

## Up-Regulation of Cluster of Differentiation (CD) 11b Expression on the Surface of Canine Granulocytes with Human Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)

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**ABSTRACT.** Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a pleiotropic cytokine, sharing a common beta subunit (CDw131) with interleukins 3 and 5. GM-CSF is important for its direct and indirect involvement in host defense. In veterinary medicine, human (h) GM-CSF has been used as a substitute for canine GM-CSF to stimulate canine granulocytes and macrophages. In this study, we compared the effects of three distinct hGM-CSFs produced by bacteria, yeasts and Chinese hamster ovary (CHO) cells with those of *Escherichia* (E) *coli*-produced canine GM-CSF on the cluster of differentiation 11b (CD11b) expression in canine granulocytes. The median effective dose (ED<sub>50</sub>) of hGM-CSFs from bacteria, yeasts and CHO cells was 3.09, 4.09 and 4.27 ng/ml, respectively, with no significant difference among three. In contrast, a significant difference was observed between ED<sub>50</sub> of canine GM-CSF (0.56 ng/ml) and three hGM-CSFs according to the paired *t*-test (*P*<0.05). We conclude that hGM-CSF can activate canine granulocytes, but the average activity of the three rhGM-CSFs was approximately 15% of that of canine GM-CSF.

**KEY WORDS:** canine, CD11b, flow cytometry, granulocyte-macrophage colony-stimulating factor, median fluorescence intensity, xenostimulation  
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Human granulocyte-macrophage colony-stimulating factor (hGM-CSF) is a protein of 144 amino acids (AA), including the signal peptide of 18 AA, and is produced by various types of cells. The protein is monomeric, but its active form basically takes a noncovalent homodimer in nature. Although GM-CSF is a major cytokine for hemopoiesis like granulocyte colony-stimulating factor, macrophage colony-stimulating factor and erythropoietin, the cytokine has been known to be involved in the enhancement of eosinophil chemotaxis [7], maturation of macrophages and dendritic cells [17], granulocyte activation [1], adjuvant effect [3] and inhibition of apoptosis [4].

Cluster of differentiation molecule 11b (CD11b), known as its integrin  $\alpha$  M subunit, consists of macrophage-1 antigen (Mac-1) with CD18. The molecule is expressed in many types of cells, and the CD11b expression on the surfaces of granulocytes and macrophages is increased by their activation, playing an important role in host defense. Mac-1 has been reported to support neutrophil immobilization and migration [6] and is also known as complement receptor 3 (CR3) that binds to iC3b, eliminating pathogens and

immune complexes by neutrophils, macrophages and the reticuloendothelial system. CD11b is rapidly elevated by the activation of neutrophils and macrophages, and the amount of CD11b in neutrophils correlates with their activation and inflammation [11].

Clinical trials of the adjuvant therapy and the prevention form leukocytopenia with GM-CSF in veterinary cancer medicine have been started, but the preparation of canine GM-CSF for clinical use is still unavailable. Thus, we just have to choose that of human GM-CSF (hGM-CSF) at the present time. Because hGM-CSF is active in canine cells, it has been empirically employed as a substitute for canine GM-CSF [18, 22]; however, its quantitative activity in canine cells has not been elucidated. Here, we compared the effects of hGM-CSF to those of canine GM-CSF in canine granulocytes and also measured the median effective doses (ED<sub>50</sub>) of three different rhGM-CSFs in canine granulocytes.

Anti-CD11b (M1/70) conjugated with allophycocyanin-Cy7, Gr-1 with allophycocyanin and anti-human CD14 with phycoerythrin were purchased from BioLegend Co., Ltd. (San Diego, CA, U.S.A.; provided by Tomy Digital, Tokyo, Japan). Molgramostim; *Escherichia* (E) *coli*-produced recombinant human GM-CSF (rhGM-CSF), sargramostim produced by yeasts and canine recombinant GM-CSF were obtained from Amoytop Biotech (Xiamen, Fujian, People's Republic of China), Genzyme corporation (Cambridge, MA, U.S.A.) and R&D systems (Minneapolis, MN, U.S.A.), respectively. JCR Pharmaceuticals Co., Ltd. (Akashi, Japan) donated rhGM-CSF produced by Chinese hamster ovary (CHO) cells.

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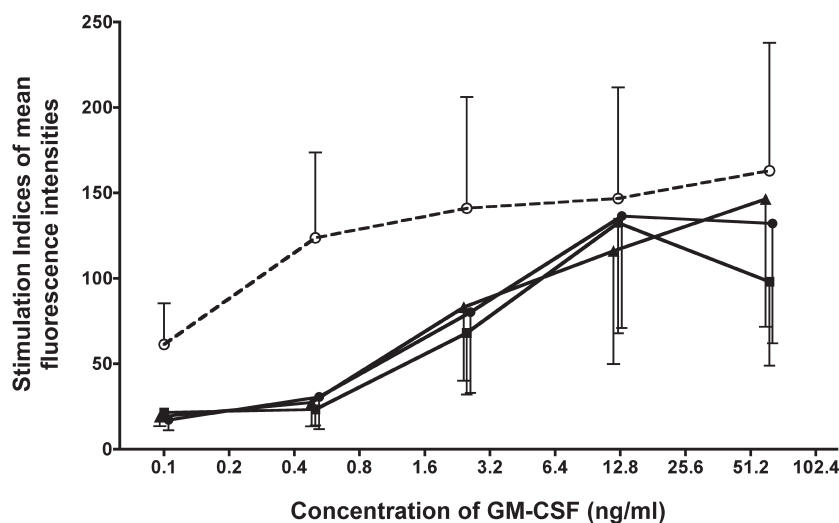


Fig. 1. Dose-response curves of CD11b expression with various granulocyte-macrophage colony-stimulating factors in canine granulocytes. Sigmoid curves represent dose-responses of molgramostim (solid circle), sargramostim (solid square), CHO-produced hGM-CSF (solid triangle) and canine GM-CSF (open circle and broken line). The points and bars show the average values and standard deviations from 4 animals, respectively. X and Y axes show the concentrations of GM-CSF (ng/ml) and stimulation indices, respectively.

Heparinized canine blood was obtained from 2 male and 2 female beagles for practical trainings of students at Nippon Veterinary and Life Science University. These beagles were individually housed, fed dog chows once a day and drank water *ad libitum*. These bloods were transported to our laboratory and processed at room temperature within 1 hr. Briefly, 100- $\mu$ l aliquots of the blood were aseptically placed in 2.0-ml sterile microtubes, to which various amounts of canine or hGM-CSF were added at final concentrations of 0.02–62.5 ng/ml or macrophage-serum free medium (macrophage-SFM; Invitrogen Corporation, Carlsbad, CA, U.S.A.) alone. Subsequently, all the samples were incubated for 15 min at a 37°C in a 5% CO<sub>2</sub> incubator without shaking.

After stimulation, antibody cocktail was added to each tube, which was then incubated for 30 min at 4°C. The blood was hemolyzed with 0.15 M ammonium chloride containing 1 mM KHCO<sub>3</sub> and 0.1 mM EDTA 4Na (pH 7.3), washed twice with flow cytometer buffer (PBS containing 2% BSA and 0.1% sodium azide) and then fixed in FluoroFix™ buffer (BioLegend), as per the manufacturer's instructions. The cells were re-suspended in 100  $\mu$ l flow cytometer buffer (PBS containing 2% BSA and 0.05% sodium azide). Data were acquired using FACSArray (BD Bioscience, San Jose, CA, U.S.A.), gating the granulocyte area on a forward vs. side scatter. The median fluorescence intensities (MFIs) of CD11b<sup>+</sup> population were obtained under the gate of granulocytes at SSC vs. FSC scatter and CD14<sup>+</sup>. The indices of MFIs were determined by dividing MFIs from GM-stimulated cultures by MFI from PBS-cultured granulocytes. ED<sub>50</sub>, determined from MFI values using the probit method, was statistically analyzed using paired *t*-tests at every GM-CSF dose.

Three hGM-CSFs revealed increased CD11b expression on canine granulocytes in a dose-dependent manner (Fig. 1). ED<sub>50</sub> of molgramostim, sargramostim and hGM-CSF from CHO cells was 3.09, 4.09 and 4.27 ng/ml, respectively; moreover, no significant difference was observed among these rhGM-CSFs (Table 1). In contrast, ED<sub>50</sub> of canine rGM-CSF was 0.56 ng/ml, which was significantly different from the three rhGM-CSFs according to the paired *t*-test results ( $P < 0.05$ ). Further, ED<sub>50</sub> of molgramostim, sargramostim and rhGM-CSF from the CHO cells was 18.1%, 13.7% and 13.1%, respectively, compared with the canine rGM-CSF for canine granulocytes.

GM-CSF is not only an important hemopoietic cytokine, but also involved in the upregulation of the immune system and host-defense [5, 19, 21], because immune cells express its receptor [10, 14]. In experiments using dogs, rhGM-CSF has been employed as a substitute for the canine reagent [2, 16].

GM-CSF activity is usually measured by the proliferation of cells that are GM-CSF-dependent; e.g. TF-1 for hGM-CSF [9]. The detection of augmented CD11b with GM-CSF is rapid and easy. CD11b expression on the surface of neutrophils has been reported to elevate by GM-CSF stimulation [12, 15]. Uchida *et al.* have reported that the quick elevation of CD11b expression on human neutrophils by GM-CSF stimulation was caused by its endogenous molecules but not de novo synthesis [20]. According to a modified Uchida method [20], we detected the activities of rhGM-CSFs in canine neutrophils in a dose-dependent manner. We conclude it may not be a problem to employ rhGM-CSF to canine experiment. This technique doesn't require any GM-CSF-dependent cell line and is applicable to every animal species.

Table 1. Median effective doses of various granulocyte-macrophage colony-stimulating factors in expression of CD11b in canine granulocytes

GM-CSF	ED <sub>50</sub> (ng/ml)	Specific activities (units/ $\mu$ g)	Relative activities (%) to canine GM-CSF in ED <sub>50</sub>
Molgramostim	3.09 $\pm$ 1.18 <sup>a</sup>	323.6	18.1
Sargramostim	4.09 $\pm$ 1.56 <sup>a</sup>	244.5	13.7
CHO hGM-CSF	4.27 $\pm$ 1.51 <sup>a</sup>	234.2	13.1
Canine GM-CSF	0.56 $\pm$ 0.46 <sup>a</sup>	1,785.7	—

a) Median effective dose (ED<sub>50</sub>) of canine granulocyte-macrophage colony-stimulating factor (GM-CSF) significantly differed from those of hGM-CSFs according to the paired *t*-test results (*P*<0.05).

Furthermore, it has been reported that some mouse cells are not stimulated by hGM-CSF. However, McClure *et al.* proved that rhGM-CSF activated the BaF-B03 mouse cell line transfected with human GM-CSF receptor  $\alpha$  subunit gene [13]. The intracytoplasmic region of the subunit did not participate in the signal transduction [23], which suggests that the  $\alpha$  subunit plays an important role in binding species-specifically to GM-CSF. Therefore, the  $\alpha$  subunit of canine GM-CSF may have an effective affinity to rhGM-CSF, although rhGM-CSFs had a weaker impact on canine granulocytes compared with canine rGM-CSF in this study. Therefore, to obtain an effect equivalent to an expected activity in dogs with hGM-CSF, we must employ an approximately septuplet dose of rhGM-CSF (Table 1). Nevertheless, this indicates that rhGM-CSF can be a valuable tool for a canine study.

In addition, we also compared GM-CSFs from three different sources: *E. coli*, yeasts and CHO cells; although no significant difference was determined in ED<sub>50</sub> for the three sources, *E. coli*-produced rhGM-CSF (molgramostim) revealed the highest activity. Moreover, Kelleher *et al.* determined that *E. coli*-produced hGM-CSF had higher efficacy with regard to the proliferation of TF-1 cells compared with that of CHO protein [8]. Although we are not able to explain why molgramostim exhibited the highest activity in our study, Kelleher *et al.* suggested that the difference was the result of the higher affinity of *E. coli* protein [8]. Molgramostim is not much different from the other two types investigated without their glycosylation, which may be involved in their 3-D conformation and homodimer formation and/or interfere with their interactions with GM-CSFR, affecting GM-CSF activity. Thus, the differences in glycosylation may be responsible for their varied activities.

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