Induction of different activated phenotypes of mouse peritoneal macrophages grown in different tissue culture media

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Abstract The role of activated macrophages in the host defense against pathogens or tumor cells has been investigated extensively. Many researchers have been using various culture media in in vitro experiments using macrophages. We previously reported that J774.1/JA-4 macrophage-like cells showed great differences in their activated macrophage phenotypes, such as production of reactive oxygen, nitric oxide (NO) or cytokines depending on the culture medium used, either F-12 (Ham's F-12 nutrient mixture) or Dulbecco modified Eagle's medium (DMEM). To examine whether a difference in the culture medium would influence the functions of primary macrophages, we used BALB/c mouse peritoneal macrophages in this study. Among the activated macrophage phenotypes, the expression of inducible NO synthase in LPS- and/or IFN- γ -treated peritoneal macrophages showed the most remarkable differences between F-12 and DMEM; i.e., NO production by LPS- and/or IFN- γ -treated cells was far lower in DMEM than in F-12. Similar results were obtained with C57BL mouse peritoneal macrophages. Besides, dilution of F-12 medium with saline resulted in a slight decrease in NO production, whereas that of DMEM with saline resulted in a significant increase, suggesting the

possibility that DMEM contained some inhibitory factor(s) for NO production. However, such a difference in NO production was not observed when macrophage-like cell lines were examined. These results suggest that phenotypes of primary macrophages could be changed significantly with respect to host inflammatory responses by the surrounding environment including nutritional factors and that these altered macrophage phenotypes might influence the biological host defense.

Keywords Mouse peritoneal macrophage · Macrophage activation · Nitric oxide · Cytokine · Culture medium

Introduction

Macrophages play pivotal roles in host defense through inflammation, tumor regression, anti-viral and -bacterial activities, and also in the development of organs and tissues as well (Wynn et al. 2013). Among them, the most remarkable one is the activated macrophage phenotype induced by pathogenic components such as lipopolysaccharide (LPS) and/or cytokines such as interferon (IFN)- γ derived from host immune cells (Mantovani et al. 2004; Mosser 2003). Activated macrophages attack intracellular/extracellular pathogens by secreting effector molecules such as reactive oxygen species and nitric oxide (NO) (Bogdan 2015; Green et al. 1991). NO is mainly

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produced by inducible NO synthase (iNOS) in the macrophages; and this enzyme is not present in the resting cells but is rapidly induced upon stimulation of the macrophages with inflammatory cytokines, bacterial products or infection (Aktan 2004; Bogdan 2015; Xie and Nathan 1994; Zamora et al. 2000). NO produced by iNOS plays major roles in protection of the host from parasites, mycobacteria, fungi, and viruses (Green et al. 1991; Xie and Nathan 1994). However, the sustained production of NO sometimes causes adverse effects such as vascular dilatation, sepsis or organ failure (Wollert and Drexler 2002; Xie and Nathan 1994; Zamora et al. 2000). In addition, activated macrophages secrete pro-inflammatory cytokines such as interleukin (IL)-1, IL-6 or tumor necrosis factor (TNF), some of which are involved in fever and others in acute inflammatory responses (Arango Duque and Descoteaux 2014; Mantovani et al. 2004).

Many researchers have been using various culture media in macrophage experiments performed in vitro. In fact, macrophages are often cultured in Roswell Park Memorial Institute (RPMI)-1640 medium, Dulbecco's modified Eagle medium (DMEM), Ham's F-12 nutrient mixture or DMEM/F-12 medium. The choice of the culture medium in each study is commonly considered on the basis of cell growth and stability of cellular phenotypes. In the preceding paper, we showed that the activated macrophage phenotypes differed greatly when the cells were grown in different culture media (Kawakami et al. 2016). In brief, cells of the macrophage-like cell line showed higher expression of iNOS and production of TNF- α and IL-1 β after LPS + IFN- γ -treatment when cultured in DMEM than in F-12, whereas the production of NO showed no significant difference between these 2 media. Therefore, these results seemed to suggest that the choice of the culture medium was not only important but also influenced the results of the study, leading to different conclusions. Until now, it has been reported that macrophages are regulated by environmental factors such as cytokines, chemokines, pattern-recognition molecules, hormones, and radiation, resulting in numerous distinct functional phenotypes (Chiang et al. 2008; Stout et al. 2005; Stout and Suttles 2004, 2005). However, it is still largely unknown whether the type of tissue culture medium *per se* might influence the activity and the functions of the macrophages other than their growth and viability.

In this study, we examined whether the activated macrophage phenotypes of mouse peritoneal macrophages would be affected by the types of culture medium used. We found that the expression of iNOS elicited by LPS and/or IFN- γ was quite different in F-12 and DMEM and that iNOS was scarcely induced in the cells cultured in DMEM. On the contrary, TNF- α production was induced to a greater extent in DMEM than in F-12. Therefore, macrophage activation was influenced largely by distinct culture media, implying that inflammatory responses or cellular cytotoxicity of mouse macrophages was affected by nutrient factors in these media.

Materials and methods

Materials

F-12, DMEM, and FBS were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Saline and recombinant murine IFN- γ were generous gifts from Otsuka Pharmaceutical Co. Ltd (Tokyo, Japan) and TORAY (Tokyo, Japan), respectively. Penicillin and streptomycin mixed solution was purchased from Nacalai Tesque (Kyoto, Japan). LPS (*Escherichia coli* 055:B5) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Griess-Romijn nitrite reagent was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan), and ELISA kits for TNF- α and IL-1 β were purchased from R&D Systems (Minneapolis, MN, USA).

Culture of mouse peritoneal macrophages and macrophage cell lines

Isolation of peritoneal macrophages. Female BALB/c and C57BL/6J mice (6 weeks of age) were obtained from Japan SLC (Shizuoka, Japan) as specific pathogen-free animals. The mice were intraperitoneally administered 5 mL of ice-cold normal saline, and then the peritoneal cell suspension was re-drawn with the same needle. The collected peritoneal cells were centrifuged at 700 $\times g$ for 3 min, and the cell pellet was suspended in 1 volume of $\times 1/3$ saline for 1 min to disrupt the contaminating red blood cells, with this disruption followed by the addition of 2 volumes of normal saline to recover normal osmolality. After centrifugation at 700 $\times g$ for 3 min, the cells

were suspended in F-12 supplemented with 10% heat-inactivated FBS, 50 U/mL penicillin, and 50 µg/mL streptomycin; and then the cell number was counted. More than 98% of adherent cells into culture dishes after incubation for 1 h were macrophages as judged from the Fc receptor mediated phagocytosis and Giemsa staining, as reported previously (Karahashi and Amano 2000). The isolation and use of mouse peritoneal macrophages was approved by the Animal Committee of Osaka University of Pharmaceutical Sciences, and animals were handled in accordance with the principles and guidelines established by the committee.

Culture of cell lines. Mouse macrophage-like cell lines J774.1/JA-4 (Amano and Akamatsu 1991) and RAW264.7 (Tanaka et al. 1989) were maintained and cultured in F-12 supplemented with 10% heat-inactivated FBS, 50 U/mL penicillin, and 50 µg/mL streptomycin at 37 °C in a CO₂ incubator (5% CO₂—95% humidified air). The cells were passaged every 1–3 days and were maintained up to the 25th passage without any significant changes in cell morphology or biological responses.

Reverse transcription-PCR

Cells were seeded at 1×10^6 cells/2.5 mL/dish of F-12 into 35-mm culture dishes. The non-adherent cells were removed after 1–1.5 h of incubation at 37 °C. The adherent cells were then incubated at 37 °C for various times (0–20 h) with 2.5 mL of fresh F-12 or DMEM medium containing no additive, LPS (100 ng/mL), and/or IFN- γ (10 U/mL). Total RNA was isolated from the cells by using a High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions, then reverse-transcribed with ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). Analysis of mRNA was performed with a StepOnePlus Real-time PCR system (Thermo Fisher Scientific Inc.), as described previously (Kawakami et al. 2016). Expression of mRNA was quantitatively calculated by use of the delta–delta–Ct method. Ct data were normalized to the internal control GAPDH mRNA. The forward and reverse primer sequences for each gene were as follow: NOS2 (iNOS): 5'-CTTTGCCACGGACGAGAC-3' and 5'-TCATTGTACTCTGAGGGCTGAC-3'; IL1B (IL-1 β): 5'-AGTTGACGGACCCCAAAAG-3' and 5'-AGCTGGATGCTCTCATCAGG-3'; IL6

(IL-6): 5'-GCTACCAAACTGGATATAATCAGGA-3' and 5'-CCAGGTAGCTATGGTACTCCAGAA-3'; IL10 (IL-10): 5'-CAGAGCCACATGCTCCTAGA-3' and 5'-TGTCCAGCTGGTCCTTTGTT-3'; IL18 (IL-18): 5'-CAACCTTCCAAATCACTTCCT-3' and TCCTTGAAGTTGACGCAAGA; NFKBIA (IkB α): 5'-ACGAGCAAATGGTGAAGGAG-3' and 5'-ATGATTGCCAAGTGCAGGA-3'; RELA (p65): 5'-CCCAGACCGCAGTATCCAT-3' and 5'-GCTCCAGGTCTCGCTTCTT-3'; NFKB1 (p105): 5'-GACCACTGCTCAGGTCCACT-3' and 5'-TGTCATATCCCGGAGTTCA-3'; ARG1 (arginase 1): 5'-GAATCTGCATGGGCAACC-3' and 5'-GAATCCTGGTACATCTGGGAAC-3'; ARG2 (arginase 2): 5'-TATGGTCCAGCTGCCATTC-3' and 5'-CCAAAGTCTTTTAGGTGGCATC-3'; GAPDH: 5'-CAAGGAGTAAGAAACCCTGGACC-3' and 5'-CGAGTTGGGATAGGGCCTCT-3'.

Measurement of production of NO, TNF- α , and IL-1 β

Cells were seeded at 1×10^5 cells/0.25 mL/well of F-12 into 48-well plates, and then incubated at 37 °C for 1–1.5 (peritoneal macrophages) or 2–4 h (cell lines) to obtain adherent cells. The culture medium was replaced with 0.25 mL of fresh F-12 or DMEM medium containing no additive, LPS (100 ng/mL), and/or IFN- γ (10 U/mL), followed by incubation at 37 °C for 20 h. For measurement of NO, TNF- α , and IL-1 β production, cell culture supernatants were collected. NO (NO₂⁻) in the cell culture supernatants was measured by performing the Griess assay. TNF- α and IL-1 β were quantitated by ELISA according to the manufacturer's instructions, as described previously (Kawakami et al. 2016).

Western blot analysis

Cells were seeded at 2×10^6 cells/5 mL/dish of F-12 into 60-mm culture dishes, and then incubated at 37 °C for 1–1.5 h. The medium was replaced with 5 mL of fresh F-12 or DMEM medium containing no additive, LPS (100 ng/mL), and/or IFN- γ (10 U/mL); and then the cells were incubated further for 20 h. Preparation of whole cell extracts and subsequent SDS-PAGE/Western blot analysis were performed as described previously (Kawakami et al. 2016). In brief, 10-µg aliquots of the cell extracts were electrophoresed through a 5–20% gradient polyacrylamide gel (ATTO, Tokyo, Japan) and thereafter transferred to Immobilon

polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were probed with mouse monoclonal anti-iNOS/NOS type II antibody (BD Biosciences, San Jose, CA, USA), goat polyclonal anti-IL-1 β /IL-1F2 antibody (R&D Systems), and mouse anti- β -actin antibody (Sigma-Aldrich) used as an internal control. Horseradish peroxidase-labeled secondary antibody (Cell Signaling Technology, Danvers, MA, USA) and Pierce Western blotting substrate (Thermo Fisher Scientific Inc.) were used for antibody detection. Chemiluminescence signal intensity was quantified by using Image J software (version 1.48v).

Re-incubation of macrophages after activation

Cells were activated in F-12 or DMEM medium containing LPS (100 ng/mL) and IFN- γ (10 U/mL) at 37 °C for 20 h. They were then washed with pre-warmed phosphate-buffered saline (PBS), and then re-incubated further for various times (0–9 h) after the addition of either fresh F-12 or DMEM without LPS or IFN- γ . At each time point of the incubation, cell culture supernatants were collected for use in the Griess assay.

Statistical analysis

Results were expressed as the mean $\pm$ SE of at least 3 independent experiments. The difference between 2 groups was analyzed by using Student's *t* test; and a multiple comparisons test performed by using the Tukey–Kramer method with the significance of difference being set at $p < 0.05$.

Results

Effect of culture medium on mRNA expression and morphology of the activated peritoneal macrophages

When the macrophages were activated by LPS, expression of a variety of genes including iNOS and pro-inflammatory cytokines was observed (Fig. 1). The LPS-treated peritoneal macrophages showed different expression of the genes after incubation in F-12 or DMEM at 37 °C for 4 h. Among them, the expression of iNOS, IL-1 β , and IL-18 mRNA was significantly lower in DMEM than in F-12. The

greatest difference was seen in iNOS expression. Although mRNA expression of pro-inflammatory cytokines such as IL-1 β , IL-6, and IL-18 tended to be lower ($p = 0.013$, 0.053 , <0.001 , respectively) in DMEM than in F-12, IL-10, known as an anti-inflammatory cytokine, showed higher expression ($p = 0.087$) in DMEM. The expression of NF- κ B-related mRNA was also significantly higher in DMEM than in F-12. These results show that activation of inflammatory genes expressed in peritoneal macrophages was different in these culture media.

However, no significant differences in the morphology including cell size, shape or damage were observed after incubation with/without LPS and/or IFN- γ in F-12 or DMEM medium (Fig. 2).

Effect of the culture medium on NO production and iNOS expression

Like expression of iNOS mRNA (Fig. 1), NO production was significantly lower in DMEM than in F-12 after treatment with LPS and/or IFN- γ (Fig. 3a): NO production from LPS + IFN- γ -treated macrophages during incubation in F-12 was about 7-fold higher than that in DMEM. Besides, the expression of iNOS protein in these cells was much higher in F-12 than in DMEM (Fig. 3b). Quantitative analysis of iNOS mRNA during the course of macrophage activation also showed significantly higher expression in F-12 at 4 h (treated with LPS or LPS + IFN- γ) and 8 h (treated with LPS) than in DMEM (Fig. 3c). To ascertain the low activity in DMEM, we changed the ratios of F-12/DMEM at 5 grades from 10/0 to 0/10 during the macrophage activation. An increase in the proportion of DMEM resulted in a dose-dependent decrease in NO production (Fig. 3d). These results suggest the possibility of the presence of some components in these media that might regulate the expression of iNOS.

The expression of iNOS was reported to be regulated by arginase, an enzyme of arginine metabolism, to produce ornithine and urea (Corraliza et al. 1995). So we examined the expression of arginase-1 and -2 in LPS-treated cells cultured in these 2 media. While arginase-1 mRNA of the LPS-treated macrophages in DMEM was reduced to 35% of that in F-12, arginase-2 mRNA was elevated to 150% (Fig. 4a). Moreover, as previously reported for J774.1 macrophages (Kawakami et al. 2016), NO production during

Fig. 1 Effects of culture medium on mRNA expression of iNOS, ILs, and NFκB-related factors in mouse peritoneal macrophages without or with LPS in F-12 or DMEM. BALB/*c* mouse peritoneal macrophages were incubated in F-12, and then the medium was replaced with either fresh F-12 or DMEM in the absence or presence of LPS (100 ng/mL). The cells were incubated at 37 °C for 4 h, and then total RNA was extracted. The expression of iNOS, IL-1β, IL-6, IL-10, IL-18, IκBα, p65,

and p105 mRNA was analyzed by using RT-PCR to obtain cDNA synthesized from total RNA. The results are shown relative to those for the LPS-treated cells in F-12 after normalization to the internal control, GAPDH mRNA in each experiment. The results are expressed as the mean ± SE for 4 independent experiments. **p* < 0.05 between indicated pairs of F-12 and DMEM

Fig. 2 Morphology of activated peritoneal macrophages in F-12 or DMEM. Peritoneal macrophages were incubated in F-12, and then the medium was replaced with either F-12 or DMEM containing no additive, LPS (100 ng/mL), and/or IFN-γ

(10 U/mL). The cells were incubated at 37 °C for 20 h. Representative photographs from repeated experiments are shown. Magnification, ×400

the re-incubation of the activated peritoneal macrophages in either of these media showed a higher value in F-12 than in DMEM (Fig. 4b). These results indicate that mouse peritoneal macrophages underwent low activation not only in terms of iNOS induction but also in terms of NO production when cultured in DMEM as compared with F-12.

Effect of the culture medium on IL-1β and TNF-α production

We next examined the difference in IL-1β and TNF-α production by mouse peritoneal macrophages in each culture medium. While IL-1β production in DMEM was significantly lower than that in F-12 only in LPS-

Fig. 3 Effects of culture medium on NO production and iNOS expression by the activated peritoneal macrophages in F-12 or DMEM. **a** NO₂⁻ in the culture supernatants of peritoneal macrophages after incubation with/without LPS (100 ng/mL) and/or IFN- γ (10 U/mL) in F-12 or DMEM for 20 h. NO₂⁻ was quantitated by performing the Griess assay as described in the text. The results are expressed as the mean $\pm$ SE for 4 independent experiments. * p < 0.05 between indicated pairs of F-12 and DMEM. **b** Western blot analysis of iNOS and β -actin protein conducted by using total protein extracts of the cells cultured in F-12 or DMEM in the presence or absence of LPS (100 ng/mL) and/or IFN- γ (10 U/mL). **c** Time course of iNOS mRNA induction in LPS- (left) or LPS + IFN- γ - (right)

treated cells in F-12 or DMEM. The results are shown relative to those for the LPS + IFN- γ -treated cells at 4 h in F-12 after normalization to the internal control, GAPDH mRNA, in each experiment. The results are expressed as the mean $\pm$ SE for 4 independent experiments. * p < 0.05 between indicated pairs of F-12 and DMEM. **d** NO₂⁻ in the culture supernatants of the peritoneal macrophages after incubation for 20 h with/without LPS (100 ng/mL) or LPS + IFN- γ (10 U/mL) in medium with varied F-12/DMEM ratio (a 10:0; b 9:1; c 5:5; d 1:9; e 0:10). NO₂⁻ was determined by use of the Griess assay. The results are expressed as the mean $\pm$ SE for 4 independent experiments. * p < 0.05 between indicated pairs of F-12/DMEM ratio for the medium

treated cells (Fig. 5a), TNF- α production in DMEM was significantly higher than that in F-12 in both LPS- and LPS + IFN- γ -treated cells (Fig. 5b). Although the expression of pro-IL-1 β protein at 20 h was not so different between these cells (Fig. 5c), a time course study on the induction of IL-1 β mRNA in the LPS-treated macrophages revealed significantly lower expression of it in DMEM than in F-12 at 4 h of incubation (Fig. 5d).

Effect of the culture medium on NO and TNF- α production by various macrophages

In this study, we demonstrated that NO production by peritoneal macrophages was lower in DMEM than in F-12. However, cells of the macrophage-like cell line

J774.1/JA-4 cells did not show these differences (Kawakami et al. 2016). Therefore, we examined NO production by using other macrophages, including C57BL mouse peritoneal macrophages and another macrophage-like cell line, RAW264.7 cells. Although the peritoneal macrophages from the both BALB/c and C57BL mice produced significantly lower NO in DMEM than in F-12 by treatment with LPS + IFN- γ (Fig. 6a, b), both macrophage-like cell lines, J774.1 and RAW264.7, showed no significant difference in NO production after treatment with LPS + IFN- γ in these culture media (Fig. 6c, d). However, all macrophages except those from the peritoneum of C57BL mice showed significantly higher TNF- α production in DMEM than in F-12 after LPS + IFN- γ -treatment (Fig. 6e–h).

Fig. 4 Effects of culture medium on expression of arginase mRNA and NO production by activated peritoneal macrophages in F-12 or DMEM. **a** Expression of arginase-1 and -2 mRNAs in peritoneal macrophages after incubation with/without LPS (100 ng/mL) for 4 h in F-12 or DMEM. The results are shown relative to those for the LPS-treated cells in F-12 after normalization to the internal control, GAPDH mRNA in each experiment. The results are expressed as the mean $\pm$ SE for 4

independent experiments. $*p < 0.05$ between indicated pairs of F-12 and DMEM. **b** Time course of NO production during re-incubation of the activated macrophages. NO₂⁻ in the culture supernatants of the activated peritoneal macrophages, pre-treated with LPS + IFN- γ in F-12 or DMEM for 20 h, was estimated during re-incubation in fresh F-12 or DMEM. NO₂⁻ was determined by performing the Griess assay. The results are expressed as the means for 4 independent experiments

Fig. 5 Effects of culture medium on induction of IL-1 β and TNF- α during peritoneal macrophage activation in F-12 or DMEM. **a**, **b** IL-1 β and TNF- α in the culture supernatants of the peritoneal macrophages after incubation for 20 h with/without LPS (100 ng/mL) and/or IFN- γ (10 U/mL) in F-12 or DMEM. IL-1 β and TNF- α were assayed by ELISA. The results are expressed as the mean $\pm$ SE for 4 independent experiments. $*p < 0.05$ between indicated pairs of F-12 and DMEM. **c** Western blot analysis of pro-IL-1 β and β -actin proteins. The

cells were incubated in F-12 or DMEM in the presence or absence of LPS (100 ng/mL) and/or IFN- γ (10 U/mL) for 20 h. **d** Time course of IL-1 β mRNA induction in LPS- (left) or LPS + IFN- γ - (right) treated cells in F-12 and DMEM. The results are relative to those for the LPS + IFN- γ -treated cells at 4 h in F-12 after normalization to the internal control, GAPDH mRNA, in each experiment. The results are expressed as the mean $\pm$ SE for 4 independent experiments. $*p < 0.05$ between indicated pairs of F-12 and DMEM

Fig. 6 Effects of culture medium on NO and TNF- α production by various activated macrophages. **a–h** NO₂⁻ (**a–d**) and TNF- α (**e–h**) in the culture supernatants of mouse peritoneal macrophages or macrophage-like cell lines after incubation for 20 h with LPS (100 ng/mL) and IFN- γ (10 U/mL) in F-12 or DMEM. NO₂⁻ was determined by using the Griess assay and

TNF- α , by ELISA. The results were obtained from culture supernatants of mouse peritoneal macrophages from BALB/c (**a, e**) and C57BL/6 J (**b, f**), and from mouse macrophage-like cell line, J774.1/JA-4 (**c, g**) and RAW264.7 (**d, h**). The results are expressed as the mean $\pm$ SE for 3–5 independent experiments. * $p < 0.05$ between indicated pairs of F-12 and DMEM

Effect of dilution of culture medium with saline on NO production from the activated macrophages

As shown in Figs. 3 and 6, NO production from peritoneal macrophages was greatly different between the cells incubated in F-12 or DMEM after macrophage activation. To investigate the medium components, we examined whether dilution of the culture media with saline might influence NO production of these macrophages (Fig. 7). NO production in F-12 was reduced significantly from 100 to 87% by dilution with saline to 50%, whereas that in DMEM was reduced to a greater extent, to 53%. To the contrary, NO production in DMEM was increased significantly from 13 to 35% by dilution with saline to 50%, and was also increased to 53% by dilution with F-12.

These results show that replacement of DMEM with saline significantly increased NO production and that of F-12 with saline decreased it less significantly, suggesting a possibility that some dominantly suppressing factor(s) on NO production might have been

included in DMEM, and other stimulating factor(s), in F-12.

Discussion

This study demonstrates the influence of culture medium on the activated phenotypes of mouse peritoneal macrophages. Among them, iNOS expression in LPS-treated peritoneal macrophages showed the most remarkable differences between incubation in DMEM and that in F-12 (Fig. 1): NO production and the level of iNOS protein were also far lower in DMEM than in F-12 after treatment with LPS and/or IFN- γ (Fig. 3). Furthermore, changes in the composition of F-12/DMEM ratios dose-dependently influenced the NO production by the activated macrophages (Fig. 3d). These results clearly show that selection of culture medium as either F-12 or DMEM led to great differences in iNOS expression and NO production in BALB/c mouse peritoneal

Fig. 7 Effect of dilution of culture medium on NO production from activated macrophages. NO₂⁻ in the culture supernatants of peritoneal macrophages after incubation for 20 h with LPS (100 ng/mL) and IFN- γ (10 U/mL) in F-12 or DMEM medium diluted or not with saline or the other medium containing FBS. The ratio of dilution is indicated at the bottom of each bar. NO₂⁻

was determined by performing the Griess assay. On the ordinate, NO production in F-12 without saline is indicated as 100% in each experiment. The results are expressed as the mean $\pm$ SE for 3 independent experiments. * p < 0.05 between indicated pairs

macrophages activated with LPS and/or IFN- γ . Similar results were obtained with the peritoneal macrophages from C57BL mouse (Fig. 6a, b). However, somewhat different results were obtained with a macrophage-like cell line, J774.1/JA-4 cells, as previously reported (Kawakami et al. 2016). In another macrophage-like cell line, RAW264.7, the cells showed a similar NO production profile as J774.1 cells, showing very modest effects of these 2 culture media (Fig. 6c, d).

Therefore, it seems feasible that peritoneal macrophage can be more easily influenced by the composition of the surrounding medium, probably due to some specific and sensitive receptors and/or effector molecules involved in the regulation of iNOS activity. As a candidate, we focused on arginase, which hydrolyzes an iNOS substrate, arginine, to ornithine and urea. The expression of arginase has been reported to differ in individual macrophages (Louis et al. 1998). The arginases genes were examined, and the results showed that the mRNA of arginase-1 was decreased by LPS-treatment but that that of arginase-2 was increased (Fig. 4a). However, due to incongruous expression of arginase-1 and arginase-2, this phenomenon of the medium effect does not seem to be

explained by arginases alone. F-12 contains higher (2.5-fold) amounts of L-Arginine than DMEM. We examined the influence of L-Arginine addition to the mouse peritoneal macrophages culture. However, the amount of L-Arginine was not effective to the high or low NO production by the macrophages (data not shown). Both media probably contain enough amount of L-Arginine to produce NO. Therefore, these results suggested that arginase expression does not seem to have significant effect on NO production in this study.

Furthermore, we examined which culture medium exerted more influences on NO production in the activated mouse peritoneal macrophages (Fig. 4b). The results suggest that F-12 induced higher NO production than DMEM when used during macrophage activation with LPS + IFN- γ and that exchange of the culture medium from F-12 to DMEM reduced NO-producing activity in the macrophages pre-activated in F-12, whereas that from DMEM to F-12 enhanced this activity in the cells pre-activated in DMEM. Besides, the results in Fig. 4b also suggest that the influence of the culture medium on NO-producing activity appeared to be more pronounced during macrophage activation than during re-incubation of the activated macrophages. To ascertain

these effects of the medium during incubation, we replaced part of the culture medium with saline. While NO production in saline-diluted F-12 was slightly decreased, that in the saline-diluted DMEM was markedly increased (Fig. 7). These results suggest the possibility that DMEM contained some inhibitory factor(s) affecting NO production, whereas F-12 contained some stimulatory factor(s). DMEM contains higher amounts of D-glucose, calcium chloride, phenol red, amino acids, vitamins, and others compared with F-12. On the other hand, some factors, such as hypoxanthine, linoleic acid, lipoic acid, putrescine, pyruvate, and thymidine, are contained only in F-12. Each of these components may have affected NO production of peritoneal macrophages. As for phenol red, DMEM contains higher amounts of phenol red as a non-physiological factor than F-12 (concentration of phenol red is 1.2 mM in F-12 and 15 mM in DMEM, respectively). We examined the influence of phenol red on the response of mouse peritoneal macrophages in F-12 that contained the same amount of phenol red as DMEM. However, there appeared no effect of 15 mM phenol red on NO production in stimulated macrophages (data not shown). So far, we have not identified the critical factor(s) that affect NO production even after extensive studies by addition to or removal from the factors described above.

Besides, we further examined the influence on the macrophages activation in RPMI-1640, with which the macrophages culture is often used. Interestingly, NO production from stimulated macrophages in RPMI-1640 was similar to that in DMEM (data not shown).

In this study, the activated macrophage phenotypes were influenced by distinct culture medium. Some kinds of macrophage responses might be influenced by their environment, including nutritional components and other elements. Besides the macrophage-like cell lines as J774.1 and RAW264.7 cells, mouse peritoneal macrophages were much more extensively influenced by the difference in the culture medium, implying that the macrophages in host animals, including humans, might show altered responses to the surrounding nutritional components other than specific cytokines or hormones. This point of view would seem to be very important for understanding macrophage responses and related phenotypes in terms of physiological meaning.

Pro-inflammatory cytokines such as TNF- α , IL-1, and IL-6 may induce increased vascular permeability and recruitment of inflammatory cells (Arango Duque and Descoteaux 2014). The inflammatory response might be beneficial for the host when the pro-inflammatory cytokines are produced in appropriate amounts, but it becomes harmful when they are produced in excess under deregulated production. For example, excessive production of TNF- α , IL-1, and IL-6 triggers an acute systemic inflammatory response or cachexia (Illman et al. 2005; Watkins et al. 1995). iNOS-derived NO is involved in various physiological, anti-microbial, anti-viral, anti-tumoral, and immunoregulatory activities (Akarid et al. 1995; Wink et al. 1998; Zamora et al. 2000). In contrast, aberrant iNOS induction may have detrimental results, and seems to be involved in the diseases such as asthma, multiple sclerosis, neurodegenerative disease, tumor development, and septic shock (Kleinert et al. 2003; Kroncke et al. 1998). Therefore, nutritional changes around macrophages may lead to excess production of NO or pro-inflammatory cytokines, resulting in the various aforementioned diseases.

Macrophages mostly originate from blood monocytes whose progenitor cells, pro-myelocytes, reside in the bone marrow. Although our study was performed with mouse peritoneal resident macrophages, the influence of the culture medium on blood monocytes and bone marrow-derived macrophages would seem to be worthy of study, because these cells become tissue macrophages after entering various tissues. These cells are thought to be present in tissues including alveoli and in the peritoneal cavity of all mammals, although this study was performed in vitro with mouse peritoneal macrophages. Because there is a distinct possibility that phenotypes of various macrophages might be changed by surrounding environmental components, we are going to perform experiments using mouse peritoneal macrophages in vivo in the future.

In conclusion, this study provides novel evidence that activated phenotypes of mouse peritoneal macrophages are influenced by the types of culture medium, which influence may lead to changes in host inflammatory responses, changes elicited by environmental nutrition factors. Besides, these changes in macrophage phenotypes might be a target of the host biological responses that are modified by nutrition factors, drugs or both.

Conclusion

This study shows that phenotypes of primary macrophages from mouse peritoneum could be changed significantly depending on the culture medium used, either F-12 or DMEM, in response to LPS- and/or IFN- γ -treatment. Among the activated macrophage phenotypes, the expression of iNOS showed the most remarkable differences in these media, and NO production by LPS- and/or IFN- γ -treated cells was far lower in DMEM than in F-12. However, such a difference in NO production was not observed when macrophage-like cell lines were examined. These results from the in vitro experiments suggest that the activation phenotypes of the macrophages are altered by the types of the culture media used, and primary macrophages are more susceptible to the culture media compared with macrophage-like cell lines.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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