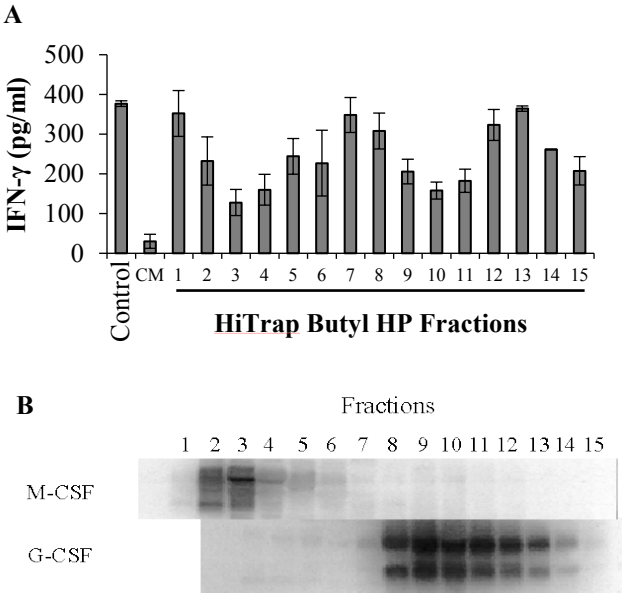

Fig. 1. Detection of M-CSF and G-CSF. A) Suppressive activity of different fractions. CM, conditioned media. B) Western blotting analysis.

To identify the proteins present in the active fractions, samples were further fractionated by several column chromatography steps, and the active fractions were analyzed by MALDI/TOF-MS. The Mascot search results showed M-CSF to be the major protein in fractions 3 and 4 as it had the highest score among the different proteins that matched the submitted data.

As shown by the TOF-MS PMF search, M-CSF was detected by Western blotting in both the fraction 3 and 4 while fractions 9 to 11 indicated the presence of G-CSF (Fig. 1B). Although in our previous study M-CSF was not detected in 4T1 CM using cytokine tip assay, in this study, detection of M-CSF was enhanced by concentration and purification of the CM. Tumor-derived G-CSF has been reported to facilitate tumor growth through the generation of myeloid-derived suppressor cells (MDSCs). MDSCs are well characterized and have been shown to suppress T cell proliferation in *in vitro* studies[17]. However, the immunosuppressive activity of M-CSF is not well characterized and our study, therefore, aimed to explore the immunosuppressive activity of M-CSF.

3.2 M-CSF suppresses IFN-γ secretion in a dose-dependent manner

To verify the suppressive activity of M-CSF, we used recombinant murine M-CSF and tested its activity in a splenocyte assay. M-CSF suppressed IFN-γ secretion in a dose-dependent manner (Fig. 2). To determine whether the suppressive activity was at the level of extracellular secretion or protein synthesis of IFN-γ, we analyzed IFN-γ levels both in the conditioned media and in the cell lysate. Results indicated that M-CSF slightly reduced the protein level of IFN-γ in the cell, but it is not significant (Fig. 2).

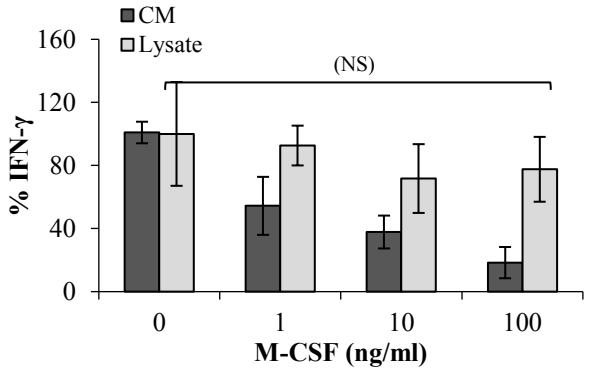

Fig. 2. Level of IFN-γ in the conditioned media and cell lysate in the presence of M-CSF. CM, conditioned media; NS, no significance.

To further characterize the activity of M-CSF in the splenocyte assay, we analyzed the kinetics of M-CSF suppressive activity for 72 hours. Splenocytes were cultured with or without 100 ng/ml M-CSF and the levels of IFN-γ released were detected at 12-hour intervals. The level of IFN-γ was comparable with or without M-CSF after 24 hours, and the significant differences were observed from about 36 hours after the incubation. While

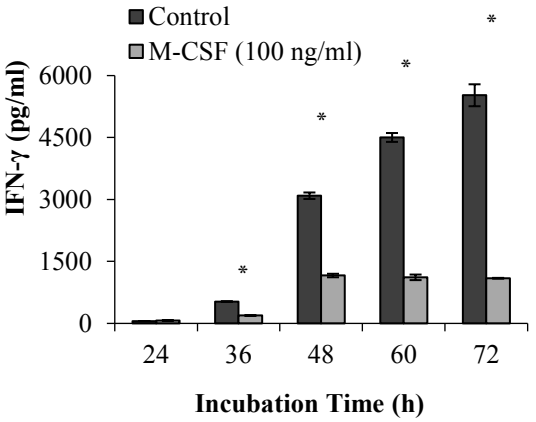

Fig. 3. Time-course suppressive activity of M-CSF. *$p < 0.05$.

the level of IFN-γ continued to increase until 72 hours after the incubation in the control experiment that of M-CSF-treated stagnated after 48 hours (Fig. 3).

We further determined whether the effect of M-CSF resulted in a permanent change to the target cells. To this end, splenocytes were cultured in the presence of 100 ng/ml M-CSF, and after 48 hours the media was changed to one without M-CSF. The cells were then cultured for another 48 hours, and the level of IFN-γ was determined. As shown in Fig. 4, withdrawal of M-CSF failed to alleviate the suppression of IFN-γ, which was induced during the first 48 h culture by M-CSF. Together these results suggest that M-CSF is conferring a permanent change to its target cells that cannot be alleviated by the simple withdrawal of M-CSF from the culture medium. It is still not clear whether the permanent change was on the side of T cells or the target cells that are interacting with M-CSF.

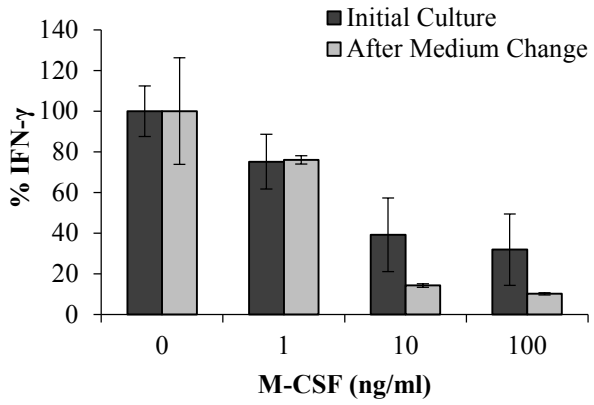

Fig. 4. Effect of M-CSF withdrawal on IFN-γ secretion.

3.3 Suppressive activity of M-CSF is through the emerging myeloid cells during tumor growth

M-CSF failed to suppress IFN-γ secretion in the splenocytes derived from normal mice, while strong activity was observed in the splenocytes from tumor-bearing mice. These results suggest that M-CSF is working through some cells that are emerging during tumor progression. However, the suppressive activity of M-CSF and its correlation with the emerging myeloid cells during tumor progression are not well reported. To test whether M-CSF activity is dependent on the newly emerged myeloid cells that are associated with developing tumors, the activity of M-CSF was tested using splenocytes from one-week tumor-bearing mice. High levels of IFN-γ were detected at this stage without additional stimulation indicating that T cells are already activated. However, M-CSF showed no suppressive activity (Fig. 5A).

The number of emerging myeloid cells in a spleen one-week after inoculation is still low and could explain the lack of M-CSF activity in this condition. The hypothesis was then tested by determining M-CSF activity in a co-culture system using T cells isolated from tumor-bearing mice and T cell-depleted splenocytes from normal mice. Significant differences were observed in the co-culture of T cells isolated from tumor-bearing mice with T cell-depleted splenocytes from normal mice compared to that of co-cultured with T cell-depleted splenocytes from tumor-bearing mice both at 10 and 100 ng/ml M-CSF (Fig. 5B).

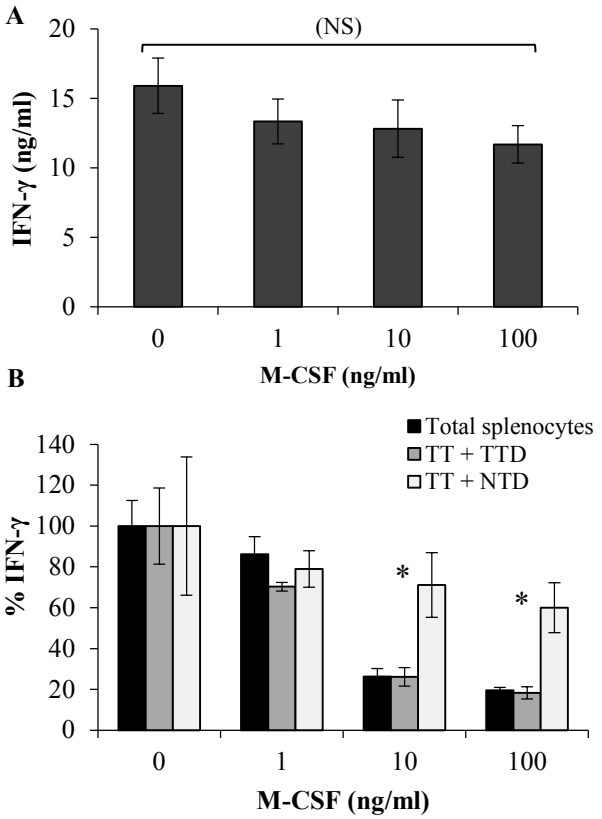

Fig. 5: M-CSF activity is mediated by emerging myeloid cells. A) M-CSF activity using splenocytes from one-week tumor-bearing mice. B) M-CSF activity in a co-culture system. TT, T cells from tumor-bearing mice; TTD, T cell-depleted splenocytes from 2-week tumor-bearing mice; NTD, T cell-depleted splenocytes from normal mice; NS, no significance. *$p < 0.05$.

The results confirmed that indeed the emerged myeloid cells during tumor progression are the target cells mediating the immunosuppressive activity of M-CSF. Myeloid cells emerging during tumor growth have been shown to suppress T cell activity through several mechanisms. These mechanisms include but are not limited to depletion of arginine through the activity of arginase 1 and nitric oxide synthase enzymes[18], depletion of tryptophan by indoleamine 2,3-dioxygenase activity[19] and generation of reactive oxygen species[17]. Although the target cells of M-CSF are yet to be identified, it is speculated that some of these mechanisms could be involved in the observed M-CSF suppressive activity.

4. Conclusion

This research investigated the suppressive activity of

M-CSF as one of the soluble factors secreted by 4T1 tumor cells. We showed that M-CSF suppresses IFN-γ secretion but not its protein synthesis in a dose-dependent manner. The suppressive activity was evident at 36 hours after incubation, and the suppressive effect was not relieved after M-CSF withdrawal. The suppressive activity was dependent on the days after tumor inoculation, which may correlate with an increase in the number of myeloid cells in the spleen during tumor growth. In this report, M-CSF target cells have not been identified, and, therefore, further studies are needed to determine the specific cells for the observed M-CSF-mediated suppressive activities and elucidate the mechanism of the action.

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