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Early effect of *Mycobacterium tuberculosis* infection on Mac-1 and ICAM-1 expression on mouse peritoneal macrophages

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Abbreviations: ICAM-1, intercellular adhesion molocule-1; MCF, mean channel fluorescence

Abstract

Effect of *M. tuberculosis* infection was studied on the expression of intercellular adhesion molocule-1 (ICAM-1) and Mac-1 markers on murine peritoneal macrophages. Intraperitoneal administration of *M. tuberculosis* resulted in a marked increase in the proportion of Mac-1$^+$ cells whereas the proportion of ICAM-1$^+$ cells declined sharply 4 h post infection. Absolute numbers of Mac-1$^+$ and ICAM-1$^+$ cells however increased at all time points after the infection. Comparison of kinetics of changes observed in Mac-1$^+$ and ICAM-1$^+$ cell populations with differential leukocyte counts in peritoneal cells indicated that these alterations could be due to cellular influx, especially that of neutrophils, or up regulation of these markers on macrophages and other peritoneal cells. In adherent peritoneal macrophages infected *in vitro* with *M. tuberculosis*, proportion of Mac-1$^+$ and ICAM-1$^+$ cells increased markedly within 24 h of infection. Mean expression of these markers on per cell basis also increased significantly. Similar results were obtained by using RAW 264.7 mouse macrophage cell line, suggesting that the enhanced expression of Mac-1 and ICAM-1 markers was a direct effect of *M. tuberculosis* infection and not mediated by contaminating cell types present in adherent macrophage preparations. Mac-1 and ICAM-1 expression was further studied on macrophages that had actually engulfed *M. tuberculosis* and compared with bystander macrophages without intracellular *M. tuberculosis.* For this purpose *M. tuberculosis* pre-stained with DiIC18 fluorescent dye were used for infecting adherent peritoneal macrophages. Mac-1 and ICAM-1 expression on gated DiIC18 positive and negative cell populations was analyzed. Our results indicate that the expression of Mac-1 and ICAM- 1 markers was significantly enhanced on all macrophages incubated with *M. tuberculosis* but was more pronounced on macrophages with internalized mycobacteria. Taken together, our results suggest that the expression of Mac-1 and ICAM-1 markers is significantly up regulated as a result of exposure and infection with *M. tuberculosis*. Since these markers play important role in the uptake of mycobacteria as well as in the process of antigen presentation by macrophages, their upregulation may be beneficial for generation of a protective immune response to *M. tuberculosis.*

Keywords: flow cytometry; intercellular adhesion molocule-1; macrophages; Macrophage-1; *Mycobacterium tuberculosis*; phagocytosis

Introduction

Macrophages play a crucial role in immunity to tuberculosis. Activated through receptors like TLR2 which recognize pathogen associated molecular patterns of *Mycobacterium tuberculosis* (Underhill *et al.,* 1999), macrophages secrete a variety of cytokines (Giacomini *et al.,* 2001), phagocytose mycobacteria (Rabinovitch, 1995) and process/present mycobacterial antigens to T cells (Ullrich *et al.,* 2000; Jiao *et al.,* 2002). T cell cytokines like IFNγ further activate macrophages to kill the intracellular mycobacteria (Diang *et al.,* 1988). In susceptible individuals, mycobacteria can subvert the protective mechanisms initiated by macrophages and survive inside macrophages. *In vitro* studies with mononuclear phagocytes have established that *M. tuberculosis* adheres either opsonincally or non-opsonically to different receptors on the host cell surface (Schlesinger *et al.,* 1990, 1994; Downing *et al.,* 1995; Peterson *et al.,* 1995). Receptor molecules on macrophages that participate in the uptake of mycobacteria include Mac-1, a complement receptor of integrin family. Mac-1 not only participates in phagocytosis of opsonized *M. tubercu-*

losis but also promotes phagocytosis of non-opsonized mycobacteria by providing two distinct binding sites for *M. tuberculosis* (Le Cabec *et al.,* 2002). Mac-1 is also involved in extravasation of macrophages and monocytes across the endothelial cells lining blood vessels (Van oud Alblas *et al.,* 1979; Rosseau *et al.,* 2000). ICAM-1 is another molecule expressed on macrophages, which belongs to immunoglobulin superfamily and has an important role as a costimulatory molecule during antigen presentation to T cells (Van Seventer *et al.,* 1990). Recently macrophages from ICAM-1 knockout mice were shown to have decreased phagocytic activity indicating an additional role of this molecule in phagocytosis (Paine *et al.,* 2002).

Mac-1 and ICAM-1 molecules may thus play important role in different phases of macrophage response to mycobacterial infection. Enhanced expression of these molecules during early phases of *M. tuberculosis* infection may be beneficial for inducing a protective immune response. In order to ensure its own survival, *M. tuberculosis* may subvert the expression of these molecules. Lopez-Ramirez et al reported that *M. tuberculosis* infection increased ICAM-1 expression significantly and that of Mac-1 molecule nominally in a human monocyte cell line THP1 (Lopez-Ramirez *et al.,* 1994). Significant upregulation of ICAM-1 expression was reported in peritoneal cells (PCs) from Balb/c mice three days after mycobacterial infections (Saha *et al.,* 1994; Tomioka *et al.,* 2000). Similar effect of *M. tuberculosis* infection on ICAM-1 and Mac-1 expression on lung or bone marrow derived dendritic cells was also reported (Gonzalez-Juarrero and Orme, 2001). Bodner et al. however did not find upregulation of ICAM-1 on bone marrow derived macrophages infected with *M. tuberculosis* (Bodnar *et al.,* 2001). Hamerman and Aderem reported a down regulation of Mac-1 expression on murine peritoneal macrophages 48 h after BCG infection (Hamerman and Aderem, 2001). Early effects of mycobacterial infection on the expression of Mac-1 and ICAM-1 on macrophages may be important to determine the course of infection, but this information is not available in literature. We started our investigations of these early effects on macrophages following an intraperitoneal infaction of *M. tuberculosis*. Similar work on resident peritoneal macrophages has not yet been reported. We found a marked increase in absolute recoveries of Mac-1 and ICAM-1 bearing cells within 4 h of intraperitoneal administration of *M. tuberculosis*. This effect was however not solely due to changes in macrophages. In order to assess the direct effect of *M. tuberculosis* infection on macrophages, we continued the investigations by using *in vitro* infection model. Our results indicate that *M. tuberculosis* infection causes early upregulation of Mac-1 and ICAM-1 expression on macrophages. Additionally, the effect on Mac-1 and ICAM-1 expression was more prominent on macrophages that had actually ingested mycobacteria in comparison to the bystander macrophages.

Materials and Methods

Animals

Inbred C57BL/6 mice (8-12 weeks old) were used throughout this study. Animals were bred and maintained in the animal house facility at Jawaharlal Nehru University, New Delhi or obtained from the National Institute of Nutrition, Hyderabad. The JNU Institutional Animal Ethical Committee approved all experimental protocols requiring the use of animals.

Reagents and other supplies

All tissue culture reagents and Tween-80 were purchased from Sigma Chemicals (St. Louis, MO). Sources of other reagents were: Fetal calf serum, Hyclone Laboratories Inc, USA; Middlebrook 7H11 Agar, Difco Laboratories, MI, USA. Anti-mouse CD16/CD32, anti-mouse Mac1-FITC and anti-mouse ICAM1-FITC monoclonal antibody were purchased from Pharmingen. DiLC18 stain was purchased from Molecular Probes, USA. All the syringes and needles were procured from Becton Dickinsion, Singapore. Costar (Cambridge, MA) was the source of all plastic disposable culture ware.

Culture of mycobacteria

The H37Ra strain (ATCC 2517) of *M. tuberculosis* provided by Dr. Katoch (Japanese Association for Leprosy Mission Aided Institute, JALMA, Agra) was grown in Sauton's medium (with 0.05% Tween 80). At late log phase bacterial cells were harvested and frozen at -70^{o}C in medium with 20% glycerol. Viability was determined by plating different dilutions of the bacterial suspension on Middlebrook 7H11 agar (enriched with OADC supplement). Colonies were counted after three to four weeks and CFU of the frozen stock were calculated.

Cell culture medium

Murine peritoneal cells and RAW 264.7 (TIB 71 from ATCC) cells were maintained in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 20 mM Hepes, 300 μg/ml glutamine, 20 μM 2-mercaptoethanol and 60 μg/ml gentamycin.

Intra-peritoneal infection of mice

A frozen stock of *M. tuberculosis* (H37Ra) of known CFU/ml was thawed and the bacilli diluted in PBS to a concentration of 1×10^8/ml. The suspension was passed through 24 G needle several times to break the clumps and prepare a single cell suspension of bacteria. Intra- peritoneal infection was initiated by injecting 50×10^6 CFUs of *M. tuberculosis* per mouse (in a volume of 500 μl).

Differential leukocyte count

Differential leukocyte count was carried out by making smears of peritoneal cells on glass slides. Cell smears were air dried and the slides were fixed by dipping into absolute methanol for 30 min. Commercial Giemsa stain (Qualigens, India) diluted 10 times with PBS were added drop by drop on the fixed smear and kept for 20 min. Then the slide was washed with flowing water and air dried. The slide was examined under a light microscope to study the differential staining of various leukocytes and a total of 200 cells from each sample were counted.

In vitro infection of Peritoneal cells and RAW 264.7 cells

Peritoneal cells harvested from C57BL/6 mouse were allowed to adhere in a 24 well culture plate (2×10^6 cells/ml/well) for 2 h. Adherent cells were washed and infected with 20×10^6 *M. tuberculosis* (H37Ra) for varying time periods. RAW 264.7 cells cultured in a 24-well plate (0.2×10^6 cells/ml) were similarly infected with 2×10^6 *M. tuberculosis* (H37Ra) for varying time periods. At different time points cells were harvested from wells, stained with anti mouse Mac-1-FITC or ICAM-1-FITC antibody and analyzed by flowcytometry.

Flow cytometric analysis of cell surface receptors

Cells to be stained (0.25×10^6/in 50 μl volume) were incubated with anti mouse CD16/CD32 Fc block (1 μg/10^6 cells) for 10 min prior to staining. Cells were then incubated with anti mouse Mac-1-FITC or ICAM-1-FITC antibody (1 μg/10^6 cells) for 30 min on ice. Cells were washed twice with staining buffer (PBS without Ca^{2+} and Mg^{2+} containing 1% FBS and 0.09% sodium azide) and fixed in 200 μl of 0.1% paraformaldehyde. Flow cytometric analysis was carried out by using a Coulter-EPICS flow cytometer and WINMDI software.

DiLC18 staining of *M. tuberculosis*

M. tuberculosis (1×10^8) were resuspended in 500 μl of PBS containing 10 μM DiLC18 dye and incubated in dark at 37°C for 2 h followed by three washings with PBS.

Statistical analyses

For each set of data, arithmetic mean and standard deviation were calculated. Data sets were compared by using unpaired Student's *t*-test for independent variables.

Results

Early upregulation of Mac-1 and ICAM-1 expression on peritoneal cells (PCs) from mice infected intraperitoneally by *M. tuberculosis*

ICAM-1 (CD54) and Mac-1 (CD11b/CD18) are amongst the surface expressed molecules on macrophages that are involved in the processes of migration of these cells to the site of infection, phagocytosis of mycobacteria, and presentation of mycobacterial antigens to T cells. It is therefore important to determine if the expression of these membrane markers is modulated during mycobactrial infection. Because of the ease in isolating the leukocyte populations from peritoneal cavity, we studied the effect of intraperitoneal administration of *Mycobacterium tuberculosis* on the expression of Mac-1 and ICAM-1 molecules on peritoneal leukocytes. Results in Figure 1A show that the percentage of PCs expressing Mac-1 doubled from $38.8 \pm 5.3\%$ to $82.8 \pm 4.6\%$ within four hours of administration of *M. tuberculosis* and remained elevated till 48 h post infection. Percentage of ICAM-1 expressing PCs however declined sharply at 4 h time point but increased steadily thereafter (Figure 1A). Increase in the absolute recoveries of Mac-1 expressing cells was more dramatic. Mac-1 positive cell population increased from about $1 \pm 0.18\times10^6$ cells /mouse to about $14.1 \pm 2.5\times10^6$ cells/mouse 4 h after the infection. Absolute numbers of Mac-1^+ PCs declined thereafter, but was still $8.8 \pm 1.6\times10^6$ cells /mouse 48 h post infection. In contrast, the absolute recovery of ICAM-1^+ PCs steadily increased from less than $1.4 \pm 0.2\times10^6$ cells/control mouse to about $8.1 \pm 1.8\times10^6$ cells/mouse 48 h post infection. Changes in proportions and absolute recoveries of PCs bearing Mac-1 and ICAM-1 markers were distinctly different and could result from differential migration of different classes of leukocytes to the site of infection. Differential leukocyte counting (DLC) was therefore carried out with PCs isolated from control and infected mice. Results in Figure 2A indicate that in PC preparations from control mice, macrophages and lymphocytes were almost equally represented with very few neutrophils. In infected mice however, neutrophils accounted for $80.2 \pm 6\%$ of all PCs.

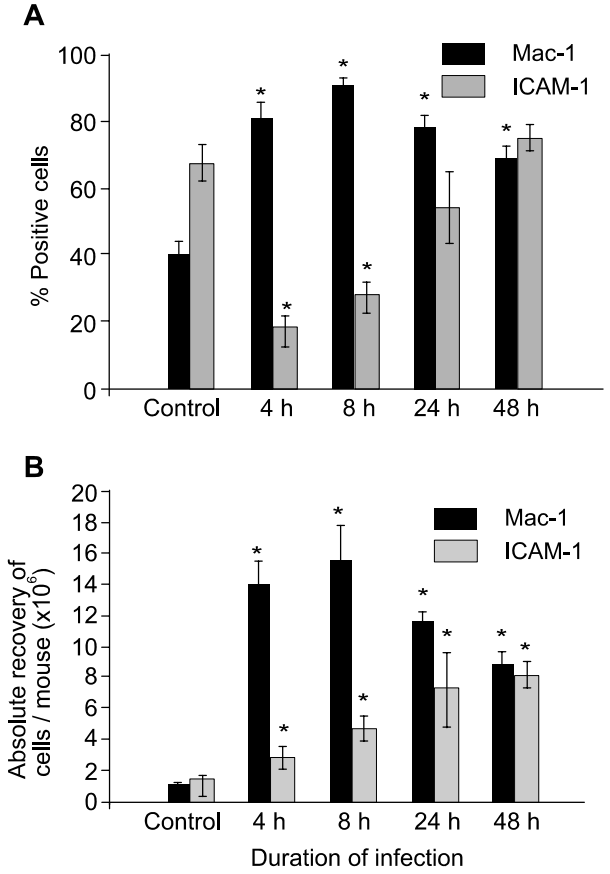

Figure 1. Time kinetics of Mac-1 and ICAM-1 expression after *M. tuberculosis* infection. 50×10^6 bacteria (*M. tuberculosis*) were injected intra-peritoneally in each mouse. At different time points after the infection peritoneal cells were harvested from mice, stained with anti-mouse Mac-1 or ICAM-1 monoclonal antibody and analyzed on a flow cytometer. (A) Percent Mac-1^+ and ICAM-1^+ cells, and (B) total recoveries of Mac-1^+ and ICAM-1^+ cells per mouse. All comparisons of infected and control cells were done by unpaired *t*-test (*$P < 0.05$). Each bar represents mean of five experiments ± SD.

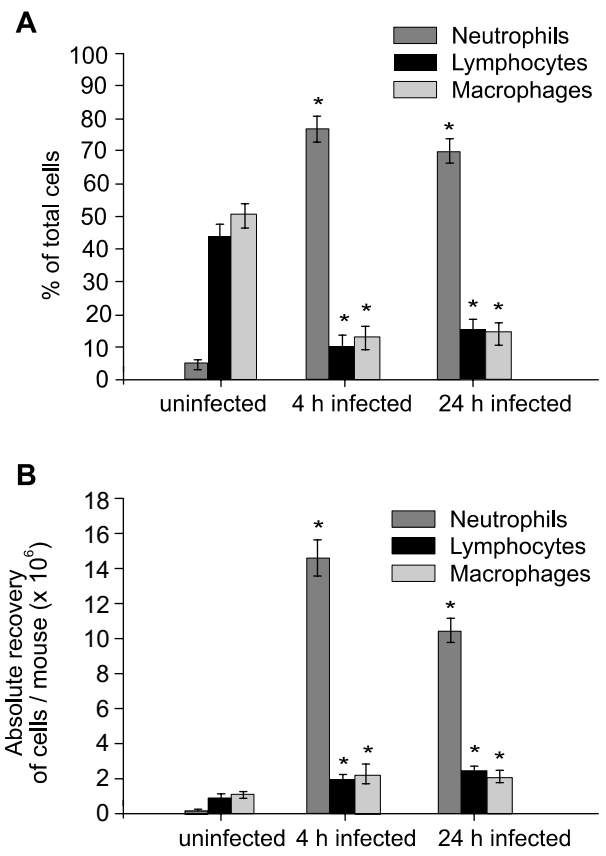

Figure 2. Effect of *M. tuberculosis* infection on differential leukocyte count in mouse peritoneal cells. 50×10^6 bacteria (*M. tuberculosis*) were injected intraperitoneally in each mouse. At different time points after infection peritoneal cells were harvested from mice and differential leukocyte count were done as described in Materials and Methods. (A) Percentage of different types of leukocytes in peritoneal cell preparations, and (B) total recoveries of different types of leukocytes per mouse. Comparisons of values for infected and control mice were done by unpaired *t*-test. (*$P < 0.05$). Each bar represents mean of three experiments ± SD.

Figure 2B shows the absolute recoveries of lymphocytes, macrophages and neutrophils in PCs from control and infected mice. Neutrophils constituted the major population in PCs derived from infected mice, but absolute numbers of lymphocytes and macrophages also increased significantly in PC preparations from infected mice. A comparison of results in Figure 1B and 2B shows that while the average absolute recovery of macrophages from infected mice was around $2.4 \pm 0.2\times10^6$ cells/mouse, absolute recoveries of Mac-1^+ and ICAM-1^+ cells reached as high as $14.1 \pm 2.5\times10^6$ and about $8.1 \pm 1.8\times10^6$ cells/mouse respectively. Clearly therefore cells other than macrophages contributed to the substantial increase in Mac-1^+ and ICAM-1^+ cells seen in the PC preparations from infected mice. In order to study the direct effect of *M. tuberculosis* infection on the expression of ICAM-1 and Mac-1 markers on macrophages, *in vitro* infection experiments were carried out to avoid the interference caused by migrated cell populations *in vivo*.

In vitro effect of *M. tuberculosis* infection on Mac-1 and ICAM-1 expression on murine peritoneal macrophages

Adherent macrophage enriched populations of mouse PCs were infected *in vitro* with *M. tuberculosis*. Results in Figure 3A show Mac-1 and ICAM-1 expression on control and *M. tuberculosis* infected adherent PCs. In control cell preparations, 40.9 ± 8.5 and $52.3 \pm 4.7\%$ cells expressed Mac-1 and ICAM-1 markers respectively. These values increased in a time dependent manner and were 87.5 ± 4.6 and

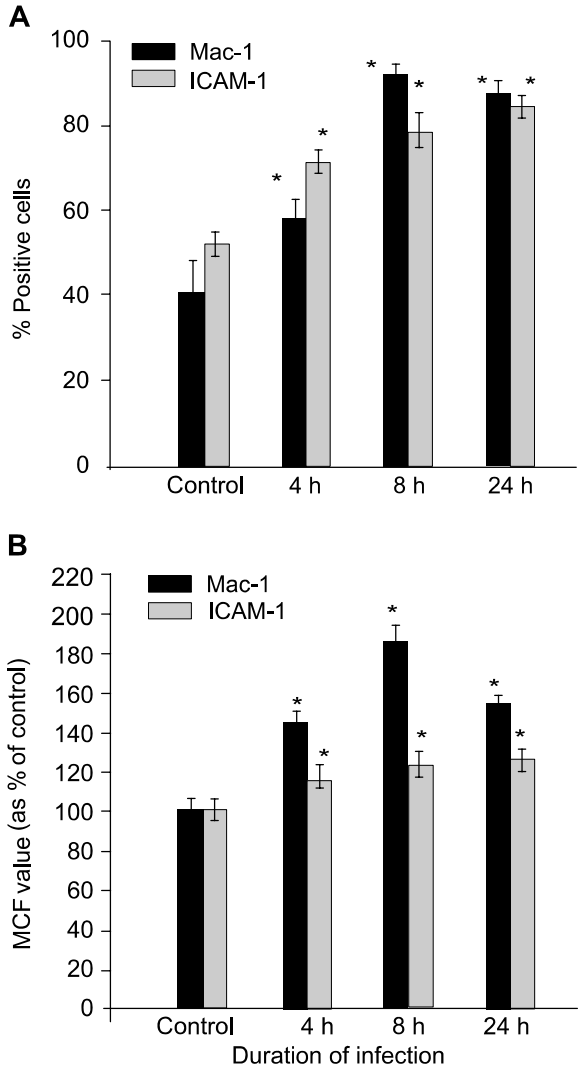

Figure 3. Effect of *M. tuberculosis* infection on Mac-1 and ICAM-1 expression of adherent peritoneal cells. Peritoneal cells harvested from C57BL / 6 mice were allowed to adhere in a 24-well culture plate (2×10^6 cells/ml/well) for 2 h. Adherent cells were washed and infected with *M. tuberculosis* as described in materials and methods. At different time points adherent cells were harvested from wells, stained with anti-mouse Mac-1 or ICAM-1 monoclonal antibody and analyzed by flow cytometry. (A) Percent cells expressing Mac-1 and ICAM-1 after *M. tuberculosis* infection, and (B) changes in mean channel fluorescence (MCF) as percentage of control. All comparisons of infected and control cells were done by unpaired *t*-test ($^*P < 0.05$). Each bar represents mean of five experiments ± SD.

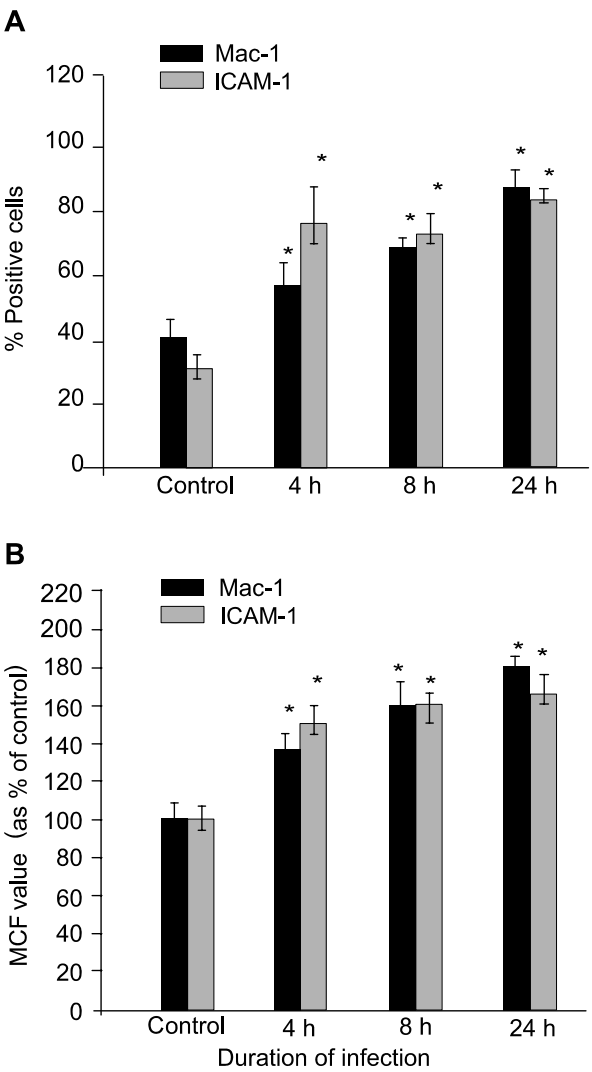

Figure 4. Effect of *M. tuberculosis* infection on Mac-1 and ICAM-1 expression of RAW 264.7 cells. RAW 264.7 cells were cultured in a 24-well plate (0.2×10^6 cells/ml) and infected with *M. tuberculosis* after different time periods. Control and infected cells were harvested, stained with anti mouse Mac-1 or ICAM-1 antibody and analyzed by flow cytometry. (A) Percent positive cells, and (B) Mean channel fluorescence (MCF) as percentage of control. All comparisons of infected and control cells were done by unpaired *t*-test ($^*P < 0.05$). Each bar represents mean of five experiments ± SD.

84.6 ± 5.9% respectively 24 h after the infection (Figure 3A). Average expression of Mac-1 and ICAM-1 on control and infected adherent PCs is shown in Figure 3B. While percentage of cells staining with Mac-1 and ICAM-1 antibodies increased significantly, average expression of Mac-1 per cell as assessed by mean fluorescence on stained cells, also increased significantly in *M. tuberculosis* infected cells (Figure 3B). Average ICAM-1 expression increased only marginally (Figure 3B). Adherent PCs are predominantly macrophages, Yet some contamination from other cell types is expected. In order to further confirm if the effect of infection with *M. tuberculosis* was a direct effect on macrophages, *in vitro* infection experiments were repeated with RAW 264.7 mouse macrophage cell line. Results in Figure 4A and B indicate that the proportion of Mac-1 and ICAM-1 expressing RAW 264.7 cells as well as the average expression of these markers on RAW 264.7 cells increased in a

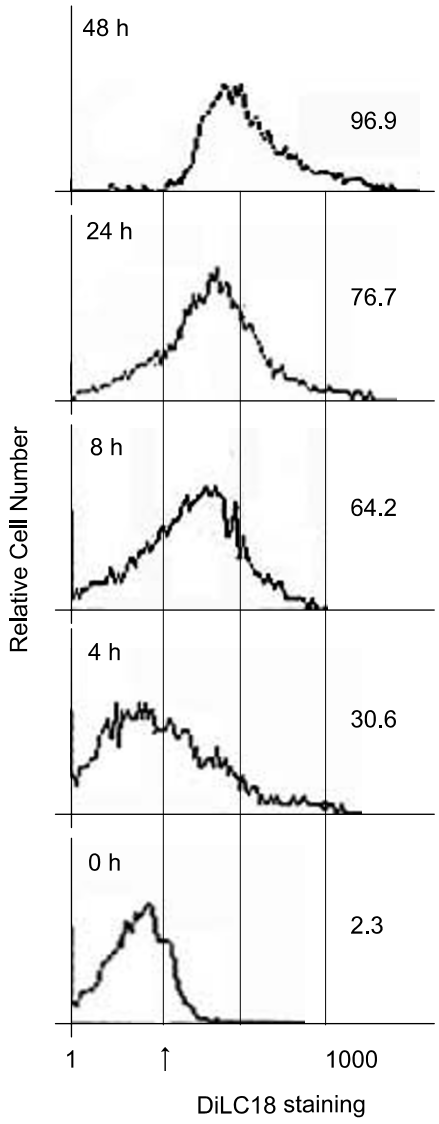

Figure 5. *In vitro* uptake of bacteria by adherent mouse peritoneal cells after infection with DiLC18 stained *M. tuberculosis*. Peritoneal cells harvested from C57BL/6 mice were cultured for 2 h. Adherent cells were washed and infected with DiLC18 stained *M. tuberculosis* for varying time periods. At different time points adherent cells were washed and analyzed by flow cytometry. An arrow on *x*-axis indicates the position of gate determined by using uninfected cells. Percentage cells positive for DiLC18 have been indicated in the individual histograms.

time dependent manner after *M. tuberculosis* infection.

Expression of Mac-1 and ICAM-1 molecules on peritoneal macrophages with or without ingested *M. tuberculosis*

It was of interest to determine if the enhanced expression of Mac-1 and ICAM-1 markers was confined to macrophages that ingested *M. tuberculosis*sis or was uniformly seen on all cells exposed to *M. tuberculosis*. For this purpose, macrophages were infected with *M. tuberculosis* pre-stained with DiLC18, a fluorescent dye which binds the bacterial cells irreversibly. Macrophages with or without fluorescent *M. tuberculosis* could be gated on a flow cytometer and expression of Mac-1 and ICAM-1 populations could be studied on both populations of cells. Results of a typical experiment showing the uptake of DiLC18 stained *M. tuberculosis* by adherent PCs are given in Figure 5. About 64% macrophages had taken up *M. tuberculosis* 8 h post infection. By 48 h more than 95% macrophages were positive for DilC18 stained *M. tuberculosis* (Figure 5). Results of a representative experiment showing the expression of Mac-1 and ICAM-1 markers on control macrophages and macrophages infected with DiLC18 stained *M. tuberculosis* are given in Figures 6. Proportions of Mac-1^+ and ICAM-1^+ cells within DiLC18 positive and negative adherent PCs are shown in Figures 6A and 6B. These results show that there was a significant increase in Mac-1 and ICAM-1 expression on macrophages with or without ingested *M. tuberculosis*. The increase in expression of these receptors was however more pronounced on macrophages with bacteria (Figures 6C and 6D).

Discussion

The basic aim of this study was to assess the effect of *M. tuberculosis* infection on expression of two selected markers *i.e.* Mac-1 and ICAM-1 on macrophages. These markers participate in the process of migration of macrophages to the site of infection and facilitate the phagocytic activity of the macrophages. Mac-1 is probably the first known receptor which has the unique ability to mediate type I phagocytosis of mycobacteria under non-opsonic conditions and type II under opsonic conditions (Le Cabec *et al.,* 2002). ICAM-1 plays an important role in transmission of signal between APCs and T cells (Van Seventer *et al.,* 1990). Sustained increase of ICAM-1 expression on macrophages is necessary for maintaining the structure of tubercular granuloma (Johnson *et al.,* 1998). Macrophages treated with blocking ICAM-1 antibodies or derived from ICAM-1 knockout mice show significantly decreased phagocytosis of microbeads (Paine *et al.,* 2002).

Our data suggest that there was an early migration of leukocytes in the peritoneal cavity after the initiation of *M. tuberculosis* infection. Number of Mac-1^+ cells also increased but the increase was significantly greater than the number of macrophages actually present in peritoneal cavity indicating that Mac-1^+ cells other than macrophages contributed to

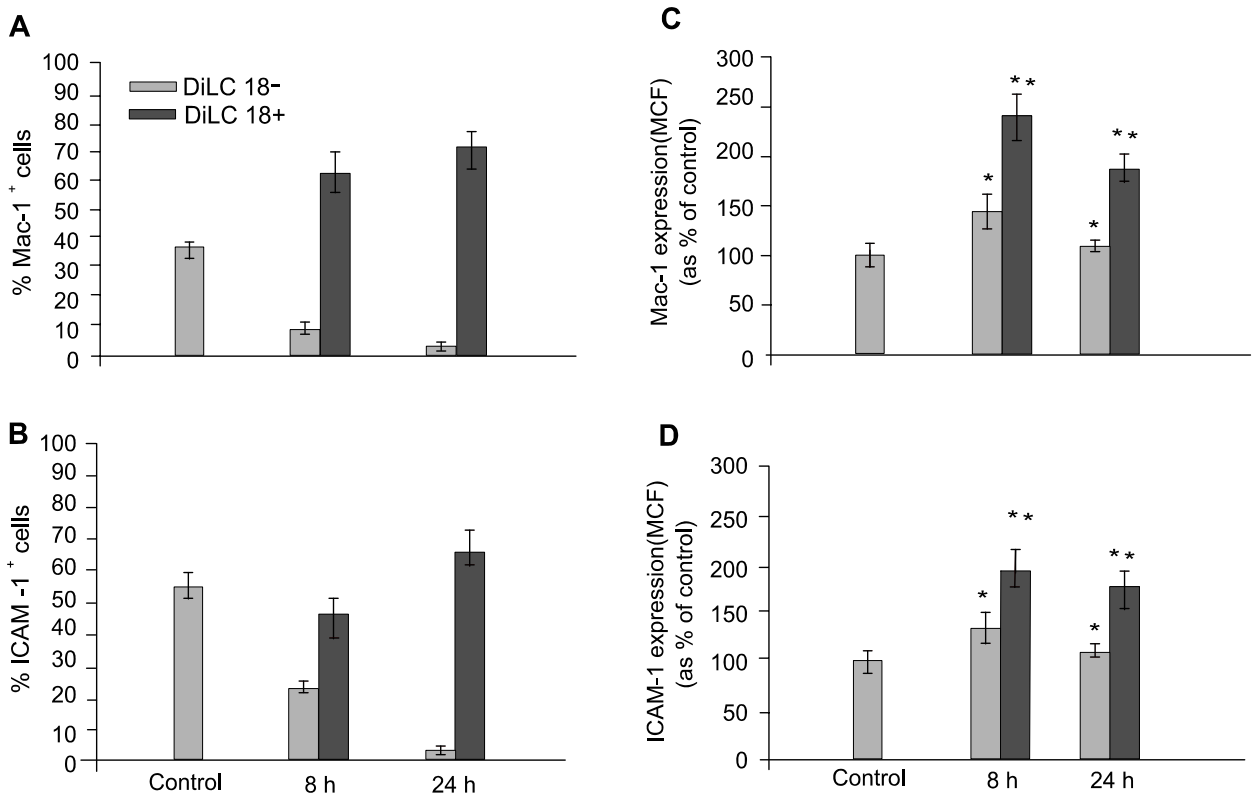

Figure 6. Mac-1 and ICAM-1 expression on *M. tuberculosis* positive and negative populations of adherent mouse peritoneal cells infected *in vitro*. Peritoneal cells were harvested from C57 BL / 6 mouse and cultured in a 24 well culture plate (2×10^6 cells / ml / well) for 2 h. Adherent cells were washed and infected with DIL C18 stained *M. tuberculosis*. After 8 and 24 h, adherent cells were harvested from wells, stained with anti mouse Mac-1 or ICAM-1 FITC antibody and analyzed by flow cytometry. (A, B) Percentage of Mac-1 and ICAM-1 positive cells, and (C, D) Changes in mean fluorescence (MCF) of Mac-1 and ICAM-1 expression as percentage of control. All the comparisons of infected and control cells were done by unpaired *t*-test (*, $P < 0.05$ and **$P < 0.01$). Each bar represents mean of five experiments ± SD.

the increase. In contrast, percentage of ICAM-1 bearing cell sharply declined 4 h after the infection and then gradually increased. Early decline may be due to the dilution of ICAM-1 bearing cells by the influx of ICAM-1 negative neutrophils. In absolute terms, ICAM-1 positive cells increased steadily and half of the PCs after 48 h of the infection were ICAM-1 positive. Considering that macrophages constituted about 15.6 ± 3.7% of PCs at 48 h time point, it is likely that increased ICAM-1 expression could be due to influx of ICAM-1^+ cells and/or induction of this marker on macrophages as well as on other types of PCs including neutrophils. Accumulation of high numbers of Mac-1 and ICAM-1 bearing cells during early phases of infection may be due to influx of cells bearing these markers or induction of these markers on cells which migrated to peritoneal cavity, or both. Neutrophil accumulation was confirmed by performing differential leukocyte counts which indicated that the recovery of neutrophils went up from negligible to about $14.4 \pm 1.7\times10^6$ neutrophils per mouse, 4 h after infection. A similar influx of neutrophils was observed in peritoneal cavity of mice inoculated with M. bovis BCG (Appelberg, 1992) An earlier report claimed that the injection of M. bovis BCG into mouse pleural cavity induced an intense biphasic inflammatory reaction, which peaked at 24 h and 15 days. An influx of neutrophils occurred at 4 h, but was maximal at 24 h. At this time an intense influx of eosinophils and mononuclear cells were also observed (Menezes-de-Lima-junior *et al.,* 1997). In our studies, the neutrophils influx was maximum at earlier time points and significant accumulation of eosinophils in peritoneum not noticed in *M. tuberculosis* infected mice. The difference in two studies could be due to different sites of infection. Early non-adaptive immune responses may play an important role in imparting protection until an adaptive immune response can be mounted. One mechanism of protection in this early phase is to recruit more phagocytic cells and effector molecules to the site of infection through the release of a battery of cytokines and chemokines. It is possible that the upregulation of Mac-1 and ICAM-1 markers on PCs from *M. tuberculosis* infected mice was a consequence of altered cytokine milieu in the peritoneal cavity due to inflammatory response, and

not a direct effect of interaction of macrophages with bacteria. *In vitro* experiments were carried out to assess the direct effect of *M. tuberculosis* infection on the expression of Mac-1 and ICAM-1 markers on macrophages. Infection of adherent peritoneal macrophages with *M. tuberculosis* caused an increase in percentage of Mac-1$^+$ and ICAM-1$^+$ cells as well as an increase in mean channel fluorescence (MCF) indicating that there was an induction as well as upregulation of these markers in infected macrophages. These results were further confirmed by using a mouse macrophage cell line RAW 264.7. The increase in the MCF value after infection in case of both the markers was quite pronounced just after 4 h of infection. Lopez Ramirez *et al.* found that infection of human monocyte/ macrophage cell line, THP-1 with live *M. tuberculosis* showed significant increase in surface expression of ICAM-1 and a nominal increase in Mac-1 expression 24 h after the infection (Lopez-Ramirez *et al.,* 1994). In human PBLs however, M. avium induced a dose dependent down-regulation of ICAM-1 expression (Mohaghegpour *et al.,* 1997). An initial upregulation and a latter decline in ICAM-1 expression were observed in mouse peritoneal macrophages in response to M. avium (Maw *et al.,* 1996; Tomioka *et al.,* 2000). TNFα is responsible for the initial upregulation of ICAM-1, where as TGFβ and IL-10 appears to be responsible for the decline (Tomioka *et al.,* 2000).

The increase in the absolute number of cells expressing these markers in experiments involving *in vivo* infections was however too large to be explained by only induction/upregulation of these markers. We therefore conclude that the increase observed in the cell population expressing these two markers could be due to migration of cells bearing these markers into the peritoneal cavity as well as due to an upregulation of these markers on cells present in the peritoneal activity. Furthermore, in experiments where fluorescence labeled *M. tuberculosis* was used as an infection agent, which enabled us to analyze markers on bacteria positive and bacteria negative macrophages. The increase in Mac-1 and ICAM-1 expression was found to be significantly more on macrophages with intracellular *M. tuberculosis*. These observations suggested that a direct interaction of macrophages with *M. tuberculosis* may be an important factor in determining the expression levels of Mac-1 and ICAM-1 markers on macrophages. In view of the demonstrated role of these two markers, it is likely that the macrophages exposed to *M. tuberculosis* may acquire enhanced phagocytic activity and enhanced ability to present mycobacterial antigens to T cells for an effective immune response.

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