

# Properties of immunosuppressive macrophages generated by *Mycobacterium intracellulare* infection in *M. intracellulare*-susceptible and resistant mice

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## SUMMARY

Splenic macrophages (M $\phi$ s) generated in *Mycobacterium intracellulare* (Min)-infected mice exhibit suppressor activity against T cell mitogenesis. We examined profiles of the Min-induced generation of immunosuppressive M $\phi$ s (Min-M $\phi$ s) in Min-susceptible BALB/c (*bcg*<sup>s</sup>) and resistant CBA/JN (*bcg*<sup>r</sup>) mice. Min infection in BALB/c mice caused a more rapid generation of the immunosuppressive M $\phi$ s, which expressed M $\phi$  markers such as CD11b and F4/80, exhibited an increased ability to generate reactive oxygen intermediates, and inhibited IL-2 receptor expression by mitogenic T cells, than did Min infection in CBA/JN mice. Thus, the *bcg* gene may be related to the generation of Min-M $\phi$ s in host mice.

**KEY WORDS:** Immunosuppressive macrophages, *Mycobacterium intracellulare*, *bcg* gene

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The world-wide increase in the incidence of mycobacterial infections, namely tuberculosis and *Mycobacterium avium-intracellulare* complex (MAIC) infections, associated with AIDS has resulted in serious health problems in many countries. The generation of immunosuppressive macrophages (M $\phi$ s) is frequently encountered in hosts with such mycobacterial infections, and leads to depressed cellular host immunity in the advanced stages of infection (Ellner, 1978; Edwards *et al.*, 1986; Tomioka, 2009). We previously found that immunosuppressive M $\phi$ s produced in the spleens of *Mycobacterium intracellulare* (Min) - infected mice (designated Min-M $\phi$ s) caused strong suppression of concanavalin A (Con A) - or T cell receptor ligation-induced T cell mitogenesis (Tomioka *et al.*, 1990; Shimizu *et al.*,

2004) and that the Min-induced generation of immunosuppressive M $\phi$ s *in vivo* was mediated by TNF- $\alpha$  in combination with IFN- $\gamma$  or IL-1 $\beta$  (Tomioka *et al.*, 1996). In addition, the suppressor activity of Min-M $\phi$ s was mediated by soluble factors, including reactive nitrogen intermediates, prostaglandin E and TGF- $\beta$  and partly by cell-to-cell contact with target T cells (Tomioka *et al.*, 1995, Maw *et al.*, 1997; Shimizu *et al.*, 2004; Cai *et al.*, 2006). Notably, in Min-infected mice, the immunosuppressive M $\phi$ s play important roles in the establishment of the unresponsive host T cells that are occasionally encountered in the advanced stages of MAIC infection (Gilbertson *et al.*, 1999; Tomioka, 2009). The immunosuppressive M $\phi$ s are thought to affect T cell functions to generate proinflammatory cytokines, especially M $\phi$ -activating cytokines such as IFN- $\gamma$  and GM-CSF, as in the case of tumor-associated immunosuppressive M $\phi$ s (Loercher *et al.*, 1999), thereby causing down-regulation of the host's antimycobacterial defense mechanisms (Tomioka, 2004; Tomioka, 2009). Here, we studied profiles of the generation of immunosuppressive M $\phi$ s in MAIC-susceptible

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BALB/c (*bcg<sup>s</sup>*) and MAIC-resistant CBA/JN (*bcg<sup>r</sup>*) mice (Vidal *et al.*, 1995) during the course of Min infection and examined some cellular properties of the Min-M $\phi$ s.

Suppressor activity of Min-M $\phi$ s was measured by Method 1 or 2, as described (Tomioka *et al.*, 1995). Method 1: Spleen cells (SPCs) harvested from mice infected intravenously with  $1 \times 10^8$  CFUs of a Min N-260 strain (serovar 16) (in some experiments, a Min 31F093T strain was used) at 2 to 3 weeks after infection and cultured in 0.2 ml of RPMI-medium containing 5% fetal bovine serum (FBS) in flat-bottom microculture wells at a density of  $5 \times 10^5$  to  $2 \times 10^6$  cells/well at 37°C for 2 h. The wells were vigorously rinsed with 2% FBS-Hanks' balanced salt solution (FBS-HBSS) and then filled with 0.1 ml. Method 2, SPCs from infected mice were cultured in 5% FBS-RPMI medium (8 ml) in a 90-mm culture dish at a density of  $4 \times 10^7$  cells/dish at 37°C for 2 h. After rinsing with 2% FBS-HBSS with vigorous vibration using a vibrator, adherent cells were harvested with a rubber policeman, resuspended in 5% FBS-RPMI medium and used as the Min-M $\phi$ s. Then,  $1.25 \times 10^5$  normal SPCs were co-cultivated with the Min-M $\phi$ s in 0.2 ml of RPMI-medium containing 2  $\mu$ g/ml of Con A for 72 h and pulsed with 0.5  $\mu$ Ci of  $^3$ H-TdR for the final 6 to 8 h. Cells were harvested onto glass fiber filters and radioactivity was measured. Second, Min-M $\phi$ s and Con A-stimulated splenic T cells were subjected to flow cytometric analysis as described previously (Shimizu *et al.*, 2004). Briefly, the test cells were treated with individual monoclonal antibodies (mAbs) for 1 h and then stained with FITC-conjugated goat anti-mouse IgG antibody for 1 h. After washing and subsequent fixation with paraformaldehyde, test cells were subjected to flow cytometry using a FACStar or EPICS ELITE flow cytometer. Third, the generation of reactive oxygen intermediates (ROIs) by Min-M $\phi$ s was measured in terms of chemiluminescence (CL) as follows. Test cells suspended in phenol red-free HBSS containing 0.1 mM luminol were incubated at 37°C in the presence of 100 ng/ml of phorbol myristate acetate (PMA) and the photoemission was measured in a lumiphotometer.

First, we examined profiles of the generation of immunosuppressive M $\phi$ s during the course of Min infection in MAIC-susceptible BALB/c (*bcg<sup>s</sup>*) and

MAIC-resistant CBA/JN (*bcg<sup>r</sup>*) mice. As shown in Figure 1A, bacterial loads in the spleens of Min-infected BALB/c as well as CBA/JN mice decreased during the first 2 weeks after infection, indicating M $\phi$ -mediated bacterial elimination in the early phase of infection. The bacterial loads in the spleens of both strains thereafter increased due to the regrowth of Min organisms, indicating that the antimicrobial activity of host splenic M $\phi$ s was decreased after week 2. Notably, BALB/c mice showed greater bacterial loads throughout the 10-week observation period than did CBA/JN mice, indicating that the degree of M $\phi$  activation in terms of antimycobacterial activity is greater in *bcg<sup>r</sup>* mice than in *bcg<sup>s</sup>* mice. This difference was more obvious in the later stages of infection (weeks 4 to 10). Figure 1B shows changes in the proliferative response of host SPCs to Con A during the course of Min infection. The Con A-induced mitogenic response of SPCs of both strains was markedly reduced at week 2. Notably, in CBA/JN mice, the observed reduction in mitogenesis was thereafter rapidly overcome returning to a normal level at week 4. Meanwhile, in BALB/c mice, the reduced mitogenic responsiveness of SPCs lasted much longer, with only a 40% recovery achieved even at week 10.

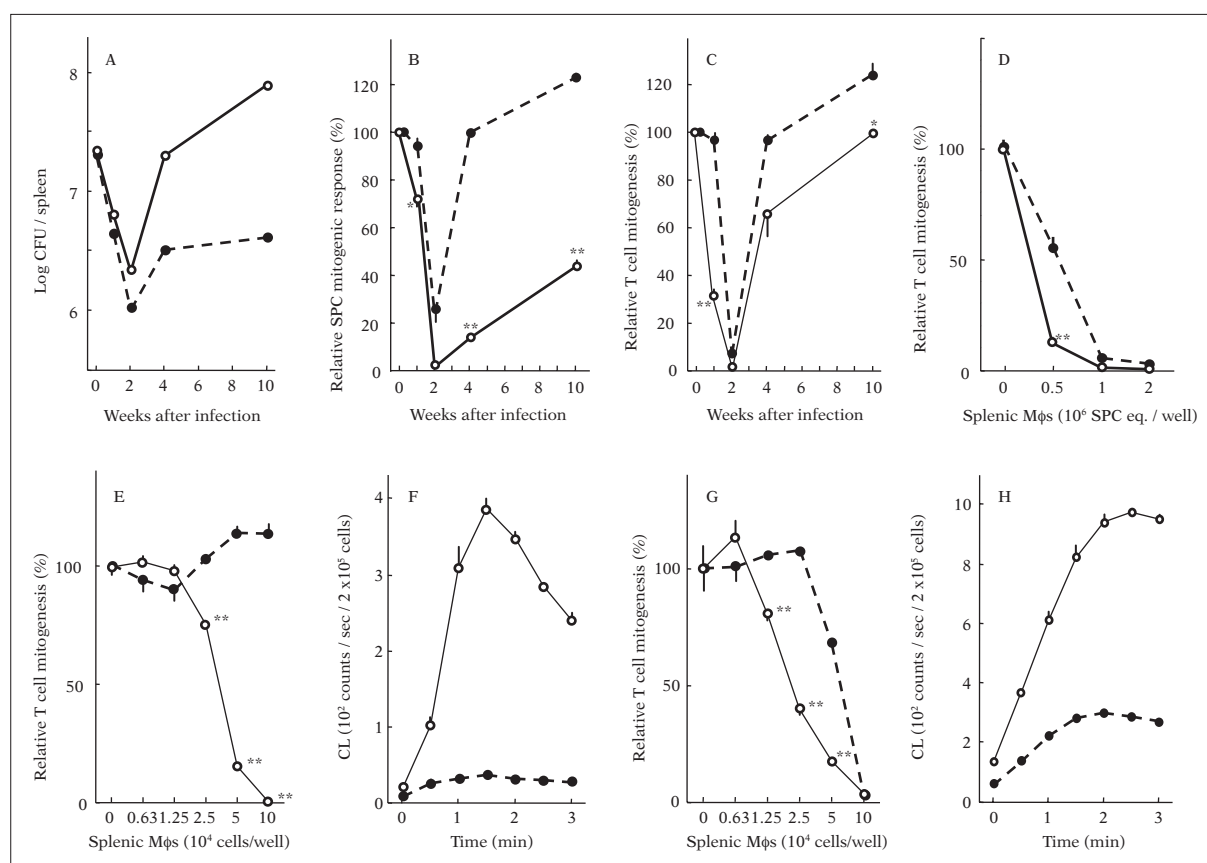
Next, using Method 1, we examined the suppressor activity of immunosuppressive M $\phi$ s generated in a specific number of host SPCs, which were harvested from BALB/c and CBA/JN mice at weeks 1, 2, 4 and 10 after Min infection (Figure 1C). At week 1 after infection, immunosuppressive M $\phi$ s were detected in the spleens of BALB/c mice, but not in CBA/JN mice. This indicates that immunosuppressive M $\phi$ s are generated more quickly in *Bcg<sup>s</sup>* mice than in *Bcg<sup>r</sup>* mice after Min infection. Next, at week 2, Min infection caused the generation of immunosuppressive M $\phi$ s in both strains, though the suppressor activity was somewhat (but significantly) greater in the BALB/c mice (Figure 1D). From week 2, the immunosuppressive M $\phi$ s were progressively sequestered until by week 10, essentially no suppressor activity was detected in SPCs of either strain. Notably, the suppressor activity of M $\phi$ s was diminished more promptly in CBA/JN mice than in BALB/c mice. Taken together, in both strains, the suppressor activity of splenic M $\phi$ s was greatest at week 2 and thereafter declined, having completely diminished by week 10. Notably, the activity was

induced more rapidly and more marked in BALB/c mice than CBA/JN mice.

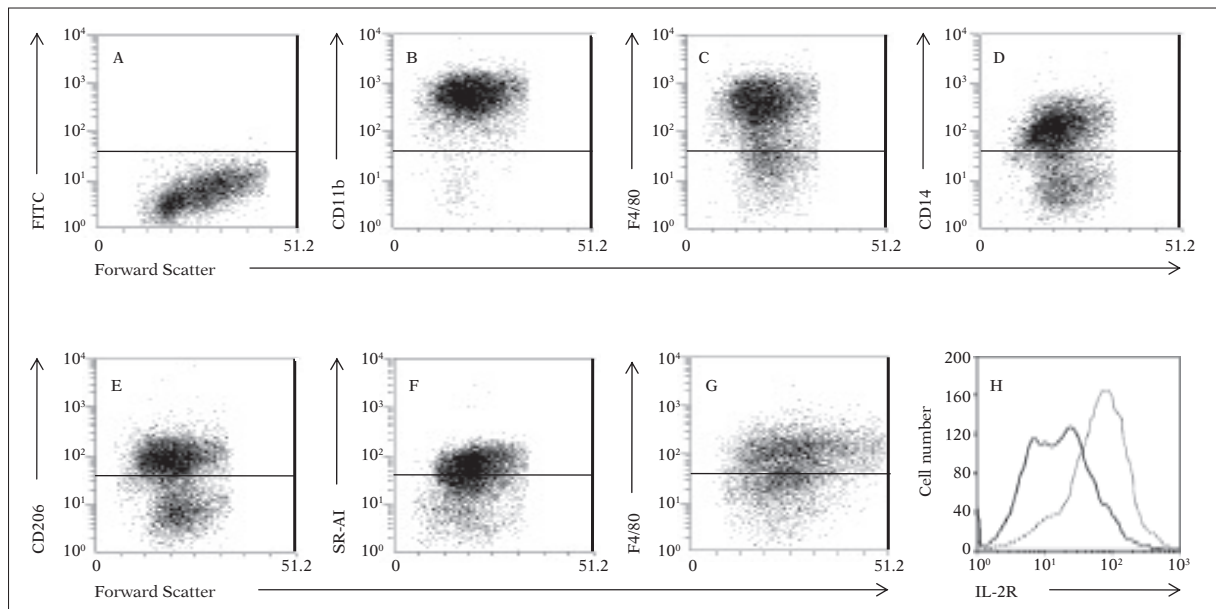
Next, using Method 2, we compared the per cell suppressor activity of splenic M $\phi$ s obtained from BALB/c and CBA/JN mice at weeks 1 and 2 after Min infection. At week 1 after infection, the splenic M $\phi$ s of BALB/c mice exhibited strong suppressor activity, while those of CBA/JN mice essentially lacked such activity (Figure 1E). In this case, the splenic M $\phi$ s of BALB/c mice concomitantly exhibited strong CL (a measure of the ability of M $\phi$ s to produce ROI), whereas those of

CBA/JN mice showed only weak CL (Figure 1F). At week 2 after infection, the splenic M $\phi$ s of BALB/c mice showed stronger suppressor activity and CL, than those of CBA/JN mice (Figures 1G and 1H).

Next, we examined the profiles of expression of M $\phi$ -specific surface markers on Min-M $\phi$ s by flow cytometry using mAbs specific to CD11b (Mac-1), F4/80 (M $\phi$ -specific differentiation antigen), CD14 (LPS receptor), CD206 (mannose receptor C Type 1), and SR-AI (M $\phi$ -specific scavenger receptor) (Figure 2). The majority of Min-M $\phi$ s gen-



**FIGURE 1 -** Changes in bacterial loads in the spleens, Con A mitogenic responsiveness of SPCs, and generation of immunosuppressive M $\phi$ s in the spleens of BALB/c (open circles) and CBA/JN (closed circles) mice during the course of Min infection. (A) Bacterial loads in the spleens of the both strain mice during the course of Min 31F093T infection. (B) Con A-induced mitogenesis of SPCs obtained from BALB/c and CBA/JN mice at intervals after Min 31F093T infection. (C) Generation of suppressor M $\phi$  activity against T cell mitogenesis during the course of Min 31F093T infection. Each plot indicates relative Con A mitogenesis of T cells, when co-cultured on the M $\phi$  monolayer prepared by seeding  $1 \times 10^6$  SPCs harvested at indicated time after infection. (D) Relative Con A mitogenesis of T cells, when co-cultured on the M $\phi$  monolayer prepared by seeding  $5 \times 10^5$  to  $2 \times 10^6$  SPCs harvested at week 2 post Min 31F093T infection. (E to H) Suppressor activities (E, G) and PMA-induced CL (F, H) of splenic M $\phi$ s obtained from host SPCs at week 1 (E, F) and week 2 (G, H) after Min N-260 infection. Each symbol indicates the mean  $\pm$  standard error ( $n=3$ ). \*, \*\*Significant difference was found between BALB/c and CBA/JN mice (\* $P<0.05$ , \*\* $P<0.01$ ). These data represent one of two experiments that were performed with similar results.



**FIGURE 2 - Characterization of Min-Mφs by flow cytometry.** (A to F) Flow cytometry of the expression of Mφ marker molecules by Min-Mφs of BALB/c mice. Tested Min-Mφs induced by Min N-260 infection (week 2 post infection) were stained with mAbs specific to CD11b (B), F4/80 (C), CD14 (D), CD206 (E), and SR-AI (F). (G) Flow cytometry of F4/80 expression by Zymosan A-induced mouse peritoneal Mφs. The Mφs were stained with anti-F4/80 mAb. (H) Profiles of IL-2R expression by Con A-stimulated T cells, which were co-cultured with normal splenic Mφs (broken line) or Min-Mφs (solid line). Con A-stimulated SPCs were cultured on the Mφ monolayers prepared by seeding  $2 \times 10^6$  SPCs from normal mice or Min N-260-infected mice (week 2 post infection) for 48 h.

erated in BALB/c mice were positive for these Mφ markers (Figures 2B to 2F). Peritoneal Mφs were also positive for these markers (data not shown), but the intensity of F4/80 expression was less than that of Min-Mφs (Figure 2G versus Figure 2C). We previously found that Min-Mφs suppressed generation of IL-2-reactive T cells from naive SPCs in response to Con A (Tomioka *et al.*, 1990). Therefore, we examined the effects of Min-Mφs on the expression of the IL-2 receptor (IL-2R) by splenic T cells in response to Con A stimulatory signals. As shown in Figure 2H, Min-Mφs strongly suppressed the expression of IL-2R by target T cells, indicating that they exert their suppressor activity by blocking the receptor's expression. The present findings indicate the following. First, Min infection in BALB/c (*b6g<sup>s</sup>*) mice led to a more potent and rapidly formed immunosuppressive Mφ population in host spleen, compared to the case with CBA/JN (*b6g<sup>r</sup>*) mice. It appears that this is in part due to differences in bacterial load in the spleens of the host mice, as BALB/c mice showed greater bacterial loads throughout the observation period than did CBA/JN mice (Figure

1A). Second, the reduction in the Con A mitogenic activity of SPCs at week 2 post infection was firmly linked to the generation of immunosuppressive Mφs in host spleens at the same time point of infection (Figure 1B versus Figure 1C). Third, in BALB/c mice, the suppressed state of SPC mitogenic activity thereafter lasted for long periods until week 10 (Figure 1B), while suppressor Mφ activity was rapidly diminished after week 2 (Figure 1C). This implies that immunosuppressive Mφs generated around week 2 after infection play important roles in the establishment of the subsequent hypo-responsiveness of host SPCs to Con A mitogenic signals. Taken together, it is thought that the marked and prolonged generation of suppressor Mφs in BALB/c (*b6g<sup>s</sup>*) mice compared to CBA/JN (*b6g<sup>r</sup>*) mice is closely related to insufficient T cell-mediated immunity against MAIC infection in the *B6g<sup>s</sup>* genotype. This may result in a lowered level of acquired immunity-dependent host resistance to MAIC pathogens, thereby causing the failure to effectively inhibit the growth of pathogens at sites of infection.

In this context, *bcg* gene is essentially equivalent to the *Nramp1* gene encoding natural resistance-associated macrophage protein 1 (Nramp1) (Courville *et al.*, 2006). The natural Nramp1 homologs form a family of proton-coupled transporters that facilitate the cellular absorption of divalent metal ions. Since highly active immunosuppressive M $\phi$ s were generated in Min-infected BALB/c (*bcg<sup>s</sup>/Nramp1<sup>s</sup>*) mice, it appears that the expression of suppressor activity by Min-induced M $\phi$ s is not dependent on Nramp1 protein, indicating that the membrane transport of divalent metal ions plays no major role in the suppressor activity of Min-M $\phi$ s. On this point, current studies using Nramp1-deficient mice are under way.

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