Degalactosylated/Desialylated Human Serum Containing GcMAF Induces Macrophage Phagocytic Activity and

In Vivo Antitumor Activity

（GcMAF 含有ヒト血清のマクロファージ貪食活性
化能評価方法の開発）

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Chapter 1 : Introductory chapter

Macrophages are known to have a critical role in antitumor immunity, can infiltrate into tumors and are found in most tumor sites. Macrophage also has a function as an effector of cell immunity via presentation of an antigen to T cell and production of interleukin 1. Accordingly, it is important to activate macrophage for treatment and prevention of a cancer and an infectious disease, and the activation of macrophage makes it possible to carry out treatment and prevention of a cancer and an infectious disease.

A factor for activating macrophage is, for example, an interferon, and its clinical application has been carried out. In addition, it is known that a certain kind of polysaccharides has an immunostimulating activity, and some of them are expected to be developed as an antiviral agent and an anticancer agent.

The group-specific component (Gc) protein, also known as vitamin D-binding protein (DBP) or Gc globulin, is a 53-kDa human plasma glycoprotein (1). Inflammation results in the hydrolysis of terminal galactose and sialic acid of the Gc protein and this is mediated by membrane-bound-galactosidase present on the activated B cell and sialidase on T cell to produce Gc protein-derived macrophage-activating factor (GcMAF) (2). GcMAF has been shown to possess several biological activities, such as

macrophage activation via superoxide generation (3, 4) and phagocytic activation (5), anti-angiogenesis effect (6, 7), and antitumor activities (8-10). Moreover, it has been demonstrated that GcMAF administration has clinical benefits in patients with metastatic colorectal, metastatic breast, and prostate cancers, and nonanemic HIV-infected patients, (11-14).

However, a major problem associated with the clinical use of purified GcMAF. In previous studies, GcMAF was purified from human serum using an affinity column modified with 25-hydroxy-vitamin D_3 (15). However, it is difficult to prevent contamination when a column is repeatedly used. Additionally, purified GcMAF is unstable in the presence of oxygen at room temperature and in the absence of antioxidants such as albumin and uric acid that abundantly occur in blood (16). To overcome problems associated with GcMAF purification, we prepared unpurified GcMAF by using autologous serum as a source. GcMAF-containing human serum demonstrates remarkable clinical effects. Saisei Mirai will have treated more than 1,000 patients with GcMAF-containing human serum, both with and without conventional therapies (17, 18).

Thus, we propose a novel method for preparation of autologous serum containing GcMAF by degalactosylation and desialylation and report the potential role of

autologous GcMAF in stimulating phagocytosis in macrophages and *in vivo* antitumor

activity.

Colostrum is a type of milk produced by the mammary glands of mammals just prior to

giving birth. It contains serum proteins and antibodies such as albumin, insulin-like

growth factor (IGF), epidermal growth factor (EGF), nerve growth factor (NGF),

lactoferrin, immunoglobulin G (IgG), immunoglobulin A (IgA), and immunoglobulin M

(IgM) to protect the newborn against various infectious diseases; however, it contains

lower amounts of carbohydrates and lipids than does mature milk (19). IgA, in

particular, is known to protect against various kinds of infection, and to interact with an

Fc receptor called FcαRI (or CD89) to initiate inflammatory reactions (20). In addition,

IgA has an *O*-linked sugar chain, and the binding property for the Fc receptor decreases

if there are many sialic acid residues (21). It has been reported that the number of

GalNAc attached to IgA *O*-linked glycans was significantly decreased in patients with

Crohn's disease and was strongly correlated with clinical activity (22).

 Gc protein has an *O*-linked sugar chain and produce GcMAF with an

N-acetylgalactosamine (GalNAc) moiety (2).

We hypothesized that colostrum could be a macrophage-activator if enzymatically

modified IgA and Gc protein had activity similar to that of GcMAF. Therefore, we

propose a novel macrophage-activator function for degalactosylated/desialylated bovine

colostrum and report the potential role of this modified bovine colostrum in stimulating

phagocytosis in macrophages *in vitro* and *in vivo*.

Chapter 2 : Materials and Methods

Preparation of GcMAF-containing human serum.

Gc protein and GcMAF were prepared as previously reported by Uto *et al*. (23, 24).

Human serum containing 1f1f-subtype of Gc protein (100 µL, 100 µg/µL) was obtained

from human volunteers and each serum sample was incubated with 65 mU of

β-D-galactosidase (from *Escherichia coli*; WAKO) and neuraminidase (sialidase, from

Clostridium perfringens; SIGMA) in 100 mM sodium phosphate buffer (pH 7.0) at

37°C for 3 h. The reaction mixture was heated at 60°C for 10 min and the solution was

concentrated using a microcon-concentration unit (10,000 MWCO; Millipore

Corporation, Billerica, MA, USA). The protein concentrations were determined using a

BCA protein assay kit (Rockford, IL, USA).

And, GcMAF is also prepared from the serum of the cancer patient as described above.

Preparation of degalactosylated/desialylated bovine colostrum.

Bovine colostrum was obtained from Jun Sei Co. Ltd. (Tokyo, Japan). One milligram of

bovine colostrum powder was dissolved in 1 mL of 50 mM sodium phosphate buffer

(pH 7.0) and incubated with 65 mU of β-D-galactosidase (from *Escherichia coli*;

WAKO) either with or without 65 mU of neuraminidase (sialidase, from *Clostridium*

perfringens; SIGMA-ALDRICH) at 37°C for 1 h. The reaction mixture was then heated

at 60°C for 10 min to deactivate the enzymes. The protein concentrations were

determined using a BCA protein assay kit (Rockford, IL, USA).

Analysis of the constituent of human serum and bovine colostrum

The content of total protein, albumin, immunoglobulin G, immunoglobulin A and

immunoglobulin M and the ratio of albumin: globulin in human serum or bovine

colostrum was measured in BML.

SDS-PAGE and western blotting.

The GcMAF-containing human serum and degalactosylated/desialylated bovine

colostrum were subjected to sodium dodecyl-polyacrylamide gel electrophoresis

(SDS-PAGE; XV PANTERA GEL MP, 7.5–15%; DRC Co., Ltd., Tokyo, Japan) under

reducing conditions and subsequently electroblotted onto a nitrocellulose membrane.

Non-specific binding was blocked by overnight incubation in Tris-buffered saline (pH

7.4) containing 0.1% Tween 20 and 1% BSA at 4°C. The membranes were then probed

with anti-human Gc globulin (DakoCytomation Japan) and with biotin-conjugated *Helix*

pomatia agglutinin (HPA) lectin (Sigma-Aldrich, St. Louis, MO,USA)specific for

GcMAF with *N*-acetylgalactosamine (GalNAc) moiety. After membrane washing, the

blots were incubated with horseradish peroxidase (HRP)-labeled anti-rabbit IgG (GE

Healthcare Life Sciences, Uppsala, Sweden) as a secondary antibody. The blots were

developed using an ECL western blotting detection system (GE Healthcare). The

visualization and quantification of the western blot bands was achieved using an ECL

western blotting detection system (GE Healthcare), a LumiCube chemiluminescence

analyzer, and JustTLC image analysis software (Liponics, Tokyo, Japan).

Isolation and culture of mouse peritoneal macrophages.

Mouse peritoneal adherent cells containing macrophages were collected from

8-week-old female ICR mice (Japan SLC, Hamamatsu, Japan), as previously reported

by Uto *et al*. (23, 24) and cultured in 24-well plates at a density of 5×10^5 cells/well in

serum-free RPMI 1640 (Life Technologies, Carlsbad, CA, USA) for 1 h. The cultured

cells were then washed 3 times with serum-free RPMI 1640 to separate adherent

macrophages from non-adherent cells such as T- and B-cells. The cultured macrophages were treated with various concentrations of serum proteins for 15 h and used for *in vitro* phagocytosis assay as described below.

In vitro phagocytosis assay.

Mouse peritoneal macrophages were layered onto coverslips in a 24-well plate. After 3 h of human serum treatment, the cultures were assayed for phagocytic activity. Sheep red blood cells (SRBCs; Nippon Bio-Supp. Center, Tokyo, Japan) were opsonized by rabbit hemolytic serum (anti-sheep red blood cells, Cosmo Bio Co., Tokyo, Japan). Opsonized SRBCs (0.5%) in serum-free RPMI 1640 were overlaid onto each macrophage-coated coverslip and cultured for 1.5 h. The non-internalized erythrocytes were lysed by immersing the coverslip into a hypotonic solution (1/5-diluted phosphate-buffered saline). The macrophages were fixed with methanol, air-dried, and stained with Giemsa stain. The number of phagocytosed erythrocytes per cell was determined microscopically; 250 macrophages were counted for each data point. The data were expressed in terms of the phagocytosis index (PI), which was defined as the percentage of macrophages with ingested erythrocytes multiplied by the mean number

of erythrocytes ingested per macrophage.

In vivo antitumor activity assay.

ICR female mice weighing 20–25 g were housed in polycarbonate cages under standard laboratory conditions (24 ± 1°C, 12 h light/dark cycle) with food and water *ad libitum*. All procedures used for animal experimentation were approved by the Animal Research Committee of the University of Tokushima (TokuDobutsu12025). The Ehrlich ascites carcinoma (EAC) cells were obtained from Cancer Research Institute of Kanazawa University and were maintained in the peritoneal cavity of mice by injecting 0.1 mL of ascitic fluid every 7 days. Ascitic tumor cell counts were done in a Neubauer hemocytometer using the trypan blue dye exclusion method. Tumor cell suspensions were prepared in phosphate buffer saline (PBS).

Ten-week old female ICR mice were inoculated with 1×10^7 EAC cells (0.2 mL/mouse) intraperitoneally (i.p.). The day of tumor implantation was assigned as day '0'. On day 1, the animals were randomized and divided into 5 groups (n = 5) and administered i.p. with PBS, GcMAF (40 ng/kg/d), or GcMAF-containing human serum (172, 517, and 1,552 μg protein/kg/d) for 7 days. The animals with ascitic tumor were weighed every

day. Kaplan-Meier survival curves were analyzed by the log-rank test.

In vivo phagocytosis assay.

Seven-week-old female C57BL/6 mice under anesthesia were injected with 300 μL of degalactosylated/desialylated bovine colostrum directly into the small intestine. After 1 h, 300 μL of AF488-labeled ovalbumin (OVA) protein (Invitrogen, Tokyo, Japan) was injected into the small intestine. After 1 h, mice were sacrificed, and the small intestine was extracted. Fat and a Peyer's patch were removed, washed by PBS, and then stirred at 37°C in 20 mL of FACS buffer for 20 min. Collagenase (Roche Diagnostics K.K., Tokyo, Japan) was also added and then cut off it. After stirring at 37°C for 1 h, EDTA was added and stirred for an additional 5 min. The supernatant was filtered with a cell strainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and the residue was suspended in 10 mL of FACS buffer. The supernatant was removed by centrifugation. The cell pellet was suspended in 10 mL of 40% percoll (GE Healthcare), followed by addition of 5 mL of 75% percoll to the bottom and centrifugation. After the supernatant was removed, the residue was suspended in 9 mL of FACS buffer and then centrifuged again. After the supernatant was removed, anti-mouse F4/80,

anti-mouse/human CD11b, and anti-mouse/human CD16/32 (BioLegend, Inc., San

Diego, CA, USA) antibodies were added and reacted for 15 min. After the supernatant

was removed, wash buffer was added and the mixture was subjected to a final

centrifugation. 7-Aminoactinomycin D (Sigma-Aldrich) was added and analyzed by

FACSCantoII (Becton, Dickinson and Company).

Flow cytometry assay.

Mouse peritoneal macrophages were cultured in 24-well plates at a density of 5×10^5

cells/well in serum-free RPMI 1640 for 15 h. The cultured cells were washed two times

with serum-free RPMI 1640 and then treated with degalactosylated/desialylated bovine

colostrum for 24 h. The supernatant (50 μL) was added to a mixture of capture bead

diluent (48 μL), mouse IL-1β capture bead E5 (1 μL), and mouse TNF-α capture bead

C8 (1 μL) (Becton, Dickinson and Company). After the solution containing beads were

incubated at room temperature for 1 h, the mixture of capture bead diluent (48 μL),

mouse IL-1β PE detection reagent (1 μL), and mouse TNF-α PE detection reagent (1 μL)

was added. The solution containing beads were incubated at room temperature for 1 h,

the beads were washed with 1 mL of wash buffer (Becton, Dickinson and Company)

and centrifuged at 200 g for 5 min. The supernatant was removed and then 300 μL of

wash buffer was added and analyzed using FACSVerse.

Statistical analysis.

Data are expressed as mean and standard deviation. The statistical significance of the

differences between the results of the independent experiments was analyzed using

Student's t test. A P value of <0.05 was considered statistically significant.

Chapter 3 : Results

Preparation and identification of GcMAF-containing human serum.

We obtained 8.8 mg of serum protein from 100 µL of healthy human serum by

degalactosylation/desialylation and concentration using a microcon centrifugal filter.

Figure 1A shows the western blot of the degalactosylated/desialylated human serum.

Four bands (70 kDa, 55 kDa, 51 kDa, 17 kDa) were detected on the blot membranes by

using an HPA lectin, which recognizes GalNAc moiety. Since the 55-kDa protein band

was specifically detected by anti-human Gc globulin, it was confirmed that GcMAF was

present in the degalactosylated/desialylated human serum. The signal intensity of the

55-kDa protein was significantly higher than that of the control serum as shown in

Figure 1B. Therefore, it was clearly demonstrated that degalactosylation/desialylation of

human serum is a useful method to produce GcMAF.

Stimulating activity of GcMAF-containing human serum on phagocytic activity of

mouse peritoneal macrophages.

We examined phagocytic activation by using GcMAF-containing human serum against

mouse peritoneal adherent cells containing macrophages. Figure 2A shows significant

phagocytic activation with 1 to 1000 ng of GcMAF-containing human serum as

compared with that by the control. The maximum phagocytic index (PI) of 1.73 for 10

ng of GcMAF-containing human serum corresponded to that of10 ng of GcMAF (PI =

1.59). No phagocytic activation was shown in peritoneal macrophages incubated with

non-treated human serum (Figure 2B).

And, we prepared GcMAF from the serum of the cancer patient. Figure 3 shows

comparison of phagocytic activation with GcMAF from cancer patient's serum and

healthy people serum compared with that by the control. GcMAF prepared from the

serum of the cancer patient shows high activity as GcMAF prepared form the serum of

healthy people.

In vivo antitumor activity of GcMAF-containing human serum in EAC model.

Figure 4 shows the antitumor activity of GcMAF-containing human serum against EAC

inoculated i.p. into mice. Treatment of EAC-bearing mice with 1,552 μg protein/kg

dosage of GcMAF-containing human serum resulted in significant prolongation of life

span, and the T/C (%) value was 138% compared to that of the EAC-bearing control

group.

Analysis of the constituent of human serum and bovine colostrum

Figure 5 shows the content of total protein, albumin, immunoglobulin G (IgG),

immunoglobulin A (IgA), and immunoglobulin M (IgM) and the ratio of albumin:

globulin in three kind of each human serum or bovine colostrum.

Preparation and identification of degalactosylated/desialylated bovine colostrum.

We first checked the digestion activity of the *O*-linked sugar chain of the glycoprotein

included in the bovine colostrum. Figure 6A shows the Coomassie Brilliant Blue (CBB)

stain and Figure 6B shows the western blot of the bovine colostrum (lane 1),

degalactosylated bovine colostrum (lane 2), and degalactosylated/desialylated bovine

colostrum (lane 3). Five bands (180, 90, 75, 63, 28 kDa) were detected on the CBB stain,

but only three bands (180, 75, 28 kDa) were detected by using an HPA lectin, which

recognizes the GalNAc moiety.

Stimulating activity of degalactosylated/desialylated bovine colostrum on phagocytic activity of mouse peritoneal macrophages.

We examined phagocytic activation by using degalactosylated and degalactosylated/desialylated bovine colostrum against mouse peritoneal macrophages. Figure 7 shows significant phagocytic activation with 10 ng of degalactosylated and degalactosylated/desialylated bovine colostrum, as compared with that observed with the control. Degalactosylated/desialylated bovine colostrum (PI = 1.80) showed significantly more potent phagocytic activation than did degalactosylated bovine colostrum (PI = 1.64). Ten nanograms of non-treated bovine colostrum showed significant phagocytic activation as compared with that observed with the control; however, its activity was relatively weak (PI = 1.23).

Stimulating activity of degalactosylated/desialylated bovine colostrum on the phagocytic activity of mouse intestinal macrophages.

We examined *in vivo* phagocytic activation of degalactosylated/desialylated bovine colostrum against mouse intestinal macrophages. In Figure 8, 1 mg/kg of degalactosylated/desialylated bovine colostrum exhibited a proportion of phagocytic

macrophage number (C; 25.5%) that was higher than those exhibited by non-treated

bovine colostrum (B; 4.5%) and GcMAF-containing human serum (D; 7.2%), and the

value was equal to the lipopolysaccharide (LPS) (A; 26.9%) of the positive control.

Stimulating activity of degalactosylated/desialylated bovine colostrum on induction of

inflammatory cytokines.

We checked whether inflammatory cytokines were stimulated in phagocytic activated

macrophages by treatment with degalactosylated/desialylated bovine colostrum. As seen

in Figure 9, neither IL-1β(A) nor TNF-α (B) was produced as a major inflammatory

cytokine from mouse peritoneal macrophages stimulated with

degalactosylated/desialylated bovine colostrum, similar to that observed with

GcMAF-containing human serum.

Chapter 4 : Discussion

We prepared GcMAF-containing human serum by treating human serum with

β-galactosidase and sialidase and then evaluated GcMAF-containing serum for its

ability to activate peritoneal macrophage phagocytosis and its *in vivo* antitumor activity.

The western blot analysis with HPA lectin showed 4 positive bands, including a GcMAF

positive band. On the basis of the molecular weights of the other 3 bands (70 kDa, 51

kDa, 17 kDa), we speculated that the bands may be serum glycoproteins

α1T-glycoprotein (75 kDa), immunoglobulin (Ig) heavy chain (50–70 kDa), and

α1-antitrypsin (51 kDa). However, it has been reported that α1T-glycoprotein has no

GalNAc moiety (25). Therefore, the 70-kDa band may be IgA or IgD heavy chain with

O-linked oligosaccharides that consist of GalNAc, galactose, and sialic acid with the

same sugar chain composition as Gc protein. IgA is the most common immunoglobulin

in the blood, and the plasma of a healthy adult contains about 90–400 mg/dL of

immunoglobulins. Additionally, plasma contains about 3–5 mg/dL of Gc protein.

FcαRI (or CD89), the Fc receptor for IgA, is expressed on immune effector cells and it

initiates inflammatory reactions (26, 27). Additionally, it has been demonstrated that the

number of GalNAc attached to IgA *O*-linked glycans was significantly decreased in

patients with Crohn's disease and was strongly correlated with clinical activity (28).

These data suggest that GcMAF as well as IgA are activated by GalNAc that is presented by β-galactosidase and sialidase treatment. If this hypothesis is correct, it may explain the mechanisms by which 10 ng of GcMAF-containing serum protein induced phagocytic activation comparable to that of 10 ng of purified GcMAF.

Also of interest to us is IgA deficiency, which is the most common immune deficiency disorder (29). People with this disorder have low or absent levels of IgA which affects the proper functioning of the immune system and increases episodes of bronchitis, conjunctivitis (eye infection), gastrointestinal inflammation including ulcerative colitis, Crohn's disease and upper respiratory tract infections (29, 30). Whether or not IgA deficiency has implications for endogenous GcMAF levels has yet to be elucidated. In addition, exogenous GcMAF treatment for people with selective IgA deficiency disorder is also of interest. Further investigation is required to elucidate whether active glycoproteins, other than GcMAF, are present in human serum.

Taking into account the real clinical application with GcMAF-containing human serum, the exogeneous preparation of GcMAF artificially is used with a sample of the patient's serum or serum from his or her close relatives. Because treatment using GcMAF must pay attention to each patient's immune specificity, possibly caused by differences of Gc protein types, GcMAF level, and immune state of each subject.

GcMAF prepared from the serum of the cancer patient shows the high phagocytic activity. Accordingly, we propose GcMAF-based immunotherapy considering the patient's individual immune status.

We also evaluated degalactosylated/desialylated bovine colostrum for its ability to activate mouse peritoneal and intestinal macrophage phagocytosis *in vitro* and *in vivo*.

The colostrum contains serum proteins and antibodies such as albumin, immunoglobulin G (IgG), immunoglobulin A (IgA), and immunoglobulin M (IgM).

Therefore it is suggested that colostrum could be a macrophage-activator similar to that of GcMAF containing human serum.

Three HPA-positive bands of the degalactosylated/desialylated bovine colostrum are shown in Figure 6B, and it was suggested that the band of 75 kDa corresponds to glycoprotein-a constituting IgA and the band of 28 kDa corresponds to DBP (31, 32). IgA and DBP has an *O*-linked sugar chain corresponding to the higher HPA-positive band, present when treated with β-galactosidase and sialidase, as compared to when treated with β-galactosidase alone. This result correlated to potency of the macrophage phagocytic activity of degalactosylated or degalactosylated/desialylated bovine colostrum in Figure 7. Degalactosylated/desialylated bovine colostrum also activated

intestinal macrophages *in vivo* (Figure 8); however, it is generally believed that it does not undergo intestinal absorption when the molecular weight of the material is greater than 500 Da. In contrast, it is reported that some peptides of relatively high molecular weight (~15,000 Da) could be absorbed in the mouse intestinal tract (33). Therefore, it is suggested that even a glycoprotein having a high molecular weight included in the bovine colostrum can be absorbed. Unlike LPS and interferon γ (IFN-γ), the degalactosylated/desialylated bovine colostrum does not induce production of inflammatory cytokines such as TNF-α and IL-1β as shown in Figure 9. We believe that this is a positive result indicating an association between inflammatory cytokines and the risk of autoimmune disease. Degalactosylated/desialylated bovine colostrum can become an effective therapeutic agent for autoimmune disease if it can suppress the production of inflammatory cytokines. In particular, it has been recently reported that the etiology of the autoimmune condition inflammatory bowel disease (IBD) involves Th17 cells that mediate the production of inflammatory cytokines (34). We will continue to investigate the relationships of various disease conditions with the macrophage activation mechanism of degalactosylated/desialylated bovine colostrum in the future.

In conclusion, we propose that GcMAF-containing human serum and

degalactosylated/desialylated bovine colostrum can be used as an effective phagocytosis

activator for macrophages that does not induce inflammatory cytokines and antitumor

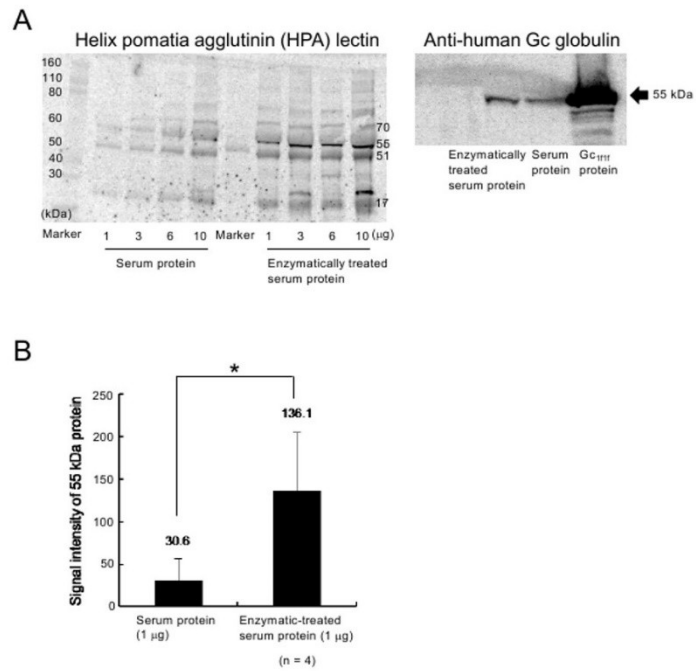agent for cancer immunotherapy.

Figure legends

Figure 1. Analysis of group-specific component protein-derived macrophage-activating factor (GcMAF) generated by the enzymatic treatment of human serum. (A) Western blots probed with anti-human Gc globulin and *Helix pomatia* agglutinin (HPA) lectin. (B) Quantification of the signal intensity of the detected protein bands was performed using JustTLC image analysis software.
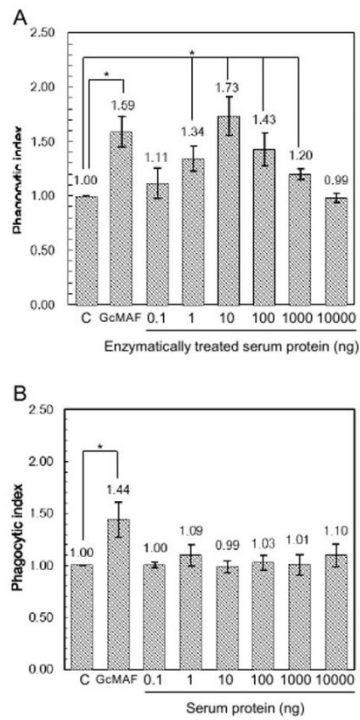
Figure 2. Phagocytic activity of mouse peritoneal macrophages observed using enzymatically treated human serum protein (A) or serum protein (B). All experiments were performed in triplicates. Each error bar represents the standard deviation. Number on each bar indicates the mean value. $*P < 0.05$.
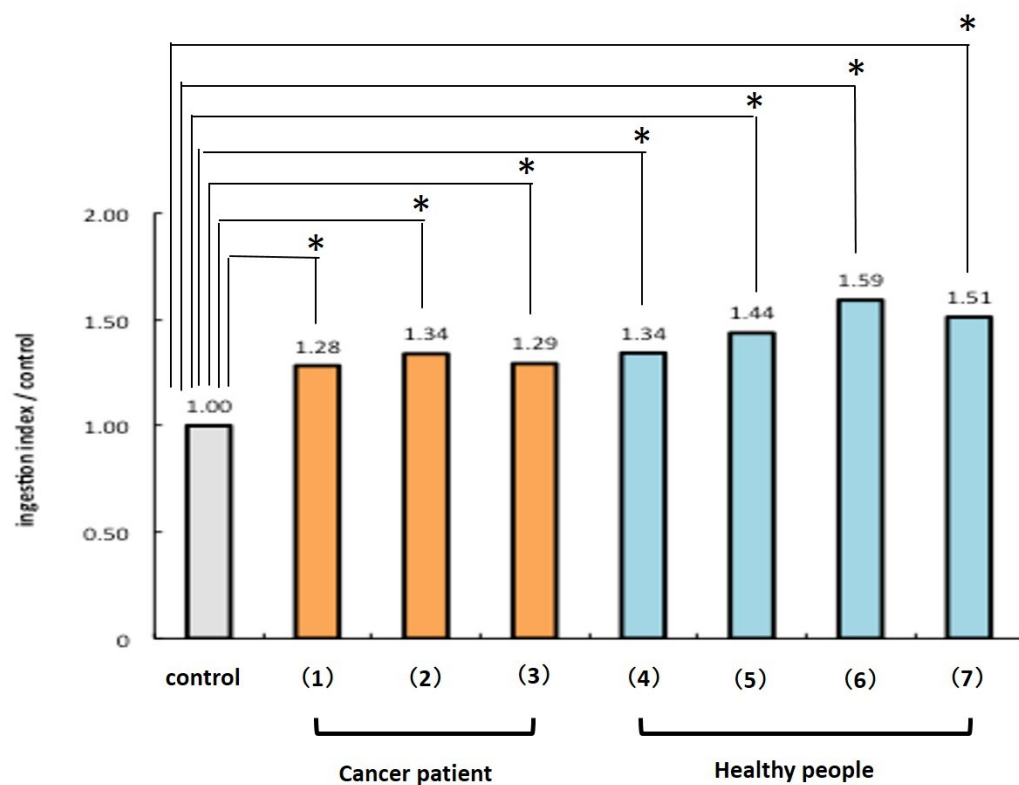
Figure 3. Comparison of phagocytic activity of GcMAF from cancer patient's serum (orange bar: sample 1 to 3) and healthy people serum (blue bar: sample 4 to 7). All experiments were performed in triplicates. Each error bar represents the standard deviation. Number on each bar indicates the mean value. *$P < 0.05$.
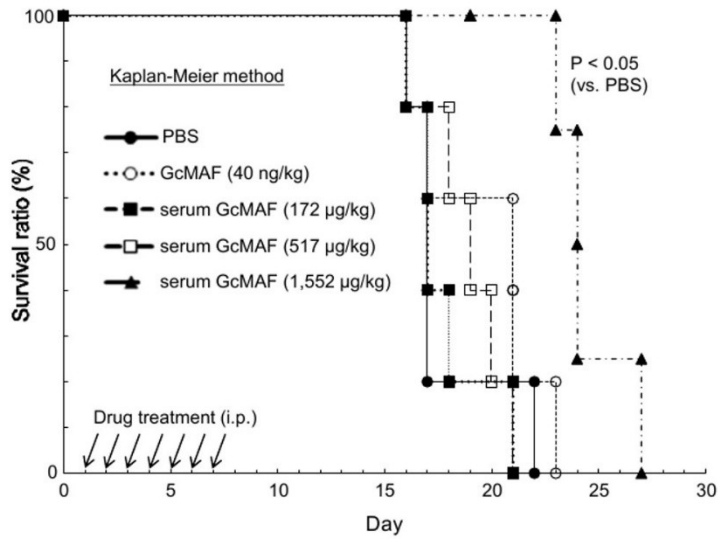
Figure 4. *In vivo* antitumor activity assessed using enzymatically treated human serum

protein and GcMAF. All experiments were performed in triplicates. Each error bar

represents the standard deviation. Number on each bar indicates mean value. *$P < 0.05$.

	Units	Range	初乳 ①	初乳 ②	初乳 ③	血清 ①	血清 ②	血清 ③
TP	g/dl	6.5 - 8.2	6.5	4.5	4.1	6.2	5.8	6.2
Alb	g/dl	3.7 - 5.5	2.0	1.4	0.6	3.8	3.7	4.0
A/G		1.55 - 2.55	0.44	0.45	0.17	1.64	1.97	1.96
IgG	mg/dl	820 - 1740	871	456	924	1139	907	1019
IgA	mg/dl	90 - 400	42	36	132	281	171	97
IgM	mg/dl	31 - 200	27	21	259	115	136	62

Figure 5. The measurement of serum proteins and antibodies such as albumin,

immunoglobulin G (IgG), immunoglobulin A (IgA), and immunoglobulin M (IgM) in

human serum and bovine colostrum. The sample of three kinds of each was measured.
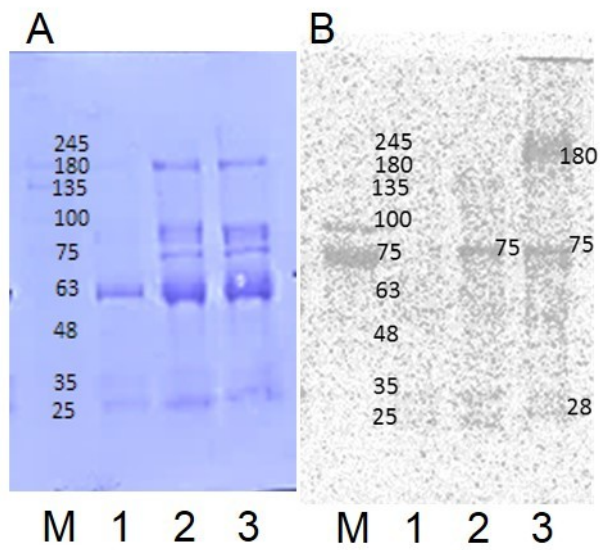
Figure 6. SDS-PAGE of degalactosylated and degalactosylated/desialylated bovine colostrum. (A) CBB-stain and (B) western blots probed with anti-human Gc globulin and *Helix pomatia* agglutinin (HPA) lectin. M: marker; lane 1: bovine colostrum; lane 2: degalactosylated bovine colostrum; lane 3: degalactosylated/desialylated bovine colostrum.
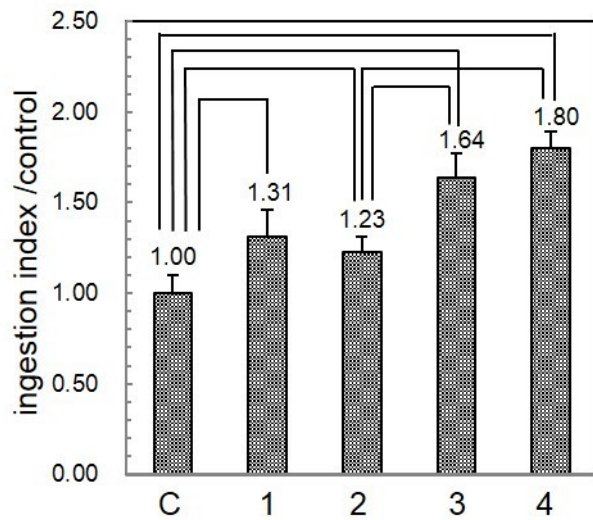
Figure 7. *In vitro* phagocytic activity of mouse peritoneal macrophages observed using degalactosylated/desialylated bovine colostrum and GcMAF-containing human serum. C: control; 1: 1 μg of LPS; 2: 10 ng of non-treated bovine colostrum; 3: 10 ng of degalactosylated bovine colostrum; 4: 10 ng of degalactosylated/desialylated bovine colostrum. All experiments were performed in triplicate. Each error bar represents the standard deviation. The number on each bar indicates the mean value. *$P < 0.05$.
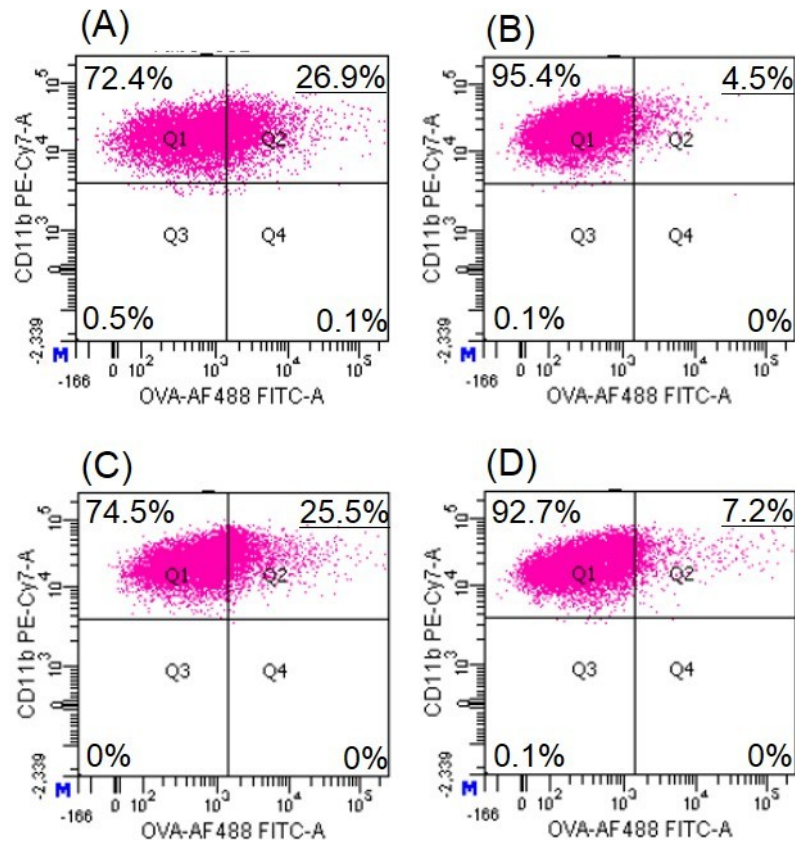
Figure 8. *In vivo* phagocytic activity of mouse intestinal macrophages observed using degalactosylated/desialylated bovine colostrum and GcMAF-containing human serum. The macrophage cells gated by expression of F4/80 and CD11b. Q2 area express the phagocytic macrophage. (A) 1 mg/kg of LPS; (B) 1 mg/kg of bovine colostrum; (C) 1 mg/kg of degalactosylated/desialylated bovine colostrum; (D) 1 mg/kg of GcMAF-containing human serum.
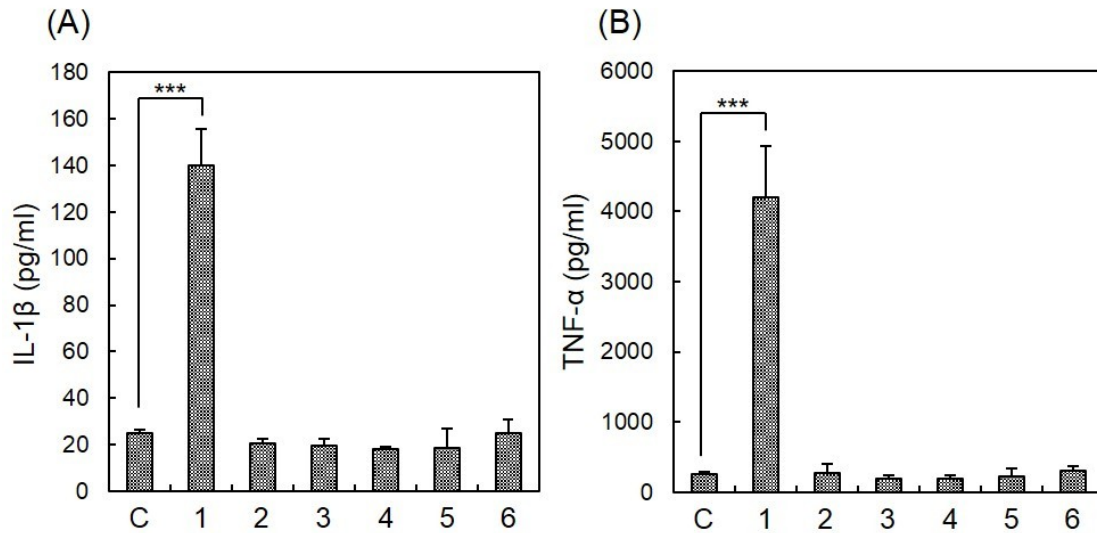
Figure 9. Induction of inflammatory cytokines by mouse peritoneal macrophage treated with degalactosylated/desialylated bovine colostrum or GcMAF-containing human serum. (A) IL-1β and (B) TNF-α. C: control; 1: 1 μg of LPS and 10 ng of interferon-γ; 2: 10 ng of non-treated bovine colostrum; 3: 10 ng of degalactosylated bovine colostrum; 4: 10 ng of degalactosylated/desialylated bovine colostrum; 5: 10 ng of non-treated human serum; 6: 10 ng of degalactosylated/desialylated human serum. All experiments were performed in triplicate. Each error bar represents the standard deviation. The number on each bar indicates mean value. ***$P < 0.005$.

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