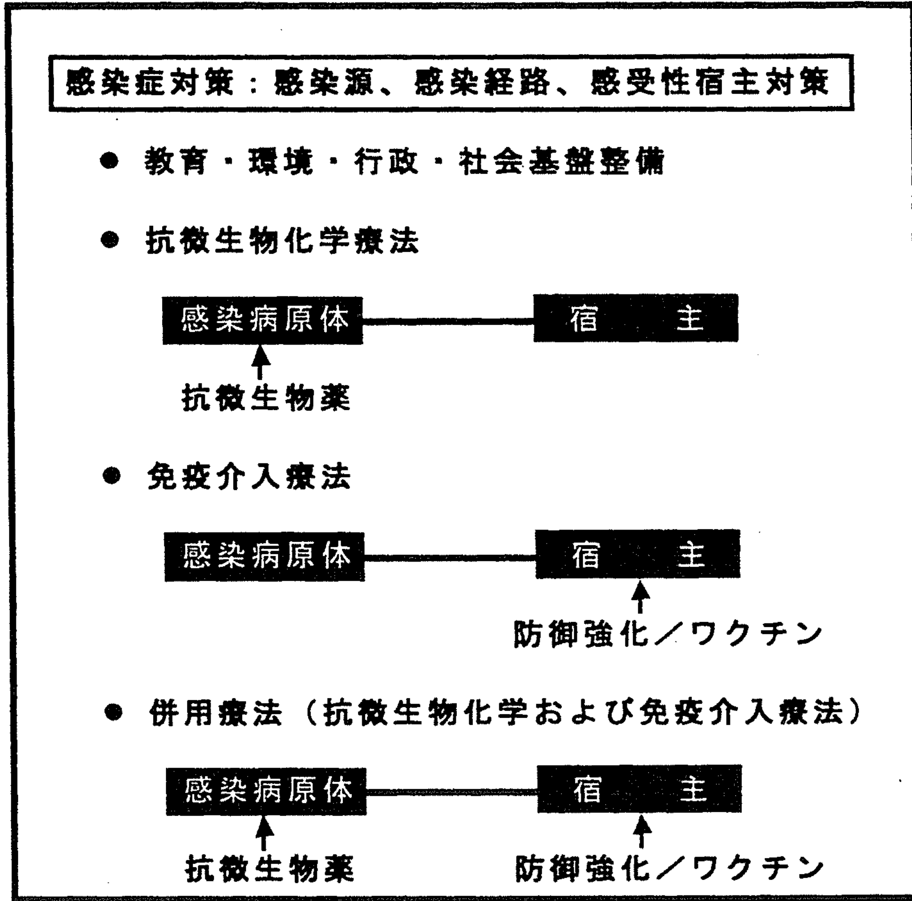

図—2　感染症制圧戦略

後，これら病原体感染による悪性腫瘍の減少が期待される[15]。

野生型ポリオ根絶，次いで，麻疹，百日咳，ジフテリア，結核の制圧や根絶に人類は邁進するであろう。しかし，微生物界は新興病原体や薬剤耐性微生物を産出して，人間社会に侵入し，健康に対する脅威を提供することが反復されるだろう。さらに，腸管出血性大腸菌感染症，メチシリン耐性ブドウ球菌感染症や結核などの集団・病院を含めた施設内感染，さらに，生物テロも脅威であり，感染症危機管理体制の確立は急務の課題である。

21世紀の世界や人類は感染症と戦い，制圧／根絶，あるいは共存を指向

15) Steinbrook, R.2006. The potential of human papillomavirus vaccines. N. Engl. J. Med. 354 : 1109–1112.

した新しい道を模索することになるが，この過程には多くの困難や障害が待ち受けていることだろう。教育・環境・行政・社会基盤整備に加えて，人類の叡智が感染症を克服することを，そして，健康被害を減少させることを期待している。

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Macrophage scavenger receptor down-regulates mycobacterial cord factor-induced proinflammatory cytokine production by alveolar and hepatic macrophages

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Abstract

We aimed to reveal the regulatory function of macrophage scavenger receptor-A (MSR-A) in proinflammatory cytokine production by macrophages stimulated with mycobacterial cord factor (CF). By the culture with CF, MSR-A (+/+) alveolar macrophages and Kupffer cells produced TNF-α/MIP-1α in a time- and dose-dependent manner. However, the amounts of cytokines produced by them were much less compared to those produced by MSR-A (−/−) macrophages. Consistent with this, treatment of MSR-A (+/+) macrophages with anti-MSR-A antibody increased TNF-α production. Binding of CF to MSR-A was demonstrated by measuring the binding affinity. These results indicate that CF binds MSR-A, and MSR-A down-regulates TNF-α/MIP-1α production by activated macrophages, suggesting the role of this receptor in suppression of excessive inflammatory responses during mycobacterial infection.
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Keywords: Cord factor; Macrophage scavenger receptor; Tumor necrosis factor alpha; Macrophage inflammatory protein-1 alpha; Alveolar macrophage; Kupffer cell

1. Introduction

In mycobacterial infection, the cell-surface cord factor (CF), or trehalose dimycolate, plays an important role in the development of granulomatous inflammation. This function is mediated by tumor necrosis factor-alpha (TNF-α) and macrophage inflammatory protein 1 alpha (MIP-1α), both potent inflammatory cytokines, secreted by activated macrophages [1,2]. In vitro experiments demonstrated TNF-α/MIP-1α production by direct stimulation of cultured macrophages with CF [3], and in vivo studies revealed high levels of TNF-α/MIP-1α in CF-induced pulmonary granulomas in mice [4]. These data indicate that granulomatous inflammation induced by mycobacterial infection seems to be closely related to TNF-α/MIP-1α production by CF-activated macrophages. There are, however, few reports on the regulatory mechanism of cytokine production induced by CF stimulation.

It is recently demonstrated that mice lacking class-A macrophage scavenger receptor (MSR-A) [5] or cyclophilin C-associated protein, a member of scavenger receptor cysteine-rich domain superfamily [6], are more susceptible than wild-type mice to endotoxin shock due to overproduction of TNF-α.

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This fact indicates a suppressive effect of MSR-A on lipopolysaccharide (LPS)-induced TNF-α production. MSR-A was originally reported as receptors that recognize LPS of gram-negative bacteria [7] and lipoteichoic acid of gram-positive bacteria [8]. MSR-A deficient mice were accordingly more susceptible than wild-type mice to infection with *Listeria monocytogenesis* [9] and *Staphylococcus aureus* [10].

In mycobacterial infection as well, MSR-A seems to be engaged in binding of *Mycobacterium tuberculosis* to macrophages [11]. Consistent with this interpretation, macrophages obtained from pulmonary granulomas in the patients with tuberculosis highly express MSR-A [12] and contain numerous bacilli [13]. There are, however, few reports on the macrophage receptors involved in the recognition of CF. In this study, we aimed to reveal the binding of CF to MSR-A and a regulatory role of MSR-A in TNF-α/MIP-1α production by CF-stimulated macrophages, using alveolar macrophages (AMs) and Kupffer cells (KCs) prepared from MSR-A (+/+) and (−/−) mice.

2. Results

2.1. Time- and dose-dependent production of TNF-α by MSR-A (+/+) AMs and KCs after stimulation with CF

We first determined the optimal culture condition for TNF-α production by cultured macrophages. Both AMs and KCs from MSR-A (+/+) mice produced large amounts of TNF-α by treatment with CF, while they produced negligible amounts without stimulation (Fig. 1). Cytokine levels in the culture medium reached a plateau at 24 h after stimulation. When compared to KCs, AMs secreted approximately 7-fold higher amounts of TNF-α. Such TNF-α production was dose-dependent, ranging from 0.1 to 1 μg CF/well, in both AMs and KCs. It, however, considerably decreased at 5 μg/well possibly due to CF cytotoxicity [14]. From these results, we cultured AMs and KCs with a dose of 1 μg/well of CF, and measured TNF-α/MIP-1α at 24 h in the following studies.

2.2. Production of TNF-α/MIP-1α by MSR-A (+/+) and (−/−) AMs and KCs after stimulation with CF

Both AMs and KCs from MSR-A (+/+) and those from MSR (−/−) mice produced large amounts of TNF-α/MIP-1α by treatment with CF, whereas they did not produce them without stimulation (Fig. 2). Interestingly, CF-stimulated macrophages from MSR-A (−/−) mice produced significantly higher levels of TNF-α/MIP-1α than those from MSR-A (+/+) mice except for MIP-1α production by KCs. There was no significant difference in cell viability between MSR-A (+/+) and MSR-A (−/−) macrophages or between AMs and KCs (data not shown). Differences in TNF-α/MIP-1α production seen here were 'therefore' not due to differences in susceptibility of macrophages to CF toxicity.

2.3. Binding of CF to MSR-A

To determine whether CF binds MSR-A on cultured macrophages in the present experiment, we conducted a binding assay using a macrophage cell line J774. By stimulating with 1 μg/ml of LPS, J774 cells showed enhanced expression of MSR-A as demonstrated by western blot analysis (Fig. 3a). We cultured these LPS-stimulated J774 cells in either

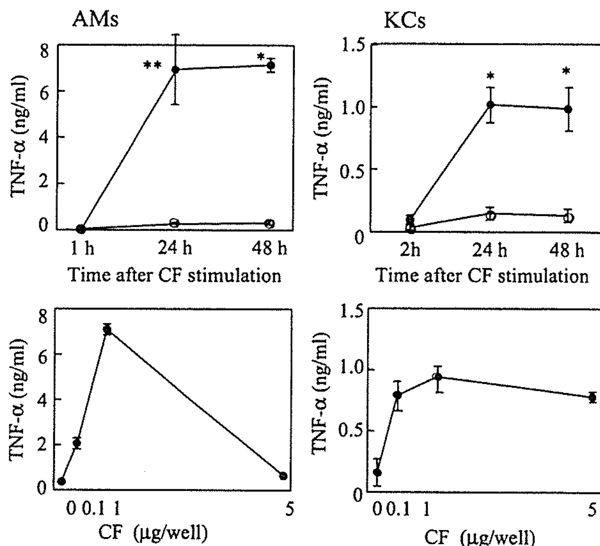

Fig. 1. (Upper columns) Timecourse of TNF-α production by AMs and KCs from MSR-A (+/+) mice after the culture on non-coated (○) and 1 μg CF-coated (●) wells. (Lower columns) Dose-response of TNF-α production by AMs and KCs from MSR-A (+/+) mice at 24 h after the culture. After plating 1.2×10^5 AMs or KCs on each well, the medium was subjected to ELISA analysis. Data represent the mean ± SD for three experiments. *$p<0.01$, **$p<0.05$ vs. non-coated wells.

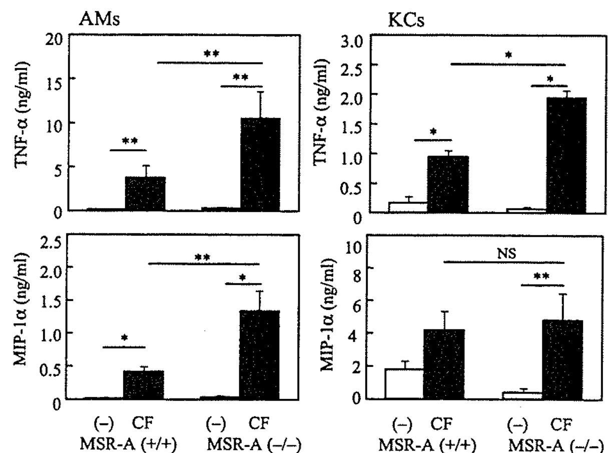

Fig. 2. TNF-α and MIP-1α production by AMs and KCs from MSR-A (+/+) and MSR-A (−/−) mice after incubation for 24 h in the presence (CF) or absemce (−) of 1 μg CF. Data represent the mean ± SD for three experiments. *$p<0.01$, **$p<0.05$. NS, not significant.

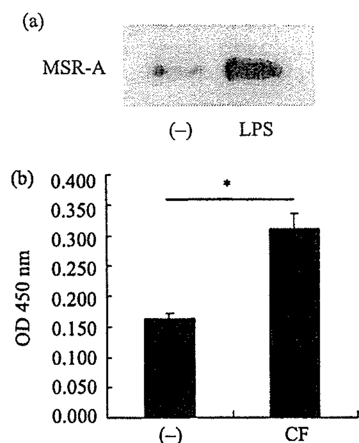

Fig. 3. Binding of CF to MSR-A. a. MSR-A expression is enhanced in LPS-stimulated J774. Western blot analysis. b. Binding affinity of CF to MSR-A. LPS-stimulated J774 was incubated for 24 h in the presence (CF) or absence (−) of 1 μg CF. Binding affinity was determined by ELISA using anti-MSR-A mAb 2F8. Data represent the mean ± SD for three experiments. $*p<0.01$.

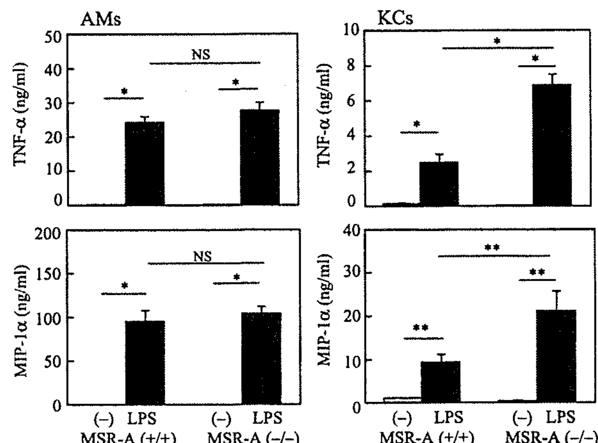

Fig. 5. TNF-α and MIP-1α production in AMs and KCs from MSR-A (+/+) and MSR-A (−/−) mice after incubation for 24 h in the presence (LPS) or absence (−) of 1 μg LPS. Data represent the mean ± SD for three experiments. $*p<0.01$, $**p<0.05$. NS, not significant.

CF-coated or uncoated wells, and measured MSR-A that was bound to the wells. The amount of MSR-A bound to CF-coated wells was significantly larger than that of uncoated wells (Fig. 3b).

2.4. Augmentation of TNF-α production by blocking MSR-A with mAb

In order to demonstrate that enhanced TNF-α production by MSR-A (−/−) macrophages is due to absence of MSR-A, we measured TNF-α in MSR-A (+/+) AMs after treatment with anti-MSR-A mAb 2F8. Twenty four hours after the culture in CF-coated wells, mAb 2F8-treated AMs produced larger amounts of TNF-α than AMs treated with control IgG-treated ones (Fig. 4).

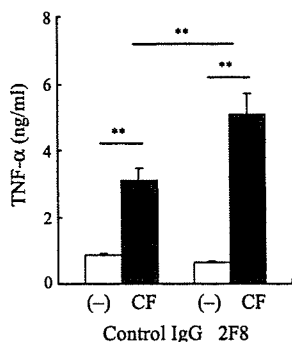

Fig. 4. Increased TNF-α production by blocking MSR-A with mAb. AMs from MSR-A (+/+) stimulated in the presence (CF) or absence (−) of 1 μg CF were treated with 20 μg/ml anti-MSR-A mAb 2F8 or control IgG2b. Data represent the mean ± SD for three experiments. $**p<0.05$.

2.5. Production of TNF-α and MIP-1α by MSR-A (+/+) and (−/−) AMs and KCs after stimulation with LPS

We next compared CF-induced TNF-α/MIP-1α production with LPS-induced one in MSR-A (+/+) and (−/−) AMs and KCs. By stimulation with 1 μg/well of LPS, AMs and KCs from either MSR-A (+/+) or (−/−) mice produced large amounts of TNF-α and MIP-1α (Fig. 5). The amount of TNF-α and MIP-1α generated by LPS stimulation was larger than that produced by CF stimulation. There was no significant difference in TNF-α and MIP-1α production from AMs between MSR-A (+/+) and (−/−) mice, whereas MSR-A

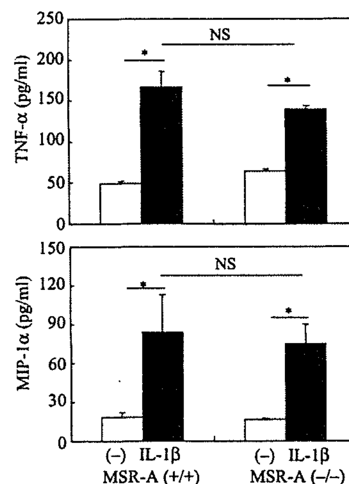

Fig. 6. TNF-α and MIP-1α production in AMs and KCs from MSR-A (+/+) and MSR-A (−/−) mice after incubation for 24 h in the presence (IL-1β) or absence (−) of 1 μg/ml IL-1β. Data represent the mean ± SD for two experiments. $*p<0.01$. NS, not significant.

(−/−) KCs showed enhanced production of TNF-α/MIP-1α compared to MSR (+/+) ones. Toll-like receptor (TLR)-4 is the receptor for lipopolysaccharide (LPS) and mediates signals via myeloid differentiation protein (MyD88) [15], although MSR-A cannot bind to IL-1. In addition, IL-1 utilizes MyD88 as signal transduction [16,17]. To clarify the role of MSR-A, we have examined the effect of IL-1β on macrophages derived from MSR-A (+/+) and (−/−) mice. The results showed that the levels of TNF-α and MIP-1α production from MSR-A (+/+) mice were comparable to those from MSR-A (−/−) mice (Fig. 6). Thus, there were no differences in the production between wild-type and MSR-A deficient mice.

3. Discussion and conclusion

It has been demonstrated that mycobacteria bind multiple distinct macrophage receptors, i.e. complement receptors [18], CD44 [19], CD14 [20], surfactant protein A receptors [21], mannose receptors [20,22], TLR-2 [23] and MSR-A[11]. Among mycobacterial components, CF is essential for the development of granulomatous inflammation because it directly stimulates macrophages to produce TNF-α and MIP-1α [3]. However, macrophage receptors that recognize CF have not been identified. In this study, we have demonstrated that CF binds MSR-A, and induces TNF-α/MIP-1α production in macrophages. Furthermore, experiments using MSR-A (−/−) and (+/+) macrophages revealed that MSR-A down-regulates CF-induced TNF-α/MIP-1α production. Such down regulation of CF-induced TNF-α production was blocked by treatment with anti-MSR-A mAb and the level of TNF-α/MIP-1α production induced by IL-1 β stimulation was not affected by MSR-A that could not bind to IL-1. These findings suggest that absence of MSR-A, but neither the defect of associated molecules like MyD88 in the downstream of a signaling pathway nor compensation by other receptors, is responsible for enhanced TNF-α/MIP-1α production in MSR-A (−/−) mice.

Several studies have demonstrated that the amount of TNF-α produced by LPS binding to MSR-A is much less compared to that produced by binding to CD14 or TLR [5,24,25], and most of LPS binds to MSR-A and remaining free LPS binds CD14 [5,6]. It is thus likely that LPS preferentially binds to MSR-A in MSR-A (+/+) mice, resulting in low production of TNF-α. By contrast, LPS binds to CD14 in MSR-A (−/−) mice, leading to more abundant TNF-α production. This is consistent with our present results. [Different from TNF-α/MIP-1α production by LPS-stimulated KCs, that by AMs was not affected by the presence of MSR-A, probably because AMs produce larger amounts of TNF-α and MIP-1α than KCs [26], and MSR-A might not sufficiently inhibit CD14-mediated TNF-α/MIP-1α production [25]. Similar mechanism might be present in the down-regulation of TNF-α/MIP-1α production by MSR-A from CF-stimulated macrophages. Thus, CF might bind to other receptors, for example TLR of MSR-A (−/−) macrophages, and produce more abundant TNF-α. However, there is still controversy about the involvement of TLR, because mycobacterial lipids, including CF, induce TNF-α production in a TLR-2-dependent manner [27], and similar cytokine production is observed in wild-type mice and in mice lacking either TLR-2 or TLR-4 [28].

In conclusion, the present study implies that MSR-A down-regulates TNF-α/MIP-1α production by macrophages stimulated with CF and LPS, and plays a preventive role in excessive inflammatory responses during microbial infection.

4. Materials and methods

4.1. Mice

MSR-A (+/+) and (−/−) mice, 15–20 weeks old, were gifted from Chugai Pharmaceut. Co. Ltd (Tokyo, Japan). They were housed in a specific pathogen-free condition and fed chow pellet and water ad libitum. Experiments were conducted according to the guideline for animal experiments of Osaka City University Graduate School of Medicine.

4.2. Preparation of CF and LPS

CF was prepared as previously described [4]. Briefly, *M. tuberculosis* AOYAMA-B was cultivated in Sauton medium for 5–6 weeks at 37 °C. Bacterial cells were autoclaved at 121 °C for 30 min, and then harvested. The lipids were serially extracted with chloroform–methanol (4:1, vol/vol), chloroform-methanol (3:1, vol/vol) and chloroform–methanol (2:1, vol/vol). CF (C78-88, C26-α-unit) was purified by developing the lipids on a thin-layer plate of silica gel with chloroform–methanol–acetone–acetic acid (90:10:6:1, vol/vol/vol/vol) and then with chloroform–methanol–water (90:10:1, vol/vol/vol). Purified CF did not contain detectable levels of LPS. LPS of *Salmonella enteritidis* by a phenol–water method was purchased from Sigma-Aldrich (St Louis, MO).

4.3. Isolation of AMs and KCs

AMs were obtained by a repeated lavage of the lung with phosphate-buffered saline (PBS) of anesthetized mice with an intraperitoneal injection of pentobarbital. Lavaged cells were centrifuged at $200\times g$ for 10 min and suspended in Tris–NH4Cl solution to remove erythrocytes. After washing with PBS twice, they were suspended in RPMI 1640 containing 10% fetal calf serum and 100 μg streptomycin and 100 U of penicillin per milliliter. KCs were isolated by the pronase–collagenase method [29]. The liver was perfused via the portal vein with SC-1 solution (NaCl 8 g/l, KCl 400 mg/l, NaH_2PO_4–$2H_2O$ 88 mg/l, Na_2HPO_4 120 mg/l, HEPES 2,380 mg/l, $NaHCO_3$ 350 mg/l, EGTA190 mg/l, glucose 900 mg/l, pH 7.4) for 3 min, with SC-2 solution (NaCl 8 g/l, KCl 400 mg/l, NaH_2PO_4–$2H_2O$ 88 mg/l, Na_2HPO_4 120 mg/l, HEPES 2,380 mg/l, $NaHCO_3$ 350 mg/l, CaCl2–$2H_2O$ 560 mg/l, pH 7.4) containing 0.05% pronase E for 5 min, and then with SC-2 solution containing 0.05% collagenase for 10 min. The liver was excised, cut into small pieces, and incubated in the SC-2 solution containing 0.05% pronase E, 0.05% collagenase, and 20 μg/ml of DNase for 45 min at 37 °C. The cell suspension

was filtered through a steel mesh with a pore size of 150 μm, and centrifuged on 17% Nycodenz cushion. A non-parenchymal cell-enriched fraction containing KCs was obtained from the upper layer. This fraction was subjected to the elutriation with a CR20B2 elutriator (Hitachi, Tokyo, Japan) at a pump speed of 40–68 ml/min in 200 ml. Cells were cultured on a plastic dish in RPMI 1640 containing 10% fetal calf serum for 45 h. More than 95% of AMs and KCs obtained by these procedures phagocytozed 0.8 μm latex beads, and the purity was always more than 95%. Cells obtained from 3 to 5 mice were pooled, and used for each experiment.

4.4. Stimulation of macrophages with either CF or LPS

Ninety six-well plastic plates were coated with 0.1, 1, 5 μg/well of CF dissolved in isopropyl alcohol. Control plates were prepared by placing isopropyl alcohol alone. These plates were dried overnight in a biosafety cabinet. AMs or KCs in the number of 1.2×10^5 were cultured. The similar number of AMs or KCs was stimulated with 1 μg/well of LPS or 1 μg/ml of IL-1β (Peprotech EC, UK). The culture supernatant was stored at −80 °C until cytokine assays. After removing the culture supernatant, the cell viability was determined by measuring the quantity of formazan product using a Cell Proliferation Assay Kit (Promega, Madison, WI).

4.5. Cytokine assay

The concentrations of TNF-α and MIP-1α were determined using the ELISA kit (Genzyme Techne, Minneapolis, MN). Sensitivity limits of ELISAs were as 5 pg/ml for TNF-α and 1.5 pg/ml for MIP-1α.

4.6. Western blot analysis

MSR-A expression in macrophages was analysed by western blot analysis using a mAb against MSR-A (2F8; Serotec, Oxford, UK) and ECL Western Blotting Analysis System (Amersham, Aylesbury, UK).

4.7. Assay for the binding affinity of CF to MSR-A

We used LPS-stimulated J774 cells, a mouse macrophage cell line, for the assay [30]. Twenty-four hours after the culture on CF-coated or non-coated wells, they were lysed with 1% Triton X for 1 min, and washed out with 0.05% Tween 20. MSR-A that bound coated or uncoated CF and remained on the plate after washing was colored by subsequent reactions, i.e., incubation with 2F8 overnight at 4 °C, incubation with horseradish peroxidase-conjugated anti-goat antibody for 1 h at room temperature, and addition of the substrate solution. Optical density (OD) was measured at the wavelength of 450 nm.

4.8. Blockade of MSR-A with MAb

MSR-A (+/+) AMs were incubated with 20 μg/ml anti-MSR-A mAb 2F8 or isotype-matched control antibody (Rat IgG2b, Serotec, Oxford, UK) for 10 min at room temperature and subsequent 10 min at 37 °C before the culture on CF-coated or non-coated wells [31]. After 24-h incubation, concentrations of TNF-α in the culture medium were determined by ELISA.

4.9. Statistics

The data were expressed as the mean ± standard deviation (SD). The results were analyzed by the one-way ANOVA and Scheffe test for further analysis and considered significant if P value were less than 0.05.

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