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Properties of Two-type Subpopulations of Immunosuppressive Macrophages Generated by *Mycobacterium intracellulare* Infection

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Immunosuppressive macrophages (Mϕs) generated in the spleen of *Mycobacterium intracellulare* (Min)-infected mice (Min-Mϕs) exhibit suppressor activity against T cell mitogenesis in response to concanavalin A. Here, we attempted to fractionate the Min-Mϕ population generated in Min-infected BALB/c mice at week 2 after infection. Min-Mϕs were found to consist of two distinct subpopulations differing in their adhesiveness to plastic wells. The strongly adhesive Mϕ population (SA-Mϕs) exhibited marked suppressor activity. The weakly adhesive Mϕ population (WA-Mϕs) exhibited much less suppressor activity. Both SA-Mϕs and WA-Mϕs possessed a $CD11b^+$ $F4/80^+$ $SR\text{-}AI^+$ $CD14^+$ $CD206^+$ phenotype characteristic of matured Mϕs, generated reactive oxygen intermediates, and suppressed IL-2 receptor expression by concanavalin A-stimulated T cells. Moreover, their suppressor activities were dependent on prostaglandin. These findings indicate that Min infection in mice generates two types of immuosuppressive Mϕs with different levels of suppressor activity.

Key words: immunosuppressive macrophages, *Mycobacterium intracellulare*, T cell mitogenesis, suppressor activity

INTRODUCTION

The world-wide increase in the incidence of mycobacterial infections, namely those due to *Mycobacterium tuberculosis*, *M. avium* and *M. intracellulare* (Min), associated with AIDS has resulted in serious health problems in many countries (1,2). The generation of immunosuppressive macrophages (Mϕs) is frequently encountered in hosts with such mycobacterial infections, and leads to depressed cellular host immunity in the advanced stage of infection (3-5). We previously found that immunosuppressive Mϕs generated in the spleen of Min-infected mice (Min-Mϕs) caused strong suppression of concanavalin A (Con A)- or T cell receptor ligation-induced T cell mitogenesis (6-10) and that Min-induced generation of immunosuppressive Mϕs *in vivo* was mediated by TNF-α in combination with IFN-γ or interleukin-1 (7). In addition, the suppressor activity of Min-Mϕs was mediated by soluble factors including reactive nitrogen intermediates (RNIs), free fatty acids, phosphatidylserine, prostaglandin E (PGE), and TGF-β that were produced by the Min-Mϕs themselves (10-13). In addition, cell-to-cell contact with target T cells was required for full the manifestation of suppressor activity. Notably, the transmission of suppressor signals from Min-Mϕs to target T cells via cell contact was mediated by a B7-like molecule, presumably programmed death-1 ligand 1 (PD-L1), of Min-Mϕs (9). The findings that the suppressor signals of Min-Mϕs are transmitted to target T cells through various kinds of mediators and cellular mechanisms implies that Min-Mϕs consist of Mϕ subpopulations. In the present study, we attempted to fractionate the Min-Mϕ population and successfully obtained two distinct Mϕ subpopulations having differing in adhesiveness to plastic wells. These subpopulations were examined for immunosuppressive activity and the expression of Mϕ-specific surface antigens.

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MATERIALS AND METHODS

Microorganisms

Min N-260 strain isolated from a patient with Min infection was used. This organism was identified as *M. intracellulare* by a DNA probe test.

Mice

Eight to ten-week-old male BALB/c mice (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), phorbol myristate acetate (PMA) (Sigma), mouse anti-Thy 1,2 monoclonal antibody (mAb) (Cederlane Lab, Ontario, Canada), rabbit complement (Cederlane), rat anti-IL-2 receptor alpha-chain (IL-2Rα) mAb (clone AMT13; Boehringer Mannheim Biochemica, Co, Mannheim, Germany), fluorescein isothiocyanate (FITC)- conjugated goat anti-rat IgG antibody (Ab) (Jackson Immuno Research, Lab. Inc. West Grove, PA, USA), FITC-conjugated rat anti-mouse CD11b mAb (AbD Serotec, Oxford, UK), FITC-conjugated rat anti-mouse CD206 mAb (AbD Serotec), FITC-conjugated rat anti-mouse F4/80 antigen mAb (AbD Serotec), rat anti-mouse CD14 mAb (R&D Systems, Inc. Minneapolis MN), rat anti-mouse scavenger receptor class A type I (SR-AI) mAb (R&D Systems, Inc.), and (^{3}H) thymidine (^{3}H-TdR) (New England Nuclear Corp., Boston, MA, USA), carbonyl iron (Sigma).

Medium

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

Suppressor activity of Min-Mϕs

Normal spleen cells (SPCs) (1.25 x 10^5) were co-cultivated with test immunosuppressive Mϕs (test Mϕs) in 0.2 ml of RPMI-medium containing 2 μg/ml Con A in microculture wells (96 well) for 72 h and pulsed with 0.5 μCi of ^{3}H-TdR (2 Ci/mmol) for the final 6 to 8 h. Cells were harvested onto glass fiber filters and counted for radioactivity using a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, IL, USA). Suppressor activity of MAC-Mϕs was calculated as:

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

Fractionation of strongly adherent Mϕ (SA-Mϕ) and weakly adherent Mϕ (WA-Mϕ) populations from Min-induced SPCs

Min-induced SPCs were harvested from mice infected intravenously with 1 x 10^8 colony forming units (CFUs) of Min at 2 weeks after infection. The Min-induced SPCs (3 x 10^7) suspended in 3 ml of the culture medium were seeded onto 50-mm plastic culture wells and incubated at 37℃ for 2 h. After gentle rinsing three times with Hanks' balances salt solution (HBSS) to remove non-adherent cells and subsequent addition of 1 ml of HBSS to the wells, the culture wells were vigorously vibrated using a vibrator (VL mixa, Model K-550-G), and the detached cells (WA-Mϕs) were collected by gentle washing with HBSS. Strongly adherent cells (SA-Mϕs), corresponding to the cell subset called Min-Mϕ (7), were scraped off using a rubber policeman and collected by centrifugation at 200 x g for 5 min. In order to delete T cell populations from WA-Mϕ preparation, the cell preparation was treated with anti-Thy 1,2 mAb (1:20) at 4℃ for 1 h, followed by subsequent treatment with rabbit complement (1:3) at 37℃ for 30 min. Alternatively, in order to delete Mϕ populations from WA-Mϕ preparation, the cell preparation was incubated in RPMI medium containing carbonyl iron particles at 37℃ for 1 h and the cell population phagocytosing carbonyl iron particles were removed using magnetic force.

Flow cytometric analysis

Test cells (SA-Mϕs and WA-Mϕs) were subjected to flow cytometric analysis as follows (14). After blocking with 10% (v/v) FBS-phosphate-buffered saline (PBS) of test cell suspension, the resultant cells were treated with test mAbs for 1 h, washed five times with PBS, and further stained with FITC-conjugated goat anti-mouse IgG antibody for 1h. Resultant cells were washed twice with PBS, and fixed with 1% (w/v) paraformaldehyde in PBS pH 7.2 for 10 min. After washing with PBS, the resulting cells were subjected to flow cytometry using FACStar (Becton Dickinson, Mountain View, CA, USA) or EPICS ELITE flow cytometer (Beckman Coulter, Inc., Miami, FL, USA).

Measurement for expression of IL-2 receptor (IL-2 R) by Con A-stimulated T cells

Normal SPCs (1.25×10^5) were cultivated in RPMI medium containing 2 μg/ml Con A in the presence or absence of normal splenic Mϕs (2×10^6 SPC equivalent), SA-Mϕs (2×10^5) or WA-Mϕs (4×10^5). SA-Mϕs and WA-Mϕs were prepared from SPCs of Min-infected BALB/c mice at 2 weeks after infection. After 48-h cultivation, non-adherent cells were collected, washed twice with HBSS, and then blocked with 1% bovine serum albumin (BSA) in PBS. The resultant cells were stained with rat anti-IL-2Rα mAb (1:40) at 4℃ for 30 min, washed twice with 0.1% BSA-PBS, and then stained with FITC-conjugated anti-rat IgG antibody (1:40) at 37℃ for 30 min. After three washings with 0.1% BSA-PBS, the resulting cells were subjected to flow cytometry.

Chemiluminescence (CL)

Test cells (SA-Mϕs and WA-Mϕs) were suspended in 1 ml of HBSS (free from phenol red) containing 10 mM HEPES and 0.1 mM luminol. Then 100 ng of PMA dissolved in 10 μl of dimethyl sulfoxide were added to the incubation mixture and the photoemission was measured in a lumiphotometer (Lumicounter ATP-237, Tokyo Kagaku Ind., Tokyo, Japan).

Statistical analysis

Statistical analysis was performed by Student's *t*-test.

RESULTS

Previously, we found that the suppressor activity of Min-Mϕs depended on their membrane functions (15). In the experiment shown in Table 1 and Fig. 1A, we attempted to separate the Min-Mϕ population into subpopulations on the basis of adhesiveness to plastic wells, in order to examine the relationship be-

Table 1. Comparison of SA-Mϕ and WA-Mϕ subpopulations prepared from Min-induced SPCs for their suppressor activity against Con A-induced T cell mitogenesis

Adherent cells	Number of cells added (10^4)	Con A-induced SPC mitogenesis (10^3cpm±SEM; n=3)	% Inhibition of SPC mitogenesis
None added	0	23.5 ± 0.9	
SA-Mϕs	0.8	27.4 ± 1.0	-17
SA-Mϕs	1.6	11.7 ± 0.7	50
SA-Mϕs	3.2	3.8 ± 0.6	84
WA-Mϕs	5	29.0 ± 0.6	-23
WA-Mϕs	10	21.5 ± 1.2	9
WA-Mϕs	20	1.8 ± 0.3	92
WA-Mϕs (-$Thy1^+$ cells)	5	34.2 ± 1.7	-46
WA-Mϕs (-$Thy1^+$ cells)	10	27.8 ± 0.1	-18
WA-Mϕs (-$Thy1^+$ cells)	20	9.1 ± 0.1	61
WA-Mϕs (-phagocytic cells)	5	26.8 ± 1.0	-14
WA-Mϕs (-phagocytic cells)	10	36.4 ± 0.9	-55
WA-Mϕs (-phagocytic cells)	20	44.1 ± 1.8	-88

SA-Mϕs and WA-Mϕs were prepared from Min-induced SPCs, seeded onto microculture wells at indicated cell numbers, and measured for their suppressor activity against Con A-induced T cell mitogenesis. $Thy1^+$ cell-depleted WA-Mϕ subpopulation and phagocytic cell-depleted WA-Mϕ subpopulation were prepared as mentioned in "Materials and Methods". These data represent one of three experiments that were performed with similar results.

tween suppressor activity and adhesive ability. We successfully obtained two subpopulations differing in adhesive ability (see "Materials and Methods"). One population was strongly adhesive (designated "SA-Mφs"), adhering tightly to plastic wells similar to ordinary Mφs and corresponding to the cell population which we call "Min-Mφs" (7). The other population was weakly adhesive (designated "WA-Mφs"), binding gently to plastic wells and easily detached by vigorous vibration. Not only SA-Mφs but also WA-Mφs exhibited significant levels of suppressor activity against T cell mitogenesis (Table 1). However, the suppressor activity of WA-Mφs was only about one-six that of SA-Mφs (Table 1, Fig. 1A). To determine whether the suppressor activity of weakly adhesive population depends on macrophage, we tested suppressor activity of the cell population which removed Mφ from WA-Mφ using carbonyl iron particle. Notably, the WA-Mφ population exhibited no suppressor activity when phagocytic cells were deleted by carbonyl iron treatment, but did exhibit suppressor activity when T cells ($Thy1^+$ cell) were deleted (Table 1). This finding indicates that WA-Mφs belong to the Mφ lineage. In this context, Min-Mφs (corresponding to SA-Mφs) have also been found to belong to a $Thy1^-$ phagocytic cell population (7). As shown in Fig. 1B, the SA-Mφs and WA-Mφs produced comparable levels of reactive oxygen intermediates (ROIs) in terms of CL in response to PMA. Nevertheless, SA-Mφs displayed slightly greater ROI-producing ability than WA-Mφs (Fig. 1B). This indicates that SA-Mφs are in a more highly activated state than WA-Mφs.

Next, we subjected the WA-Mφ population to a flow cytometric analysis using various mAbs specific to Mφ-specific surface antigens, including CD11b (Mac-1), F4/80 (Mφ-specific differentiation antigen), SR-AI (Mφ-specific scavenger receptor), CD14 (LPS receptor), and CD206 (mannose receptor C Type 1). As indicated in Fig. 2, the majority of the WA-Mφ population possessed a $CD11b^+F4/80^+SR\text{-}AI^+CD14^+CD206^+$ phenotype. This surface antigen profile of WA-Mφs was essentially the same as that of Min-Mφs (corresponding to SA-Mφs) previously reported (16). Therefore, it appears that the WA-Mφs as well as SA-Mφs are matured Mφs.

We previously found that Min-Mφs strongly down-regulated the expression of IL-2R by Con A-stimulated T cells (7,8). As shown in Fig. 3A, both SA-Mφs and WA-Mφs potently suppressed IL-2R ex-

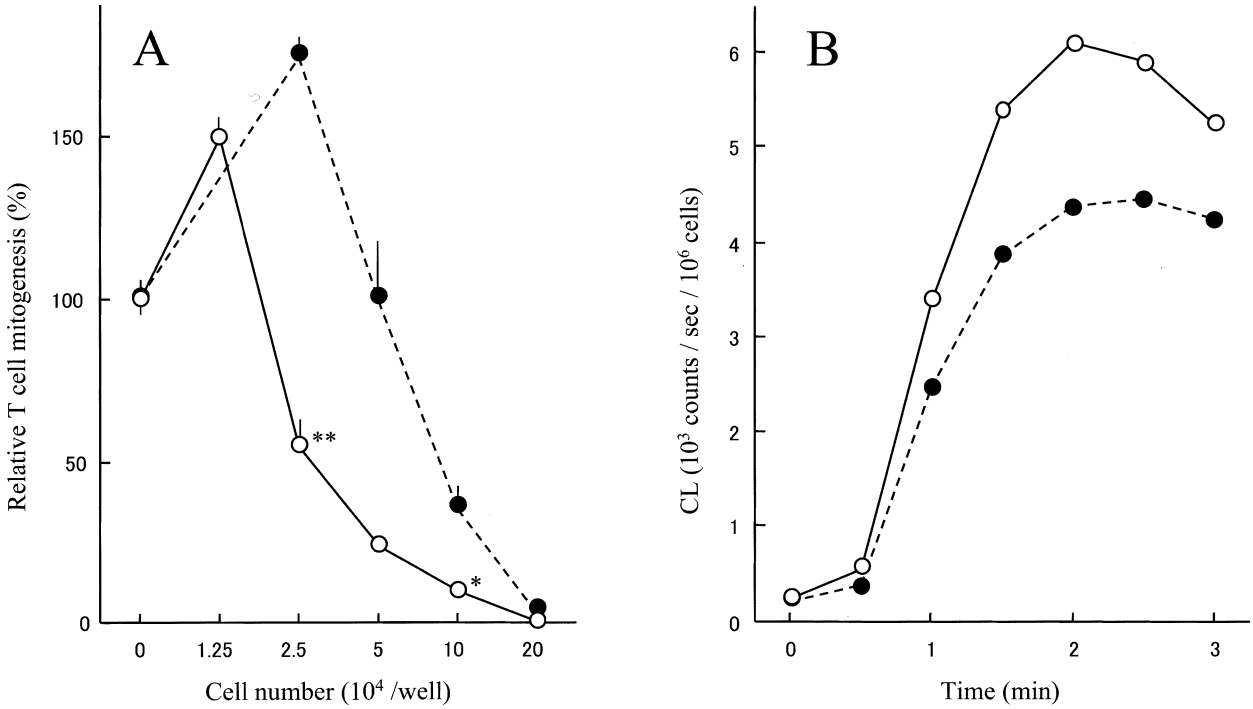

Fig. 1. Suppressor activity (A) and ROI-producing ability (B) of SA-Mφ (open circles) and WA-Mφ (closed circles) subpopulations prepared from SPCs harvested from host mice at week 2 after Min infection. *,**Significant difference was found between SA-Mφs and WA-Mφs (*P<0.05, **P<0.01). These data represent one of two experiments that were performed with similar results.

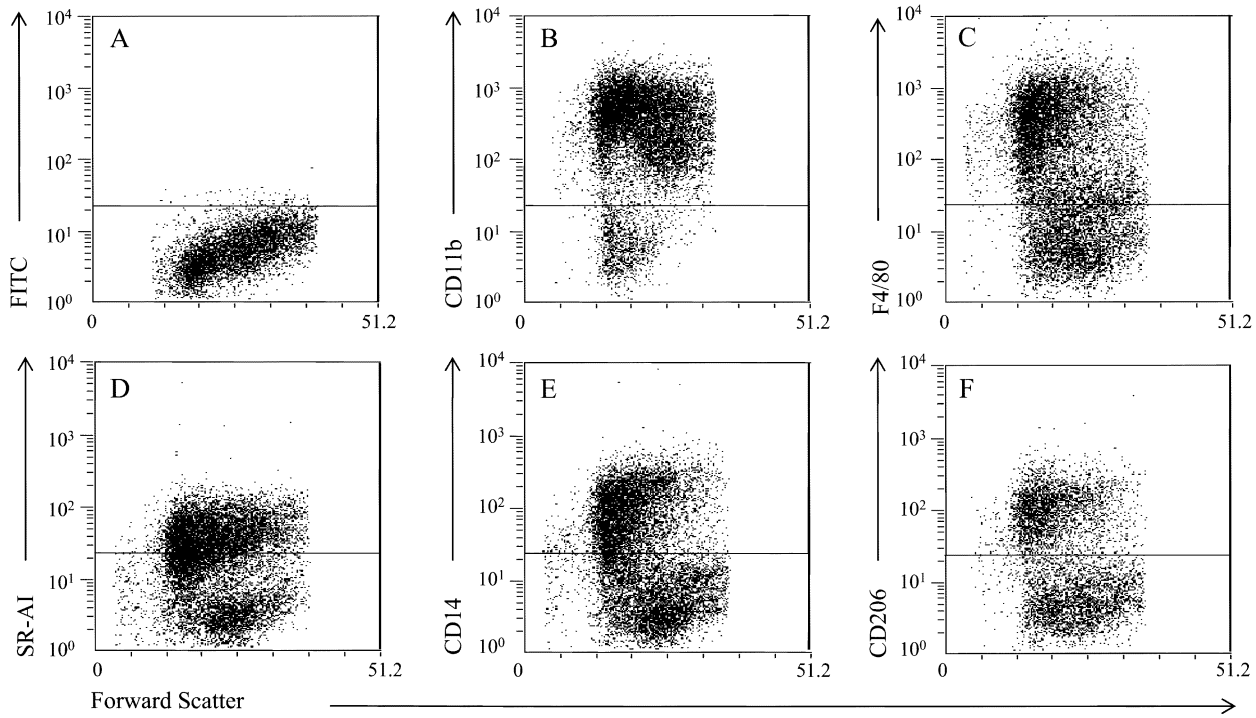

Fig. 2. Characterization of WA-Mϕs by flow cytometry for the expression of Mϕ marker molecules. Tested WA-Mϕs were not stained (A) or stained with mAbs specific to CD11b (B), F4/80 (C), SR-AI (D), CD14 (E), and CD206 (F). The other details are the same as in Fig. 1.

pression by target T cells. On the other hand, splenic Mϕs obtained from normal SPCs did not exhibit such inhibitory effects. Next, again in previous studies, we found that the suppressor activity of Min-Mϕs was mediated by PGE, since it was blocked by indomethacin (cyclooxygenase inhibitor) and exogenous PGE_2 strongly inhibited Con A-induced T cell mitogenesis (7,13). As shown in Fig. 3B, the suppressor activity of WA-Mϕs as well as SA-Mϕs was also blocked by indomethacin. These findings indicate that SA-Mϕs and WA-Mϕs display principally the same effects on target T cells, although the suppressor activity of SA-Mϕs is much greater than that of WA-Mϕs.

DISCUSSION

The present study indicated that there are at least two subsets in the Min-induced suppressor Mϕ population, a strongly adhesive subpopulation (SA-Mϕs) and a weakly adhesive subpopulation (WA-Mϕs). Notably, the SA-Mϕs exhibited much greater suppressor activity than the WA-Mϕs (Fig. 1A). In this context, we previously found that expression of the suppressor activity by Min-Mϕs was dependent on cell-to-cell contact between Min-Mϕs and target T cells and that cytochalasin B (microfilament inhibitor) strongly attenuated the suppressor activity (10,15). These findings suggest microfilament-dependent membrane function and movement to be required for the cell-to-cell interaction of Min-Mϕs with target T cells. Therefore, it appears that actin filament-mediated membrane functions, which are known to play roles in the formation of focal adhesion and stress fibers (17), are required for the suppressor activity of Min-Mϕ populations, including SA-Mϕs and WA-Mϕs. Taken together, it is thought that the suppressor activity of the two Mϕ subpopulations (SA-Mϕs and WA-Mϕs) is tightly linked to their adhesive ability, which is based on microfilament-dependent membrane function (17).

Next, WA-Mϕs were less active than SA-Mϕs in producing ROIs, as measured by PMA-triggered CL (Fig. 1B). Thus, WA-Mϕs may correspond to moderately activated Mϕ populations unlike SA-Mϕs, which are intensely activated in response to Mϕ-activating cytokines, such as IFN-γ and TNF-α generated during the course of Min infection (7,18). As shown in Fig. 3A, both SA-Mϕs and WA-Mϕs markedly suppressed IL-2R expression by target T cells. This indicates that

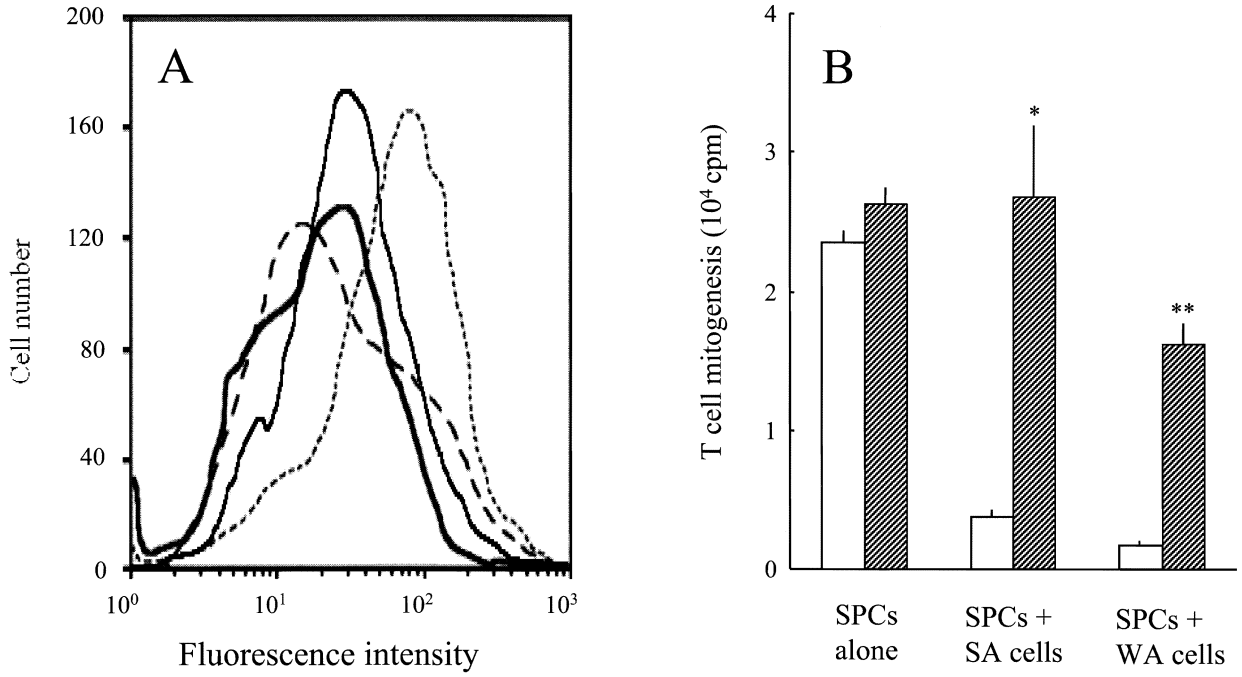

Fig. 3. Comparison for some cellular properties of SA-Mϕ and WA-Mϕ subpopulations. (A). Profiles of IL-2R expression by Con A-stimulated T cells cultivated in the presence of normal splenic Mϕs (dotted line), SA-Mϕs (thick solid line), and WA-Mϕs (thin solid line). As negative control, Con A-stimulated T cells were stained with FITC- conjugated goat anti-rat IgG Ab alone (thin broken line). (B). Effects of indomethacin on the suppressor activity of SA-Mϕs and WA-Mϕs against T cell mitogenesis. Con A-stimulated SPCs were cultured with or without SA-Mϕs (3.2×10^4) or WA-Mϕs (2×10^5) in the presence (hatched bars) or absence (open bars) of 1 μg/ml of indomethacin. *,**Significant difference was found between each set of cultures with or without indomethacin (*$P<0.05$, **$P<0.01$). The other details are the same as in Fig. 1.

both subpopulations at least in part exerted their suppressor activity by blocking the IL-2R expression of target T cells. In addition, indomethacin reduced the suppressor activity of WA-Mϕs as well as SA-Mϕs (Fig. 3B). Therefore, PGE may play roles in expression of the suppressor activity of these two immunosuppressive Mϕ subsets.

In any case, the present study revealed that Min-generated SPCs comprise two suppressor Mϕ populations differing in adhesive ability and suppressor activity, although both SA-Mϕs and WA-Mϕs are thought to belong to be matured Mϕs, on the basis of their expression of surface antigens specific to Mϕs, such as CD11b and F4/80 (Fig. 2) (16). In this context, there are mainly two types of polarized Mϕs, M1 Mϕs with an IL-12^{high}, IL-23^{high}, and IL-10^{low} phenotype and M2 Mϕs with an IL-12^{low}, IL-23^{low}, and IL-10^{high} phenotype (5,19,20). Notably, M2 Mϕs strongly express a mannose receptor (21). Since the majority of both SA-Mϕs and WA-Mϕs expressed CD206 antigen (mannose receptor), it is thought that these immunosuppressive Mϕ populations belong to M2 Mϕs. Further studies are currently underway to elucidate the roles of these suppressor Mϕ subpopulations in the establishment of immune unresponsiveness in the advanced stages of Min infections in mice.

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