Macrophages Exhibit a Large Repertoire of Activation States *via* Multiple Mechanisms of Macrophage-activating Factors

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Abstract. *Background/Aim: Macrophages are important components of human defense systems and consequently key to antitumor immunity. Human-serum macrophage activation factor (serum MAF) can activate macrophages, making it a promising reagent for anticancer therapy. Materials and Methods: We established four different macrophage subtypes through introduction of different culture conditions to THP-1- and U937-derived macrophages. We assessed phagocytic activity to understand subtype responses to typical macrophage activation factors (MAFs) and the activation mechanisms of serum MAF. Results: All four macrophage subtypes differed in their response to all MAFs. Moreover, serum MAF had two different activation mechanisms: N-acetylgalactosamine (GalNAc)-dependent and GalNAc-independent. Conclusion: Macrophage activation states and mechanisms are heterogeneous.*

Cancer immunotherapy involves activating the immune system to recognize and attack tumor cells. Among the approaches developed for this purpose, the administration of macrophage activation factors (MAFs) is a promising approach due to its patient friendliness (1). Two desirable candidates for MAF administration are Gc protein-derived macrophage activating factor (GcMAF) and serum MAF. The former is a human group-specific component (Gc) protein bearing an N-acetylgalactosamine (GalNAc) moiety, resulting from the treatment of Gc protein with β-galactosidase and neuraminidase (2), whereas the latter is β-galactosidase/neuraminidase-treated human serum and, thus, thought to contain GcMAF (3). Unfortunately, despite extensive clinical studies (4), the macrophage-activation mechanism of GcMAF is poorly understood.

However, recent years have seen substantial advancement in immunological studies of macrophages with many suggestions that mechanisms to activate macrophages are very heterogeneous, opposing the conventional concept of M1 and M2 binary activation. For example, a recent review recommended eight criteria for categorizing macrophage subtypes based on the activation mechanism (6). Moreover, pattern recognition receptors, such as Toll-like receptors 1 through 9 (TLR1 to TLR9), have specific subcellular localization to either cell or lysosomal membranes, as well as specific ligands, including microbial components, viral RNAs and CpG DNAs. Each localization and ligand category can be considered a distinct macrophage-activation mechanism (5).

We recently developed novel assays for investigating MAF activity, which contributes data towards an improved understanding of macrophage-activation mechanisms (7-9). In this study, we described differences in macrophage responses to typical MAFs. We also identified and characterized two activation pathways for serum MAF. Taken together, these results clearly demonstrated macrophage plasticity and variety in activation mechanisms.

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Materials and Methods

Cells and cell culture. The THP-1 and U937 cell lines (obtained from the RIKEN BRC through the National Bio-Resource Project of the MEXT (Tokyo, Japan) and Summit Pharmaceuticals (Tokyo, Japan), respectively) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 3% L-glutamine and 10% sodium hydrogen carbonate. Cells were cultured at 37˚C, in a 5% CO_2/95% air atmosphere that

Table I. *Phagocytic activities of four different types of macrophages.*

MAFs	Control†	MAF†	$p^{‡}$	MAFs	Control†	MAF†	$p^{‡}$
dTHP1				sTHP1			
serum MAF	51.5±3.0	61.5±2.2	0.020	serum MAF	61.8±4.5	75.1±3.9	0.035
IL-4	61.3±1.7	72.5±2.8	0.009	IL-4	61.8±4.5	63.8±4.9	0.703
IL-10	51.5±3.0	62.0±3.9	0.039	IL-10	61.8±4.5	62.5±1.4	0.860
LPS+IFN-γ	60.7±0.6	68.9±3.6	0.035	LPS+IFN-γ	60.7±4.4	61.8±2.2	0.756
dU937				sU937			
serum MAF	52.7±6.3	49.9±7.2	0.698	serum MAF	26.8±5.3	65.9±3.5	0.001
IL-4	52.7±6.3	49.2±5.1	0.571	IL-4	22.4±1.1	20.1±1.3	0.142
IL-10	52.7±6.3	50.2±7.7	0.737	IL-10	26.8±5.3	25.2±0.8	0.701
LPS+IFN-γ	57.1±4.0	50.8±4.4	0.209	LPS+IFN-γ	22.4±1.1	20.1±3.2	0.396

†Phagocytic activities with macrophage activation factor (MAF) or without MAF ("Control") were evaluated as the internalized beads ratio (IBR)±SD (n=3). ‡Probability value of the *t*-test.

was fully humidified. To induce differentiation into macrophage-like cells, we seeded THP-1 and U937 cells onto 35-mm culture dishes (5.0×10^5 cells/dish) and incubated them with 10 ng/ml 12-O-tetradecanoyl-13-acetate (TPA; Sigma-Aldrich, St. Louis, MO, USA) for 72 h and 24 h, respectively.

MAF Reagents. Human recombinant interferon (IFN)-γ, human recombinant interleukin (IL)-4 and human recombinant IL-10 were obtained from Wako (Osaka, Japan). Lipopolysaccharides (LPS) from Escherichia coli 0111:B4 were purchased from Sigma-Aldrich.

Establishment of differentiated macrophages. Although standard in cell-culture media, FBS can potentially trigger macrophage activation and is, thus, a confounding factor in this study. Therefore, we introduced a sensitization step to account for any potential effects from FBS. Sensitization consists of a 2-h incubation with FBS-free medium immediately before the phagocytosis assay. This length of time was enough to wash out non-specific adsorption of serum proteins on macrophage and substrate surfaces (data not shown). We were able to establish four types of differentiated macrophages; normally differentiated THP-1 (dTHP1) and U937 (dU937), as well as sensitized THP-1 (sTHP1) and U937 (sU937).

Phagocytosis assay. The media for macrophages were replaced with fresh medium containing MAF (5 μg/ml serum MAF, 20 ng/ml IL-4, 20 ng/ml IL-10, 1 μg/ml LPS + 5 ng/ml IFN-γ) and 90 μg of magnetic beads (Dynabeads® Protein G; Invitrogen, Oslo, Norway). Media differed depending on macrophage type: normally differentiated macrophages (dTHP1 and dU937) were cultured on FBS-supplemented medium, while sensitized macrophages (sTHP1 and sU937) were cultured on FBS-free RPMI-1640 medium. Control macrophages were added only magnetic beads in fresh medium, which fitted for each macrophage type, without MAFs. Macrophages were photographed under a bright field and phase contrast microscope at 30, 60 and 240 min after each MAF addition. All internalized and non-internalized beads were counted in these photographs. Phagocytic activities of macrophages were evaluated as the internalized beads ratio (IBR), calculated as follows:

IBR (%)=(number of internalized beads within the photograph)/(number of all beads within the photograph) ×100.

Inhibition of serum MAF activity. Serum MAF activity on dTHP1, sTHP1, dU937 and sU937 was analyzed after changing to fresh media depending on macrophage type (see "Phagocytosis assay"). All media contained 45 μg of magnetic beads, 5 μg/ml serum MAF and 10 mM of four monosaccharides that can potentially inhibit serum MAF: glucose, galactose, N-acetylglucosamine (GluNAc) and N-acetylgalactosamine (GalNAc). Control macrophages in this experiment were added only magnetic beads in fresh medium, which fitted for each macrophage type and did not contain serum MAF nor monosaccharide. Photographs were taken at 10, 30 and 60 min after each MAF addition. The activity of MAF was evaluated as the activation performance (AP) at 30 (dU937, sU937) and 60 (dTHP1, sTHP1) min after MAF addition, using this formula:

AP=(internalized beads ratio with MAF) -
(internalized beads ratio without MAF)

Immunoprecipitation and Western blotting. Using the polyclonal rabbit anti-Gc protein antibody (Dako, Tokyo, Japan), which was bound to 300 μg of Dynabeads, Gc protein was immunoprecipitated from human serum (untreated with β-galactosidase and neuraminidase) or serum MAF. Immunoadsorption (IA) was attained *via* three sequential immunoprecipitations. The efficiency of IA was estimated with Western blotting. The phagocytosis assay of these IA fractions was carried out in the same conditions as the monosaccharide experiment.

Statistical analysis. We tested for significant differences between macrophage phagocytic activities (*i.e.*, IBR) using both Student's unpaired *t*-tests and Mann-Whitney's *U*-test. The results obtained from both tests were the same in all analyses. All analyses were performed in Microsoft Excel. Significance was set at $p<0.05$; macrophages were considered activated by MAF if their phagocytic activity differed significantly from control macrophages (untreated with MAF).

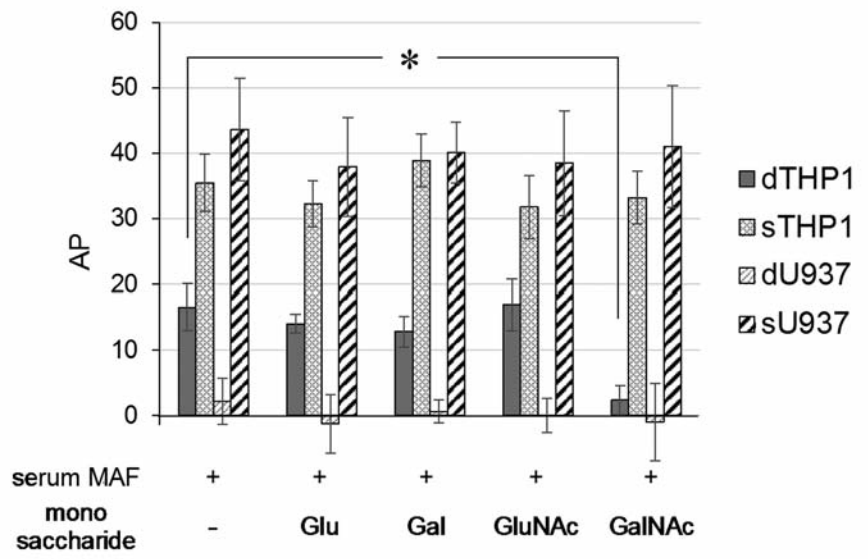

Figure 1. *Inhibitory effect of N-acetylgalactosamine (GalNAc) on serum macrophage activation factor (MAF) activation. Each monosaccharide (Glu: glucose, Gal: galactose, GluNAc: N-acetylglucosamine, GalNAc: N-acetylgalactosamine) was added with serum MAF to the culture medium of four macrophage subtypes: dTHP1, sTHP1, dU937 and sU937. The vertical axis represents activation performance (AP; difference between experimental and control (without serum MAF) internalized beads ratios (IBRs)). Error bars represent SDs (n=5 in sU937, n=3 in others). Only the difference between serum MAF and serum MAF with GalNAc was significant (*p=0.009).*

Results

Effect of FBS on macrophage activation. When we examined the control (FBS-supplemented and no-FBS) macrophages, THP-1-derived-macrophage phagocytic activity did differ significantly between FBS and no-FBS conditions (FBS: 57.8±4.9%, n=9; no-FBS: 61.2±4.5%, n=6; p=0.227 with t-test; Table I), whereas U937-derived-macrophages decreased phagocytic activity greatly in the no-FBS condition (from 54.2±6.1% to 25.4±5.3%; p=0.0008 with t-test, n=4; Table I). These results clearly demonstrated that dTHP1 and dU937 had different responses to sensitization and could be categorized as separate subtypes. The data also suggested that FBS in culture medium could influence phagocytic activity.

Effect of MAFs on four different macrophages. Macrophage responses to MAFs are summarized in Table I and exhibited clear variation across the subtypes, confirming that the four macrophages had distinct activation states. Serum MAF was able to activate dTHP1, sTHP1 and sU937, but not dU937. In the latter macrophage subtype, IBR levels were already very high in the control. Thus, we supposed that an activation threshold had been reached and serum MAF was unable to further activate dU937.

The addition of IL-4, IL-10 and LPS+IFN-γ failed to activate sTHP1, dU937 and sU937, whereas dTHP1 was activated by all aforementioned factors. Even though dTHP1 had high phagocytic activity, it still exhibited competence to all MAFs (including serum MAF).

Although the phagocytic activities of sTHP1 and sU937 were clearly different, IL-4, IL-10 and LPS+IFN-γ were unable to activate either macrophage, whereas serum MAF could significantly activate both. Two conclusions can be drawn from these results: first, sensitization had similar effects on macrophages regardless of subtypes and, second, serum MAF had a different activation mechanism from the other MAFs. We note that serum MAF consists of numerous components, with this variability being likely a factor in its activation mechanism.

Activation mechanism of serum MAF. In a previous study, GcMAF activation of macrophages was inhibited by GalNAc (10). Here, we examined whether GalNAc also inhibited the serum MAF activation of macrophages and found that the inhibition only occurred with dTHP1 (Figure 1). This result suggested that serum MAF recognized and activated dTHP1 through the GalNAc moiety of the Gc protein. In addition, GalNAc did not inhibit sTHP1 and sU937, suggesting that serum MAF exhibited some GalNAc-independent activation mechanisms. It is important to note that, since dU937 could not be activated by serum MAF, we were unable to examine the inhibitory effects of GalNAc in this macrophage.

To identify the GalNAc-independent mechanism, we analyzed serum MAF activation of sU937. We found that

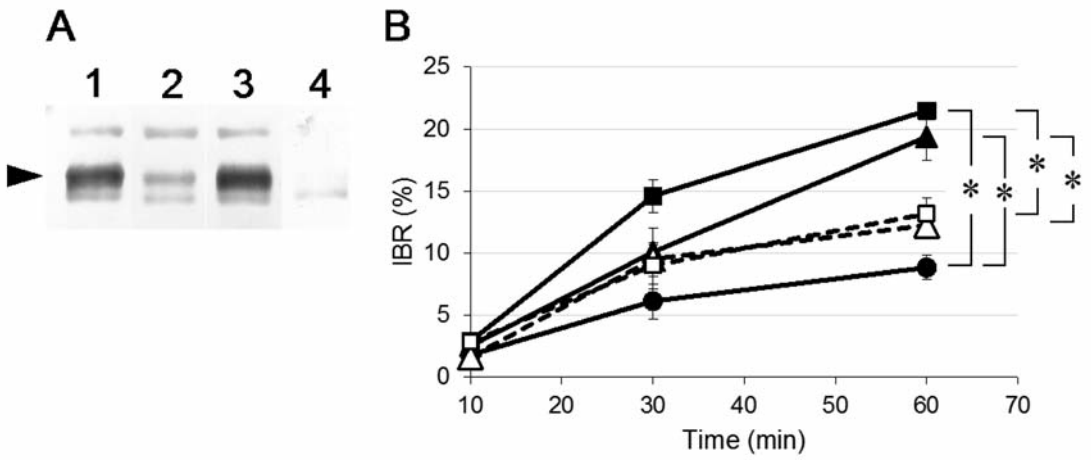

Figure 2. *The Gc protein is important for the activation of sU937. (A) Quantitative analyses of Western blotting show that Gc-protein content in the human serum (lane 1) and serum macrophage activation factor (MAF) (lane 3) conditions, respectively, decreased to 1/4 (lane 2) and 1/44 (lane 4) after immunoadsorption (IA; arrowhead). (B) Graph showing the time course of internalized beads ratios (IBRs) up to 60 min. The IBRs at 60 min post-human serum (▲) and serum MAF (■) addition were 19.4±1.9% and 21.5±0.7%. After IA, IBRs decreased to 12.2±1.1% (△) and 13.2± .2% (□), respectively. All differences between the MAF conditions (human serum, serum MAF) and the control (●) were significant (*p=0.002 (between human serum and control]), p=0.001 (between serum MAF and control); n=3). Moreover, differences between human serum and serum MAF both before and after IA were also significant (*p=0.01 (pre-IA) and p=0.001 (post-IA); n=3).*

sU937 was activated with human serum not bearing the GalNAc moiety (Figure 2; control *vs*. human serum) at levels similar to activation with serum MAF (Figure 2; control *vs*. serum MAF). Furthermore, after IA lowered the Gc-protein content of human serum (to 1/4) and serum MAF (to 1/44), sU937 phagocytic activity (IBRs) under the two treatments decreased from 19.4±1.9% to 12.2±1.1% and from 21.5±0.7% to 13.2±1.2%, respectively. Calculation of human serum and serum MAF APs (60 min post-serum MAF addition) revealed that they respectively decreased from 10.5±2.8% to 3.4±2.1% and from 12.7±1.6% to 4.3±2.2% after IA. These values corresponded to 68% and 66% decreases, suggesting that the Gc protein is important for activating sU937 and possibly the source of the GalNac-independent activation mechanism. We currently lack similar data for sTHP1 and cannot speculate on the mechanisms underlying sTHP1 activation. However, our data do seem to indicate the existence of at least two activation mechanisms for serum MAF, GalNAc-dependent and GalNAc-independent.

Discussion

In this study, we successfully established four macrophage subtypes that clearly differed in their responses to MAFs. We also demonstrated that the Gc protein was important for serum MAF activation of macrophages, which occurred *via* two pathways, GalNAc-dependent and -independent. To our knowledge, the GalNAc-independent pathway is a novel one for serum MAF. Together, these results confirmed the heterogeneity of macrophage activation states. Although we did not analyze the expression profile of subtype markers on IL-4-, IL-10- and LPS+INFγ-activated macrophages, the expression analysis of LPS-activated dTHP1 revealed that the marker gene *CXCL10* exhibited 3.3-3.8-fold higher expression than in non-activated dTHP1 (data not shown). Since *CXCL10* is a marker of *in vivo* activation, this outcome suggests that the examined MAFs triggered similar activation states *in vivo* and *in vitro*, even using macrophages derived from two separate cell lines (THP-1 and U937). Future studies could incorporate the use of more marker genes to provide more definitive evidence supporting this hypothesis.

We used macrophages differentiated from monocytic cell lines and subjected them to admittedly artificial conditions, especially as the human body is rarely serum-free. These conditions likely explain why IL-4, IL-10 and LPS+INFγ could not activate sTHP1 and sU937. However, serum MAF was able to activate these two subtypes, indicating a unique and robust activation mechanism. We considered these macrophages, with low competence to most tested MAFs, as being in a "basal state." In contrast, we designated dU937 as “fully activated” because it already exhibited very high phagocytic activity in the control and, therefore, did not respond to any of the tested MAFs. Of all the macrophage subtypes, dTHP1 was activated by all MAFs, similar to the previously described “primed macrophages,” such as peritoneal macrophages and macrophages differentiated from peripheral blood monocytes (11).

We observed high plasticity in the macrophages. Culturing in FBS-free medium for only 2-h changed the fully activated U937 back into the basal state and, subsequently, serum MAF addition was able to activate it again. This phenotypic plasticity and heterogeneity demonstrates the need for researchers to acknowledge and distinguish between macrophage subtypes. The same care should be taken when describing activation mechanisms due to the variety of receptors (*e.g.*, Toll-like, mannose, scavenger, *etc.*) that allow macrophages to recognize numerous pathogens (5). As Murray *et al*. (6) have pointed out, the type of activation mechanism alters macrophage phenotype through receptors, thus creating distinct subtypes. Our study contributes to an increasing understanding of macrophage diversity *via* documenting the differentiation of four subtypes and providing evidence of a novel serum MAF activation mechanism.

References

1 Uto Y, Yamamoto S, Takeuchi R, Nakagawa Y, Hirota K, Terada H, Onizuka S, Nakata E and Hori H: Effect of the Gc-derived macrophage-activating factor precursor (preGcMAF) on phagocytic activation of mouse peritoneal macrophages. Anticancer Res *31*: 2489-2492, 2011.

2 Yamamoto N, Naraparaju VR and Asbell SO: Deglycosylation of serum vitamin D3-binding protein leads to immuno-suppression in cancer patients. Cancer Res *56*: 2827-2831, 1996.

3 Kuchiike D, Uto Y, Mukai H, Ishiyama N, Abe C, Tanaka D, Kawai T, Kubo K, Mette M, Inui T, Endo Y and Hori H: Degalactosylated/desialylated human serum containing GcMAF induces macrophage phagocytic activity and *in vivo* antitumor activity. Anticancer Res *33*: 2881-2885, 2013.

4 Inui T, Kuchiike D, Kubo K, Mette M, Uto Y, Hori H and Sakamoto N: Clinical experience of integrative cancer immunotherapy with GcMAF. Anticancer Res *33*: 2917-2920, 2013.

5 Akira S, Uematsu S and Takeuchi O: Pathogen recognition and innate immunity. Cell *124*: 783–801, 2006.

6 Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, Gordon S, Hamilton JA, Ivashkiv LB, Lawrence T, Locati M, Mantovani A, Martinez FO, Mege JL, Mosser DM, Natoli G, Saeij JP, Schultze JL, Shirey KA, Sica A, Suttles J, Udalova I, van Ginderachter JA, Vogel SN and Wynn TA: Macrophage activation and polarization: Nomenclature and experimental guidelines. Immunity *41*: 14-20, 2014.

7 Ishikawa M, Inoue T, Inui T, Kuchiike D, Kubo K, Uto Y and Nishikata T: Novel assay system for macrophage-activating factor activity using a human U937 cell line. Anticancer Res *34*: 4577-4582, 2014.

8 Inoue T, Ishikawa M, Sumiya Y, Kohda H, Inui T, Kuchiike D, Kubo K, Uto Y and Nishikata T: Establishment of a macrophage-activating factor (MAF) assay system using human-monocytic cell line, THP-1. Anticancer Res *35*: 4441-4446, 2015.

9 Sumiya Y, Ishikawa M, Inoue T, Inui T, Kuchiike D, Kubo K, Uto Y and Nishikata T: Macrophage activation mechanisms in human monocytic cell line-derived macrophages. Anticancer Res *35*: 4447-4452, 2015.

10 Aramaki Y, Murai M and Tsuchiya S: Contribution of N-acetyl-β-D-galactsamine-specific lectin to Fc receptor-mediated phagocytosis by mouse peritoneal macrophages. Immunol *79*: 403-407, 1993.

11 Inagawa H, Kohchi C and Soma G: Usefulness of oral administration of lipopolysaccharide for disease prevention through the induction of priming in macrophages. Anticancer Res *34*: 4497-4501, 2014.

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