Macrophage Activation Mechanisms in Human Monocytic Cell Line-derived Macrophages

YU SUMIYA[1], MAMI ISHIKAWA[1], TAKAHIRO INOUE[1], TOSHIO INUI[2,3,4], DAISUKE KUCHIIKE[2,3], KENTARO KUBO[3], YOSHIHIRO UTO[2] and TAKAHITO NISHIKATA[1]

[1]*Frontiers of Innovative Research in Science and Technology (FIRST), Konan University, Kobe, Japan;*
[2]*Department of Life System, Institute of Technology and Science, Graduate School, Tokushima University, Tokushima, Japan;*
[3]*Saisei Mirai Cell Processing Center, Osaka, Japan;*
[4]*Inui Immunotherapy Clinic, Osaka, Japan*

Abstract. *Background: Although the mechanisms of macrophage activation are important for cancer immunotherapy, they are poorly understood. Recently, easy and robust assay systems for assessing the macrophage-activating factor (MAF) using monocytic cell line-derived macrophages were established. Materials and Methods: Gene-expression profiles of U937- and THP-1-derived macrophages were compared using gene expression microarray analysis and their responses against several MAFs were examined by in vitro experiments. Results: Activated states of these macrophages could not be assigned to a specific sub-type but showed, however, different unique characteristics. Conclusion: The unique of monocytic cell line-derived macrophages could provide clues to understand the activation mechanism of macrophages and, therefore, help to develop effective cancer immunotherapy with MAFs.*

Macrophages are phagocytic cells that play an essential role in providing innate immunity by internalizing pathogens and releasing cytokines. Macrophages are currently of great interest because they possess the possibility of killing and eliminating cancer cells (1-3). Activation of macrophages is important for their function. In response to various signals, macrophages undergo various modifications in their signaling pathways and develop into various sub-types of activated forms (4, 5). However, the details of the activation mechanism are yet to be determined. Macrophages are usually classified into M1 and M2 macrophages (5); however, heterogeneity of the activated macrophages was reported in 2014 (6). The authors suggested that activated states of human macrophages can be classified into 7 types based on their activation signals.

Recently, we established a novel assay protocol for assessing the macrophage-activating factor (MAF) activity using the monocytic cell lines U937 and THP-1 (7, 8). Thus, this is the first step in standardization of the MAF assay protocol. Characterization of these macrophages, which are derived from monocytic cell lines, is valuable for understanding the function of MAFs. In the present study, we compared gene-expression profiles of these monocytic cell line-derived macrophages using gene expression microarray analysis, focusing on the activation mechanism, and examined their responses against several MAFs by *in vitro* experiments.

Materials and Methods

Cells and cell culture. The U937 and THP-1 cell lines were maintained 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, U937 and THP-1 cells were seeded onto 35-mm culture dishes (5.0×10^5 cells/dish) and incubated for 72 h and 24 h, respectively, with 10 ng/ml 12-o-tetradecanoyl-13-acetate (TPA; Sigma-Aldrich, St. Louis, MO, USA). TPA-treated U937 cells were then pre-treated with serum-free RPMI-1640 medium for 2 h (sensitization) before the phagocytosis assay.

Gene expression profiles. The gene expression profiles of U937 cells, THP-1 cells, differentiated and sensitized U937-derived macrophages (sU937) and differentiated THP-1-derived macrophages (dTHP) were analysed by Hokkaido System Science (Sapporo, Japan) using the

Correspondence to: Takahito Nishikata, Frontiers of Innovative Research in Science and Technology (FIRST), Konan University, 7-1-20, Minatojima-minamimachi, Chuo-ku, Kobe 605-0047, Japan. Tel: +81 783031349, Fax: +81 783031495, e-mail: nisikata@konan-u.ac.jp

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SurePrint G3 Human GE Microarray Kit 8x60k (Agilent, Santa Clara, CA, USA), with version 1 being used for U937 cells and version 2 for THP-1 cells. For inter-array comparison, the 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 microarrays is not valid.

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

Phagocytosis assay. The media for sU937 macrophages and dTHP macrophages were replaced by fresh medium containing MAF and 45 μg magnetic beads (Dynabeads® Protein G; Invitrogen, Oslo, Norway). Macrophages were photographed under a bright field and phase contrast microscopes at 30, 60 and 240 min after MAF addition. All the internalized and non-internalized beads were counted in these photographs. Phagocytic activities of macrophages were evaluated as the internalized beads ratio (IBR). This index was calculated by the following formula:

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

Results

Sub-type assignment of differentiated macrophages. In 2014, Murray *et al*. (6) suggested the classification of activated states of human macrophages into 7 types: M(IL-4), M(IL-10), M(GC), M(GC+TGF-β), M(LPS), M(LPS+IFN-γ) and M(IFN-γ). They described several marker genes for each subtype. In the present study, expression of each Murray's subtype-marker gene in sU937 and dTHP was analysed by gene expression microarray analysis (Table I). Both macrophages expressed *ALOX5AP, CCL4, CCL5, IL1B, IL4R, IRF5, MMP9, RGS1, STAT1, TGFB1, TGFBR2* and *TNF*. The marker genes of all sub-types were expressed, while some genes of all sub-types, such as *IRF4, SOCS1, MMP12, SMAD2, IL6* and *CCL18*, were hardly expressed. Thus, these macrophages could not be assigned into a single sub-type based on the marker gene expression. Although sU937 and dTHP have similar expression profiles for these marker genes, *CD163, CXCL10, MARCO* and *MMP1* were dominantly expressed in sU937, whereas *CD40, KYNU, STAB1* and *TGM2* were dominantly expressed in dTHP. This suggested that the two macrophage types had different phenotypes.

Activated states of macrophages predicted by the cytokine-signalling pathway. Signalling pathways of cytokines are well-characterized (4, 5). When a certain cytokine signal is activated, all the genes involved in that pathway, including the receptors, adapters and transcription factors, are expressed. The expression of these factors of each signalling pathway was examined by gene expression microarray analysis (Table II). The gene with normalized intensity value more than 1.5 was considered to be expressed. According to this, IL-4, IL-10 and LPS signaling pathways could be activated in both sU937 and dTHP. However, in the IFN-γ pathway, although *IFNGR1, IFNGR2, STAT1* and *STAT2* were expressed, the expression of the adaptor proteins JAK1 and JAK2 was very low. These results also suggested that the activated state of the differentiated macrophages could not be assigned to a specific sub-type.

The response of differentiated macrophages to the activation signals. The results of the response of the macrophages are summarized in Figure 1. When the phagocytic activity of sU937 was measured with and without IL-4, the IBR values were 22.4±5.1% and 20.1±6.6%, respectively. The probability value (*p*-value) of the *t*-test of this difference was 18.3 (n=3). Thus, sU937 was not activated by IL-4. However, the phagocytic activity of dTHP was elevated from 61.3±2.8% to 72.5±3.9% by addition of IL-4. The *p*-value of this difference was 0.009 (n=3). Therefore, it showed that IL-4 activated the dTHP.

The phagocytic activities of sU937 with and without IL-10 were 26.8±19.9% and 25.2±3.4%, respectively, and the *p*-value of this difference was 0.63 (n=3). When the dTHP was cultured with and without IL-10, the phagocytic activities were 51.5±5.9% and 62.0±6.3%, respectively, and the *p*-value of this difference was 0.04 (n=3). Thus, the sU937 were not activated, whereas dTHP were activated by IL-10 under 95% confidence limits.

Finally, we examined the phagocytic activities of macrophages in the presence of LPS, IFN-γ or both and in the absence of activating factors. The phagocytic activities of sU937 under these conditions were 22.4±5.1%, 18.4±18.7%, 20.8±28.0% and 20.1±15.7%, respectively. The *p*-values of these differences with and without activating factor(s) were 0.19, 0.72 and 0.40, respectively. On the other hand, the phagocytic activities of dTHP under these conditions were 60.7±1.0%, 68.3±8.5%, 65.8±8.5% and 68.9±5.3%, respectively. The *p*-values of the difference with and without activating factor(s) were 0.14, 0.27 and 0.03, respectively. Thus, only simultaneous addition of LPS and IFN-γ to the dTHP resulted in activation under 95% confidence limits.

Discussion

Both *in vitro* and *in silico* analyses showed that neither sU937 nor dTHP could be assigned to a single sub-type of Murray's classification criteria (6). At the same time, both analyses revealed differences between sU937 and dTHP. These results simply suggested that these differentiated macrophages were unknown sub-types and might not exist at all *in vivo* for the reason that these macrophages were differentiated from cancer cell lines (9, 10). However, these

Table I. *Expression profile of marker genes in differentiated macrophages*.*

Gene name¶	U937	sU937†	THP-1	dTHP†
M(IL-4)				
CCL4	0.069	79.184	0.173	123.772
TGFB 1	3.587	13.452	4.980	25.752
TGFBR2	2.500	11.756	3.381	6.088
MMP1	0.890	437.047	0.047	1.044
CD163	0.008	4.034	0.038	0.659
MARCO	1.018	3.223	1.031	0.065
TGM2	0.007	0.036	0.036	5.458
STAB1	0.018	0.095	0.036	4.357
MRC1	0.014	0.024	0.040	0.050
MMP12	0.007	0.178	0.038	0.049
CCL18	0.008	0.739	0.037	0.045
GATA3	0.041	0.009	0.034	0.040
IL17RB	0.012	0.009	0.032	0.038
IRF4	0.007	0.009	0.032	0.038
M(IL-10)				
IL4R	15.892	32.018	7.462	40.977
SOCS3	0.027	0.273	0.145	0.727
M(GC)				
TGFB1	3.587	13.452	4.980	25.752
TGFBR2	2.500	11.756	3.381	6.088
MMP1	0.890	437.047	0.047	1.044
CD163	0.008	4.034	0.038	0.659
MARCO	1.018	3.223	1.031	0.065
STAB1	0.018	0.095	0.036	4.357
MMP12	0.007	0.178	0.038	0.049

Gene name¶	U937	sU937†	THP-1	dTHP†
M(GC+TGF-β)				
ALOX5AP	2.792	15.158	7.798	77.853
RGS1	0.007	14.844	0.037	68.771
TGFBR2	2.500	11.756	3.381	6.088
SMAD2	0.163	0.093	0.321	0.293
M(LPS)				
MMP9	0.508	361.541	0.437	1416.597
IL1B	1.275	298.979	0.192	251.574
TNF	1.263	17.071	3.463	19.357
IRF5	7.376	9.543	23.217	10.658
CXCL10	0.016	5.125	0.073	0.935
IL6	0.007	0.017	0.036	0.043
M(LPS+IFN-γ)				
IL1B	1.275	298.979	0.192	251.574
CCL5	8.056	52.544	9.573	83.435
STAT1	1.253	27.375	1.065	7.346
TNF	1.263	17.071	3.463	19.357
IRF5	7.376	9.543	23.217	10.658
CXCL10	0.016	5.125	0.073	0.935
CD40	0.011	0.025	0.234	4.365
IL6	0.007	0.017	0.036	0.043
M(IFN-γ)				
STAT1	1.253	27.375	1.065	7.346
IRF5	7.376	9.543	23.217	10.658
CCL18	0.008	0.739	0.037	0.045

*Normalized intensity values are indicated. ¶, *ALOX5AP*, arachidonate 5-lipoxygenase-activating protein; *CCL4*, chemokine, CC motif, ligand 4; *CCL5*, chemokine, CC motif, ligand 5; *CCL18*, chemokine, CC motif, ligand 18; *CD40*, CD40 antigen; *CD163*, CD163 antigen; *CXCL10*, chemokine, CXC motif, ligand 10; *CXCL11*, chemokine, CXC motif, ligand 11; *GATA3*, gata-binding protein 3; *IL1B*, interleukin 1-β; *IL6*, interleukin 6; *IL4R*, interleukin 4 receptor; *IRF4*, interferon regulatory factor 4; *IRF5*, interferon regulatory factor 5; *MARCO*, macrophage receptor with collagenous structure; *MMP1*, matrix metalloproteinase 1; *MMP9*, matrix metalloproteinase 9; *MMP12*, matrix metalloproteinase 12; *MRC1*, mannose receptor, c-type 1; *RGS1*, regulator of G protein signalling 1; *SMAD2*, homolog of mothers against dpp, 2; *SOCS3*, suppressor of cytokine signalling 3; *STAB1*, stabilin 1; *STAT1*, signal transducer and activator of transcription 1; *TGFB1*, transforming growth factor β-1; *TGFBR2*, transforming growth factor-β receptor, type II; *TGM2*, transglutaminase 2; *TNF*, tumour necrosis factor. †The values of highly expressed marker genes (>5.000) in both differentiated macrophage are underlined. The values of dominantly expressed marker genes in one macrophage (>2.000 and 5-times more than the other) have dotted underlining.

cells being human cells is an important fact as human cell line-derived macrophages could represent the molecular mechanism of human macrophages more precisely than mouse cells, which are used for many MAF assays (11), since human cells share the same gene set or genome set. U937- and THP-1 cell line-derived macrophages could offer a new stable assay system for MAFs because the conditions for macrophages can be reproduced. Moreover, as these monocytic cell lines can be fully differentiated into macrophages by the addition of TPA (12), preparation of these macrophages could be rapid and easy.

At the same time, the characters of sU937 and dTHP were unique. Gene expression microarray analyses showed that the gene expression profiles were different. For example, although *MMP9* was greatly expressed in both macrophage lines, *MMP1* was almost specific for sU937; especially in the *in vitro* experiments, responses against the activation signals were largely different. Although differentiated macrophages were predicted to be activated by most of the activation signals from the pathway analysis, dTHP fitted this prediction but sU937 did not, as it was not activated. As reported in our previous articles (7, 8), sU937 could be drastically activated by another activating factor, serum MAF (prepared by the β-galactosidase and neuraminidase treatment of human serum; 11). The dU937, which was not pre-treated with serum-free medium, actively

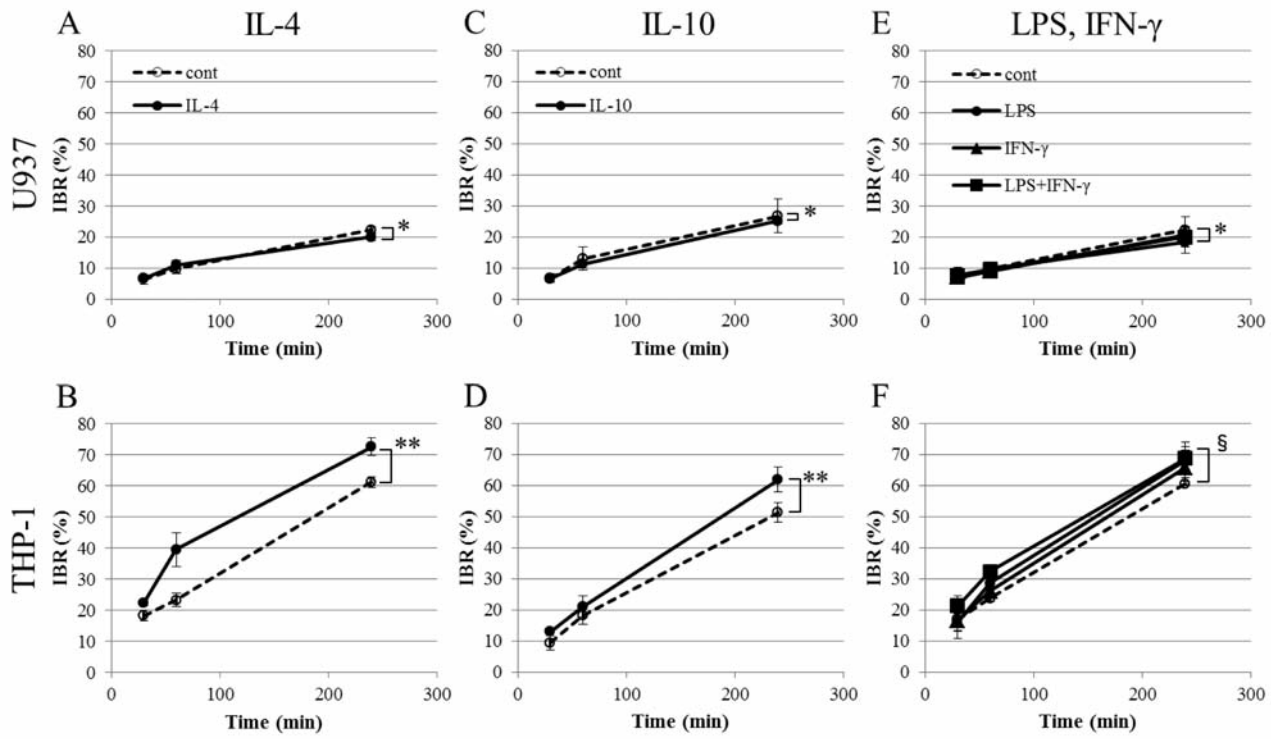

Figure 1. *Phagocytic activities of U937- and THP-1-derived macrophages treated with MAFs. sU937 (A, C, E) and dTHP (B, D, F) were cultured in the presence of MAFs. MAFs used in this experiment are as follows: 20 ng/ml IL-4 (A, B), 20 ng/ml IL-10 (C, D), 1 μg/ml LPS and/or 5 ng/ml IFN-γ (E, F). Phagocytic activity was evaluated as the internalized beads ratio (IBR). Error bars represent SD (n=3). *: Insignificant differences between with and without MAFs (A, C, E). **Significant differences between with and without MAFs (B, D). §Only the difference between with and without LPS+IFN-γ was significant (F).*

Table II. *Gene expression of molecules in signalling pathway*.*

	IL-4			IL-10			LPS			IFN-γ		
	Genes[¶]	sU937	dTHP	Genes[¶]	sU937	dTHP	Genes[¶]	sU937	dTHP	Genes[¶]	sU937	dTHP
receptors	*IL-4R*	32.02	40.98	*IL-10RA*	7.04	15.86	*TLR2*	10.08	15.91	*IFNGR1*	6.99	35.11
				IL-10RB	8.70	11.14	*TLR4*	5.45	2.73	*IFNGR2*	6.16	9.01
adapters	*JAK1*	1.15	0.47	*JAK1*	1.15	0.47	*MyD88*	2.91	5.73	*JAK1*	1.15	0.47
	JAK3	12.21	2.71	*TYK2*	1.88	4.32	*JAK2*	0.29	0.78			
				MAPK14	2.02	1.79						
TFs[†]	*STAT6*	8.17	15.17	*STAT3*	0.92	1.12	*JUN*	23.43	8.67	*STAT1*	27.38	7.35
				JUN	23.43	8.67	*FOS*	9.43	4.35	*STAT2*	42.30	59.76
				FOS	9.43	4.35						
Prediction[§]		○	○		○	○		○	○		×	×

*Normalized intensity values were indicated. [¶]*FOS*, v-fosfbj murine osteosarcoma viral oncogene homolog; *IFNGR1*, interferon-γ receptor 1; *IFNGR2*, interferon-γ receptor 2; *IL-4R,* interleukin 4 receptor; *IL-10RA*, interleukin 10 receptor, α; *IL-10RB*, interleukin 11 receptor, β; *JAK1*, Janus kinase 1; *JAK2*, Janus kinase 2; *JAK3*, Janus kinase 3; *JUN*, v-jun avian sarcoma virus 17 oncogene homolog; *MAPK14*, mitogen-activated protein kinase 14; *MyD88*, myeloid differentiation primary response gene 88; *STAT1*, signal transducer and activator of transcription 1; *STAT2*, signal transducer and activator of transcription 2; *STAT3*, signal transducer and activator of transcription 3; *STAT6*, signal transducer and activator of transcription 6; *TLR2*, toll-like receptor 2; *TLR4*, toll-like receptor 4; *TYK2*, tyrosine kinase 2. [†]Transcription factors. §: ○: As the one set of molecules in each pathway (receptor, adapter, and TFs) was expressed (>5.00), the pathway was predicted to be activated. ×: When no molecule of each part of the pathway (receptor or adapter or TFs) was expressed sufficiently, the pathway was predicted to not be activated.

phagocytized beads (7). These results suggested several interesting points regarding the activation mechanisms of macrophages. (i) Foetal bovine serum contains some factors that are necessary for macrophage activation. (ii) Without such factor(s), some inhibitory effects were exerted on the phagocytic activity of macrophages. (iii) Activation mechanism of serum GcMAF was different from other MAFs. (iv) The culture condition easily changed the activation states of macrophages. Therefore, comprehensive studies on sU937 and dTHP are needed to clarify the activation mechanisms of macrophages with the ultimate goal to interfere in the development of effective cancer immunotherapy.

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