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The role of B7 molecules in the cell contact-mediated suppression of T cell mitogenesis by immunosuppressive macrophages induced with mycobacterial infection

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SUMMARY

We found previously that immunosuppressive macrophages (Mϕs) induced by *Mycobacterium intracellulare* infection (MI-Mϕs) transmitted their suppressor signals to target T cells through cell contact with target T cells. In this study, we examined what kinds of Mϕ surface molecules are required for such cell–to–cell interaction. First, it was found that a B7-1-like molecule (B7–1LM) recognizable with one of three test clones of anti-B7-1 monoclonal antibodies (mAbs) was required for expression of the Mϕ suppressor activity. Neither anti-B7-2, anti-ICAM-1, nor anti-VCAM-1 mAb blocked the Mϕ suppressor activity. Second, MI-Mϕs increased the expression of B7–1LM in parallel with the acquisition of the suppressor activity. Moreover, MI-Mϕs bound with target T cells in a B7–1LM-dependent fashion. Third, mAb blocking of CTLA-4 on target T cells did not reduce the suppressor activity of MI-Mϕs, suggesting the role of a putative molecule on target T cells other than CTLA-4 as the receptor for B7–1LM of MI-Mϕs. Fourth, concanavalin A (Con A) stimulation of MI-Mϕs was needed for effective cell contact with target T cells and subsequent expression of the suppressor activity of MI-Mϕs. Fifth, the Con A-induced increase in the suppressor activity of MI-Mϕs was inhibited by KN-62 but not by herbimycin A, H-7, nor H-88, indicating that Con A-induced up-regulation of MI-Mϕ function is mediated by calmodulin-dependent protein kinase II or ATP/P2Z receptors, but independent of protein tyrosine kinase, protein kinase C, and protein kinase A. These findings indicate that a B7/CTLA-4-independent mechanism is needed for the transmission of the suppressor signals from MI-Mϕs to target T cells.

Keywords suppressor macrophage T cell mitogenesis B7-1 molecules cell contact *Mycobacterium intracellulare*

INTRODUCTION

Intractable mycobacterioses including multidrug resistant tuberculosis and *Mycobacterium avium* complex (MAC) infections are frequently encountered in AIDS patients [1,2]. During the course of mycobacterioses in humans and experimental animals, generation of immunosuppressive macrophages (Mϕs) is frequently encountered [3,4]. These Mϕs suppress T cell functions, including proliferative response and Th1 cytokine production responding to T cell receptor ligation, causing suppression of cellular immunity in the advanced stages of infection [5–7]. Previously, we found that immunosuppressive Mϕs were induced in the spleens of *M. intracellulare*-infected mice and that such Mϕ populations (designated MI-Mϕs) displayed potent suppressive activity against Con A-induced mitogenesis of splenic T cells [8,9]. The suppressor activity of the MI-Mϕs was mediated by humoral mediators including reactive nitrogen intermediates, transforming growth factor-β, prostaglandin E_2, free fatty acids, which were produced by MI-Mϕs themselves in response to Con A stimulation [10–12].

Recently, we found that cell contact of MI-Mϕs with target T cells is required for efficacious manifestation of the suppressor activity of MI-Mϕs [12,13]. The suppressor signals of MI-Mϕs, which are transmitted to the target T cells via cell contact, principally cross-talk with the early signalling events before the activation of protein kinase C and/or intracellular calcium mobilization [13]. In the present study, we examined which kinds of Mϕ cell surface molecules are responsible for the cell–to–cell interaction between MI-Mϕs and target T cells. We found that a B7-1-like molecule (designated B7–1LM) on MI-Mϕs plays important roles in the transmission of suppressor signals from MI-Mϕs to target T cells through a cell–to–cell interaction.

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MATERIALS AND METHODS

Microorganisms

M. intracellulare N-260 strain isolated from a patient with MAC infection was used.

Mice

Eight to 10-week-old male BALB/c (Japan Clea Co., Osaka, Japan) were used.

Special agents

Special agents used in this study were as follows: Con A (Sigma Chemical Co., St. Louis, MO, USA), genistein (Sigma), herbimycin A (Sigma), H-7 (Sigma), H-88 (Seikagaku Industry Co., Tokyo, Japan), KN-62 (Sigma), hamster anti-mouse B7-1 (CD80) monoclonal antibody (mAb) (clone 16–10A1) (Pharmingen Co., San Diego, CA, USA), rat anti-mouse B7-1 mAb (clone RMMP-1) (PBL Biomedical Laboratories, New Brunswick, NJ, USA), rat anti-mouse B7-1 mAb (clone 1G10) (Southern Biotechnology Associates, Inc., Birmingham, AL, USA), rat anti-mouse B7-2 (CD86) mAb (Pharmingen), hamster anti-mouse CTLA-4 mAb (Pharmingen), rat anti-mouse CD54 (ICAM-1) mAb (Seikagaku), rat anti-mouse CD106 (VCAM-1) mAb (Serotec Ltd, Oxford, UK), rat anti-mouse CD3 mAb (Serotec), alkaline phosphatase (ALP)-conjugated goat anti-hamster IgG antibody (Ab) (Southern Biotechnology Associates), ALP-conjugated mouse anti-rat IgG Ab (Jackson Immuno Research Laboratories, West Grove, PA, USA), rat IgG (Organon Teknika Corp., Durham, NC, UK), hamster IgG (Organon Teknika), horseradish peroxidase (HRP) conjugated-goat anti-hamster IgG mAb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), HRP conjugated-goat anti-rat IgG mAb (Santa Cruz Biotechnology, Inc.) and [^{3}H] thymidine (^{3}H-TdR) (NEN Life Science Products Inc., Boston, MA, USA),

Medium

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

Suppressor activity of MI-Mϕs

Spleen cells (SPCs) were harvested from mice infected intravenously with 1×10^8 CFUs of *M. intracellulare* at 2–3 weeks after infection and cultured in 0·2 ml of the medium in four wells each of flat-bottom 96 well microculture plates (Corning, NY, USA) at the cell densities of 5×10^5–2×10^6 cells/well at 37°C in a CO_2 incubator (5% CO_2-95% humidified air) for 2 h. The wells were vigorously rinsed with Hanks' balanced salt solution containing 2% (v/v) FBS by pipetting and then 0·1 ml of the medium was poured onto the resulting wells. This procedure usually gave more than 90% pure Mϕ monolayer cultures, with active pinocytic ability of neutral red and with phagocytic ability against latex particles, containing about 6×10^4 cells per culture well from 2×10^6 of *M. intracellulare*-induced SPCs (MI-SPCs). Then, $2{\cdot}5 \times 10^5$ of normal SPCs in 0·2 ml of the medium containing 2 μg/ml Con A were poured onto the resultant Mϕ cultures. SPCs were then cultivated at 37°C in a CO_2 incubator for 72 h and pulsed with 0·5 μCi of ^{3}H-TdR (2 Ci/mmol) for the final 6–8 h. Cells were harvested onto glass fibre filters and counted for radioactivity using a 1450 Microbeta Trilux scintillation spectrometer (Wallac Co., Turku, Finland). Suppressor activity of MI-Mϕs was calculated as:

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

Western blotting

SPCs (4×10^7) harvested from normal or MI-infected mice in 8 ml of 5% FBS-RPMI1640 were cultured at 37°C for 2 h in an 90-mm plastic culture dish which precoated with FBS. After rinsing with 2% FBS-HBSS (six times), adherent cells were gently scraped off using rubber policemen into 20% FBS-HBSS and collected by subsequent centrifugation at 250 × g for 5 min. The obtained Mϕs were suspended in an appropriate volume (1×10^7 cells/ml) of cell lysis buffer which consist of 50 mM Tris-HCl (pH 7·4) containing 150 mM NaCl, 1% Triton X-100, 1% (v/v) sodium deoxycholate, 0·1% (w/v) sodium dodecyl sulphate (SDS), 1 mM Na_3VO_4, 1 mM phenylmethanesulphonyl fluoride (PMSF) and 5% (v/v) protease inhibitor cocktail (SIGMA). Samples were centrifuged at 22 000 × g to remove insoluble matter. Equal volume of cell lysate were subjected to SDS – 10% polyacrylamide gel electrophoresis, and transferred to Immobilon^{PSQ} PVDF membranes (Millipore Corp. Bedford, MA, USA). Membranes were first incubated for over night at 4°C in 1% bovine serum albumin (BSA) in TBST buffer which consist of 10 mM Tris-HCl (pH 7·5) containing 100 mM NaCl and 0·1% Tween 20. The blot was then incubated with anti-B7-1 mAb (clone 1G10 or 16–10A1), diluted in 1% BSA-TBST buffer (1 : 500 dilution) for 4 h at room temperature. After rinsing, membranes were exposed to a HRP-conjugated anti-rat IgG mAb or HRP-conjugated anti-hamster IgG mAb which diluted in 1% BSA-TBST buffer (1 : 7500 dilution) for 2 h at room temperature. After rinsing, membranes were incubated in ECL plus (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) for 5 min, and then exposed to X-ray film until a signal was detected.

B7-1 expression on MI-Mϕs

B7-1 expression on MI-Mϕs was measured by ELISA and flow cytometry as follows.

ELISA. MI-Mϕs (5×10^4–2×10^5 cells) were cultured in four wells each of flat-bottom 96 well microculture plates (Corning) at 37°C for 1 h, washed with HBSS, fixed with 0·5% (v/v) glutaraldehyde, and washed again with phosphate-buffered saline (PBS). After blocking with PBS containing 1% (w/v) BSA for over night and subsequent washing with 0·05% (v/v) Tween 20-PBS, the resultant cells were stained with hamster anti-mouse B7-1 mAb (clone 16–10A1) at a concentration of 1 : 200 at room temperature for 2 h, and washed again with 0·1% BSA-PBS. The resultant Mϕs were further stained with ALP-conjugated goat anti-hamster IgG Ab for 2 h and washed with 0·1% BSA-PBS. Colour development was achieved by using *p*-nitrophenyl phosphate (*p*-NPP) tablets (Sigma) as the substrate.

Flow cytometric analysis. MI-SPCs (4×10^7) suspended in 8 ml of the culture medium were incubated in FBS-coated 90-mm cell culture dish at 37°C for 2 h. After washing with 2% FBS-HBSS, adherent cells were scraped off using a rubber policemen and collected into polypropylene tube (17 × 100 mm) by subsequent centrifugation. After washing with 1% BSA-PBS by centrifugation, the resultant Mϕs (>90% pure) (2×10^6) were subjected blocking with 1% BSA-PBS containing 10% (v/v)

inactivated BALB/c mouse serum at 0°C for 30 min and then washed with 1% BSA-PBS. The resultant cells were reacted with FITC-conjugated hamster anti-mouse B7-1 mAb (clone 16–10A1) at a concentration of 1 : 500 at 0°C for 3 h, washed with 1% BSA-PBS and thereafter with PBS, and fixed with 1% paraformaldehyde in PBS (pH 7·2) for over night. The resulting Mϕ cells were subjected to flow cytometry using FACStar (Becton Dickinson, Mountain View, CA, USA).

Assay for binding of MI-Mϕs with T cells

The monolayer cultures of MI-Mϕs prepared by seeding 4×10^6 of MI-SPCs on 16-mm culture wells (Corning) were preincubated in the medium containing 2 μg/ml of Con A at 37°C for 4 h. After the addition of anti-B7-1 mAb (final 20 or 50 μg/ml), $1{\cdot}25 \times 10^6$ of nylon wool column-purified splenic T cells suspended in the medium free from Con A were added, subjected to brief centrifugation, and allowed to bind to MI-Mϕs by cultivating at 37°C for 6 h. After gentle washing with PBS to remove T cells nonadherent to MI-Mϕs and subsequent fixation with 0·1% glutaraldehyde followed by rinsing with PBS, the resultant wells were subjected to blocking with 1% BSA-PBS for 3 h. Then, T cells which were binding to MI-Mϕs on the wells were stained with rat anti-mouse CD3 mAb at 37°C for 1 h, washed with 0·1% BSA-PBS, and further stained with ALP-conjugated mouse anti-rat IgG Ab at 37°C for 1 h. After rinsing with 0·1% BSA-PBS, colour development was achieved by using *p*-NPP tablets as the substrate.

Statistical analysis

Statistical analysis was performed using Bonferroni's multiple *t*-test.

RESULTS

B7-1-mediated expression of the suppressor activity by MI-Mϕs

Figure 1 shows the effects of anti-B7-1 (clone 16–10A1), anti-B7-2, anti-ICAM-1, and anti-VCAM-1 mAbs on the suppression by MI-Mϕs of SPC mitogenesis. It was found that anti-B7-1 mAb (clone 16–10A1) markedly reduced the suppressor activity of MI-Mϕs ($P < 0{\cdot}01$), whereas the other mAbs did not. Anti-VCAM-1 mAb slightly decreased the suppressor activity of MI-Mϕs. Notably, in separate experiments indicated that the two different clones of anti-B7-1 mAbs (clones RMMP-1 and 1G10) failed to display such significant efficacies in blocking the suppressor activity of MI-Mϕs as in the case of the anti-B7-1 mAb (clone 16–10A1) (data not shown). These findings suggest that a B7-1-like molecule (B7–1LM), which shared in part the same epitopes with B7-1 molecules, is required for expression of the suppressor activity of MI-Mϕs through cell-to-cell contact with target T cells. This concept is supported by the following findings.

As shown in Fig. 2, Western blotting experiments using the two clones of anti-B7-1 mAb (1G10, 16–10A1) revealed that clone 1G10 mAb bound to 33-kD, 38-kD, 48-kD, 52-kD and 62-kD proteins in MI-Mϕ cell lysate. The multiplicity of detected bands is not enigmatic, because B7-1 protein is expressed in various molecular weights due to differential glycosylation and mRNA splicing among various types of cells [14–16]. For instance, it has been reported that 1G10 mAb binds to a 46- and 52-kD proteins in the cell lysates of B cell lineages [15]. As shown in Fig. 2, 16–10A1 mAb bound to 33-, 38-, 48-, 52- and 56-kD proteins in MI-Mϕ cell lysate. Notably, Western blotting experiment indicated that 16–10A1 mAb specifically bound to 56-kD protein. In addition, the expression of 56-kD protein was markedly increased in MI-Mϕs compared to the control Mϕs. On the contrary, the expression of 33-, 38-, 48- and 52-kD proteins did not increase in MI-Mϕs compared to that in control Mϕs. These findings indicate that the 56-kD protein recognized by 16–10A1 mAb corresponds to B7–1LM protein.

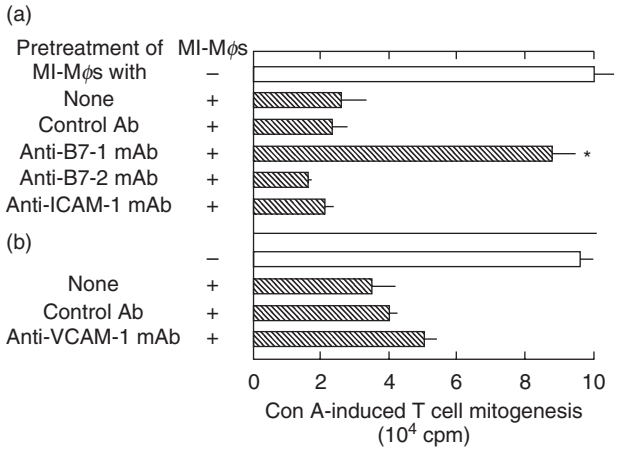
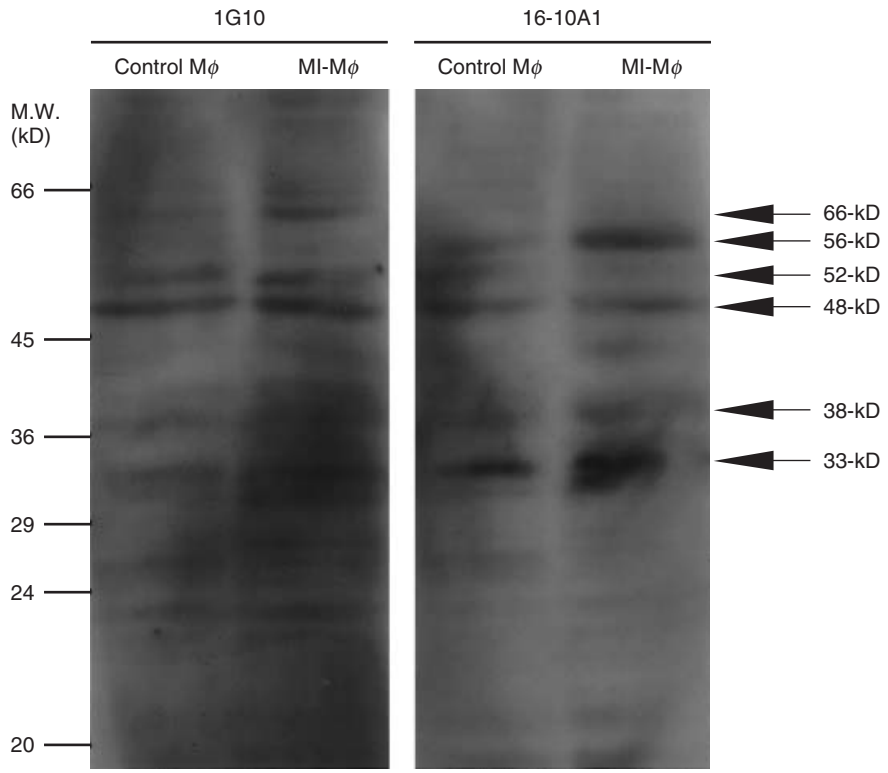


Fig. 1. Blocking of the suppressor activity of *M. intracellulare*-induced macrophages (MI-Mϕs) with anti-B7-1 mAb. MI-Mϕs on microculture wells were pretreated with (a) the indicated mAb anti-B7-1 mAb (clone no. 16–10A1), anti-B7-2 mAb, anti-ICAM-1 mAb, or control Ab (hamster IgG) at 50 μg/ml each or (b) treated with 25 μg/ml of anti-VCAM-1 mAb, or control Ab (rat IgG) at 25 μg/ml each for 2 h. After washing with 2% FBS-HBSS, SPCs were added onto the resultant Mϕs (4×10^4/well) and measured for their Con A mitogenic response. Each bar indicates the mean ± SEM ($n = 4$). *Significantly reduced compared to the control value (+control Ab) ($P < 0{\cdot}01$). Results are representative of five independent experiments.

Figure 3a shows the relationship between Mϕ suppressor activity and the extent of Mϕ expression of whole B7-1 (B7-1 and related proteins) and B7–1LM. The level of expression of whole B7-1 and particularly B7–1LM were increased in MI-Mϕs than in control Mϕs, and this increase clearly paralleled with the increase in their suppressor activity. Furthermore, when the level of whole B7-1 expression by MI-Mϕs was determined by flow cytometric analysis, significantly increased whole B7-1-highly positive cell populations were detected in MI-Mϕs (Fig. 3b). The mean fluorescence intensity of MI-Mϕs (11·7) was almost double that of control Mϕs (6·5). These findings confirm the concept that the suppressor activity of MI-Mϕs is associated with the increase in their B7–1LM expression.

Profiles of B7–1LM-dependent cell contact between MI-Mϕs and target T cells

Next, we examined profiles of the binding of MI-Mϕs with target T cells. As shown in Fig. 4a, the binding of splenic T cells to MI-Mϕs (cluster formation of T cells around MI-Mϕs) was observed by microscopy, when MI-Mϕs had been stimulated with Con A for 4 h prior to cocultivation with T cells. In this case, the average number of T cells binding to MI-Mϕs was 2·7 ± 0·2 (range 0–8). As indicated in Fig. 4b. T cell binding to MI-Mϕs was significantly inhibited by the addition of the anti-B7-1 mAb (clone 16–10A1) in a dose-dependent manner. In this case, the inhibition rate due to the treatment with 50 μg/ml of the anti-B7-1 mAb was



Band intensity of anti-B7-1 mAb (clone 16-10A1)-bound 33-, 38-, 48-, 52-, 56-kD proteins*

M.W. of the protein (kD)	Band intensity	
	Control Mϕ	MI-Mϕ
33	1·0	1·1
38	1·0	1·0
48	1·0	1·2
52	1·0	0·6
56	1·0	4·2

* The relative intensity of each protein expression in terms of MI-Mϕ/control Mϕ ratios were estimated from the data indicated by Western blotting.

Fig. 2. Profiles of B7-1 protein expression in *M. intracellulare*-induced macrophages (MI-Mϕs). MI-Mϕ cell lysate was analysed by Western blotting using anti-B7-1 mAbs (clone 1G10 and 16–10A1). Cell lysate of control Mϕs was prepared from splenic Mϕs without MI infection. In the bottom table, band intensities of the 33-, 38-, 48-, 52- and 56-kD proteins which are recognized by the 16–10A1 mAb using densitometer are indicated.

$25{\cdot}8 \pm 2{\cdot}8\%$ $(n = 3)$ $(P < 0{\cdot}05)$. These findings indicate that MI-Mϕs are capable of binding to target T cells through B7–1LM.

Receptor on target T cells for B7–1LM

It is known that B7 molecule-mediated suppressor signals are transmitted to T cells through B7/CTLA–4 interaction [17,18]. It is thus of interest to examine whether or not MI-Mϕ derived suppressor signals are transmitted to target T cells through an interaction between B7–1LM (MI-Mϕs) and CTLA-4 (T cells). As shown in Fig. 5, pretreatment of target T cells with anti-CTLA-4 mAb failed to block the expression of MI-Mϕ suppressor activity. Moreover, in separate experiments, CTLA-4 Ig was incapable of blocking the suppressor activity of MI-Mϕs even when added at 20 μg/ml (data not shown). Therefore, the possibility is excluded that CTLA-4 acts as a B7–1LM receptor on target T cells and plays crucial roles in the transmission of MI-Mϕ suppressor signals through the interaction with B7–1LM. It thus appears that there exists unknown receptor(s) on for B7–1LM on target T cells other than CTLA-4.

Profiles of Con A stimulation of MI-Mϕs required for cell contact with target T cells

As shown in Fig. 6, binding of MI-Mϕs with target T cells was markedly enhanced when MI-Mϕs had been stimulated with Con A. It thus appears that Con A stimulation of MI-Mϕs is required for the effective cell contact between MI-Mϕs and target T cells. In this context, we previously found that Con A stimulation of MI-Mϕs is required for the effective expression of their suppressor activity, since TNF-α and IFN-γ produced by Con A stimulated MI-Mϕs augment the Mϕ suppressor activity in an autocrine fashion [11]. Figure 7 shows the effects of some metabolic inhibitors on the suppressor activity of MI-Mϕs. A panel of metabolic

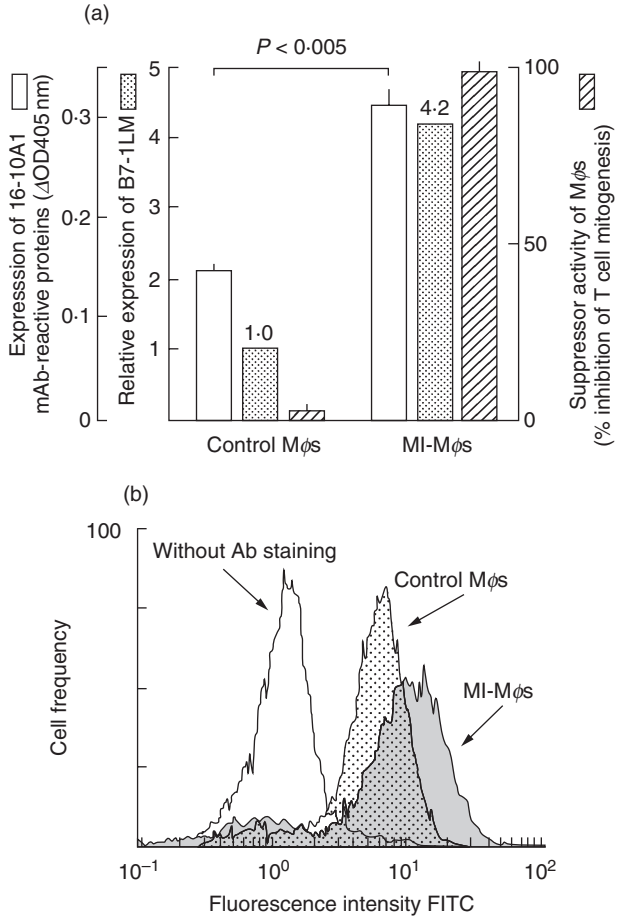


Fig. 3. Expression of whole B7-1 and B7–1LM by *M. intracellulare*-induced macrophages (MI-Mϕs). (a) MI-Mϕs or control Mϕs (splenic Mϕs from normal mice) were measured for the expression of whole B7-1 and B7–1LM by ELISA and Western blotting analysis, respectively, using 16–10A1 mAb (□ and ⊡, respectively) and suppressor activities in terms of percentage inhibition of Con A-induced SPC mitogenesis (▨). Each bar indicates the mean ± SEM ($n = 4$). Results are representative of three or four independent experiments. (b) Whole-B7-1 expression on MI-Mϕs (■) and control Mϕs (⊡) were measured by flowcytometric analysis using FITC-conjugated anti-B7-1 mAb (clone no. 16–10A1). Open histogram indicates the basal level of fluorescence on MI-Mϕs without anti-B7-1 mAb staining. Results are representative of two independent experiments.

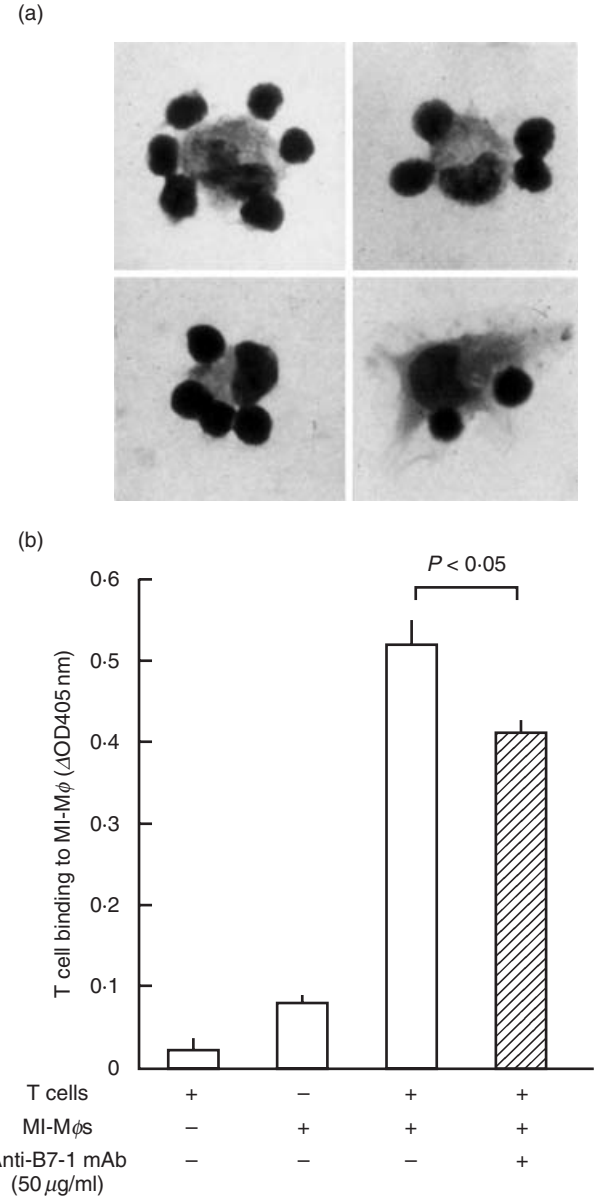


Fig. 4. T cell-binding ability of *M. intracellulare*-induced macrophages (MI-Mϕs). (a) Cluster formation of MI-Mϕs and target T cells. MI-Mϕs are tightly surrounded by T cells. MI-Mϕs were stimulated with Con A for 2 h and then co-cultured with SPCs for 3 h. After gentle rinsing, the culture wells were fixed with glutaraldehyde and subjected to Giemsa staining. Representative aspects are shown. (b) MI-Mϕs were stimulated with Con A for 4 h, then cocultured with purified splenic T cells in the presence or absence of anti-B7-1 Ab (clone no. 16–10A1) at 50 μg/ml for 6 h, and measured for T cell binding ability by ELISA as described in 'Materials and methods'. Each bar indicates the mean ± SEM ($n = 3$). Results are representative of three independent experiments.

inhibitors was used in attempt to identify the signalling pathways responsible for Mϕ Con A-stimulation. These included herbimycin A (an inhibitor of protein tyrosine kinase (PTK)), H-7 (an inhibitor of protein kinase C (PKC)), H-88 (an inhibitor of protein kinase A (PKA)) and KN-62 (an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)). MI-Mϕs were pretreated with them at the concentration of 10 μM, which can inhibit these protein kinases without exhibiting cytotoxicity against Mϕs [19]. As shown in Fig. 7, KN-62 partly but significantly diminished expression of the suppressor activity by Con A-stimulated MI-Mϕs ($P < 0{\cdot}05$). In contrast, the other protein kinase inhibitors did not exhibit such an inhibitory effect. It thus appears that CaMKII may play important roles in signalling pathways of Con A-stimulated MI-Mϕs to exhibit their suppressor activity.

DISCUSSION

In the present study, we examined which kinds of surface molecules on MI-Mϕs are responsible for cell–to–cell interaction between MI-Mϕs and target T cells, and the following findings were obtained. First, MI-Mϕ suppressor activity was blocked by an anti-B7-1 mAb but not by anti-B7-2, anti-ICAM-1, nor anti-

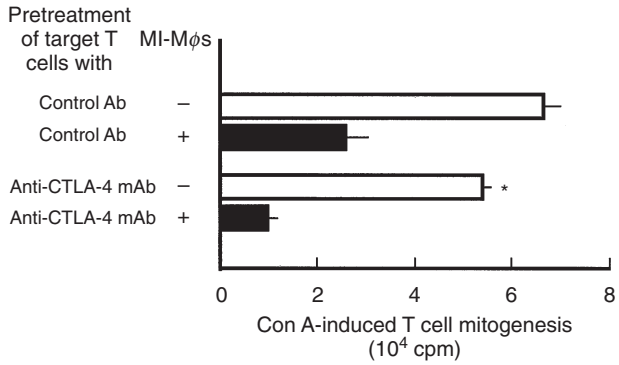


Fig. 5. Failure of treatments of target splenic T cells with anti-CTLA-4 mAbs in blocking the suppressor activity of *M. intracellulare*-induced macrophages (MI-Mϕs). SPCs were pretreated with the indicated mAb (100 μg/ml each) for 2 h. After washing with 2% FBS-HBSS, the resultant SPCs were co-cultured with MI-Mϕs (2×10^4/well) and measured for their Con A mitogenic response. Each bar indicates the mean ± SEM ($n = 4$). *Significantly different from the value of T cells treated with control Ab (hamster IgG) ($P < 0{\cdot}05$). Results are representative of four independent experiments.

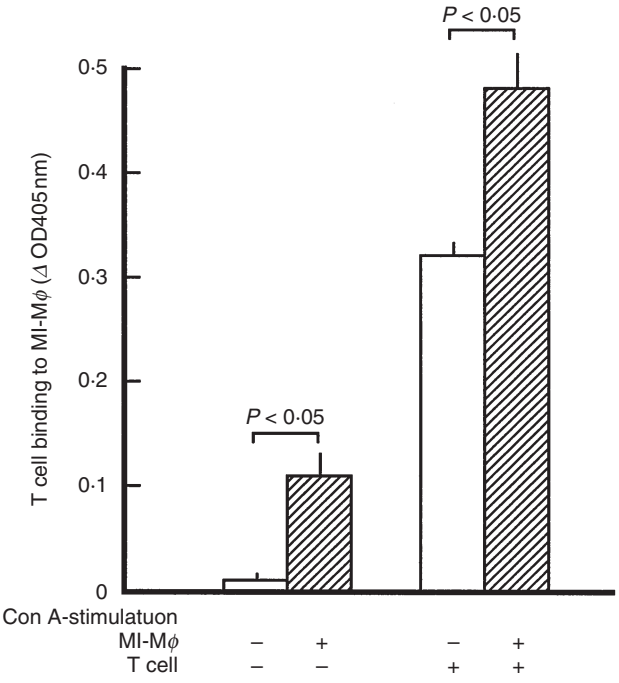


Fig. 6. Enhancement of binding of *M. intracellulare*-induced macrophages (MI-Mϕs) with target T cells by Con A-stimulation. MI-Mϕs were stimulated with Con A at 2 μg/ml for 4 h, then co-cultured with purified splenic T cells in the presence or absence of Con A at 2 μg/ml for 16 h, and measured for T cell binding ability by ELISA as described in 'Materials and methods'. Each bar indicates the mean ± SEM ($n = 3$).

VCAM-1 mAb. Notably, only one (clone 16–10A1) of the three test clones of anti-B7-1 mAbs was capable of blocking expression of the suppressor activity by MI-Mϕs. These findings suggest that transmission of the suppressor signals from MI-Mϕs to target T cells via cell contact was dependent on a novel B7-1-like molecule (B7–1LM), which shares in part the same epitope with B7-1. This

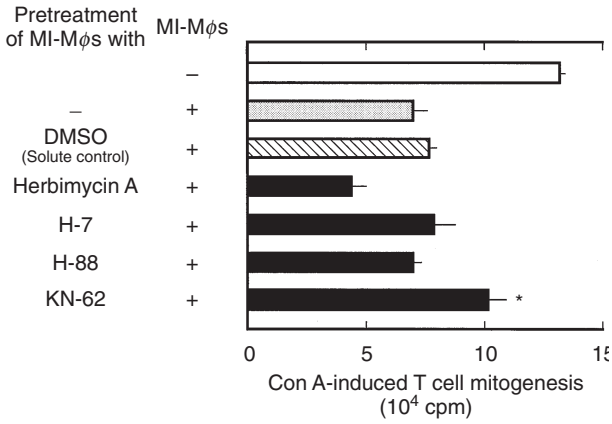


Fig. 7. Effects of inhibitors of various types of protein kinases (PTK, PKC, PKA, and CaMKII) on the expression of the suppressor activity by Con A-stimulated *M. intracellulare*-induced macrophages (MI-Mϕs). MI-Mϕs were pretreated with indicated inhibitors at 10 μg/ml at 37°C for 2 h. After washing with 2% FBS-HBSS, the resultant MI-Mϕs were cocultured with SPCs ($2{\cdot}5 \times 10^5$) in the presence of 2 μg/ml of Con A in order to measure their suppressor activity. Each bar indicates the mean ± SEM ($n = 4$). *Significantly greater than the value of the solute control [MI-Mϕs were pretreated with 0·1% dimethyl sulphoxide (DMSO)] ($P < 0{\cdot}05$). Results are representative of four independent experiments.

concept is further supported by the following findings. First, only 16–10A1 mAb binds 56-kD protein, the expression of which is markedly increased in MI-Mϕs. Second, the expression of B7–1LM on MI-Mϕs was correlated with their suppressor activity. Third, cell-to-cell binding of MI-Mϕs with target T cells was inhibited by the anti-B7-1 mAb (clone 16–10A1). Fourth, the mAb blocking of CTLA-4 molecules on target T cells did not attenuate the MI-Mϕ suppressor activity, indicating that CTLA-4 does not act as a B7–1LM receptor, and that MI-Mϕ-derived suppressor signals are transmitted to target T cells through the interaction of B7–1LM with unknown receptor(s) on T cells other than CTLA-4. Separate experiments indicated that CD28 also does not act as a B7–1LM receptor (data not shown). As shown in Fig. 7, neither herbimycin A, H-7, nor H-88 exerted inhibitory effects against the expression of the suppressor action by MI-Mϕs in response to Con A stimulation. The negative results obtained with these metabolic inhibitors suggest that Con A signal-associated expression of the suppressor activity by MI-Mϕs does not involve signalling pathways which are mediated by PTK, PKC, or PKA. On the other hand, the CaMKII inhibitor KN-62 partially attenuated the suppressor activity of MI-Mϕs, indicating that CaMKII-mediated signalling events may play important roles in the activation of MI-Mϕs to exhibit their suppressor activity in response to Con A signals.

In this context, it is noteworthy that KN-62 has an inhibitory activity against ATP/P2Z (P2X7) receptors [19,20]. Recently, it has been reported that ATP-induced stimulation of P2Z receptors on Mϕs is associated with marked increase in the activity of phospholipase D, causing potentiation of Mϕ antimycobacterial activity [21–23]. Notably, it has been reported that ATP-induced microbicidal activity of Mϕs is attenuated by KN-62 but not by inhibitors of PTK, PKC, and adenylate cyclase [19]. Therefore, it is possible that ATP/P2Z interaction on MI-Mϕs is needed for efficacious expression of their suppressor activity against target T cells.

In summary, the present study indicated that suppressive signals from MI-Mϕs are transmitted to target T cells through cell contact between a novel B7–1LM on MI-Mϕs and certain receptor(s) on target T cells other than CTLA-4. Further studies are currently underway to identify the B7–1LM.

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REFERENCES

1 Hart CA, Beeching NJ, Duerden BI. Tuberculosis into the next centry. J Med Microbiol 1996; **44**:1–34.
2 Falkinham JO. Epidemiology of infection by nontuberculous mycobacteria. Clin Microbiol Rev 1996; **9**:177–215.
3 Ellner JJ. Suppressor adherent cells in human tuberculosis. J Immunol 1978; **121**:2573–9.
4 Edwards CK, Hedegaar HB, Zlotnik A *et al.* Chronic infection due to *Mycobacterium intracellulare* in mice: association with macrophage release of prostaglandin E_2 and reversal by injection of indomethacin, muramyl dipeptide, and interferon-γ. J Immunol 1986; **136**:1820–7.
5 Turcotte R, Lemieux S. Mechanisms of action of *Mycobacterium bovis* BCG-induced suppressor cells in mitogen induced blastogenesis. Infect Immun 1982; **36**:263–70.
6 Edwards D, Kirkpatrik CH. The immunology of mycobacterial diseases. Am Rev Respir Dis 1986; **134**:1062–71.
7 VanHeyningen TK, Collin HL, Russell DG. IL-6 produced by macrophages infected with *Mycobacterium* species suppresses T cell responses. J Immunol 1997; **158**:330–7.
8 Tomioka H, Saito H, Yamada Y. Characteristics of immunosuppressive macrophages induced in spleen cells by *Mycobacterium avium* complex infections in mice. J General Microbiol 1990; **136**:965–74.
9 Tomioka H, Saito H. Characterization of immunosuppressive functions of murine peritoneal macrophages induced with various agents. J Leukoc Biol 1992; **51**:24–31.
10 Tomioka H, Sato K, Maw WW *et al.* The role of tumor necrosis factor, interferon-γ, transforming growth factor-β, and nitric oxide in the expression of immunosuppressive functions of macrophages induced by *Mycobacterium avium* complex infection. J Leukoc Biol 1995; **58**:704–12.
11 Tomioka H, Kishimoto T, Maw WW. Phospholipids and reactive nitrogen intermediates collaborate in expression of the T cell mitogenesis inhibitory activity of immunosuppressive macrophages induced in mycobacterial infection. Clin Exp Immunol 1996; **103**:219–25.
12 Maw WW, Shimizu T, Sato K *et al.* Further study on the roles of the effector molecules on immunosuppressive macrophages induced by mycobacterial infection in expression of their suppressor function against mitogen-stimulated T cell proliferation. Clin Exp Immunol 1997; **108**:26–33.
13 Ogasawara K, Tomioka H, Shimizu T *et al.* Profiles of cell–to–cell interaction of *Mycobacterium intracellulare*-induced immunosuppressive macrophages with target T cells in terms of suppressor signal transmission. Clin Exp Immunol 2002; **129**:272–80.
14 Freeman GJ, Gray GS, Gimmi CD *et al.* Structure, expression, and T cell costimulatory activity of the murine homologue of the human B lymphocyte activation antigen B7. J Exp Med 1991; **174**:625–31.
15 Chen C. Faherty DA, Gault A *et al.* Monoclonal antibody 2D10 recognizes a novel T cell costimulatory molecule on activated murine B lymphocytes. J Immunol 1994; **152**:2105–14.
16 Inobe M, Linsley PS, Ledbetter JA *et al.* Identification of an alternatively spliced form of the murine homologue of B7. Biochem Biophys Res Commun 1994; **200**:443–9.
17 Walunas TL, Lenschow DJ, Bakker CY *et al.* CTLA-4 can function as a negative regulator of T cell activation. Immunity 1994; **1**:405–13.
18 Tivol EA, Boyd SD, McKeon S *et al.* CTLA4 Ig prevents lymphoproliferation and fatal multiorgan tissue destruction in CTLA-4-deficient mice. J Immunol 1997; **158**:5091–4.
19 Lammas DA, Stober C, Harvey CJ *et al.* ATP-induced killing of mycobacteria by human macrophages is mediated by purinergic P2Z (P2X7) receptors. Immunity 1997; **7**:433–44.
20 Humphreys BD, Dubyak GR. Induction of the P2Z/P2X7 nucleotide receptor and associated phospholipase D activity by lipopolyshaccharide and IFN-γ in the human THP-1 monocytic cell line. J Immunol 1996; **157**:5627–37.
21 el-Moatassim C, Dubyak GR. A novel pathway for the activation of phospholipase D by P2Z purinergic receptors in BAC1.2F5 macrophages. J Biol Chem 1992; **267**:23664–73.
22 Exton JH. Phospholipase D. enzymology, mechanisms of regulation, and function. Physiol Rev 1997; **77**:303–20.
23 Kusner DJ, Adams J. ATP-induced killing of virulent *Mycobacterium tuberculosis* within human macrophages requires phospholipase D. J Immunol 2000; **164**:379–88.