Medical Research Archives

ESMED
European Society of Medicine

OPEN ACCESS

Published: January 31, 2023

Citation: Fumio Amano, 2022. Alteration of Activated Phenotypes of the Macrophages Treated with Lipopolysaccharide and Interferon-γ by Sodium Bicarbonate in the Culture Medium, Medical Research Archives, [online] 11(1). https://doi.org/10.18103/mra.v11i1.3493

DOI
https://doi.org/10.18103/mra.v11i1.3493

ISSN: 2375-1924

RESEARCH ARTICLE

Alteration of Activated Phenotypes of the Macrophages Treated with Lipopolysaccharide and Interferon-γ by Sodium Bicarbonate in the Culture Medium

Fumio Amano, Ph.D.

Former Address: Professor, Laboratory of Biodefense & Regulation, Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan
Present address: Guest Professor, Division of Health Sciences, Osaka University graduate School of medicine, 1-7 Yamadaoka, Suita, Osaka 565-0871, Japan

Email: fumio230amano45@yahoo.co.jp

ABSTRACT

Macrophage is the immune phagocytic cell, playing variety of immunological and inflammatory reactions. The macrophage activation has been extensively studied *in vitro* using culture media like Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium (F-12) in response to bacterial infection, tumor development, cytokines and so on. In the study of macrophage activation during co-culture with EL-4 tumor cells, we found different phenotypes of J774.1 macrophage-like cell line in F-12 and DMEM, when treated with lipopolysaccharide (LPS) and interferon-γ (IFN- γ). Among these phenotypes, nitric oxide (NO) production with corresponding inducible NO synthase (iNOS) expression was remarkable, showing higher in F-12 than in DMEM. Besides, O_2^-generating activity and production of interleukin-1β (IL-1β) were also higher in F-12 than DMEM, although production of tumor necrosis factor-α (TNF- α) was higher in DMEM than F-12. RT-PCR analysis revealed significantly higher expression of mRNA of iNOS, IL-1 β, IL-18, IκBα in F-12, but higher that of p65 and p105 in DMEM after treatment with LPS + IFN-γ, suggesting these differences being induced at the transcriptional levels. Through investigation of critical factor(s) in these culture media that influence the activation phenotypes of the macrophages, we found that sodium bicarbonate ($NaHCO_3$) concentrations in these culture media, 14 mM in Ham's F-12 and 44 mM in DMEM, were the key. Culture medium-induced differences in macrophage activation were also observed in RAW264.7 macrophage-like cell line and in mouse peritoneal macrophages. The recent studies suggested involvement of carrier (SLC) transporter gene expression and subsequent elevation of JAK/STAT signaling cascades in these $NaHCO_3$ responses. Taken together, these results provide evidence for the importance of $NaHCO_3$ in the culture medium in the macrophage activation *in vitro*, implying important insights to the $NaHCO_3$ concentration *in vivo* in the body of patients suffering from inflammation, tumor development or immune disorders where macrophage activation is involved.

Introduction

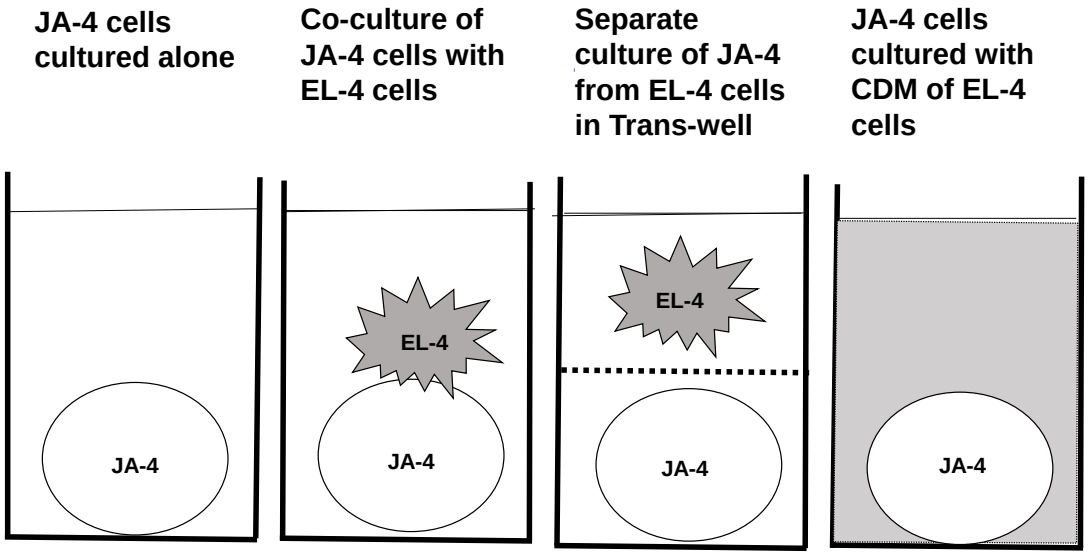
Macrophages are monocytic phagocytes involved in innate immune responses toward foreign materials, microbes, tumor cells and apoptotic cells in health and diseases[1-4]. In tumor immunity, macrophages play pivotal roles[1,2], showing cytotoxic effects toward target tumor cells through interactions not only through direct attachment but also by secretion of humoral mediators like tumor-necrosis factor (TNF). One of the most characteristic properties of the macrophages is "macrophage activation", that is the process of acquisition and expression of various phenotypes of the armed cells participating in self-defense[5,6]. Many studies of macrophage activation have been performed in vitro using cell culture systems with chemically defined medium such as Dulbecco modified Eagle's medium (DMEM), Ham's F-12 nutrient mixture (F-12), or Roswell Park Memorial Institute (RPMI)-1640 medium (RPMI 1640) and so on. Because these media are commercially available world-wide and have suitable such properties for the monocyte/ macrophage culture as stable growth and/or maintenance of the cellular phenotypes, each researcher usually selects only one cell culture medium to examine macrophage functions and mechanisms of macrophage activation. Although the choice of culture medium showed different effects on the growth and differentiation of human periodontal ligament cells[7], human dental pulp-derived cells[8], and dog periosteum-derived cells[9], there have been little reports describing the effects of different culture medium on macrophage activation. On the other hand, macrophages are known to be regulated by many kinds of environmental factors, such as pattern recognition molecules of microbes and chemicals, cytokines, chemokines, hormones, stresses of temperature, radiation, which leads to variation and numerous different phenotypes of the cells[6,10-13].

This review describes the facts that selection of culture medium, either F-12 or DMEM, influences on differences in the activated macrophage phenotypes, and that concentration of sodium bicarbonate is the key to cause these differences. It is also emphasized that much attention is necessary to be paid in the selection of culture medium before starting macrophage activation studies.

2 Macrophage activation during co-culture with tumor cells

In the previous study of macrophage activation *in vitro* with lipopolysaccharides (LPS) and monosaccharide precursors of Lipid A, we found that mouse peritoneal macrophages and a macrophage-like cell line, J774.1, showed cytotoxic effects toward EL-4 mouse thymoma cells during co-culture of the macrophages with EL-4 cells[14]. In the culture wells, attachment of the activated macrophages to the target EL-4 cells was frequently observed and production of superoxide (O_2^-) from the macrophages increased, suggesting the role of O_2^- in the killing of the target cells[15]. Besides, soluble factors like TNFα were produced by these activated macrophages, suggesting its involvement of the tumor cell killing as well[16].

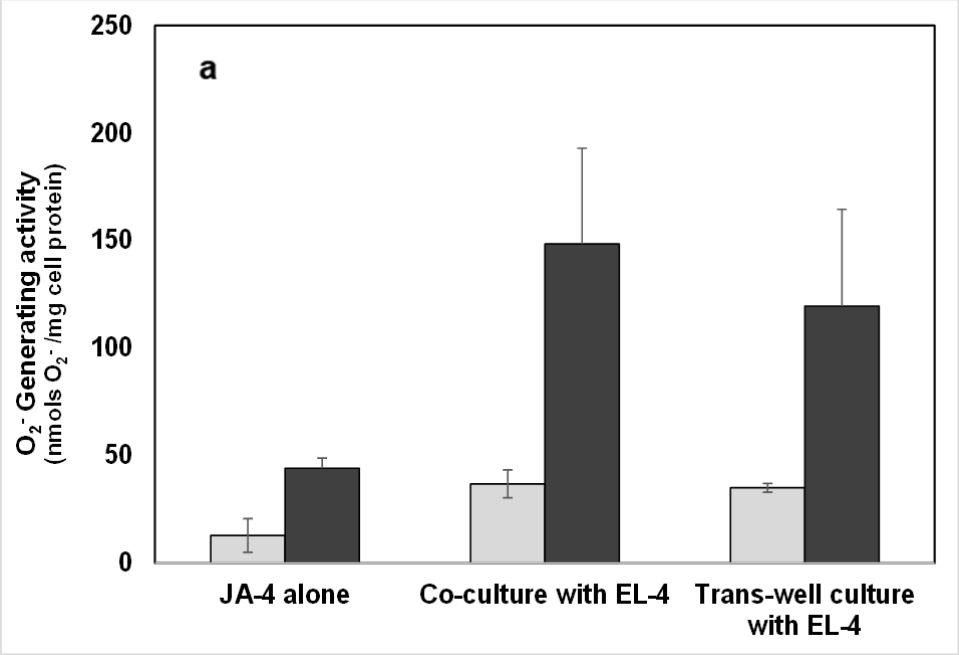
Macrophage activation during co-culture with tumor cells



Macrophage activation during co-culture with tumor cells summarizes the flow of the experiments leading to find out such soluble factor(s) of EL-4 tumor cells as inducing macrophage activation. In the presence of 10 U/mL interferon-γ (IFN- γ), co-culture of JA-4 cells with EL-4 tumor cells in Ham's F-12 medium resulted in spreading of the macrophages, adhesion of the macrophages to EL-4 cells, and induction of O_2^--generating activity of the macrophages. Because the separate culture of the macrophages from EL-4 cells using Trans-well^R culture system also induced spreading and induction of O_2^--generating activity of the macrophages, we examined conditioned medium (CDM) of EL-4 cells whether it might induce macrophage activation. Details of the experiments are shown in the following two figures 1a and 1b.

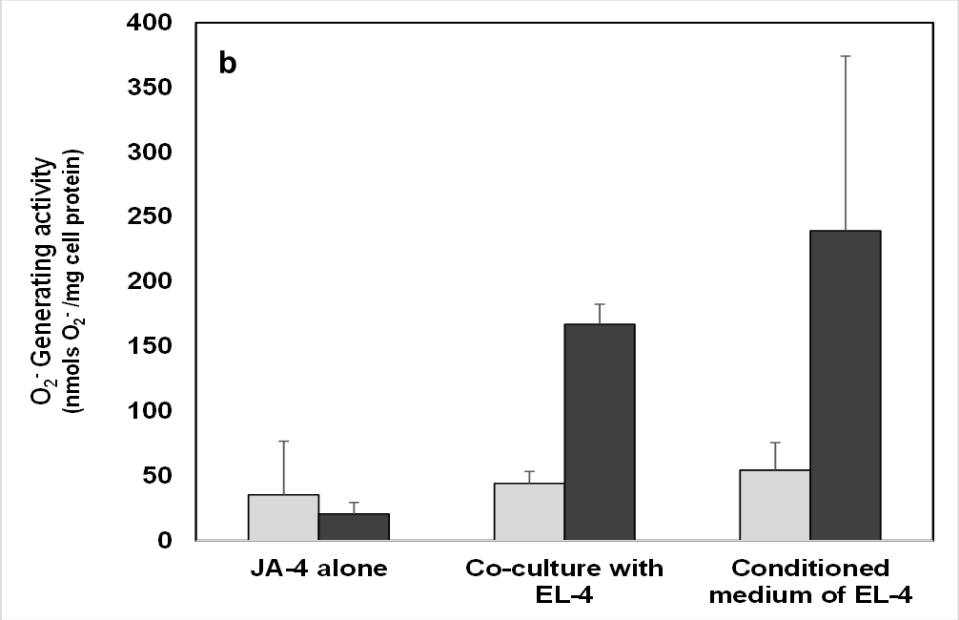
Based on these findings, we developed a co-culture system between JA-4 cells, a subline of J774.1 cell line[17], and EL-4 cells in a cell culture dish. In this experiment, although the macrophages had not been pre-treated with LPS, addition of EL-4 cells to the macrophage culture resulted in the activation of O_2^- generating activity of the macrophages, and addition of 10 U/mL interferon-γ (IFN- γ) to the co-culture enhanced the activity significantly (Fig. 1). These results suggested that direct attachment of the macrophages to EL-4 cells might have induced the elevation of the O_2^- generating activity of the macrophages. However, separate culture of EL-4 cells from the macrophages using Trans-well culture system also induced the activity (Fig. 1a), suggesting some of the soluble factors produced by EL-4 cells might be responsible for the activation of the macrophages. Then we examined the culture medium of EL-4 cells, which was prepared by culturing of EL-4 cells in F-12 medium overnight, and then collected, centrifuged, filtrated through 0.45mm sterile filter. These EL-4 culture supernatants, named as conditioned medium (CDM), was added by 20% to the macrophage culture, which showed induction of the O_2^- generating activity of the macrophages (Fig. 1b). These results suggested a possibility of the presence of certain macrophage-activation factor(s) in the CDM.

Fig. 1 a



In **Fig. 1a**, JA-4 cells were precultured and suspended in F-12 medium, then seeded and incubated at 37C for 4-8 h. The medium was replaced with fresh one with (black histogram) or without (gray histogram) 10 U/mL IFN-γ, and the macrophages were incubated for 16 h. Then the cells were rinsed with phosphate buffered saline (PBS) thoroughly, and 0.1 mL/ well of fresh F-12 medium was added, together with 0.1 mL/ well of either the medium alone or the same number of EL-4 tumor cells for co-culture, and the cells were incubated at 37C for 24 h, before assay of O_2^--generating activity as described before[17]. Concerning EL-4 cells, they were maintained in DMEM + 10% FBS, but for this assay, they were rinsed thoroughly with F-12 and the culture medium was exchanged with F-12 + 10% FBS and incubated at 37C for 16-18 h. EL-4 cells were then washed and suspended in fresh F-12 + 10% FBS, the same culture medium for JA-4 macrophages. For the Trans-well culture, JA-4 cells were seeded at the bottom of the 4 wells-clustered Trans-well plate, incubated in the presence or absence of 10 U/mL IFN-γ, followed by incubation with upper chamber containing the same number of EL-4 tumor cells simultaneously, and the incubation was performed for 24 h, before assay of O_2^--generating activity.

Fig. 1 b



In **Fig. 1b**, the methods were as described in the legends above, except for the assay of the conditioned medium (CDM) of EL-4, which was collected from the overnight culture of EL-4 cells in F-12 + 10% FBS, and then filtrated through 0.45 μm sterile membrane filter. The assay was performed by incubation of the preincubated macrophages together with 20% (v/v) of either CDM or the culture medium alone at 37C for 24 h.

Thereafter, we started to isolate such macrophage-activating factor from the CDM. At the beginning of the study, we exchanged the culture medium of EL-4 cells from DMEM, the maintenance medium of EL-4 cells, to F-12 every time, to facilitate adaptation of EL-4 cells to co-culture with macrophages. However, preparation of the CDM in a larger scale required more quick and easy protocols for EL-4 cell culture, and then we revised the methods of the CDM preparation to use EL-4 cell culture medium directly after incubation of the cells in fresh DMEM containing 10% fetal bovine serum (FBS) at 37C for 18 h, without subsequent exchanging with F-12. Although this CDM of EL-4 cells significantly induced macrophage activation, showing increased O_2^- generation, nitric oxide (NO) and inflammatory cytokines with elevation by IFN-γ addition, we found unexpected effects of DMEM added to the macrophage culture. When fresh DMEM was added instead of CDM as a negative control by 20% to the macrophage culture containing fresh F-12, it caused variation of the activated macrophage phenotypes not only by CDM but also by fresh DMEM; Decreased O_2^- generation and NO production, and increased release of TNFα and IL-1β, were observed. These results suggested a possibility that the macrophage activation might be varied in the different culture medium, either F-12 or DMEM. This is the time point that we decided to start investigation of the effects of culture medium whether they influence on the studies of macrophage activation.

3 Effect of culture medium on macrophage activation

In the subsequent studies, we focused on the culture medium *per se* with special interests on the macrophage activation. Among the activated macrophage phenotypes[18], NO generation was one of the remarkable phenotypes that were influenced by the selection of the culture medium either F-12 or DMEM, we mainly examined NO generation and inducible nitric oxide synthase (iNOS) induction in this study[19,20]. J774.1/JA-4 macrophages were preincubated at 37C either in F-12 or DMEM medium containing 10% FBS in a CO_2 incubator (5% CO_2-95% humidified air), then the medium was changed with fresh ones and the cells were incubated further with 10 ng/mL LPS with or without 10 U/mL IFN-γ. After 20 h, the culture supernatants were collected and assayed for nitrite anion (NO_2^-) by using Griess reagent. For estimation of iNOS protein, macrophages were collected, and the cell extracts were tested by Western blot analysis using an anti-mouse iNOS/NOS Type II antibody. The cells were also examined iNOS mRNA by RT-PCR by using iNOS (NOS2) primers.

As reported by Kawakami et al[18], NO production was slightly higher in DMEM than that in F-12, although induction of iNOS protein and iNOS mRNA was significantly higher in J774.1/JA-4 macrophages treated by LPS and IFN-γ in DMEM than those in F-12, as summarized in Table I. Time-course study of iNOS proteins and mRNA in these macrophages showed significantly higher induction in these cells in DMEM than in F-12, showing that the selection of the culture medium influenced on activation processes as well as expression of macrophage phenotypes[19]. The further analysis of these macrophages revealed, however, the extents of the induction concerning O_2^- generating activity in the macrophages were higher in the cells incubated in F-12 than those in DMEM (Table I.), indicating differences among activation phenotypes of the macrophages[18]. Besides, induction of TNF-α and IL-1β in these macrophages showed differences in these culture medium; TNF-α release and induction of pro-TNF-α and TNF-α mRNA were higher in DMEM than in F-12, although IL-1β release was higher in these cells at 12 h in F-12 but lower at 20 h than in DMEM with corresponding changes in IL-1β protein and IL-1β mRNA levels (Table I.). These results showed that the activation of macrophage varied among the phenotypes dependently on time-course and the culture medium.

Table I. Differences in activation phenotypes of J774.1 macrophages in F-12 and DMEM medium induced by 100 ng/mL LPS and 10 U/mL IFN-γ

J774.1/JA-4 cells were seeded and preincubated in F-12 or DMEM medium at 37C for 4 h, then the medium was changed with fresh each medium, respectively, followed by incubation in the absence or the presence of 100 ng/mL LPS and 10 U/mL IFN-γ. After incubation at 37C for 4-20 h., culture supernatants were collected and assayed for NO_2^-, TNF-α and IL-1β, and the cells were examined for protein of iNOS, pro-TNF-α and pro-IL-1β by Western blot analysis, and for mRNA of each by RT-PCR, as described in Kawakami et al[18]. The superscripts with parentheses in the table mean the culture time of the cells before assays.

	F-12			DMEM		
Phenotypes	Products or activity	Western blot	RT-PCR	Products or activity	Western blot	RT-PCR
NO_2^- /iNOS /iNOSmRNA	++$^{20h)}$	++$^{20h)}$	++$^{4h)}$	++~+++$^{20h)}$	++++$^{20h)}$	++++$^{4h)}$
O_2^- generating activity	+++$^{20h)}$			+$^{20h)}$		
TNF-α/ pro-TNF-α/ pro-TNF-α mRNA	++$^{20h)}$	+~++$^{20h)}$	++$^{4h)}$	+++$^{20h)}$	+$^{20h)}$	++$^{4h)}$
IL-1β /pro- IL-1β / pro- IL-1 β mRNA	++$^{20h)}$	+$^{20h)}$	+++$^{4h)}$	++++$^{20h)}$	++++$^{20h)}$	+$^{4h)}$

In addition to the influences on the transcriptional regulation of macrophage activation, the culture medium showed different effects at the post-transcriptional levels; The macrophages were incubated either in F-12 or DMEM at 37C for 20 h in the presence or absence of LPS + IFNγ, followed by medium changes with both F-12 and DMEM, and the 2nd incubation was performed by additional incubation for 2-6 h in the absence of LPS + IFN-γ. NO_2^- production during the 2nd incubation was always higher in F-12 than DMEM irrespectively to the medium used in the 1st incubation[18]. Analysis of the amounts of iNOS/NOS II protein by Western blot analysis, however, indicated that more iNOS protein was induced and retained in the activated macrophages that had been incubated in DMEM in the 1st incubation with LPS + IFN-γ, than those in F-12. These apparently opposite results might be explained by the level of NAPDH, co-factor of iNOS, which was significantly reduced and became less in DMEM than in F-12[18]. These results suggested that selection of F-12 or DMEM also influenced on the functions of the activated macrophages post-translationally.

Besides J774.1/JA-4 macrophage-like cell line, the effects of the culture medium on macrophage activation were examined using other macrophages including RAW264.7 macrophage-like cell line and primary macrophages from BALB/*c* and C57BL mouse peritoneum[19]. Similar results were obtained in these macrophages with reduced production of NO_2^- and increased release of TNF-α in DMEM than in F-12 in response to LPS + IFNγ. Because the difference of NO_2^- production from BALB/*c* mouse peritoneal macrophages between the culture in F-12 and DMEM was remarkable, the effects were examined precisely using these macrophages. The resident macrophages were collected and incubated in F-12 medium with 10% FBS at 37C for 1-1.5 h, and then the culture medium was changed with fresh F-12 or DMEM containing 10% FBS in the presence or

absence of 100 ng/mL LPS and /or 10 U/mL IFNγ. The culture supernatants and the cells were collected after incubation at 37C for 0-20 h, followed by assays of NO production, TNF-α release, and IL-1β release in the supernatants, and by analyses of mRNA and protein expression concerning iNOS and IL-1β, respectively. mRNA expression was also estimated concerning inflammatory cytokines like IL-6, IL-10, and IL-18, as well as such responsible proteins as IκBα, $p65^{Phox}$, $p105^{Phox}$, and arginase-1 and -2[19]. The results showed that many of the LPS-induced mRNA expression was lower in DMEM than that in F-12, suggesting influence of the culture medium, either F-12 or DMEM, on LPS-induced inflammatory responses of the mouse peritoneal macrophages at the transcriptional levels (Table II).

Table II. Effects of culture medium on mRNA expression of iNOS, IL-1β, IL-6, IL-10, IL-18, IκBa, p65, p105 and arginase-1/2 in BALB/*c* mouse peritoneal macrophages in the presence or absence of 100 ng/mL LPS

BALB/*c* mouse peritoneal macrophages were seeded and preincubated in F-12 or DMEM medium at 37C for 4 h, then the medium was changed with fresh each medium, respectively, followed by incubation in the absence or the presence of 100 ng/mL LPS. After incubation at 37C for 4 h, the cells were harvested and mRNA contents of each were examined by RT-PCR, as described in Kawakami et al[19]. The results are shown relatively with arbitrary scores with (-) to (+++++++), and the ratios between the results obtained from the culture with F-12 and those with DMEM are shown with arrows in the right end of the table.

	F-12		DMEM		DMEM/ F-12
mRNA	**- LPS**	**+LPS**	**- LPS**	**+LPS**	**+/- LPS**
iNOS	-	+++++	-	+	↓↓↓
IL-1β	-	+++++	-	++++	↓
IL-6	-	+++++	-	++++	↘
IL-10	+/-	+++++	+/-	++++++	↗
IL-18	++	+++++	+	++	↓↓↓
IκBα	+	+++++	+/-	++++	↓
p65	+++++	+++++	++++	+++++++	↑↑
p105	++	+++++	++	+++++++	↑↑
arginase-1	+++++++	+++++	++	++	↓
arginase-2	++	+++++	++++	+++++++	↑

4 Identification of sodium bicarbonate ($NaHCO_3$) as the key molecule responsible for altered responses of activated macrophages in different culture medium

To study the causes for the different activation phenotypes of the macrophages substantially, mixed medium with F-12 and DMEM at different ratios was prepared and NO production was assayed. Addition of DMEM to F-12 at and more than 50% resulted in the decline of NO production

dose-dependently, suggesting presence of suppressive factor(s) in DMEM on the macrophage activation[19]. Besides, mixing of saline with DMEM at 1:1 resulted in significantly higher production of NO than DMEM alone from the activated macrophages, also suggesting the presence of suppressive factor(s) in DMEM itself[19].

The chemical components of F-12 and DMEM at the molar bases are shown in Table III. Among them, some of the amino acids and vitamins, glucose, $CaCl_2$, KCl, $NaHCO_3$, and phenol red are higher in DMEM than in F-12. We prepared modified F-12, comprised with F-12 medium and the additives of the above to adjust the final concentrations of each to those of DMEM. Induction of iNOS proteins in J774.1/JA-4 macrophages cultured in these modified F-12 media in the presence of LPS + IFN-γ for 20 h showed increase in iNOS protein dependently on the doses of $NaHCO_3$ (Table I)[20]. The time-course and the dose-response experiments with the modified F-12 and DMEM, composed with 14 mM and 44 mM $NaHCO_3$, showed that addition of 44 mM $NaHCO_3$ in F-12 resulted in the increase of iNOS protein, iNOS mRNA and NO_2^- production, compared with the culture with standard F-12 with 14 mM $NaHCO_3$, and that addition of 14 mM $NaHCO_3$ in DMEM, the decrease of them from the levels in DMEM with 44 mM $NaHCO_3$, respectively[20]. These results suggested a possibility that $NaHCO_3$ is the key molecule in the culture medium responsible for induction of the differences in macrophage activation.

Table III. Composition of F-12 and DMEM medium

Composition of Ham's F-12 and DMEM was referred to the components list of each in Sigma Aldrich site (https://www.sigmaaldrich.com/US/en/search/culture-medium...), which is converted to be shown at the molar bases.

Components	F-12 (mM)	DMEM (mM)
Amino acids		
Glycine	0.100	0.400
L-Alanine	0.100	-
L-Arginine hydrochloride	1.000	0.398
L-Asparagine H_2O	0.100	-
L-Aspartic acid	0.100	-
L-Cysteine hydrochloride H_2O	0.200	-
L-Cystine 2HCl	-	0.201
L-Glutamic acid	0.100	-
L-Glutamine	1.000	4.000
L-Histidine hydrochloride H_2O	0.100	0.200
L-Isoleucine	0.031	0.802
L-Leucine	0.100	0.802
L-Lysine hydrochloride	0.199	0.798
Methionine	0.030	0.201

L-Phenylalanine	0.030	0.400
L-Proline	0.300	-
L-Serine	0.100	0.400
Threonine	0.100	0.798
L-Tryptophan	0.010	0.078
L-Tyrosine disodium salt dihydrate	0.030	0.398
L-Valine	0.100	0.803
Vitamines		
Biotine	0.00003	-
Choline chloride	0.1000	0.0286
D-Calcium panthothenate	0.0010	0.0084
Folic acid	0.0029	0.0091
Nicotinamide	0.0003	0.0328
Pyridoxine hydrochloride	0.0003	0.0194
Riboflavin	0.0001	0.0011
Thiamine hydrochloride	0.0009	0.0119
Vitamine B_{12}	0.0010	-
i-Inositol	0.1000	0.0400
Inorganic salts		
Calcium chloride ($CaCl_2$) (anhyd.)	0.3	1.8
Cupric sulfate (CuSO4 5H2O) (anhyd.)	0.00001	-
Ferric sulfate (FeSO4 $7H_2O$)	0.003	-
Ferric nitrate ($Fe(NO_3)3\ 9H_2O$)	-	0.0002
Magnesium chloride (anhyd.)	0.6023	-
Magnesium sulfate ($MgSO_4$) (anhyd.)	-	0.8139
Potassium chloride (KCl)	3.0	5.3
Sodium bicarbonate ($NaHCO_3$)	14.0	44.0
Sodium chloride (NaCl)	131.0	110.3
Sodium phosphate dibasic (Na_2HPO_4) (anhyd.)	1.0	-
Sodium phosphate monobasic ($NaH_2PO_4\ H_2O$)	-	0.9058
Zinc sulfate ($ZnSO_4\ 7H_2O$)	0.003	-
Other components		
D-Glucose	10.0	25.0
Hypothanthine Na	0.0300	-

Linoleic acid	0.0003	-
Lipoic acid	0.0010	-
Phenol red	0.0032	0.0399
Putrescine 2HCl	0.0010	-
Sodium pyruvate	1.0	-
Thymidine	0.0029	-

As for the pH changes, F-12 medium containing 14 mM $NaHCO_3$ showed pH 7.35 and that containing 44 mM $NaHCO_3$ showed pH 7.80. When pH of the former was adjusted with NaOH to 7.85, and that of the latter, with HCl to 7.40, iNOS protein levels of the activated macrophages showed similarly high levels as the cells cultured in F-12 with 44 mM $NaHCO_3$. Instead, when pH of F-12 medium with 14 mM $NaHCO_3$ was adjusted to 7.75 by lowering gas concentration of the CO_2 incubator from 5% to 3.5%, the cells resulted in no increase in iNOS protein expression. These results suggested that not the pH *per se* but the concentration of $NaHCO_3$ was responsible for the changes in iNOS expression and subsequent production of NO from the activated macrophages[20].

Besides, TNFα and IL-1β release was also higher in DMEM than in F-12 in the activated J774.1 macrophages (Table I). Because sodium bicarbonate (NaHCO3) is comprised with sodium ion (Na^+) and bicarbonate ion (HCO_3^-), we examined which ion was really responsible to raising the different activated macrophage phenotypes between in F-12 and DMEM. When F-12 medium containing 14 mM $NaHCO_3$ was added with 30 mM $KHCO_3$ or NaCl to raise concentration of HCO_3^- or Na^+ to 44mM, respectively, the activated macrophages produced similarly high NO_2^- in these two modified media as in F-12 with 44 mM $NaHCO_3$ but they produced similarly high TNFα only in F-12 that had been added with 30 mM $KHCO_3$, and non-increased IL-1β in neither of the modified medium (Table IV). These results showed the diverse effects of $NaHCO_3$ concentration on the induction of the activated macrophage phenotypes, showing that NO production might be regulated by both Na^+ and HCO_3^-, and TNFα by HCO_3^-, but IL-1β by neither of them alone but by $NaHCO_3$ itself[20].

Taken together, $NaHCO_3$ is the crucial factor that regulates the macrophage activation dependently on its doses in F-12 and DMEM.

Table IV. Influence of $NaHCO_3$, Na^+ and HCO_3^- on the induction of differences in activation phenotypes of J774.1 macrophages in the modified F-12 medium containing 100 ng/mL LPS and 10 U/mL IFN-γ

J774.1/JA-4 cells were seeded and preincubated in F-12 at 37C for 4 h, then the medium was changed with fresh F-12 medium containing 14 mM $NaHCO_3$ with or without addition of 30 mM $KHCO_3$ or NaCl, respectively, or F-12 medium containing 44 mM $NaHCO_3$, followed by incubation in the presence of 100 ng/mL LPS and 10 U/mL IFN-γ. After incubation at 37C for 20 h, culture supernatants were collected and assayed for NO_2^-, TNF-α and IL-1β, as described in Kawakami et al[18]. The results are shown relative with + ~ ++++, arbitrarily.

$NaHCO_3$	14 mM	44 mM	14mM	
Addition	(-)	(-)	30mM $KHCO_3$	30 mM NaCl
NO_2^-	++	++++	++++	++++
TNF-α	+++	++++	++++	+++
IL-1β	+	++++	+~++	+

5 Possible mechanisms underlying the novel effects of sodium bicarbonate on variation of macrophage responses

As for the mechanisms underlying induction of the different phenotypes of activated macrophages, Kawakami et al focused on the transporters and subsequent signals involved in regulation by $NaHCO_3$[21]. RAW264.7 macrophage-like cell line was mainly used in their study instead of J774.1/JA-4 cell line, because RAW264.7 cell line also exhibited different activation phenotypes between F-12 and DMEM[19], and because RAW264.7 cell line was suitable for gene-transfer experiments but J774.1/JA-4 cell line has many difficulties in them. RAW264.7 cells were maintained and incubated in $NaHCO_3$-free DMEM, and the activation was performed in the DMEM with 0, 10, 20 and 40 mM $NaHCO_3$ in the presence or absence of 100 ng/mL LPS + 10 U/mL IFN-γ. mRNA levels of IL-6, iNOS and COX-2 were examined at 6 h of the incubation, and the results showed significant increase in mRNA levels of IL-6, iNOS and COX-2 after incubation with 40 mM $NaHCO_3$ as well as 40 mM $KHCO_3$. Transport of bicarbonate ion (HCO_3^-) is known to be controlled by solute carrier (SLC) transporters[22]. Among them, SLC4 and SLC26 were reported to regulate acidification of phagosomes in macrophages[23,24]. Analysis of SLC4 and SLC26 mRNA expression in RAW264.7 macrophages showed expression of SLC4A2, SLC4A7, SLC26A6, and SLC26A11 mRNA[21]. Addition of 4, 4'-diisothiocyanate-2, 2'-stilbenedisulfonic acid disodium salt (DIDS), a broad inhibitor of SLC transporters, or S0859, an SLC4A7-selective inhibitor at 0, 50 or 100 mM to the culture of RAW264.7 macrophages with LPS+IFN-γ, resulted in dose-dependent inhibition of the increase in IL-6, iNOS and COX-2 mRNA. These results suggested that SLC transporters, especially SLC4A and SLC26A, were involved in the regulation of macrophage activation by $NaHCO_3$[21]. Besides, pretreatment with either DIDS or S0859 significantly inhibited the increase of intracellular pH of RAW264.7 macrophages caused by 40 mM $NaHCO_3$, suggesting the involvement of $NaHCO_3$/SLC transporter in the

regulation of macrophage activation through rise of intracellular pH.

Analysis of signaling pathways in the LPS+IFN-γ-treated macrophages, phosphorylation of mitogen-activated kinases (MAP kinases) [25, 26] including p38, Erk (extracellular regulated kinase) and JNK (c-Jun N-terminal kinase) was similarly induced both in the cells incubated in 10 mM and 40 mM $NaHCO_3$, but the extents of phosphorylation of STAT1 (signal-transducing activator of transcription 1) were significantly higher in the cells incubated in 40 mM $NaHCO_3$ than those in 10 mM $NaHCO_3$. Addition of DIDS or S0859 significantly inhibited this increase, suggesting the involvement of $NaHCO_3$/SLC transporter in JAK/STAT signaling cascade in the regulation of LPS+IFN-γ -mediated macrophage activation as well[21]. The further study with gene-transfer experiments concerning SLC4A2 and/or other SLC genes seem to be needed for elucidation of their crucial roles on the regulation of macrophage activation by $NaHCO_3$, as Sedlyarov et al[23] showed the role of SLC4A7 in macrophage phagosome acidification.

6 Discussion and perspectives in the future studies in macrophage biology and pathology

In this review, I summarized a series of our studies on the novel effects of $NaHCO_3$ on the induction of different phenotypes of activated macrophages, which were started with a small but important finding that J774.1/JA-4 macrophages behaved differently in F-12 and DMEM in response to LPS+IFN-γ, as described in the previous section (2 Macrophage activation during co-culture with tumor cells). This finding was given by the control experiments with DMEM alone to estimate the CDM of EL-4 tumor cell culture that had been prepared from DMEM, concerning macrophage activation assayed in F-12. The phenotypes of the activated macrophages were O_2^- generation, NO production, and secretion of TNFα and IL-1β (Table I). Surprising was the distinct effect of DMEM from F-12, despite the experiments of the same protocol using the same macrophages and the same macrophage activation factors, LPS+IFN-γ. The results were reproducible, showing induction of the different activation phenotypes of macrophages in these two media[18]. These studies finally showed that $NaHCO_3$ was the key molecule causing the differences[19,20], and its concentration, 14 mM in F-12 or 44 mM in DMEM, was suggested to regulate intracellular pH rise differently through SLC transporter(s)[21].

The results seem to call important attention to the macrophage researchers not only to select their culture medium properly but also to estimate variation in biological effects and phenomena of the macrophages reported from other laboratories using different culture media. Compared with fetal bovine serum, culture medium had not been a matter for many researchers to start with in their experiments, because a lot of the culture media are produced and provided commercially in the world now with certification of the chemical components. However, now, one more attention seems necessary to be paid for promotion of their studies to avoid misunderstanding, as described in this review.

Another important point is concerning physiological as well as pathological roles of $NaHCO_3$ on the macrophage functions. Macrophages, originated from monocytes, play many important roles in the host defense being

distributed in whole body compartments, showing diversity in different tissues and organs[6,10,26,27]. One of the most important characteristics of the macrophages is the macrophage activation, that includes gaining new properties to adapt to environmental changes and also to combat against foreign substances which are threatening host[6,28,29]. Because macrophages reside in many tissues through the bodies[30-32], where concentrations of O_2 and CO_2 vary, some macrophages might be exposed to high or low concentrations of these gas molecules, being influenced by different concentrations of bicarbonate ions (HCO_3^-) through SLC4 family transporters[21-23,33]. A possibility of changing macrophage functions, including activation, by HCO_3^- is a practical issue, as described in this review[18-21].

Medicals also imply attentions to compel macrophages to unusual bicarbonate exposure. For example, bicarbonate is often used as an anti-acidosis medicine at high doses for the patients of chronic kidney diseases (CKD) or metabolic acidosis[22,34-36]. Because acidosis and/or extracellular acidic environment sometimes affects homeostasis of the host[36,37], immune systems are influenced[37-39] and the chances of inflammation[39,40], infection of microbes[41-42] or progression of cancer[43,44] will be increased.

It seems important to remind that the respiratory gas balances impose many basic points to facilitate understandings of a series of these problems on the differences of the activated macrophage phenotypes caused by the different culture media, as described in this review. Dynamism of carbon dioxide (CO_2) in the body[45,46] influences immune systems not only in mammals[46,47] but also in insects[49], through microenvironments of the organs, the tissues and the cells. The further studies are necessary to understand the mechanisms of acidosis under variety of metabolic changes[31], together with the changes in innate immune systems focusing especially on regulation of intracellular pH not only in cytosols but also in some specialized organelles including endosomes[23].

7 Conclusion

In conclusion, activation of macrophages treated with LPS+IFN-γ *in vitro* is critically regulated by $NaHCO_3$ in the culture medium through SLC transporters.

Acknowledgements

This work was supported by a Grant-in-Aid for the Promotion of Science from the Ministry of Education, Culture, Sports, Science and Technology of Japan (JSPS KAKENHI) Grant Numbers 24590165, 12672147 and 15K08051. The first part of the study was mainly performed by Mr. T. Sakai, and the latter part, by Dr. T. Kawakami, which constructed the body of Dr. Thesis of T. Kawakami. I thank the members of the Laboratory of Biodefence and Regulation, Osaka University of Pharmaceutical Sciences, for many experiments and discussion.

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