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Glycolytic pathway affects differentiation of human monocytes to regulatory macrophages

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ABSTRACT

Cellular metabolic state and individual metabolites have been reported to regulate the functional phenotype of immune cells. Cytokine production by regulatory and inflammatory macrophages is thought to mainly involve fatty acid oxidation and glycolysis, respectively, which fuel mitochondrial oxidative phosphorylation. However, the association between metabolic pathways and the acquisition of specific macrophage phenotypes remains unclear. This study assessed the relationship between glycolysis and the differentiation of regulatory macrophages. Human monocytes derived from peripheral blood were cultured *in vitro* in the presence of macrophage colony-stimulating factor to yield regulatory macrophages (M-Mϕs). M-Mϕs had a regulatory macrophage phenotype and produced substantial IL-10 following stimulation with lipopolysaccharide. To analyze the role of glycolysis, glycolysis inhibitors (2-deoxy-D-glucose or dichloroacetate) were added during M-Mϕ differentiation. These cells cultured with glycolysis inhibitors produced significantly lower amounts of IL-10, but produced significantly higher amounts of IL-6 compared to M-Mϕs differentiated without glycolysis inhibitors. Such phenotypic change of M-Mϕs differentiated with glycolysis inhibitors was associated with the alteration of the gene expression pattern related to macrophage differentiation, such as *CSF1*, *MMP9* and *VEGFA*. M-Mϕs differentiated with glycolysis inhibitors seemed to retain plasticity to become IL-10 producing cells. Furthermore, increased level of pyruvate in culture medium was found to partially reverse the effects of glycolysis inhibitors on cytokine production of M-Mϕs. These results indicate the importance of glycolytic pathway in macrophage differentiation to a regulatory phenotype, and pyruvate may be one of the key metabolites in this process.

1. Introduction

Macrophages, which are widely distributed in a variety of organs [1], play important roles in regulating inflammatory responses in a context dependent manner [2]. Phenotypes of macrophages are determined by environmental stimuli. Mirroring the Th1-Th2 polarization of T cells, polarized macrophages are often referred to as inflammatory (M1 or classically activated) and regulatory (M2 or alternatively activated) macrophage [3]. Inflammatory macrophages are involved in anti-microbial responses and express high levels of pro-inflammatory cytokines, including tumor necrosis factor α (TNF-α), interleukin (IL)-6, IL-12, and IL-23. In contrast, regulatory macrophages are thought to have critical roles in the termination of inflammation by producing the

Abbreviations: M-CSF, macrophage colony-stimulating factor; IFN-γ, interferon-gamma; M-Mϕ, macrophage differentiated from CD14 positive monocytes in the presence of M-CSF; Mγ-Mϕ, macrophage differentiated from CD14 positive monocytes in the presence of M-CSF and IFN-γ; 2-DG, 2-deoxy-D-glucose; DCA, dichloroacetate; TCA, tricarboxylic-acid; LPS, lipopolysaccharide; C type 1;IDO1, indoleamine 2,3-dioxygenase 1; CSF1, colony stimulating factor 1; MMP9, matrix metallopeptidase 9; VEGFA, vascular endothelial growth factor A; PDK, pyruvate dehydrogenase kinase.

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Table 1
Quantitative PCR primers used in this study.

Gene Symbol	Forward (5′-3′)	Reverse (5′-3′)
CCL22	GGTATTTGAACCTGTGGAATTGGAG	CAGGCCCTGGATGACACTGA
IDO1	AGACTGTGTCTTGGCAAACTGGAA	TGCATTGCCTTGAATACAGTAGGAA
CSF1	TCGGAGTACTGTAGCCACATGATTG	TGGCACGAGGTCTCCATCTG
MMP9	CCCTGGAGACCTGAGAACCAA	CATCTCTGCCACCCGAGTGTA
VEGFA	CTGGAGTGTGTGCCCACTGA	CATTCACATTTGTTGTGCTGTAGGA
18S rRNA	ACTCAACACGGGAAACCTCA	AACCAGACAAATCGCTCCAC

anti-inflammatory cytokine IL-10 [4–8]. It has also been reported that regulatory macrophages promote tissue repair, contribute to metabolic homeostasis and are involved in immunity against parasitic helminth infections [1,9–11].

Metabolic pathways, including oxidative phosphorylation, glycolysis and lipolysis, are actively involved in determining cell fate and in the effector functions of immune cells [12,13]. Activation of mature inflammatory and regulatory macrophages is characterized by distinct metabolic states. Activation of inflammatory macrophages is dependent on aerobic glycolysis, a process important for the production of reactive oxygen species for host defenses against microbes [14]. In addition, some of the metabolites resulting from glycolysis and the tricarboxylic-acid (TCA) cycle are important in regulating the immunological reactions of inflammatory macrophages [15–17]. In contrast, activation of regulatory macrophages involves the use of fatty acid oxidation to fuel mitochondrial oxidative phosphorylation [18,19].

Although glycolysis has an established role in the activation of inflammatory macrophages, little is known about the association between glycolysis and acquisition of the regulatory macrophage phenotype. Unraveling the relationships between metabolism and the differentiation of regulatory macrophages would have important implications for the homeostatic roles of these cells [1,2].

This study therefore analyzed the roles of glycolysis in the *in vitro* differentiation of human monocytes to regulatory macrophages. Our results suggest that glycolysis is essential for macrophages to differentiate into an IL-10 producing phenotype, and that pyruvate is a key metabolite during the process of regulatory macrophage differentiation.

2. Materials and methods

2.1. In vitro *monocyte differentiation*

This study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the ethics committees of Keio University School of Medicine (Approval Number; 20090259). Signed informed consent forms were obtained from all donors and all data were analyzed anonymously throughout the study. Ten healthy donors participated in this study. Peripheral blood mononuclear cells collected from healthy donors were isolated from 30 mL of heparinized peripheral blood samples by density gradient centrifugation using LymphoPrep (Nycomed Pharma, Oslo, Norway). Cells were aspirated from the gradient interface, washed with PBS, and resuspended in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO) containing 10% heat-inactivated fetal bovine serum (Life Technologies, Carlsbad, CA), 100 U/ml penicillin and 100 mg/ml streptomycin (Life Technologies). Monocytes were purified using a magnetic cell separation system (MACS; Miltenyi Biotec, Auburn, AL) with anti-human CD14 microbeads (Miltenyi Biotec), and were seeded in 6-well plates at 1×10^6 cells/well. The culture medium contained 50 ng/ml macrophage colony-stimulating factor (M-CSF; R&D Systems, Minneapolis, MN) to induce the production of M-CSF differentiated macrophages (M-Mϕs), or 50 ng/ml M-CSF and 100 ng/ml interferon-gamma (IFN-γ; R&D) to induce the production of M-CSF and IFN-γ differentiated macrophages (Mγ-Mϕs). Glycolysis was inhibited during differentiation by adding 2-deoxy-D-glucose (2-DG; Sigma-Aldrich) or dichloroacetate (DCA; Sigma-Aldrich) at indicated concentrations. After 6 days, the cells were trypsinized and harvested with a cell scraper; these cells were defined as completely differentiated macrophages.

2.2. *Stimulation of* in vitro *differentiated macrophages*

The differentiated macrophages described above were seeded in 96-well culture dishes at 1×10^5 cells/well and stimulated for 20 h with 100 ng/ml of lipopolysaccharide from Escherichia coli 0111:B4 (LPS; Sigma Aldrich), and the supernatants were collected for cytokine assays. TNF-α, IL-10, and IL-6 concentrations were measured using a Human Inflammatory Cytokine Kit (Cytometric Beads Assay series; BD Biosciences, San Diego, CA), according to the manufacturer's instructions. The cells were subsequently analyzed using a FACSCalibur system (BD Biosciences).

2.3. *Quantitative RT-PCR*

Total RNA was isolated from macrophages using NucleoSpin RNA kits (Takara Bio Inc., Shiga, Japan). For quantitative RT-PCR, a One-Step PrimeScript RT-PCR Kit (Takara Bio Inc.) was used with specific primers for *CCL22*, *indoleamine 2,3-dioxygenase 1* (*IDO1*), *colony stimulating factor 1* (*CSF1*), *matrix metallopeptidase 9* (*MMP9*), *vascular endothelial growth factor A* (*VEGFA*) and *18S rRNA* (Table 1). PCR amplifications were performed using a CFX96 Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA), and relative expression was determined by normalization to the amplification of the *18S rRNA* sequence.

2.4. *Flow cytometric analysis*

Cells were incubated with monoclonal antibodies against CD14, CD33, CD163, CD206, CD209, and HLA-DR and with isotype controls (BioLegend; San Diego, CA). The fluorescence intensity of the labeled cell surface markers was assessed using a FACSCalibur flow cytometer (BD Biosciences), and the data were analyzed using FLOWJO (FlowJo, Ashland, OR) software. Dead cells were excluded from analyses by 7-AAD (BD Biosciences) staining.

2.5. *Respiratory activity assay*

Cellular respiratory activity was measured using XF Mito Stress Test Kits (Seahorse Bioscience, North Billerica, MA) according to the manufacturer's instructions. Oxygen consumption rate (OCR, equal to mitochondrial respiration) was measured using an XF24 analyzer (Seahorse bioscience). Briefly, cells were seeded at 1.6×10^5 cells/well the day before the experiment. Basal and maximal respiratory activities were determined. Cellular maximal respiratory activity was determined by adding 0.75 μM oligomycin, an inhibitor of complex V (ATP synthase), followed by 0.75 μM carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), an uncoupler of mitochondrial oxidative phosphorylation.

Table 2
The 10 genes most up-regulated in 2DG-treated M-Mϕ compared with M-Mϕ.

Rank	Gene Symbol	Gene Name	log2 ratio, M+/M−
1	SLC6A9	solute carrier family 6 (neurotransmitter transporter, glycine), member 9	8.13
2	ASS1	argininosuccinate synthase 1	7.46
3	EEF1A2	eukaryotic translation elongation factor 1 alpha 2	7.12
4	KCNG1	potassium voltage-gated channel, subfamily G, member 1	7.09
5	WNT5A	wingless-type MMTV integration site family, member 5A	6.73
6	IL6	interleukin 6 (interferon, beta 2)	6.52
7	PSAT1	phosphoserine aminotransferase 1	6.40
8	CHAC1	ChaC, cation transport regulator homolog 1 (E. coli)	6.25
9	OR1N1	olfactory receptor, family 1, subfamily N, member 1	5.64
10	ASNS	asparagine synthetase (glutamine-hydrolyzing)	5.60

Gene expression levels from micro array analysis (Fig. 3) are presented as a ratio of normalized intensity (log2).

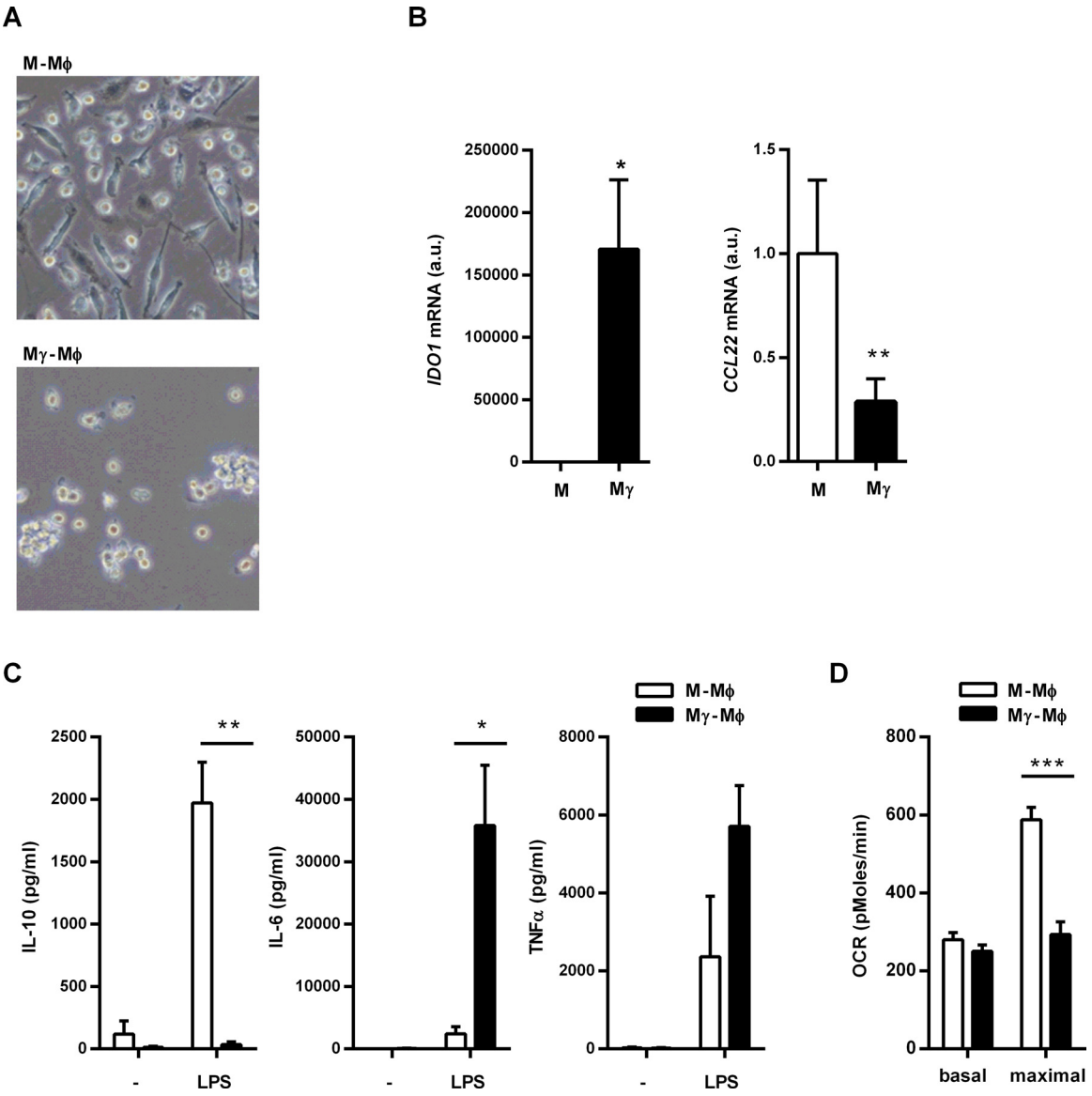

Fig. 1. M-Mϕs have a regulatory macrophage phenotype and IL-10 producing activity compared with Mγ-Mϕs. (A) Morphological phenotypes of M-Mϕs and Mγ-Mϕs differentiated *in vitro* from peripheral CD14 positive monocytes. (B) Expression levels of *IDO1* and *CCL22* mRNAs, as determined by real-time quantitative PCR and normalized relative to the amount in each sample of *18S rRNA*. Data indicate the fold expression compared with M-Mϕs (n = 3). (C) Levels of cytokines produced by stimulation of M-Mϕs and Mγ-Mϕs with 100 ng/ml LPS for 20 h (n = 4). (D) Respiratory activity of M-Mϕs and Mγ-Mϕs, as measured using Mito Stress tests (Seahorse Bioscience). The oxygen consumption rate (OCR) was determined using an XF24 analyzer (n = 5). Data represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared with M-Mϕs by Student's *t*-tests (B, C, D).

2.6. Microarray analysis

Total RNA samples were extracted from macrophages using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Equal amounts of RNA from 3 individuals were mixed. The following assays and analyses were performed at Chemical Evaluation and Research Institute (CERI, Tokyo, Japan). RNA was subjected to quality control analysis

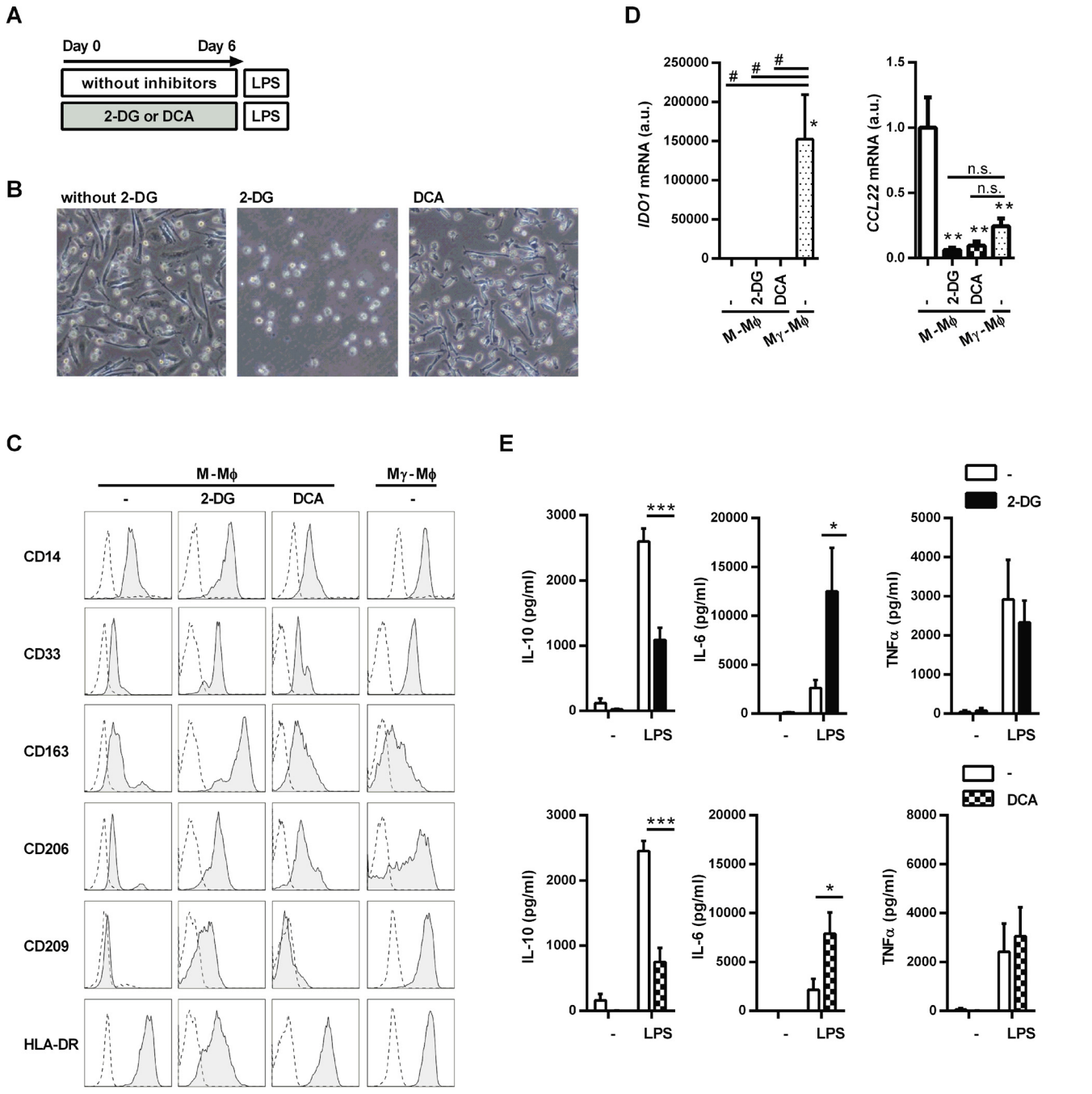

Fig. 2. Inhibition of glycolysis during M-Mϕ differentiation results in the loss of IL-10 producing phenotype and other regulatory macrophage characteristics. (A) Schematic diagram of the experiments. (B) Morphological phenotypes of M-Mϕs differentiated in the presence or absence of 2-DG or DCA. (C) Macrophages were stained with indicated mAbs and analyzed by flow cytometry. The shaded histogram shows the profiles of the indicated antibody staining and the open histogram shows staining of isotype controls. Representative data from three individual experiments are shown. (D) Levels of expression of *IDO1* and *CCL22* mRNAs were analyzed by real-time quantitative PCR and normalized relative to the amount of *18S rRNA* in the same samples. The numbers indicate fold expression compared with M-Mϕs (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with M-Mϕs by Dunnett's type multiple comparison test. #$p < 0.05$ compared with Mγ-Mϕs by Dunnett's type multiple comparison test. (E) Levels of cytokines produced by M-Mϕs differentiated in the presence or absence of glycolysis inhibitors (3 mM 2-DG or 20 mM DCA) and stimulated with 100 ng/ml LPS for 20 h (n = 5–7). Data represent mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$ compared with M-Mϕs differentiated in the absence of inhibitor by Student's *t*-tests.

and reverse transcribed and labeled by using Quick-Amp Labeling Kits (Agilent Technologies, Santa Clara, CA). The labeled cDNA was hybridized to SuperPrint G3 Human GE microarray (8 × 60 K) ver.2 (Agilent Technologies). The microarray was scanned with a DNA Microarray Scanner (Agilent Technologies) and the expression profiles were analyzed with Feature Extraction ver. 10.7.1.1 (Agilent Technologies). Signal intensities were normalized to the mean intensity of all the genes represented on the array. Microarray data are available from the National Center for Biotechnology Information's Gene Expression Omnibus under accession number GSE74182. Canonical pathway analysis was generated using QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity).

Table 3
The 10 genes most down-regulated in 2DG-treated M-Mϕ compared with M-Mϕ.

Rank	Gene Symbol	Gene Name	log2 ratio, M+/M−
1	GAL	galanin/GMAP prepropeptide	2.15×10^{-3}
2	SPC25	SPC25, NDC80 kinetochore complex component	6.77×10^{-3}
3	BIRC5	baculoviral IAP repeat containing 5	6.96×10^{-3}
4	KIAA0101	KIAA0101	7.59×10^{-3}
5	FA2H	fatty acid 2-hydroxylase	7.75×10^{-3}
6	CHIT1	chitinase 1 (chitotriosidase)	8.86×10^{-3}
7	CRABP2	cellular retinoic acid binding protein 2	8.99×10^{-3}
8	MRGPRF	MAS-related GPR, member F	9.43×10^{-3}
9	HIST2H3A	histone cluster 2, H3a	1.07×10^{-2}
10	DUSP13	dual specificity phosphatase 13	1.07×10^{-2}

Gene expression levels from micro array analysis (Fig. 3) are presented as a ratio of normalized intensity (log2).

Table 4
Top 5 canonical pathways of genes up-regulated in 2DG-treated M-Mϕ compared with M-Mϕ.

Rank	Name	p-value	Ratio
1	Hematopoiesis from Pluripotent Stem Cells	2.08×10^{-7}	12/33 (36%)
2	Primary Immunodeficiency Signaling	2.22×10^{-6}	12/40 (30%)
3	Endoplasmic Reticulum Stress Pathway	1.45×10^{-4}	7/21 (33%)
4	T Cell Receptor Signaling	1.31×10^{-3}	14/94 (15%)
5	tRNA Charging	1.53×10^{-3}	8/38 (21%)

The canonical pathway analyses were based on micro-array data (Fig. 3). Log2 ratios of gene expression were analyzed using Ingenuity Pathway Analysis (IPA® version 18841524, QIAGEN Redwood City,). P-value expresses the significance of the relationship for the pathway. The numbers of genes whose expression level are changed in each pathway are expressed as "Ratio".

2.7. Cellular metabolite analysis

Cellular metabolites were extracted with a methanol containing internal standard solution (Human Metabolome Technologies, Yamagata, Japan). Cationic metabolites were measured by capillary electrophoresis–connected time-of-flight mass spectrometry (CE-TOFMS, Agilent Technologies) and anionic compounds by positive and negative modes of CE-MS/MS (Agilent Technologies).

2.8. Statistical analysis

All data are expressed as mean ± SEM. Data were compared using Student's *t*-test or Dunnett's type multiple comparison test, with $p < 0.05$ defined as statistically significant.

3. Results

3.1. M-Mϕs have regulatory macrophage phenotypes

Phenotypes of the regulatory macrophages (M-Mϕs) used in this study were compared to the inflammatory macrophages (Mγ-Mϕs). M-Mϕs and Mγ-Mϕs were differentiated *in vitro* from monocytes in the presence of M-CSF and of M-CSF plus IFN-γ, respectively. M-Mϕs exhibited a spindle-like adhesive morphology, distinct from the spherical and non-adhesive Mγ-Mϕs (Fig. 1A) [20]. Compared with Mγ-Mϕs, M-Mϕs expressed significantly higher levels of the regulatory macrophage-related gene, *CCL22*, and a lower level of the inflammatory macrophage-related gene, *IDO1* (Fig. 1B) [21]. M-Mϕs stimulated with LPS produced larger amounts of the anti-inflammatory cytokine, IL-10, and smaller amounts of the pro-inflammatory cytokines, IL-6 and TNF-α, than LPS-stimulated Mγ-Mϕs (Fig. 1C). We also analyzed the metabolic states of macrophages using a Flux analyzer. Regulatory macrophages have been reported to mainly use mitochondrial respiration, whereas inflammatory macrophages have been found to rely on glycolysis [17,19]. The maximal potential of respiration was higher in M-Mϕs than in Mγ-Mϕs, suggesting that the former has a metabolic state closer to regulatory macrophages (Fig. 1D). Taken together, we used M-Mϕs for the analysis of the differentiation of regulatory macrophages.

3.2. Glycolysis inhibition during M-Mϕ differentiation alters its characters

To investigate the role of glycolysis during the differentiation of monocytes into regulatory macrophages, M-Mϕs were incubated with 2-DG or DCA to inhibit glycolysis [22,23]. 2-DG is phosphorylated by hexokinase, but cannot be further metabolized, resulting in the inhibition of hexokinase. In contrast, DCA inhibits pyruvate dehydrogenase kinase (PDK) by mimicking pyruvate, shifting cellular metabolism from glycolysis to oxidative phosphorylation. As the schematic diagram shown in Fig. 2A, M-Mϕs were differentiated in the presence of 2-DG or DCA and their phenotypes were determined. Cells differentiated in the presence of 2-DG lost their adhesive phenotype and showed a distinct pattern of cell surface antigens, expressing Siglec-3 (CD33), scavenger receptor (CD163), mannose receptor (CD206), and DC-SIGN (CD209) more strongly, and HLA-DR more weakly, than M-Mϕs (Fig. 2B, C) as previously reported [20,24]. The patterns of cell surface antigens of DCA-treated M-Mϕs were similar to that of M-Mϕs. Mγ-Mϕs showed different patterns of cell surface antigens from that of M-Mϕs differentiated with or without 2-DG and DCA. M-Mϕs differentiated in the presence of 2-DG or DCA showed similar expression of *CCL22* to Mγ-Mϕs (Fig. 2D). Whereas, similar to M-Mϕs differentiated without 2-DG or DCA, M-Mϕs differentiated with 2-DG or DCA showed reduced expression of *IDO1* compared to Mγ-Mϕs. Furthermore, these M-Mϕs differentiated in the presence of glycolysis inhibitors produced significantly lower amounts of IL-10, but produced significantly higher amounts of IL-6 and similar amounts of TNF-α, compared with M-Mϕs differentiated in the absence of glycolysis inhibitors (Fig. 2E). It is shown that inhibition of glycolysis during the differentiation of M-Mϕs results in the loss of their characteristic cytokine production patterns independent of other phenotypes.

3.3. Expression of genes essential for macrophage differentiation are affected by glycolysis inhibitors

The gene expression patterns of macrophages were analyzed to understand the mechanism underlying the ability of glycolysis inhibitors to regulate macrophage phenotypes. A microarray analysis identified 2345 probes up-regulated more than 2-fold, and 2284 probes down-regulated less than 0.5-fold, in M-Mϕs differentiated in the presence than in M-Mϕs differentiated in the absence of 2-DG (Fig. 3A). The expression of several genes related to metabolic pathways, such as *SLC6A9*, *ASS1*, *PSAT1*, *ASNS* and *FA2H*, differed dramatically in these two cell types (Tables 2 and 3). Hierarchical clustering showed that the gene expression pattern

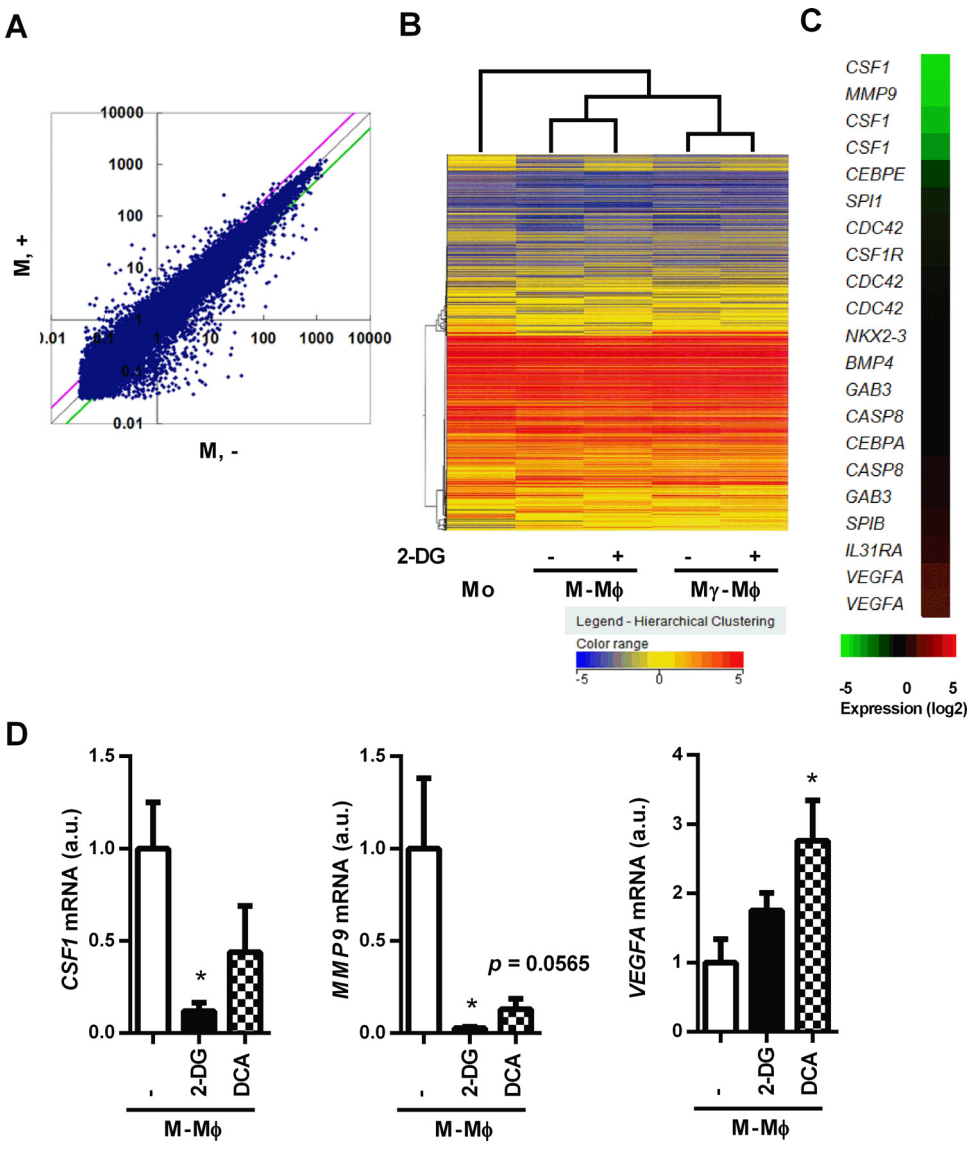

Fig. 3. M-Mϕs differentiated in the presence of 2-DG and DCA have distinct gene expression patterns (A) Scatter plot for gene expression by M-Mϕs (M, −) *versus* M-Mϕs differentiated in the presence of 2-DG (M, +). Gene expression data from DNA microarray analyses were normalized globally using GeneSpring GX 12.0 (Agilent). Purple and green lines represent >2-fold and <0.5-fold gene expression by 2-DG-treated M-Mϕs compared with M-Mϕs, respectively. (B) Ward's hierarchical clustering of gene expression patterns by monocytes (Mo), M-Mϕs (M, −), 2-DG-treated M-Mϕs (M, +), Mγ-Mϕs (M, −), and Mγ-Mϕs differentiated in the presence of 2-DG (M, +). The five samples used in this analysis detected 23,776 probes. (C) Gene expression levels in 2-DG-treated M-Mϕs relative to M-Mϕs presented as a ratio of normalized intensity (log2) by heat map. Genes related to macrophage differentiation (GO: 0030225) were selected for analysis. (D) Quantitative real-time RT-PCR of the levels of expression of *CSF-1*, *MMP9*, and *VEGFA* mRNAs. The level of expression in each sample was normalized relative to the amount of *18S rRNA* in that sample. Data indicate fold expression compared with M-Mϕs (n = 3). *p < 0.05 compared with M-Mϕs by Dunnett's type multiple comparison test.

Table 5
Top 5 canonical pathways of genes down-regulated in 2DG-treated M-Mϕ compared with M-Mϕ.

Rank	Name	p-value	Ratio
1	Dendritic Cell Maturation	6.57×10^{-9}	36/158 (23%)
2	OX40 Signaling Pathway	1.11×10^{-8}	18/48 (38%)
3	Allograft Rejection Signaling	1.60×10^{-8}	16/39 (41%)
4	LXR/RXR Activation	1.50×10^{-7}	28/119 (24%)
5	Hepatic Fibrosis/Hepatic Stellate Cell Activation	1.85×10^{-7}	38/194 (20%)

The canonical pathway analyses were based on micro-array data (Fig. 3). Log2 ratios of gene expression were analyzed using Ingenuity Pathway Analysis (IPA® version 18841524, QIAGEN Redwood City,). P-value expresses the significance of the relationship for the pathway. The numbers of genes whose expression level are changed in each pathway are expressed as "Ratio".

of 2-DG-treated M-Mϕs was closer to M-Mϕs than monocytes and Mγ-Mϕs (Fig. 3B). Canonical pathway analysis on IPA identified 1524 of the 2345 up-regulated probes and 1657 of the 2282 down-regulated probes in 2-DG-treated M-Mϕs than in M-Mϕs. Top 5 pathways significantly up-regulated or down-regulated in ascending order are listed in Tables 4 and 5. Canonical pathway

analysis revealed that 2-DG altered the pathways involved in the regulation of cellular differentiation (hematopoiesis from pluripotent stem cells and dendritic cell maturation) and inflammatory pathways (primary immunodeficiency signaling, T cell receptor signaling, dendritic cell maturation, OX40 (CD134) signaling, allograft rejection signaling, and hepatic fibrosis/hepatic stellate cell activation).

Expression levels of genes related to macrophage differentiation (GO: 0030225) in the microarray were shown in Fig. 3C. The biggest changes were found in the levels of expression of *CSF1*, *MMP9* (decrease) and *VEGFA* (increase) when expression levels of genes in cultured cells in the presence of 2-DG were compared to differentiated cells in the absence of 2-DG (Fig. 3C). Quantitative PCR analysis showed that the levels of expression of *CSF1* and *MMP9* were significantly lower in M-Mϕs differentiated in the presence than in the absence of 2-DG, with similar results observed when M-Mϕs were differentiated in the presence of DCA (Fig. 3D). The level of expression of *VEGFA* was up-regulated by glycolysis inhibitors though the increase by 2-DG was not statistically significant.

3.4. M-Mϕs differentiated with 2-DG retain plasticity

To test whether glycolysis inhibition affects cell fate permanently, the plasticity of 2-DG-treated M-Mϕs was assessed (Fig. 4A). M-Mϕs were differentiated in the presence of 2-DG for 6 days, then the differentiation medium was changed to the same medium without 2-DG, and culture was continued. On day 11 (5 days after the removal of 2-DG containing culture medium), cells had a spindle-like adhesive phenotype (Fig. 4B) and produced comparable amounts of IL-10 and IL-6 to those produced by 2-DG-untreated M-Mϕs, whereas M-Mϕs differentiated in the presence of 2-DG for 6 days produced significantly smaller amounts of IL-10 and larger amounts of IL-6 compared to 2-DG-untreated M-Mϕs (Figs. 4C). These results indicate that M-Mϕs differentiated for 6 days in the presence of 2-DG are sufficiently plastic to revert to an M-Mϕ-like phenotype by 5 days of 2-DG free condition of differentiation.

3.5. Pyruvate is a key metabolite for M-Mϕ function

We hypothesized that key metabolites which strongly regulate macrophage differentiation may exist in glycolytic pathway. To investigate this hypothesis, we used M-Mϕs differentiated in the presence of DCA rather than 2-DG, because DCA does not completely shut down the entire flux of glycolytic pathway [23]. We found that the presence of DCA affected the concentration of several metabolites related to the glycolytic pathway and TCA cycle before and after LPS stimulation (Fig. 5A). Notably, M-Mϕs differentiated in the presence of DCA showed a marked difference from cells differentiated in the absence of DCA in pyruvate concentration after LPS stimulation (Fig. 5A). Because DCA promotes pyruvate consumption by accelerating its utilization in the TCA cycle [25,26], we focused on pyruvate as a key molecule. To analyze the effect of pyruvate, pyruvate was added to the culture medium during the differentiation of M-Mϕs, with or without DCA (Fig. 5B). The addition of pyruvate alone had no effect on the index of functional differentiation of M-Mϕs, such as the production of IL-6 and IL-10. In contrast, the addition of pyruvate to M-Mϕs differentiated in the presence of DCA resulted in a significant reduction in IL-6 production, but had no effect on IL-10 production (Fig. 5C). These results may indicate that the phenotypic changes observed in M-Mϕs differentiated in the presence of DCA were due to the effect of pyruvate at least in the property to produce IL-6.

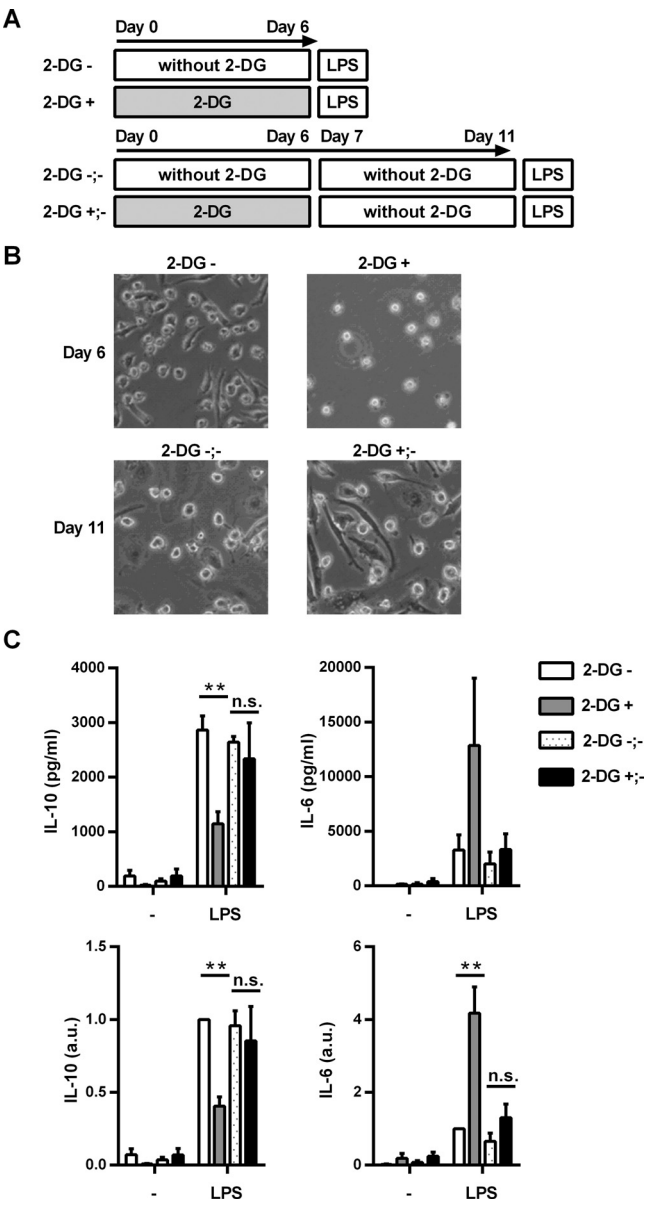

Fig. 4. M-Mϕs differentiated in the presence of 2-DG retain plasticity. (A) Schematic diagram of the experiments. (B) Morphological phenotypes of macrophages cultured in the indicated conditions on days 6 and 11. (C) Macrophages cultured in the indicated condition were stimulated with 100 ng/ml LPS for 20 h (n = 3–4) and the levels of IL-10, IL-6 and TNFα expression were measured. Data represent mean ± SEM. $^{**}p < 0.01$ compared with control by Student's *t*-tests.

4. Discussion

This study investigated the roles of glycolytic pathway in the differentiation of macrophages to a regulatory phenotype, and offered a new insight into the regulation of macrophage differentiation by glycolytic pathway. We found that inhibition of glycolysis during the differentiation of M-Mϕs resulted in the loss of its regulatory macrophage phenotypes including cytokine production patterns. Remarkably, these effects on cytokine production by glycolysis inhibitors were partially reverted by the addition of pyruvate to the culture medium. Therefore, these results indicate that glycolytic pathway is involved in the differentiation of regulatory macrophages by providing the key metabolite for their differentiation.

Using microarray and quantitative PCR analyses, we revealed the involvement of glycolytic pathway in the regulation of gene

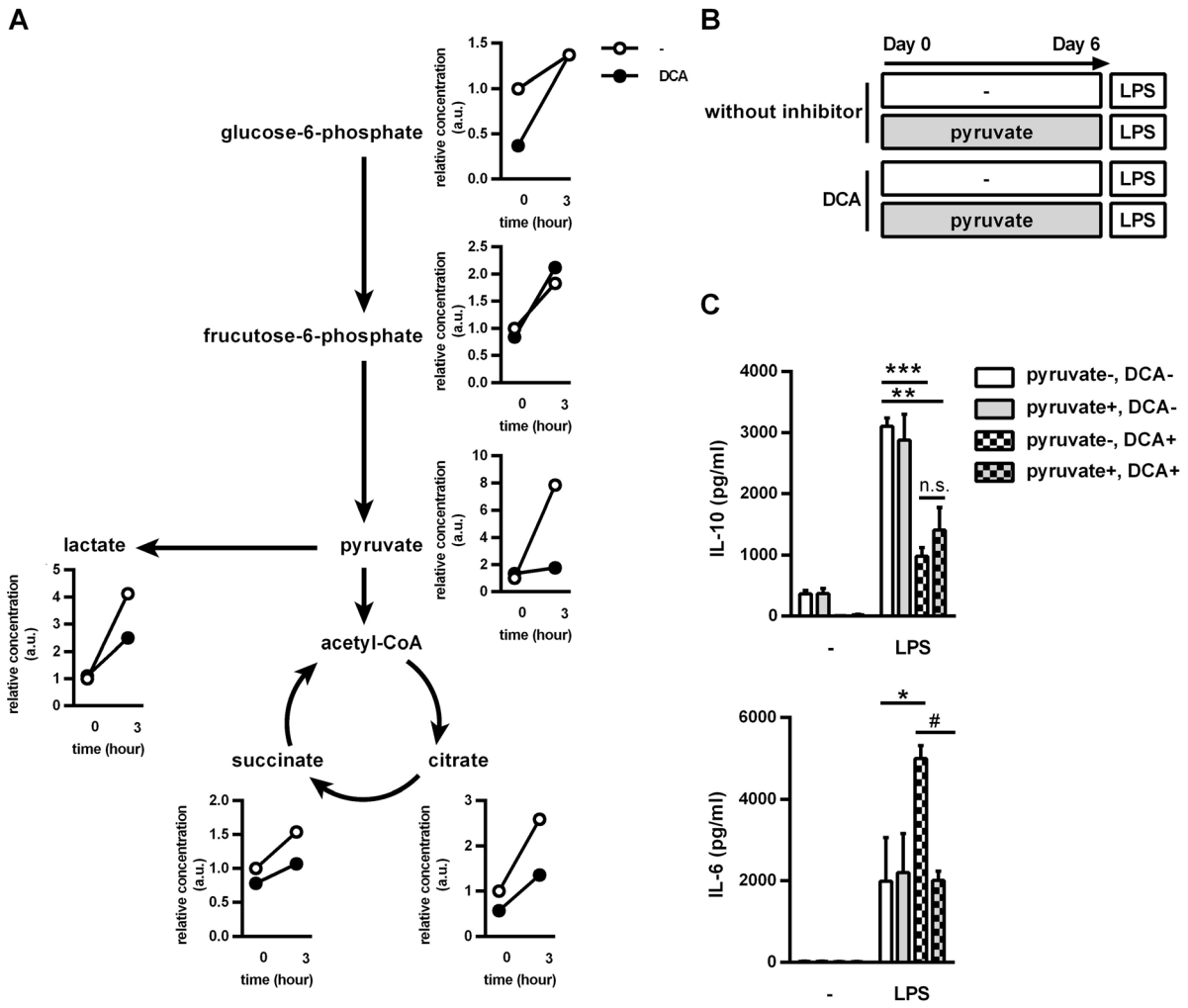

Fig. 5. Cellular metabolites are involved in the regulation of M-Mϕs (A) M-Mϕs differentiated in the presence or absence of 20 mM DCA were seeded onto a new plate and stimulated with 100 ng/ml LPS for 0–3 h. Representative metabolites in the glycolytic pathway and TCA cycle are shown. Data are expressed relative concentration to M-Mϕs differentiated in the absence of DCA and without LPS stimulation (n = 1). (B) Schematic diagram of the experiments. (C) Levels of cytokine production by M-Mϕs differentiated in the presence or absence of 20 mM DCA and 5 mM pyruvate and stimulated with 100 ng/ml LPS for 20 h (n = 4). Data represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with M-Mϕs differentiated in the absence of DCA and pyruvate by Dunnett's type multiple comparison test. #$p < 0.05$ compared with M-Mϕs differentiated in the presence of DCA and the absence of pyruvate by Dunnett's type multiple comparison test.

expression related to macrophage differentiation. *CSF1*, which encodes M-CSF, and *MMP9* were found to be down-regulated in M-Mϕs differentiated in the presence of glycolysis inhibitors. Although circulating M-CSF is thought to be secreted by endothelial cells of many organs [27], it is also locally synthesized by macrophages, mainly its membrane-spanning cell surface form [28], and locally synthesized M-CSF contributes to the differentiation of macrophages [4,29–33]. MMP-9 is preferentially expressed in macrophages and regulates key macrophage functions, such as the angiogenic capacity of M2 macrophages and cellular migration [34,35]. Down-regulation of M-CSF and MMP-9 by inhibiting glycolysis should have altered the differentiation of M-Mϕs. On the contrary, up-regulation of *VEGFA* in M-Mϕs differentiated in the presence of glycolysis inhibitors might have induced the inflammatory phenotype of M-Mϕs (increased IL-6 production or comparable gene expression of *CCL22* to Mγ-Mϕs), since *VEGFA* is downstream of NF-κB [36]. On the other hand, microarray analysis revealed that the gene expression pattern of M-Mϕs differentiated in the presence of 2-DG was closer to that of M-Mϕs than of monocytes, and Mγ-Mϕs differentiated in the presence or absence of 2-DG. Thus, global gene expression pattern suggested that inhibition of glycolysis did not promote differentiation toward an inflammatory, Mγ-Mϕ-like phenotype. However, detailed investigation of the alteration in global gene expression pattern including the results of canonical pathway analysis should be conducted in the future work.

Macrophages are thought to be highly plastic. Depending on the environmental cues, macrophages can change their phenotype from an inflammatory to a regulatory phenotype and vice versa [37–39]. Porcheray et al., demonstrated inflammatory and regulatory macrophages were rapidly and fully reversible by treating with a counterstimulatory cytokine [38]. Moreover Das et al., showed that efferocytosis switched macrophages to an anti-inflammatory phenotype through the action of microRNA21 [39]. Plasticity of macrophages is thought to be beneficial and required in circumstances where resolution of inflammation is needed, such as the process of wound healing [37]. Interestingly, the present study indicated that M-Mϕs differentiated in the presence of a glycolysis inhibitor have sufficient plasticity to become IL-10 producing, M-Mϕ-like cells. It may be possible that inhibition of glycolytic pathway reflects the circumstance of malnutrition which is associated with inflammation of whole body or local tissue such as tumors [40–42]. Collectively, our findings suggest that macrophages may have plasticity to differentiate into a particular phenotype depend-

ing not only on the cytokines but also on the metabolic state including the supply of glucose.

Several metabolites, including succinate and lactate, were shown to be involved in regulating cytokine production by macrophages [15,16]. Also, recent studies have described the anti-inflammatory properties of pyruvate or ethyl pyruvate [43,44]. Ethyl pyruvate is a hyperpermeable analog of pyruvate [45]. These molecules are reported to inhibit the production of pro-inflammatory cytokines (IL-6, TNF, *etc.*) from macrophages by acting as an effective scavenger of reactive oxygen species [44,46]. The present study showed that the addition of pyruvate alone to differentiating M-Mϕs did not suppress IL-6 production, whereas the addition of pyruvate to M-Mϕs differentiated in the presence of DCA significantly reduced the production of IL-6 enhanced by DCA. These observations provide a new insight that endogenous pyruvate plays pivotal roles in the differentiation of regulatory macrophages to acquire its characteristic cytokine production pattern. In contrast, pyruvate had no effect on IL-10 production, suggesting the involvement of other metabolites or that the externally added pyruvate was not sufficient for the complete differentiation of M-Mϕs. Although ethical and technical restriction of using human monocytes makes it difficult to study entire mechanism of our observations, more detailed investigation on the metabolic pathways including activities of regulatory enzymes and levels of key metabolites may reveal the mechanisms how pyruvate and inhibitors affect the differentiation of macrophages.

Recently, Tan et al. reported that mitochondrial respiration which is enhanced by knockdown of PDK1 stimulated M2 activation of macrophages [47]. In this paper, they treated macrophages by IL-4 for several hours to enhance M2 activation, and found that mitochondrial respiration is required for the early activation of M2 macrophages. PDK1 knockdown should have similar effects on the differentiation of macrophages to the addition of DCA which inhibits PDK [23]. However, our study indicated that DCA suppressed the differentiation of macrophages toward regulatory phenotype. In our study, we incubated monocyte with M-CSF for 6 days to differentiate into regulatory macrophages, and evaluated the effect of DCA during the whole period of differentiation. The difference of the protocol and the period of observation between these studies would account for the discrepancy between these findings. That is, DCA might induce the M2 activation of already differentiated macrophages, however, it might inhibit the differentiation of monocyte to a regulatory phenotype.

Treatment of cancers is thought to be enhanced by drugs that modulate metabolic pathways [25,48]. It may be possible that modulation of macrophage differentiation to a regulatory phenotype by the inhibition of metabolic pathways also contribute to cancer treatment, because tumor associated macrophages exhibit regulatory macrophage phenotype and play critical roles in tumor progression by constituting its microenvironment [49,50].

In summary, our results reveal a new aspect of regulatory macrophage differentiation in which glycolytic pathway facilitates this process by supplying pyruvate. Our findings suggest that macrophages will change their phenotype dramatically depending on their circumstances including its metabolic state.

5. Conclusion

In this study we showed that inhibition of glycolysis during differentiation of regulatory macrophages resulted in a loss of their characteristics including cytokine production patterns and alteration of gene expression patterns relates to macrophage differentiation. These effects were reversed by the addition of pyruvate to the culture media at least in part. It was indicated that glycolytic pathway plays important roles in macrophage differentiation to be a regulatory phenotype, and pyruvate may be one of the key metabolites in this process.

Conflict of interest

Hiroaki Suzuki is an employee of Ajinomoto Pharmaceuticals Co., Ltd. Tadakazu Hisamatsu and Takanori Kanai received research grants from Ajinomoto Co., Inc., and Ajinomoto Pharmaceuticals Co., Ltd.

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