Establishment of a Macrophage-activating Factor Assay System Using the Human Monocytic Cell Line THP-1

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Abstract. *Background: As the mechanism of macrophage activation is not well-understood, standardization of an assay system for measuring phagocytic activities of macrophages will be useful for research on macrophages. Previously, we established a novel standardized macrophage-activating factor (MAF) assay system using U937. Materials and Methods: Using the human monocytic cell line THP-1, another standardized MAF assay system was established. Characteristic gene expression of U937- and THP-1-derived macrophages was compared by gene expression microarray analysis. Results: Both U937- and THP-1-derived macrophages showed obvious phagocytic activities with unique characteristics and, therefore, could not be assigned to a single sub-type. Conclusion: Activation of macrophages is an intricate cellular process. Comparison of our two novel assay systems provides new insights into macrophage activation mechanisms.*

Macrophages, which are phagocytic cells, play an important role in innate immunity. There is growing interest in macrophages due to their interaction with cancer cells, such as the cancer-associated macrophages (CAMs) (1), and their ability to eliminate cancer cells during cancer immunotherapy (2). However, the mechanism of macrophage activation is not well-understood. One of the reasons is the lack of a defined index for activated state. The subtype classifications of macrophages with marker genes are confusing (3). Even in the phagocytic assays, various types of cells, such as mouse peritoneal macrophages (4), a mouse macrophage-like cell line (*e.g.* RAW264.7; 5) or a monocyte fraction of human peripheral blood (6), as well as different types of target substances, such as opsonized sheep-red blood cells (7), latex beads (8) or zymosans (9), were used. Since, however, it is very difficult to compare the data obtained from such diversified protocols, a standardized macrophage-activating factor (MAF) assay system is a necessity in the field of macrophage research.

Recently, we established an easy and robust MAF assay system (10) using the human leukaemia monocyte lymphoma cell line U937 and magnetic beads. The system was very effective in evaluating the activity of the group-specific component protein-derived macrophage-activating factor (GcMAF), which is known as vitamin D3-binding protein (11) and is a good candidate for cancer immunotherapy (12, 13). The results obtained from this system were stable and clear. Therefore, we proposed our system as a standard MAF assay system.

In order to make the standardized assay system more reliable, we tried to establish another assay system using another human-monocytic cell line, THP-1. The comparison of these two different, but closely related systems, should be very informative. In the present study, we compared gene-expression profiles of U937- and THP-1-derived macrophages by gene expression microarray analyses and described their characteristics. Our results showed the complexity of macrophages and suggested the importance of our novel assay system that, complementing the previous one, will be useful for studying the mechanisms of macrophage activation.

Materials and Methods

Cells and cell culture. U937 and THP-1 cells were obtained from Summit Pharmaceuticals (Tokyo, Japan) and RIKEN BRC

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(Tsukuba, Japan), respectively. Both lines were grown in RPMI-1640 medium supplemented with 10% foetal bovine serum (Hyclone, Logan, UT, USA), 3% L-glutamine and 10% sodium hydrogen carbonate. Cells were cultured in a 5% CO_2/95% air fully humidified atmosphere at 37˚C. To induce differentiation into macrophage-like cells, THP-1 cells were seeded onto 35-mm culture dishes (5.0×10^5 cells/dish) and incubated for 24 h with 10 ng/ml 12-O-tetradecanoyl-13-acetate (TPA) (Sigma-Aldrich, St. Louis, MO, USA). In the case of U937 cells, incubation time was 72 h with 10 ng/ml TPA. TPA-treated U937 cells were then pre-treated with serum-free RPMI-1640 medium for 2 h (sensitization) before the phagocytosis assay.

Phagocytosis assay. The medium was replaced by the same fresh medium and 90 μg magnetic beads (Dynabeads® Protein G; Invitrogen, Oslo, Norway) and 5 μg/ml (final concentration) serum MAF were added. Serum MAF was prepared by β-galactosidase and neuraminidase treatment of human serum (7). Macrophages were photographed under a bright-field microscope at 10, 30 and 60 min after serum MAF addition and internalized and non-internalized beads were counted. Phagocytic activities of macrophages were evaluated as the internalized beads ratio (IBR). The activity of MAF was evaluated as the activation ratio (AR) at 30 and 60 min after MAF addition. These indices were calculated by the following formulae:

IBR(%)=(number of internalized beads within the photograph)/(number of all beads within the photograph) ×100
AR=(internalized beads ratio with MAF)/(internalized beads ratio without MAF)

Gene expression profile. The gene expression profiles of monocytic cell lines (U937 and THP-1), differentiated macrophages (dU937 and dTHP), differentiated and sensitized U937 (sU937), as well as macrophages activated with serum MAF (sU937+M and dTHP+M) were analysed by Hokkaido System Science (Sapporo, Japan) using the SurePrint G3 Human GE Microarray Kit 8x60k (Agilent, Santa Clara, CA, USA), version 1 for U937 and version 2 for THP-1. For inter-array comparison, relative amount of gene expression was calculated as normalized intensity value. This method of normalization was the 75-percentile shift, which uses the 75th percentile signal value as a standard. However, quantitative comparison between different versions of microarray is not valid.

Results

Novel MAF assay system using THP-1. Our novel MAF assay system using THP-1 follows basically the same protocol as described previously by us (10) but without sensitization. In order to verify our novel assay system, activation with serum MAF was examined. The phagocytic activity of dTHP with 5 μg/ml serum MAF was measured (Figure 1A). Although, the control phagocytic activity was not negligible, serum MAF significantly activated the dTHP (AR=1.5±0.3, p=0.007; t-test, n=3). Moreover, dose dependency for serum MAF was determined. As shown in Figure 1B, a clear peak at 0.5 μg/ml could be observed. The maximum AR was 1.8±0.097. The concentration of the maximum activation was 10-times lower than that of sU937 (10). These results showed that this novel MAF assay system is also easy to use and robust and, therefore, could be used as a good counterpart of the sU937 MAF assay system.

To compare these two assay systems, we analyzed gene-expression profiles of U937, THP-1, dU937, dTHP, sU937, sU937+M and dTHP+M. First, we examined the entire change of gene expression by scatter plot analysis (Figure 2). In the case of different monocytes (U937 and THP-1) and differentiated macrophages (dU937 and dTHP), the plots were widely distributed suggesting a high magnitude of changes in gene expression. During the activation with serum MAF, *i.e.* sU937 to sU937+M and dTHP to dTHP+M, plots showed a similar convergent pattern suggesting that transcriptional changes were not obvious during activation.

In order to in-depth molecularly analyze the activation of macrophages, characteristic genes for differentiated macrophages were analyzed (Table I). *ZNF865* was highly expressed in both dU937 and dTHP. *FTH1, CCL3* and *MMP1* were highly expressed only in dU937, whereas *MMP9, SNAR-A3, GPR155* and *MAFB* were highly expressed only in dTHP (Table I). These genes were characteristic of the differentiated macrophages. Some genes, such as *MMP9* and secreted phosphoprotein 1 (*SPP1*) showed increased expression during differentiation, suggesting that they might be involved in the activation of macrophages.

One of the interesting points of these assay systems was the sensitization of dU937. Although the transcriptional changes, before and after sensitization, were not obvious, characteristic changes were evident. Among the genes, which represented significant expression (normalized intensity value >10), the genes with decreased level of expression (<0.5-fold change) during the course of sensitization did not show enough changes in the serum MAF activation. For example, amyloid beta (A4) precursor protein (*APP*), chemokine (C-X-C motif) ligand 2 (*CXCL2*), regulator of G-protein signalling 16 (*RGS16*), guanine nucleotide binding protein (G protein) alpha 13 (*GNA13*) and inhibin beta A (*INHBA*) were decreased (0.4- to 0.5-fold) during sensitization and did not exhibit sufficient expression (1.0- to 1.5-fold) after the addition of serum MAF. Similarly, most genes with increased expression (>2-fold change) during the course of sensitization did not show alterations during the serum MAF activation. For example, thrombomodulin (*THBD*), G protein-coupled receptor 68 (*GPR68*), Src-like-adaptor (*SLA*), chemokine (C-C motif) receptor 1 (*CCR1*) and chemokine (C-C motif) ligand 7 (*CCL7*) increased (2.1- to 2.8-fold) during sensitization but did not increase enough (1.0- to 1.3-fold) after the addition of serum MAF. Although the relationship between these genes and the sensitization remains ambiguous, certain receptors or signaling molecules might play a role in the process of sensitization.

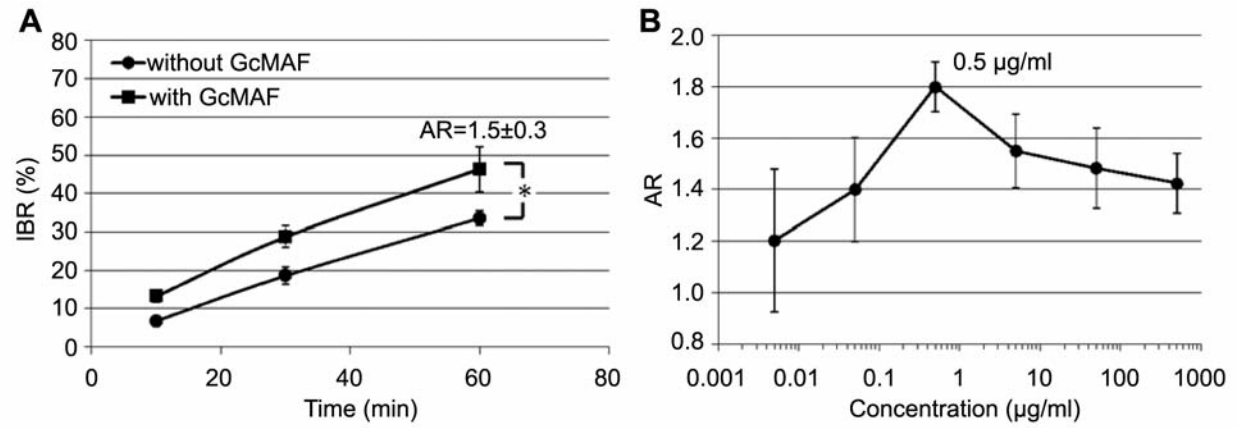

Figure 1. *Phagocytic activity of THP-1-derived macrophages treated with serum macrophage-activating factor (MAF). Phagocytic activity of differentiated THP-1 (dTHP) at 10, 30 and 60 min after serum MAF addition were evaluated as internalized beads ratio (IBR) (A). Error bars represent SD (n=3). *Significant difference between presence and absense of serum MAF (p=0.007; t-test). Dose dependency of dTHP was determined at 60 min after serum MAF addition (B), which was evaluated as activation ratio (AR). Error bars represent SD (n=3).*

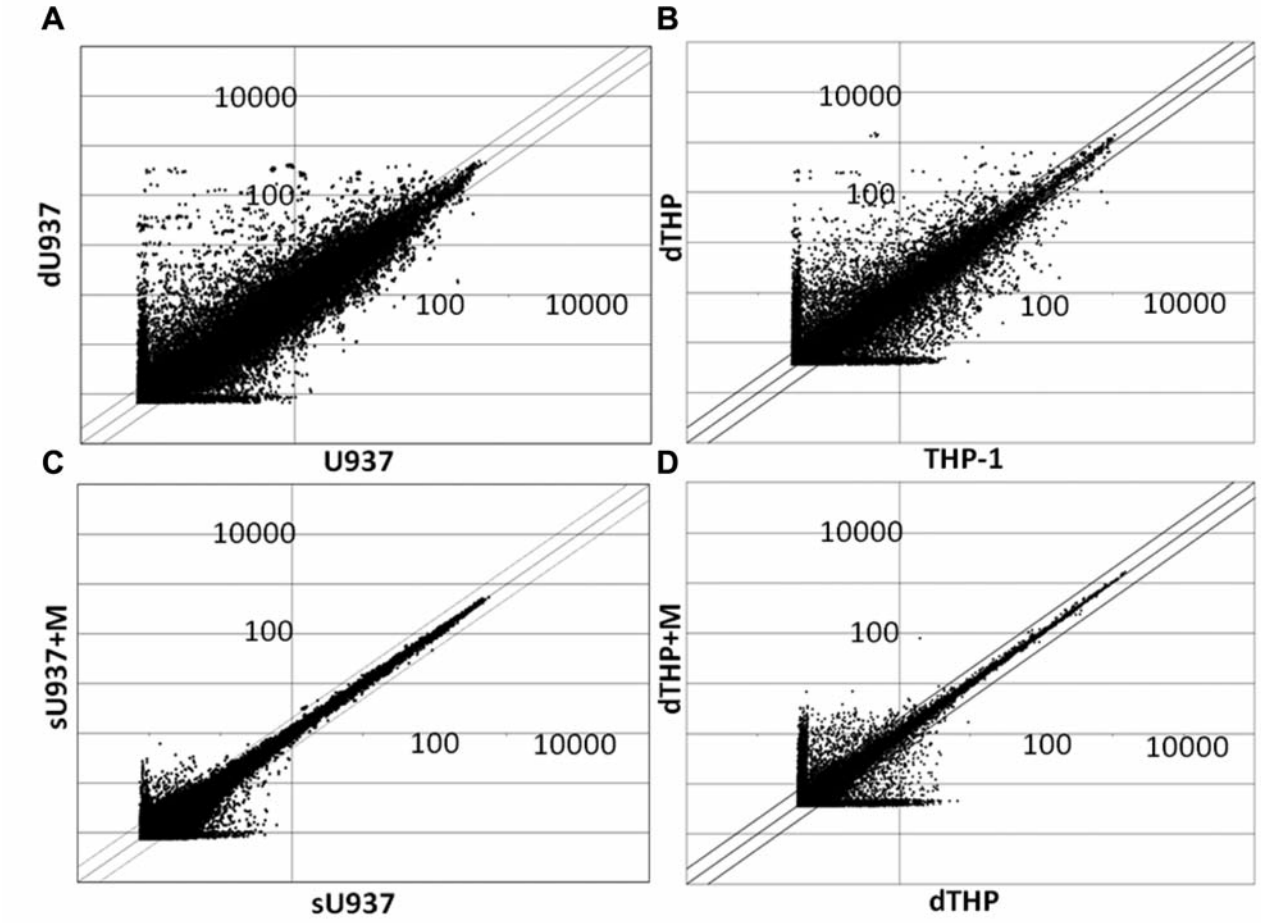

Figure 2. *Changes in gene expression in monocytes, differentiated macrophages and activated macrophages. Scatter plots of gene expression in U937 and differentiated U937 (dU937) (A), in THP-1 and differentiated THP-1 (dTHP) (B), in differentiated and sensitized U937 (sU937) and sU937 with serum macrophage activating factor (sU937+M) (C) and in dTHP and dTHP with serum macrophage activating factor (dTHP+M) (D).*

Table I. *Expression profiles of characteristic genes in differentiated macrophages**.

Gene name	Description	U937	dU937	THP-1	dTHP
ZNF865	Zinc finger protein 865	399.196	396.310	941.274	1047.972
FTH1	Ferritin, heavy polypeptide 1	40.656	398.113	126.220	755.769
CCL3	Chemokine (C-C motif) ligand 3	0.796	390.308	8.526	498.415
MMP1	Matrix metallopeptidase 1	0.890	389.879	0.047	1.044
MMP9	Matrix metallopeptidase 9	0.508	337.427	0.437	1416.597
SNAR-A3	Small ILF3/NF90-associated RNA A3	64.851	24.705	1043.060	928.317
GPR155	G protein-coupled receptor 155	338.757	351.210	780.983	915.343
MAFB	V-maf oncogene homolog B	0.906	114.467	1.272	812.005
SPP1	Secreted phosphoprotein 1	0.017	310.251	0.039	261.133
IL1B	Interleukin1beta	1.275	267.384	0.192	251.574
IL4I1	Interleukin 4 induced 1	0.557	168.147	2.170	257.623
IFI30	Interferon, gamma-inducible protein 30	23.142	123.503	0.699	238.335
CCL4	Chemokine (C-C motif) ligand 4	0.069	122.622	0.173	123.772
MMP7	Matrix metallopeptidase 7	0.011	160.738	0.036	2.650
CCL3L3	Chemokine (C-C motif) ligand 3-like 3	0.097	129.186	0.616	58.834
TDO2	Tryptophan 2,3-dioxygenase	0.008	124.889	0.034	27.365
GDF15	Growth differentiation factor 15	0.114	59.790	0.748	260.736
A2M	Alpha-2-macroglobulin	0.008	25.367	0.038	183.556

*Averages of normalized intensity values are indicated. Double line divides into two different categories: genes that are highly expressed in dU937 (>380) and/or dTHP (>800) and genes that show low expression in monocytes (<1) and increased expression in differentiated macrophages (>100). Genes included in both categories are listed only in the upper part. Dotted lines divide the genes that are expressed in both dU937 and dTHP, dominantly in dU937 and dominantly in dTHP.

Discussion

We have established two assay systems for measuring MAF activity, using the U937 and THP-1 monocytic cell lines. These two assay systems are very similar, except for their mechanisms of activation (14). U937 needs sensitization, whereas THP-1 does not. The optimum concentrations of serum MAF are different in one order of magnitude. Furthermore, certain genes are differentially activated. Thus, these two different types of MAF assay systems have two advantages in macrophage research. The first advantage is their use in drug discovery in relation to the activation of innate immunity. As these two -robust in nature- assay systems are quick and easy to perform, they can be considered effective evaluation systems for MAFs, including colostrum MAF that shows outstanding clinical effects in cancer immunotherapy (15). The second advantage is their utility in basic research. The genes listed in this study are new candidates for the study of macrophage activation mechanisms.

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