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Further study on the roles of the effector molecules of immunosuppressive macrophages induced by mycobacterial infection in expression of their suppressor function against mitogen-stimulated T cell proliferation

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SUMMARY

Previously, we found that phospholipids and reactive nitrogen intermediates (RNI) collaborated in expression of the T cell mitogenesis-inhibitory activity of immunosuppressive macrophages induced by *Mycobacterium avium-intracellulare* complex (MAIC) infection. In this study, we examined the roles of free fatty acids (FFA) and prostaglandins (PG) as effectors of MAIC-induced macrophages, and moreover, their collaborating effects with RNI. First, treatment of MAIC-induced macrophages with quinacrine (phospholipase A_2 (PLA_2) inhibitor), dexamethasone (inhibitor of PLA_2 and PG synthesis) or indomethacin (PG synthesis inhibitor) attenuated their suppressor activity against concanavalin A (Con A)-induced mitogenesis of splenocytes (SPC), indicating important roles of FFA liberated from membrane phospholipids and PG, as effectors. Second, oleic acid, PGE_2, RNI generated from NOR 4 (a new nitric oxide (NO) donor), and phosphatidylserine (PS) exhibited suppressor activity against SPC mitogenesis without showing significant cytotoxicity, in an irreversible manner. Third, the suppressor activities of RNI and PGE_2 were potentiated by combined use with oleic acid in a synergistic manner. Fourth, a dual-chamber experiment in which target SPC were separated from MAIC-induced macrophages by a Millipore filter revealed a requirement for cell-to-cell contact for expression of the suppressor function of MAIC-induced macrophages. These findings indicate that RNI, FFA, PG, and phospholipids (presumably PS) and their collaboration play central roles in expression of the T cell mitogenesis-inhibitory function of MAIC-induced suppressor macrophages.

Keywords free fatty acids prostaglandins reactive nitrogen intermediates phosphatidylserine immunosuppressive macrophages *Mycobacterium avium-intracellulare* complex

INTRODUCTION

World-wide increases in the incidence of mycobacterial infections, including tuberculosis and *Mycobacterium avium-intracellulare* complex (MAIC) infections, coincident with the AIDS epidemic have been reported [1,2]. Mycobacterial infections cause severely depressed cellular immunity of hosts in the advanced stage of infection [3]. During the course of mycobacterioses in humans and experimental animals, generation of immunosuppressive macrophages is frequently encountered [4,5]. Previously, we found that immunosuppressive macrophages were induced in the spleens of host mice around 2–3 weeks after MAIC infection, and that these macrophage populations displayed potent suppressive activity against concanavalin A (Con A)-induced mitogenesis of splenocytes (SPC) [6,7]. Concerning effector molecules of MAIC-induced suppressor macrophages (MAIC-induced macrophages), the following has been elucidated [6–11]. First, although MAIC-induced macrophages showed increased reactive oxygen species release, scavengers for oxygen radicals (superoxide dismutase and catalase) could not attenuate the suppressive activity [7]. Therefore, reactive oxygen intermediates are not involved.

In the course of screening for other effector molecules, we found that SPC mitogenesis-inhibiting activity of the MAIC-induced macrophages was in part attenuated by N^G-monomethyl-L-arginine (NMMA) and aminoguanidine [9,10], specific inhibitors of nitric oxide (NO) synthase (NOS) [12]. In our study, it was also found that reactive nitrogen intermediates (RNI), including NO radicals generated from acidified nitrite ion [13], exerted an inhibitory activity against SPC mitogenesis [9]. Since mild acidification of NO_2^- yields NO radicals and other RNI including NO_2 through formation of HNO_2 [13], the amounts of NO radicals generated in this system seem to be much smaller than in the case of NOS-mediated NO formation. In this study, we therefore examined the T cell mitogenesis-inhibitory activity of RNI by using NOR 4, a newly synthesized NO radical donor [14].

Furthermore, our previous study revealed that some phospholipids, including phosphatidylserine (PS) and phosphatidylinositol,

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exerted an inhibitory activity against SPC mitogenesis [10]. In addition, we also found that free fatty acids (FFA) such as oleic and arachidonic acids also possessed suppressive activity against SPC mitogenesis [8]. Although these findings are suggestive of the probability of participation of phospholipids and FFA in expression of the immunosuppressive function of MAIC-induced macrophages, further studies are needed to elucidate the mechanisms of the suppression of T cell proliferation by these effectors. In this investigation, we carried out detailed studies on the modes of expression of the suppressor activity by FFA, phospholipids, and also prostaglandins (PG), one of well known effectors of immunosuppressive macrophages [6], by using modulators of phospholipase A_2 (PLA_2) activity and PG synthesis. Moreover, we examined combined effects of these effector molecules in exerting suppressive activity against SPC mitogenesis. In addition, a double-chamber experiment performed in this study clearly demonstrated that cell-to-cell interaction is a prerequisite for manifestation of the suppressor activity by MAIC-induced macrophages.

MATERIALS AND METHODS

Microorganisms

MAIC N-260 strain isolated from an MAIC patient was used. This organism was identified as *M. intracellulare* belonging to serovar 16.

Mice

Female BALB/c mice, 8–10 weeks old, purchased from Japan Clea Co. (Osaka, Japan) were used.

Special agents

Quinacrine, dexamethasone, indomethacin, oleic acid, arachidonic acid, PS, prostaglandin E_2 (PGE_2), NMMA, and aminoguanidine were purchased from Sigma Chemical Co. (St Louis, MO). NOR 4, (d,1)-N-[(E9-4-ethyl-3-[(2)-hydroxyimino]-5-nitro-3-hexene-1-yl]-3- pyridine carboxamide, was obtained from Dojindo Laboratory (Kumamoto, Japan). Other agents were obtained from Wako Pure Chemical Industry (Osaka, Japan). Quinacrine, indomethacin, PS, PGE_2, and NOR 4 were dissolved or finely emulsified into dimethyl sulfoxide (DMSO) by gentle sonication at the following concentrations: quinacrine, 100 mM; indomethacin, 1 mg/ml; PS, 25 mg/ml; PGE_2, 1 mM; NOR 4, 200 mM. Oleic and arachidonic acids were finely emulsified into the FFA-solubilizing buffer, 50 mM sodium bicarbonate pH 8·3 containing 5% DMSO and 0·1% Tween 80, by vigorous mixing at 100°C at the concentration of 5 mg/ml. The resultant samples were diluted with culture medium and then entirely dissolved or finely emulsified by gentle sonication. The other agents were dissolved into distilled water and diluted with culture medium at 100-fold or more.

Medium

RPMI 1640 medium (RPMI) supplemented with 25 mM HEPES, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin G, 5×10^{-5} M 2-mercaptoethanol and 5% (v/v) heat-inactivated fetal bovine serum (FBS) (M. A. Bioproducts, Walkersville, MD) was used for cell culture.

Mitogenic response of SPC

Con A-induced mitogenesis of SPC was measured as reported previously [6]. Briefly, $2{\cdot}5 \times 10^5$ SPC were cultured in 0·2 ml of 5% (v/v) FBS–RPMI containing 2 μg/ml Con A (Miles-Yeda Ltd., Israel) in the presence or absence of test agents including NOR 4, PS, PGE_2, and FFA in flat-bottomed 96-well microcultures plates (Corning Glass Works Co., Corning, NY) at 37°C in a CO_2 incubator for 72 h, pulsed with 0·5 μCi of ^{3}H-TdR (2 Ci/mmol; New England Nuclear Corp., Boston, MA) for the final 6–8 h. Cells were harvested onto glassfibre filters and counted for radioactivity using a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, IL). Since the above agents were added into the SPC cultures at concentrations of 0·1–0·4 mM (NOR 4), 0·025–0·25 μM (PGE_2), and 12·5–50 μg/ml (PS), the final concentrations of DMSO in these cultures were 0·05–0·2%, 0·0025–0·025%, and 0·05–0·2%, respectively. In the control experiments, the addition of 0·25% or 0·5% DMSO caused no significant changes in SPC mitogenesis, as follows: −DMSO, $49{\cdot}5 \pm 2{\cdot}3 \times 10^3$ ct/min; +0·25% DMSO, $51{\cdot}6 \pm 1{\cdot}6 \times 10^3$ ct/min; +0·5% DMSO, $45{\cdot}5 \pm 3{\cdot}8 \times 10^3$ ct/min. Oleic and arachidonic acids were added at concentrations of 12·5–50 μg/ml into the SPC cultures. In the control experiments, the addition of the equivalent volumes of the FFA-solubilizing buffer (1 : 400 to 1 : 100) caused no marked changes in SPC mitogenesis, as follows: −solubilizing buffer, $68{\cdot}5 \pm 1{\cdot}2 \times 10^3$ ct/min; +solubilizing buffer at 1 : 400, 1 : 200, and 1 : 100, $76{\cdot}7 \pm 2{\cdot}9 \times 10^3$ ct/min, 79·5 ± 1·4, and $89{\cdot}1 \pm 1{\cdot}6 \times 10^3$ ct/min, respectively.

Suppressor activity of MAIC-induced macrophages

Suppressor activity of MAIC-induced macrophages was measured as described previously [6]. Briefly, SPC were harvested from mice infected intravenously with 1×10^8 colony-forming units (CFU) of MAIC 2 weeks after infection. The SPC (1×10^6–4×10^6) were cultured in 0·2 ml of 10% (v/v) FBS–RPMI in four wells each of microculture plate in a CO_2 incubator for 2 h. Wells were then vigorously rinsed with Hanks' balanced salt solution (HBSS) containing 2% (v/v) FBS, and then 0·1 ml of 5% (v/v) FBS–RPMI was poured onto the resulting wells. This procedure usually gave >90% pure macrophage monolayer cultures, with active pinocytic ability of neutral red and with phagocytic ability against latex particles, containing about 5×10^4 cells per culture well from 2×10^6 of MAIC-induced SPC. Then $2{\cdot}5 \times 10^5$ of normal SPC in 0·1 ml of the medium containing 4 μg/ml (final 2 μg/ml) of Con A were poured onto the resultant macrophage cultures, unless otherwise specified. Con A-induced mitogenesis of SPC was then measured as described above. In some experiments, the macrophage monolayer culture was pretreated with either quinacrine (12·5 μM), dexamethasone (1 μg/ml), or indomethacin (25 μg/ml) in 0·1 ml of 5% (v/v) FBS–RPMI at 37°C for 2 h, rinsed once with 2% FBS (v/v)–HBSS, and subsequently measured for its suppressor activity against Con A-induced SPC blastogenesis as described above. Suppressor activity of MAIC-induced macrophages was calculated as:

$$\%\ \text{suppression of SPC mitogenesis} = \frac{^3\text{H-uptake}\ (-\text{macrophages}) - ^3\text{H-uptake}\ (+\text{macrophages})}{^3\text{H-uptake}\ (-\text{macrophages})} \times 100$$

Measurement of NO production

Culture supernatants of SPC with or without MAIC-induced macrophages in the presence or absence of NMMA or aminoguanidine

were allowed to react with Griess reagents, and nitrite content was quantified by measuring absorption at 550 nm.

RESULTS

Participation of FFA and PG in expression of the suppressor activity of MAIC-induced macrophages against SPC mitogenesis

In our previous study, NMMA and aminoguanidine (NOS inhibitors) [12,15] attenuated the suppressor activity of the MAIC-induced macrophages. In the present study, we observed a marked reduction of RNI-producing ability of MAIC-induced macrophages (in a monolayer culture prepared by seeding 5×10^5 MAIC-induced SPC) by these NOS inhibitors, as follows ($n = 4$): control (none added), $36{\cdot}6 \pm 0{\cdot}3\,\mu$M; +NMMA (0·5 mM, $6{\cdot}45 \pm 0{\cdot}24\,\mu$M (82% inhibition); +aminoguanidine (0·5 mM), $4{\cdot}55 \pm 0{\cdot}24\,\mu$M (88% inhibition). The reduction in RNI-producing ability of MAIC-induced macrophages due to these agents was accompanied by a significant decrease in their suppressor activity, as follows ($n = 4$): SPC mitogenesis (178 080 ± 2393 ct/min) was reduced to 377 ± 187 ct/min (99·8% suppression), when co-cultured with MAIC-induced macrophages. When the target SPC were co-cultivated with the MAIC-induced macrophages in the presence of NMMA or aminoguanidine, their mitogenic response was significantly increased ($P < 0{\cdot}05$, Student's *t*-test), as follows: +NMMA, 101 152 ± 4143 ct/min (38·2% suppression); +aminoguanidine, 100 595 ± 2426 ct/min (40·6% suppression). Neither NMMA nor aminoguanidine significantly affected SPC mitogenesis in the absence of MAIC-induced macrophages: +NMMA, 163 567 ± 9982 ct/min; +aminoguanidine, 169 479 ± 6807 ct/min. These findings confirm the role of RNI in MAIC-induced macrophage-mediated suppression of SPC mitogenesis.

Next, we examined the effects of pretreatment of MAIC-induced macrophages with quinacrine (PLA_2 inhibitor) [16], dexamethasone (lipocortin-dependent inhibitor of PLA_2 and consequently PG synthesis) [17], and indomethacin (cyclo-oxygenase inhibitor causing reduction of PG synthesis (especially PGE_2)) [18]. As indicated in Table 1, short-term (2 h) treatment of MAIC-induced macrophages with these agents significantly ($P < 0{\cdot}01$; Student's *t*-test) reduced their suppressor activity. In this experiment, the recovery of viable cells of untreated control macrophages at 0 time and after 24 h cultivation was as follows ($n = 6$): 0 time, $2{\cdot}40 \pm 0{\cdot}07 \times 10^4$ cells/well; 24 h cultivation, $2{\cdot}22 \pm 0{\cdot}18 \times 10^4$ cells/well (93% of the 0 time control). Viable cell recovery after 24 h cultivation of pretreated macrophages was sufficiently high compared with that of untreated macrophages (fixed as 100%), as follows ($n = 3$): quinacrine treatment, 77 ± 14%; dexamethasone treatment, 155 ± 18%; indomethacin treatment, 113 ± 7%. Therefore, the observed reduction in suppressor activity of MAIC-induced macrophages by these agents is not due to their cytotoxic effect. It is somewhat strange to note the increase in viable cells due to treatment with dexamethasone or indomethacin. In particular, dexamethasone added at the higher dose, such as 1 μg/ml, caused a similar increase in the recovery of viable macrophages (153 ± 8%). The precise reason for this phenomenon is unknown. However, it is possible that these agents induce proliferative response of certain cell populations or prevent detachment of macrophages from culture wells during 24 h cultivation. The reproducibility of this result was confirmed by repeated experiments, and moreover, the same result was obtained when these agents were added into culture medium for Con A-stimulated SPC co-cultured with MAIC-induced macrophages (data not shown). Therefore, it is concluded that FFA and PG play important roles in expression of the immunosuppressive function of MAIC-induced macrophages. However, the possibility still remains that dexamethasone reduced the suppressor activity of MAIC-induced macrophages by reducing their RNI production, as reported by Knowles *et al.* [19].

Table 1. Effect of pretreatment of *Mycobacterium avium-intracellulare* complex (MAIC)-induced splenic macrophages with quinacrine (phospholipase A_2 inhibitor), dexamethasone (phospholipase A_2 and prostaglandin (PG) synthesis inhibitor), or indomethacin (PG synthesis inhibitor) on their suppressor activity against concanavalin A (Con A) mitogenic response of splenocytes (SPC)*

MAIC-induced macrophages	Pretreatment with	SPC mitogenesis (10^3 ct/min ± s.e.m.; $n = 4$)	Percent suppression of mitogenesis
−	—	97·2 ± 2·1	—
+	Medium alone	25·8 ± 5·1	73·5
+	Quinacrine	78·0 ± 2·5**	19·8
+	Dexamethasone	51·1 ± 2·5***	47·4
+	Indomethacin	72.3 ± 2.0**	25·6

* The macrophage monolayer culture prepared by seeding 1×10^6 MAIC-induced SPC was preincubated in the medium with or without addition of either quinacrine (12·5 μM), dexamethasone (1 μg/ml), or indomethacin (25 μg/ml) at 37°C for 2 h. Normal SPC were cultured with the resultant MAIC-induced macrophages in the medium containing 2 μg/ml Con A. In separate experiments for solute control, 0·125% or 2·5% dimethyl sulfoxide (equivalent to its concentrations in the media with the addition of 12·5 μM quinacrine and 25 μg/ml indomethacin) caused no significant effect on the suppressor function of MAIC-induced macrophages. When monolayer culture of MAIC-induced macrophages obtained by seeding 2×10^6 of MAIC-induced SPC was treated or not treated with 3·0% dimethyl sulfoxide, the resultant macrophage caused 95% and 99% suppression of SPC mitogenesis, respectively.

** $P < 0{\cdot}005$; *** $P < 0{\cdot}01$; significantly higher than the value of the control (medium alone) macrophages.

Table 2. Irreversible inhibitory effect of NOR 4-derived reactive nitrogen intermediates (RNI), oleic acid, and prostaglandin E_2 (PGE_2) against concanavalin A (Con A)-induced mitogenic response of splenocytes (SPC)

Addition*	Concentration	Preincubation of SPC	SPC mitogenesis (10^3 ct/min ± s.e.m.; $n = 4$)	Percent inhibition of mitogenesis
—	—	−†	85·1 ± 2·4	—
—	—	+‡	103 ± 10·2	—
NOR 4	0·4 mM	−†	20·2 ± 3·9	76·3
NOR 4	0·4 mM	+‡	0·18 ± 0·03	99·8
Oleic acid	50 μg/ml	−†	0·29 ± 0·02	99·7
Oleic acid	50 μg/ml	+‡	0·20 ± 0·06	99·8
PGE_2	0·25 μM	−†	42·7 ± 1·5	49·8
PGE_2	0·25 μM	+‡	60·5 ± 2·2	41·3

* In separate experiments for solute control, dimethyl sulfoxide as a solvent for NOR 4 or PGE_2 and the solubilizing buffer for oleic acid caused no significant effect on SPC mitogenic response, as mentioned in Materials and Methods.

† Fresh SPC without preincubation were measured for their mitogenic response to 2 μg/ml Con A in the presence or absence of indicated agents.

‡ SPC were preincubated in the Con A-free medium (3 ml) with or without the addition of the indicated agents at 37°C for 24 h (the first culture). Preincubated SPC were thoroughly washed with 2% fetal bovine serum–Hanks' balanced salt solution and thereafter measured for their Con A response in the absence of the agents (the second culture).

The modes of the suppressive action of RNI, FFA, PG, and phospholipids against Con A-induced SPC mitogenesis

Previously, we found PS displayed an irreversible inhibitory effect against the Con A-induced mitogenic response of SPC [10]. In Table 2, results are shown of the study of the other effectors, including RNI, FFA, and PG, for their suppressor activities against SPC mitogenesis, and whether or not their SPC mitogenesis-inhibitory activities were reversible. First, RNI generated from NOR 4 (0·4 mM) which releases approx. 0·3 mM NO radicals during 6 h [14] exerted a potent inhibitory action against Con A-induced SPC mitogenesis. This result confirms our previous findings on the role of RNI using acidified $NaNO_2$ as a NO donor [10]. Oleic acid (50 μg/ml) also caused a marked reduction in SPC mitogenesis, while PGE_2 (0·25 μM) showed a weaker activity than the former effectors. In separate experiments, these agents exerted inhibitory activity in a dose-dependent manner, and moreover arachidonic acid (25 μg/ml) also displayed suppressive activity (data not shown). When SPC ($2{\cdot}5 \times 10^5$ cells/well) were cultured in a Con A-free medium alone for 24 h, viable cell recovery was $1{\cdot}99 \pm 0{\cdot}08 \times 10^5$ viable cells (80·0% of seeded SPC). In this case, the percent recovery of viable cells in the presence of the above agents compared with the untreated control (medium alone; fixed as 100%) was as follows: NOR 4, 74 ± 7%; oleic acid, 70 ± 7%; PGE_2, 75 ± 7%. This indicates that these effectors exhibit not so marked cytotoxic action against SPC when added at the above concentrations, which caused remarkable reduction of SPC mitogenesis (Table 2). Therefore, other mechanisms than cytotoxicity may be crucial for expression of the SPC mitogenesis-suppressing activity of these effector molecules.

Next, SPC exposed to NOR 4 (0·4 mM), oleic acid (50 μg/ml), or PGE_2 (0·25 μM) at 37°C for 24 h were measured for the Con A mitogenic response, in order to know whether or not these agents acted on the target SPC in a reversible or irreversible fashion. As shown in Table 2, SPC which had been preincubated with these agents showed a reduced blastogenic response to Con A in the drug-free medium. The extent of the reduction was at a level close to that observed when Con A mitogenesis of untreated SPC was measured in the presence of the same concentration of these agents. This indicates irreversibility of the action of RNI, FFA, and PGE on SPC, as previously observed for the case of PS [10].

The next question is whether phospholipids directly act on target SPC or their immunosuppressive action is mediated by FFA molecules generated from membranous phospholipids due to enzymatic hydrolysis by PLA_2 located on the cell membrane of target SPC. As shown in Fig. 1, quinacrine (PLA_2 inhibitor) [16] did not influence PS-mediated suppression of SPC mitogenesis. Similar results were obtained when another PLA_2 inhibitor, dexamethasone, was used (data not shown). Therefore, the latter possibility is excluded.

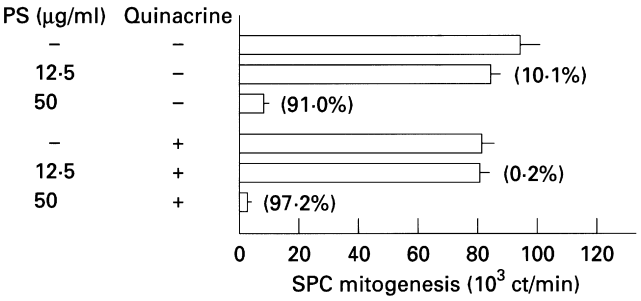

Fig. 1. Effect of quinacrine (phospholipase A_2 inhibitor) upon expression of the suppressor activity of phosphatidylserine (PS) against concanavalin A (Con A)-induced mitogenic response of splenocytes (SPC). SPC were cultured in medium containing 2 μg/ml Con A and the indicated amounts of PS in the presence or absence of 0·63 μM quinacrine. Each bar indicates the mean ± s.e.m. ($n = 4$). In parentheses, percent inhibition due to quinacrine addition is indicated. Dimethyl sulfoxide as a solvent for PS or quinacrine caused no significant effect on SPC mitogenic response, as described in Materials and Methods.

Table 3. Inhibitory activities of NOR 4-derived reactive nitrogen intermediates (RNI), oleic acid, and prostaglandin E_2 (PGE_2), or combinations of these against concanavalin A (Con A)-induced mitogenic response of splenocytes (SPC)*

NOR 4 (mM)	Oleic acid (μg/ml)	PGE_2 (μM)	SPC mitogenesis (10^3 ct/min ± s.e.m.)†	Percent inhibition of mitogenesis
—	—	—	95·6 ± 5·8	—
—	12·5	—	121 ± 5·1	−26·6
0·2	—	—	44·3 ± 2·5	53·7
0·2	12·5	—	23·3 ± 0·7‡	75·6
—	—	—	84·1 ± 4·3	—
—	—	0·25	37·2 ± 0·9	55·8
0·1	—	—	59·9 ± 2·0	28·8
0·1	—	0·25	24·8 ± 1·6‡	70·5
—	—	—	101 ± 1·7	—
—	25	—	103 ± 2·0	−2·0
—	—	0·025	68·1 ± 5·5	32·6
—	25	0·025	6·4 ± 1·3‡	93·7

* Normal SPC were cultured in medium containing 2 μg/ml Con A with or without the addition of indicated agents. Dimethyl sulfoxide as a solvent for NOR 4 or PGE_2 and the solubilizing buffer for oleic acid caused no significant effect on SPC mitogenic response, as described in Materials and Methods.

† The mean of three or four incubations.

‡ Significantly lower than the values of each agent alone ($P < 0{\cdot}005$).

Collaboration of effectors in suppression of SPC mitogenesis

In our previous work [10] we found that RNI collaborated with PS in the manifestation of its suppressive activity against SPC mitogenesis. That is, the suppressor activity of acidified $NaNO_2$-derived RNI was augmented by combination with PS in a synergistic manner. Therefore, it is of interest to know whether or not RNI collaborate with other effectors. Table 3 shows representative results of repeated experiments concerning the combined effects of some effector molecules against SPC mitogenesis. First, the suppressor activity of NOR 4 (0·2 mM)-derived RNI was significantly potentiated by combination with oleic acid (12·5 μg/ml), which alone showed no inhibitory action. This means a combined effect of RNI with oleic acid. In separate experiments, the RNI action was similarly augmented in

Table 4. Requirement for cell-to-cell contact in expression of the suppressor function of *Mycobacterium avium-intracellulare* complex (MAIC)-induced macrophages against concanavalin A (Con A)-induced mitogenesis of splenocytes (SPC)*

Cultivation of SPC in	MAIC-induced macrophages (10^7 SPC eq.)	SPC mitogenesis (10^3 ct/min ± s.e.m.; $n = 4$)	Percent suppression of mitogenesis
Bottom chamber	—	512 ± 12†	—
(16-mm well)	1·25	18·2 ± 2·3	96·4
	2·5	1·0 ± 0·1	99·8
	5·0	1·1 ± 0·03	99·8
Top chamber	—	289 ± 15†	—
(10-mm well)	1·25	272 ± 21	5·9**
	2·5	237 ± 51	18·0
	5·0	224 ± 18	22·5***

* The monolayer culture of MAIC-induced macrophages was prepared on a 16-mm culture well (bottom chamber) by seeding indicated numbers of MAIC-induced SPC. Then, normal SPC (1×10^6 cells) in 1 ml medium were seeded into the bottom chamber allowing cell-to-cell contact of SPC with the MAIC-induced macrophages, or SPC in 0·5 ml medium were seeded into a 10-mm culture well equipped with a Millipore filter (top chamber) immersed into 0·5 ml medium in the bottom chamber to separate target SPC from the MAIC-induced macrophages, and then cultured in the presence of 2 μg/ml Con A for 72 h.

† $n = 9$ or 10 in the control cultures.

** $P < 0{\cdot}01$; *** $P < 0{\cdot}025$; significantly lower than the value for the corresponding SPC culture in a bottom chamber.

combination with arachidonic acid (data not shown). Second, the suppressive activity of NOR 4 (0·1 mM)-derived RNI was merely additively increased by combined use with PGE_2 (0·25 μM). The same result was obtained when PGE_2 was added at 0·025 μM (data not shown). Third, when PGE_2 (0·025 μM) was used in combination with oleic acid (25 μg/ml) which exhibited no suppressive action against SPC mitogenesis, a marked increase in the suppressor activity of PGE_2 was observed. Similar results were obtained when PGE_2 and oleic acid were added at concentrations of 0·25 μM and 12·5 μg/ml, respectively (data not shown). This means a synergistic effect of PGE_2 with oleic acid. On the other hand, PGE_2 did not exhibit significant combined effect with PS (data not shown). Therefore, these results indicate that, of various combinations of the four effectors with each other, only the combinations of 'RNI + PS' [10] and 'RNI + oleic acid', and 'PGE_2 + oleic acid' gave a real combined inhibitory effect against SPC mitogenesis.

Cell-to-cell contact is needed for expression of MAIC-induced macrophage-mediated suppression of SPC mitogenesis

In order to discover whether the suppressor action of MAIC-induced macrophages is based on the action of the above soluble effectors released from these macrophages, or requires direct cell-to-cell contact of the effector macrophages with target SPC, the following dual-chamber experiment was done (Table 4). First, SPC were cultivated on the monolayer culture of MAIC-induced macrophages prepared in a 16-mm culture well (Corning) (designated 'bottom chamber') in 1 ml medium with the addition of 2 μg/ml Con A. Second, SPC suspended in 0·5 ml medium were added into a 10-mm well (designated 'top chamber') equipped with a 0·45-μm Millipore filter-bottom (Millipore Corporation, Bedford, MA), which was immersed in 0·5 ml medium poured onto an MAIC-induced macrophage monolayer in the bottom chamber, and cultured in the presence of 2 μg/ml Con A. By using the latter culture system, SPC in the top chamber were separated from MAIC-induced macrophages by a Millipore membrane, but this system allowed the diffusion of soluble factors between the two chambers. As shown in Table 4, expression of the suppressor activity of MAIC-induced macrophages was remarkably reduced by separating SPC from MAIC-induced macrophages by a Millipore filter in dual chambers. Therefore, expression of the suppressor action of MAIC-induced macrophages is principally dependent upon their cell-to-cell contact with target T cells.

However, at higher densities of MAIC-induced macrophages, a weak but significant suppression of SPC mitogenesis was caused by the suppressor macrophages, even when they were separated from the target SPC in the dual-chamber system. This indicates that a small portion of the suppressor action of MAIC-induced macrophages is mediated by soluble effector molecules which do not require cell-to-cell contact.

DISCUSSION

RNI have been reported to be responsible for expression of the T cell mitogenesis-inhibitory effect of some kinds of immunosuppressive macrophages and natural suppressor cells, in a number of studies using NOS inhibitors, including NMMA and aminoguanidine [9,10,20–22]. Previously, we found that acidified $NaNO_2$-derived RNI exhibited a strong suppressive activity against SPC mitogenesis [10]. In the present study we found that RNI liberated from NOR 4, a newly synthesized NO donor [14], also exerted potent suppressor activity at concentrations at which they showed no significant cytotoxicity. RNI are, therefore, considered to be important effectors for the suppressor activity of MAIC-induced macrophages. Attenuation of the suppressor function of MAIC-induced macrophages by dexamethasone (Table 1) may support this idea, since the agent prevents NO formation of macrophages [19], although this phenomenon may be also partly attributable to its inhibitory action against biosynthesis of PLA_2 and PG [17], as described below. Recently, it has been demonstrated that human alveolar macrophages do not utilize RNI in expressing their suppressive action against mitogen-stimulated T cell proliferation [23]. RNI seem, therefore, not to be the only effector molecule of immunosuppressive macrophages. Indeed, in our previous [10] and present studies, NOS inhibitors did not fully overcome the suppressor activity of MAIC-induced macrophages. Thus, it is important to identify and characterize types of effector molecules other than RNI.

Macrophages activated by mycobacterial infection release a large amount of FFA [24], which are known to cause inhibition of mitogen-induced T lymphocyte proliferation [8,25]. Oleic acid is also known to perturb transmembrane signallings of T cells, such as Con A-induced Ca^{2+} influx [26], presumably due to a physical perturbation of membrane lipid. Our present study demonstrated that quinacrine (PLA_2 inhibitor) [16] and dexamethasone (PLA_2 inhibitor) [17] significantly attenuated suppressor function of MAIC-induced macrophages (Table 1). Furthermore, oleic and arachidonic acids, the major FFA components of membrane phospholipids of murine macrophages [27], greatly reduced SPC mitogenesis in an irreversible manner (Table 2 and unpublished observation). Therefore, it can be concluded that FFA play an important role as effectors of MAIC-induced macrophages in expression of their suppressor function.

With respect to the inhibitory action of indomethacin against expression of the suppressor function of MAIC-induced macrophages, the recent finding by Gan *et al.* [28] is interesting. They found that programmed cell death of MAIC (*M. avium*)-infected macrophages was accelerated by treatment with indomethacin, concomitant with decrease in *M. avium*-dependent accumulation of mRNA encoding plasminogen activator inhibitor type-2 (PAI-2). Therefore, it is likely that part of the indomethacin-mediated inhibition of the suppressor function of MAIC-induced macrophages observed here is due to the same mechanism. However, in our study the bacterial load of MAIC-induced macrophages was about 0·18 ± 0·02 bacilli/macrophage cell, which is only 12 times smaller than the value (2·3 ± 1·3 bacilli/macrophage) of human monocytes infected with *M. avium* in the *in vitro* culture system of Gan *et al.* [28]. Therefore, the contribution of indomethacin-induced apoptosis of MAIC-infected macrophages seems not so significant in our experimental system.

Previously, we found suppressive activity of some phospholipids such as PS and phosphatidylinositol [10]. Similar suppressor activity of phospholipids was reported by Caselli *et al.* [29]. These findings strongly suggest the important role of phospholipids as immunomodulators of T cell function. In this study, quinacrine (PLA_2 inhibitor) [16] did not reduce the SPC mitogenesis-suppressing activity of PS (Fig. 1). This excludes the possibility that PS molecules are cleaved by hydrolytic action of PLA_2 which are located on the cell membrane of SPC, and FFA molecules generated from PS consequently attack the target SPC as a true effector. Thus, it seems that phospholipids themselves directly act on the target SPC. In the context of our findings on PS action, it is

noteworthy that PS transported to external leaflet of plasma membrane acts as a membrane 'flag' on apoptotic cells, resulting in the recognition and engulfment of these cells by phagocytes which possess PS receptors, as reported by Fadok *et al.* [30]. Moreover, a recent study of Martin *et al.* [31] using a Jurkat T lymphocyte leukaemia cell line, A1.1 T cell hybridoma, HL-60 promyelocytic leukaemia cell line, murine thymocytes, and human neutrophils demonstrated that PS (but not other phospholipids), which is normally confined to the internal leaflet of the plasma membrane, was externalized during apoptosis in a stimulus-independent manner. Thus, it is probably that exogenous PS molecules supplied to the target T cells by direct addition to culture medium or presented in a suppressor macrophage-dependent manner also play the same role. Therefore, it will be of interest to see if MAIC-induced splenic macrophages recognize apoptotic cells in a PS-dependent manner, or use the thrombospondin/vitronectin/CD36 system [30,32]. On this point, detailed studies are needed.

In relation to this, we previously found that the suppressor activity of MAIC-induced macrophages was reversed by cytochalasin B [6], indicating an essential role of microfilament network-dependent membrane function of these macrophages in expression of their immunosuppressive activity. In the present study, we confirmed that cell-to-cell contact is required for expression of the suppressor function of MAIC-induced macrophages, since only a weak suppression of SPC mitogenesis was observed when the target SPC were separated from the macrophages by a Millipore filter in a dual-chamber culture system (Table 4). These findings provide support for the hypothesis that the effector molecules of MAIC-induced macrophages, including RNI, FFA, PG, and phospholipids, are principally presented to the target T cells via direct cell-to-cell contact. Such cell-to-cell contact might contribute to an increase in efficacy of these four effectors to attack the target SPC, by enhancing the delivery of the effector molecules to the lymphocytes and decreasing their dilution. However, there still remains another possibility, that suppressive effectors are released from MAIC-induced macrophages only when these macrophages face the target SPC through cell-to-cell interaction and receive some signals from the SPC.

It is noteworthy that, of various combinations among the four effectors, 'RNI + PS' [10], 'RNI + oleic acid', and 'PGE_2 + oleic acid' showed a real combined inhibitory effect against SPC mitogenesis. This supports the idea that these effectors mediate the suppressor function of MAIC-induced macrophages, by collaborating with each other. Further studies are currently underway concerning effects of these effector molecules of MAIC-induced macrophages upon a signal transduction cascade in mitogen-stimulated T cells.

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