Effect of H37Ra and E. coli infection on the accumulation of leukocytes in mouse peritoneal cavity

The initial events during a primary infection with *M. tuberculosis* are poorly understood and few models exist for investigating the sequence of events that follow the first contact of the host with Mycobacteria. In the present study we utilized infection in peritoneal cavity to understand the early events following the infection. C57Bl/6 mice were infected with 50x10^6 *M. tuberculosis* (H37Ra strain) intra-peritoneally and at various time intervals peritoneal cells were harvested and counted. The injection of *M. tuberculosis* into mouse peritoneal cavity induced an intense inflammatory reaction characterized by influx of leukocytes. Normal recovery of leukocytes from peritoneal cavity of control mice was 2.2 ± 0.25 x 10^6 cells / mouse (ranging from 1.75 to 3.2 x 10^6 cells/ mouse). Four hours after *M. tuberculosis* infection, the cell count went up to 19 ± 3.1 x 10^6 cells / mouse. At later time points the cell recovery declined marginally (Fig 1).

To further study the dose response of exposure to Mycobacteria, mice were infected with different doses of *M. tuberculosis* (H37Ra strain) intra-peritoneally. Lower doses (2.5 and 10 x10^6 Mycobacteria / mouse) did not cause any increase in number of cells recovered from the peritoneum, four hours after infection. At higher doses of Mycobacteria, however, there was a sharp increase in the recovery of peritoneal cells. The yield of peritoneal cells in mice infected with 25 x10^6 and 50 x 10^6 H37Ra were 12.5 ± 2.4x 10^6 cells / mouse and 18.2 ± 2.8 x 10^6 cells / mouse respectively (Fig 2 A). In order to find out whether *E. coli* infection could generate a similar inflammatory response, different doses of *E. coli* were injected in the mice. Our results given in Fig.2 B indicate that *E. coli* was less effective than *M. tuberculosis* in inducing peritoneal cell accumulation.
FIG 1. Effect of *M. tuberculosis* infection on the yield of mouse peritoneal cells

50x10^6 bacteria (H37Ra) were injected intra-peritoneally in each mouse. At different time points after the infection, peritoneal cells were harvested from mice, washed twice with PBS and counted in a haemocytometer. Cell yields in infected and control mice were compared by unpaired t-test (*, p<0.05). Each bar represents mean cell recovery of seven observations ± SD.
FIG 2. Effect of infection with different doses of live H37 Ra or E.coli on yield of mouse peritoneal leukocytes

Different doses of bacteria (H37Ra, left panel and E.coli, right panel) were injected intra-peritoneally in each mouse. After four hours, peritoneal leukocytes were harvested and cell recovery/mouse was determined by counting the cells in a haemocytometer. All comparisons of infected and control cells were done by unpaired t-test (*, p<0.05). Each bar represents mean of three observations ± SD.
A ten fold higher dose of *E. coli* was required to induce a response comparable to *M. tuberculosis* (Fig 2 A and B).

**Effect of *M. tuberculosis* infection on the light scatter properties of mouse peritoneal cells**

The leukocytes, which accumulated in peritoneal cavity in response to H37Ra, were characterized by flow cytometry. The forward and side scatter of laser is related to size and granularity respectively of the analyzed cell population. Both forward scatter and side scatter of the peritoneal cells obtained from infected mice were found to be higher, implying that there was an increase in the size as well as granularity of these cells (Fig 3). Such results could be due to the accumulation of granulocytes in the peritoneal cavity of infected mice. This proposition was further examined by doing differential leukocyte counting.

**Differential leukocyte count of mouse peritoneal cells from control and infected mice**

Results in Fig 4 indicate that the proportion of polymorphonuclear neutrophils (PMNs) was markedly elevated 4 hours after infection. There was a concurrent decline in the proportion of lymphocytes and monocytes. The changes in the proportion of these cells observed at 4 hours post infection persisted at 24 hours time point also (Fig 4A). Absolute recovery of PMNs, lymphocytes and monocytes per mouse at 4 and 24 hours post infection are given in Fig 4B. These results clearly indicate that there is a significant influx of PMNs in peritoneal cavity of infected mice. It is interesting to know that even though the proportion of lymphocytes and monocytes were markedly lower in peritoneal cell
FIG 3. Effect of *M. tuberculosis* infection on the light scatter properties of mouse peritoneal cells

50x10⁶ bacteria (H37Ra) were injected intra-peritoneally in each mouse. At different time points peritoneal cells were harvested from mice and studied by flow cytometry. The forward scatter (FS) versus side scatter (SS) plots cell populations from control and infected mice have been shown.
FIG 4. Effect of *M. tuberculosis* infection on differential leukocyte count in mouse peritoneal cells

50×10⁶ bacteria (H37Ra) were injected intra-peritoneally in each mouse. At different time points after infection peritoneal cells were harvested from mice. A differential leukocyte count was done as described in Materials and Methods. (A) Percentage of different types of leukocytes in the population, and (B) total recovery 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 observations ± SD.
preparations derived from infected mice (Figs.4A), absolute recoveries of lymphocytes and monocytes per mouse were significantly elevated (Figs. 4 A and B).

Results

Changes in the proportion and absolute recovery of T and B lymphocytes in peritoneal cells after *M. tuberculosis* infection

Lymphocyte population consists of several subsets of cells like T, B and natural killer (NK) cells. In addition, there are sub populations within these categories of lymphocytes. It was of our interest to know the relative contributions of different subsets of lymphocytes to the changes observed in the peritoneal compartment in infected mice. For this purpose T and B cells were enumerated by flowcytometric analysis using anti mouse TCRβ and CD19 monoclonal antibody respectively. Flowcytometer histogram showing T and B lymphocyte populations at different time points post infection was given in Fig 5. These results indicate that 4 hours after infection there was a marked depletion of both T and B cells in peritoneal cells. At later time points, some recovery in the number of T and B cells was observed. A more quantitative form of these results depicted in Fig 6 indicates that the proportion of T cells fell markedly 4 hours post infection, the decline being about 80 %. At subsequent time points the proportion of T cells recovered, but remained significantly lower than control, even at 48 hours post infection (Fig 6A). Interestingly, absolute numbers of T cells in peritoneal cavity did not decline at any time point after the infection. Instead, a gradual increase in absolute number of T cells was observed, which became statistically significant at 24 hours and 48 hours post infection. (Fig 6B)

The proportion of B cells also declined markedly 4 hours post infection and remained low at later time points (Fig 6C). Unlike T cells, absolute number of B cells recovered from
FIG 5. Effect of *M. tuberculosis* infection on proportion of T and B lymphocytes in peritoneal cells

50x10^6 bacteria (H37Ra) were injected intra-peritoneally in each mouse. At different time points peritoneal cells were harvested, stained with anti mouse TCR β (panel A) and CD19 (panel B) antibody separately and analyzed on a flow cytometer as described in Materials and Methods. Gating (arrow on X-axis) and percent positive cells have been shown in individual histograms.
FIG 6. Time kinetics of changes in the number of T and B lymphocytes in peritoneal cells after *M. tuberculosis* infection

50x10^6 bacteria (H37Ra) were injected intra-peritoneally in each mouse. At different time points peritoneal cells were harvested from mice, washed twice with PBS and cell recovery / mouse was determined by counting cells in a haemocytometer. Harvested cells were stained with anti mouse TCR β and CD19 antibody and analyzed on a flowcytometer. (A) Percent T lymphocytes, (B) Recovery of TCR β positive cells, (C) Percent B lymphocytes and (D) Recovery of CD19 positive cells. All the comparisons of infected and respective uninfected control were done by unpaired t-test (*, p<0.05). Each bar represents mean of five observations ± SD.
Results

peritoneal cavity were not different in control and infected mice at any time point after infection (Fig 6D).

Changes in the proportion and number of Mac1 (CD11b/CD18) positive cells in peritoneal lavage after *M. tuberculosis* infection

Mac1 (CD11b/CD18), an adhesion molecule, is known to be expressed on granulocytes, macrophages, dendritic cells and B1 cells. Role of CD11b/CD18 in neutrophil and monocyte extravasation during inflammatory responses is well known (Rossi and Hellewell, 1994; Patarroyo, 1994). In order to study the effect of *M. tuberculosis* on Mac1 expression, peritoneal cells were stained with anti mouse Mac1 monoclonal antibody and analyzed by flowcytometry. Results in Fig. 7 indicate that about 38 % control peritoneal cells were positive for Mac1 expression. At all time points after the infection, there was a marked increase in proportion of Mac1+ cells in peritoneum. The data in Fig. 8A show that the proportion of Mac1 expressing cells doubled just 4 hours after infection, and the increase was sustained at later time points.

Absolute number of Mac1 expressing cells in peritoneal cavity increased by about 12 to 14 times 4 and 8 hours after infection (Fig 8B). This increase was lower at later time points, but still remained significantly higher than the control at all time points.

Time kinetics of CD 54 expression on peritoneal cells after *M. tuberculosis* infection

Intercellular adhesion molecules (ICAM 1) also play an important role in the extravasation of a variety of leukocytes during inflammatory responses. Expression of ICAM 1 (CD54) on peritoneal cells derived from control and infected mice is depicted in Fig 9. These
FIG 7. Effect of *M. tuberculosis* infection on proportion of Mac 1 positive population in peritoneal cells

5x10⁶ bacteria (H37Ra) were injected intra-peritoneally in each mouse. At different time points peritoneal cells were harvested, stained with anti mouse Mac1 antibody and analyzed on a flow cytometer as described in materials and methods. An arrow on X-axis indicates the position of gate determined by using uninfected cells treated with isotype control antibody. Percent positive cells have been indicated in individual histograms.
FIG 8. Time kinetics of changes in the number of Mac1 positive cells in peritoneal cell population after *M. tuberculosis* (H37Ra) infection

50x10⁶ bacteria (H37Ra) were injected intra-peritoneally in each mouse. At different time points peritoneal cells were harvested, washed twice with PBS and cell recovery /mouse was assessed by counting cells in a haemocytometer. Percentage of Mac 1 + cells was determined by anti mouse Mac1 antibody staining and flow cytometry as described in Materials and Methods. All the comparisons of infected and respective uninfected controls have been done by unpaired t-test (*, p<0.05). Each bar represents mean of five observations ± SD.
FIG 9. Effect of *M. tuberculosis* infection on CD54 expression of peritoneal cells

50x10⁶ bacteria (H37Ra) were injected intra-peritoneally in each mouse. At different time points peritoneal cells were harvested from mice, stained with anti mouse CD54 antibody and analyzed on a flow cytometer as described in Materials and Methods. An arrow on X-axis indicates the position of gate determined by using uninfected cells treated with isotype control antibody. Percent CD54 positive cells have been indicated in individual histograms.
Results

Results indicate that the proportion of CD54+ cells was 65.5% in control which declined to 15% just 4 hours after infection but recovering at 48 hours time point. However, absolute number of CD54+ cells increased in a time dependent manner following infection with *M. tuberculosis* (Fig 10B).

Time kinetics of IFNR expression on peritoneal cells after *M. tuberculosis* infection

Since IFNγ plays a very important role in the immunity to tuberculosis, expression of IFNγ receptor (IFNR) was studied. In control mice the proportion of IFNR+ cells was 71.7%. Four hours after infection, the proportion of IFNR+ cells in the peritoneal lavage came down to 24.5%, but recovered later to the control level 48 hours after infection (Fig 11). On the contrary, absolute number of IFNR+ cells in the peritoneal cavity increased at all time points after infection (Fig 12B).

Mac1, CD54 and IFNR expression may have crucial effect on macrophage functions. In the studies carried out so far, *M. tuberculosis* infection *in vivo* resulted in significant changes in expression of these molecules on mouse peritoneal cells. Changes in various markers seen in this experimental system could be due to two factors. Firstly, cells bearing defined markers may enter or exit peritoneal cavity as a result of infection. Alternatively, *M. tuberculosis* infection may directly or indirectly modulate the expression of various cell surface markers on different cell types present in the peritoneal cavity. In the experiments described below H37Ra infection was introduced in peritoneal cells cultured *in vitro* and the effect of expression on various cell surface markers studied. This system does not have the problem of cellular influx and exit, and the changes observed should therefore be related to up and down regulation of these markers in response to H37Ra infection.
FIG 10. Time kinetics of CD54 expression after *M. tuberculosis* infection

50x10⁶ bacteria (H37Ra) were injected intraperitoneally in each mouse. At different time points after peritoneal cells were harvested from mice, stained with anti mouse CD54 antibody and analyzed on a flowcytometer as described in Materials and Methods. (A) Percent CD54⁺ cells, and (B) recovery of CD54⁺ cells. All comparisons of infected and control cells were done by unpaired t-test (*, p<0.05). Each bar represents mean of five observations ± SD.
FIG 11. Effect of *M. tuberculosis* infection on IFNR expression of peritoneal cells

50x10^6 bacteria (H37Ra) were injected intra-peritoneally in each mouse. At different time points peritoneal cells were harvested from mice, stained with anti mouse IFNR antibody and analyzed on a flow cytometer as described in Materials and Methods. An arrow on X-axis indicates the position of gate determined by using uninfected cells treated with isotype control antibody. Percent positive cells have been indicated in individual histograms.
FIG 12. Time kinetics of change in IFNR expression on peritoneal cells after *M. tuberculosis* infection

50×10^6* bacteria (H37Ra) were injected intra-peritoneally in each mouse. At different time points peritoneal cells were harvested from mice, stained with anti-mouse IFNR antibody and analyzed on a flow cytometer as described in Materials and Methods. (A) Percent IFNR+ cells, and (B) recovery of IFNR positive cells. All comparisons of infected and control cells were done by unpaired t-test (*, p<0.05). Each bar represents mean of five observations ± SD.
Results

Effect of *M. tuberculosis* infection on the light scattering properties of adherent mouse peritoneal cells in culture

Adherent peritoneal macrophages were treated with *M. tuberculosis* and analyzed on a flowcytometer. Results in Fig 13 indicate some increase in the side scattering by the infected cells as compared to the control cells, which implied an increase in the granularity of the cells upon infection. These changes were however small as compared to the changes observed due to *in vivo* infection (Fig 3). It is likely that phagocytosis of *M. tuberculosis* by adherent peritoneal cells was responsible for changes in side scatter shown in Figs 13A and B.

*M. tuberculosis* induced changes in Mac1, CD54 and IFNR expressions on adherent mouse peritoneal cells in culture

Cultured adherent peritoneal cells were treated with *M. tuberculosis* and changes in the expression of Mac1, CD54 and IFNR markers were studied by flowcytometry. Our results indicate that the proportion and mean channel fluorescence intensity of Mac1 receptors on adherent peritoneal cell population increased at all time points after infection. The highest
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 hours. Adherent cells were washed and infected with *M. tuberculosis* (H37Ra) at a MOI of 1:10 for varying time periods. At different time points adherent cells were harvested from wells and analyzed by flow cytometry. (A) The forward scatter (FS) versus side scatter (SS) plots with regions delineated inside individual histograms showing gating and (B) side scatter versus cell count plots for infected and control cell populations with horizontal line to indicate the gating and vertical line to earmark the shift in the peak. Values indicate the percentage of gated cells.
FIG 14. Effect of *M. tuberculosis* infection on Mac1 expression of adherent peritoneal cells

Peritoneal cells harvested from C57Bl/6 mice were allowed to adhere in a 24 well culture plate (2x10^6 cells/ml/well) for 2 hours. Adherent cells were washed and infected with *M. tuberculosis* (H37Ra) at a MOI of 1:10 for varying time periods. At different time points adherent cells were harvested from wells, stained with anti mouse Mac1 antibody and analyzed by flowcytometry. (A) The expression profiles after H37Ra infection. M1 window contains positively stained cells, (B) percent Mac 1+ cells, and (C) 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 three observations ± SD.
FIG 15. Effect of *M. tuberculosis* infection on CD 54 expression of adherent peritoneal cells

Peritoneal cells harvested from C57Bl/6 mouse were allowed to adhere in a 24 well culture plate (2x10^6 cells/ml/well) for 2 hours. Adherent cells were washed and infected with *M. tuberculosis* (H37Ra) at a MOI of 1:10 for varying time periods. At different time points adherent cells were harvested from wells, stained with anti mouse CD54 antibody and analyzed by flowcytometry. (A) The expression profiles after H37Ra infection. M1 window contains positively stained cells, (B) percent CD 54 + cells, and (C) 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 three observations ± SD.
Results

The proportion of IFNR\(^+\) cells was increased significantly at 8 hours post infection but mean intensity of IFNR expression per cell was significantly elevated only at 24 hours time point (Fig16A, B and C).

**Effect of *M. tuberculosis* on IFNR expression by peritoneal T and B cells**

The above results indicate that *M. tuberculosis* could increase IFNR expression on adherent peritoneal cells. IFNR is however expressed on macrophages and lymphocyte populations (T, B and NK cells). It was of interest to determine if *M. tuberculosis* could alter IFNR expression on lymphocytes as well. For this purpose unfractionated peritoneal cells were cultured with or without *M. tuberculosis* followed by double staining for TCR \(\beta\) and IFNR. Two colour histograms of these results are given in Fig.17. Quantitative comparisons indicate that there was a significant increase in IFNR expression on TCR\(\beta^+\) T cells (Fig 18).

Similar experiments in which control and infected cells were double stained with CD19 and IFNR indicated that there was no upregulation of IFNR on CD19\(^+\) cells (Fig 19 and 20).

**Effect of *M. tuberculosis* treatment on Mac1, CD54 and IFNR expressions on RAW 264.7 macrophage cell line**

Results described above have shown an increase in the expression of Mac1, CD54 and IFNR on T cells, B cells and population from mouse peritoneal cells, in response to *M. tuberculosis* infection *in vitro*. This effect on adherent cells is likely to be on macrophage population. As partially purified adherent population was used in these experiments. In
FIG 16. Effect of *M. tuberculosis* infection on IFNR expression of adherent peritoneal cells

Peritoneal cells harvested from C57Bl/6 mouse were allowed to adhere in a 24 well culture plate (2x10^6 cells/ml/well) for 2 hours. Adherent cells were washed and infected with *M. tuberculosis* (H37Ra) at a MOI of 1:10 for varying time periods. At different time points adherent cells were harvested from wells, stained with anti mouse IFNR antibody and analyzed by flow cytometry. (A) The expression profiles after H37Ra infection. M1 window contains positively stained cells, (B) percent IFNR+ cells, and (C) changes in mean channel fluorescence (MCF) as percentage of control. All comparisons of infected and control cells have been done by unpaired t-test (*, p<0.05). Each bar represents mean of three observations ± SD.
FIG 17. Effect of *M. tuberculosis* (H37Ra) infections on TCR β/IFNR expression of peritoneal cells

Peritoneal cells were harvested from C57Bl/6 mouse, cultured in a teflon flask and infected with *M. tuberculosis* (H37Ra) at a MOI of 1:10 for varying time periods. After 4 and 24 hours cells were harvested, stained with anti mouse TCR β and IFNR antibodies and analyzed by flow cytometry as described in Materials and Methods.
FIG 18. Effect of *M. tuberculosis* (H37Ra) infections on TCR β/IFNR expression on peritoneal T cells

Peritoneal cells were harvested from C57Bl/6 mouse, cultured in a teflon flask and infected with *M. tuberculosis* (H37Ra) at a MOI of 1:10 for varying time periods. After 4 and 24 hours cells were harvested, stained with anti mouse TCR β and IFNR antibody and analyzed by flow cytometry as described in Materials and Methods. Panel A compares the percentage of IFNR+ T cells, and Panel B compares changes in mean fluorescence (MCF) of IFNR staining on T cells in control and infected populations. All comparisons of infected and control cells were done by unpaired t -test (*, p<0.05). Each bar represents mean of three observations ± SD.
FIG 19. Effect of *M. tuberculosis* (H37Ra) infections on CD19/IFNR expression of peritoneal cells

Peritoneal cells were harvested from C57Bl/6 mouse, cultured in a teflon flask and infected with *M. tuberculosis* (H37Ra) at a MOI of 1:10 for varying time periods. After 4 and 24 hours cells were harvested, stained with anti mouse CD 19 and IFNR antibody and analyzed by flow cytometry as described in Materials and Methods.
FIG 20. Effect of *M. tuberculosis* (H37Ra) infections on IFNR expression on peritoneal B cells

Peritoneal cells were harvested from C57Bl/6 mouse, cultured in a teflon flask and infected with *M. tuberculosis* (H37Ra) at a MOI of 1:10 for varying time periods. After 4 and 24 hours cells were harvested, stained with anti mouse CD19 and IFNR antibody and analyzed by flow cytometry as described in Materials and Methods. Panel A compares the percentage of IFNR*+* B cells, and Panel B compares changes in mean fluorescence (MCF) of IFNR staining on B cells in control and infected populations. All comparisons of infected and control cells were done by unpaired t-test (*, p<0.05). Each bar represents mean of three observations ± SD.
order to ascertain whether the effect of *M. tuberculosis* infection was indeed on macrophages, a murine macrophage cell line, RAW 264.7, was treated with *M. tuberculosis* and expression of Mac1, CD54 and IFNR assessed at 4, 8 and 24 hours post infection. Results in Figs 21, 22 and 23 indicate that all three markers were upregulated in *M. tuberculosis* treated RAW 264.7 cells. These results taken together with results in Fig 14, 15 and 16 clearly indicate that *M. tuberculosis* can directly induce Mac1, CD54 and IFNR on macrophages.

Staining of *M. tuberculosis* with fluorescent dyes

While studying the effects of *M. tuberculosis* on various markers on peritoneal cell preparations, it could not be ascertained whether the observed changes were confined to the cells which had engulfed *M. tuberculosis* or was also seen on cells without phagocytosed *M. tuberculosis*. In order to distinguish between the effects on these two types of cells we used *M. tuberculosis* tagged with a fluorescent dye so that cells with engulfed bacteria could be distinguished flowcytometrically from cells without engulfed bacteria.

For this purpose, *M. tuberculosis* were stained with DIL C18 fluorescent dye. This dye can irreversibly bind *M. tuberculosis* (Fig 24).

Time kinetics of uptake of DIL C18 stained *M. tuberculosis* by mouse peritoneal cells

When DIL C18 stained *M. tuberculosis* were injected intraperitoneally, phagocytosis of the stained *M. tuberculosis* by peritoneal macrophages could clearly be shown under the fluorescent microscope. Flowcytometric data (Fig 25) indicate that maximum percentage
RAW 264.7 cells cultured in a 24 well plate (0.2 x 10^6 cells/ml) were infected with *M. tuberculosis* (H37Ra) at a MOI of 1:10 for varying time periods. Control and infected cells were harvested, stained with anti mouse Mac1 antibody and analyzed by flow cytometry. (A) The expression profiles after infection. An arrow on X-axis indicates the position of gate determined by using uninfected cells treated with isotype control antibody. Percent positive cells have been indicated in individual histograms, (B) percent Mac 1^+^ cells, and (C) 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 three observations ± SD.

FIG 21. Effect of *M. tuberculosis* infection on Mac1 expression of RAW 264.7 cells
RAW 264.7 cells cultured in a 24 well plate (0.2 x10^6 cells/ml) were infected with *M. tuberculosis* (H37Ra) at a MOI of 1:10 for varying time periods. Control and infected cells were harvested, stained with anti mouse CD54 antibody and analyzed by flow cytometry. (A) The expression profiles after infection. An arrow on X-axis indicates the position of gate determined by using uninfected cells treated with isotype control antibody. Percent positive cells have been indicated in individual histograms, (B) percent CD 54^+^ cells, and (C) 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 three observations ± SD.
FIG 23. Effect of *M. tuberculosis* infection on IFNR expression of RAW 264.7 cells

RAW 264.7 cells cultured in a 24 well plate (0.2 x10^6 cells/ml) were infected with *M. tuberculosis* (H37Ra) at a MOI of 1:10 for varying time periods. Control and infected cells were harvested, stained with anti mouse IFNR antibody and analyzed by flow cytometry. (A) The expression profiles after infection. An arrow on X-axis indicates the position of gate determined by using uninfected cells treated with isotype control antibody. Percent positive cells have been indicated in individual histograms, (B) percent IFNR^+^ cells, and (C) 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 three observations ± SD.
FIG 24. Staining of *M. tuberculosis* with fluorescent dye DIL C18

Single cell suspensions of the bacteria (H37Ra) were stained with a fluorescent dye, DIL C18, as described in Materials and Methods and analyzed by flow cytometry. Profile of DIL C18 stained H37Ra cells (...) and unstained H37Ra cells (−) have been shown.
50x10⁶ DIL C18 stained bacteria (H37 Ra) were injected intra-peritoneally in mice. At different time points peritoneal cells were harvested and the intake of the bacteria by the cells was examined by flow cytometry. (A) Uptake of DIL C18 stained bacteria by mouse peritoneal cells. Percentage of M. tuberculosis associated cells have been indicated in the individual histograms, and (B) Kinetics of uptake of DIL C18 stained H37 Ra by peritoneal cells against corresponding time points derived from 3 independent experiments.

FIG 25. Time kinetics of uptake of DIL C18 stained M. tuberculosis by mouse peritoneal cells
(35±3.8%) of peritoneal cells associated with DIL C18 stained *M. tuberculosis* was observed at 8 hours after the infection.

**Time kinetics of uptake of DIL C18 stained *M. tuberculosis* by mouse peritoneal cells in vitro**

When adherent mouse peritoneal cells were infected with DIL C18 stained *M. tuberculosis* in cell cultures; there was a time dependent increase in the uptake of *M. tuberculosis* (Fig 26).

**Effect of treatment with different doses of α methyl mannoside on the proportion of cells associated with stained bacteria**

It was essential to know whether peritoneal cells associated with DIL C18 stained *M. tuberculosis* had actually engulfed the bacteria or the stained bacteria were simply adhering to the peritoneal cells. If the stained *M. tuberculosis* are only adhering to peritoneal cells then it should be possible to remove them by using α methyl D-mannoside, because mannose residues play an important role in the binding of *M. tuberculosis* and macrophages (Schreiber et al 1993). Results in Fig 27 indicate that treatment of peritoneal cells derived from mice injected with DIL C18 stained *M. tuberculosis*, with α methyl mannoside, did not alter the fluorescence-staining pattern. These results suggest that the stained peritoneal cells which were present in the cell preparation derived from mice infected with DIL C18 stained *M. tuberculosis*, was due to phagocytosis of *M. tuberculosis* and not due to passive adherence of the bacteria on the macrophages.
Peritoneal cells harvested from C57Bl/6 mouse were cultured in a 24 well culture plate for 2 hours. Adherent cells were washed and infected with DIL C18 stained *M. tuberculosis* at a MOI of 1:10 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. Percent positive cells have been indicated in individual histograms.
FIG 27. Effect of α methyl D mannoside on the fraction of peritoneal cells associated with DIL C18 stained *M. tuberculosis*

50x10⁶ DIL C18 stained bacteria (*M. tuberculosis*, H37Ra) were injected intra-peritoneally in each mouse. After eight hours cells were harvested and cultured in teflon flasks for 12 hours with or without different doses of α methyl mannoside. At different time points cells were harvested and analyzed by flow cytometry. An arrow on X-axis indicates the position of gate determined by using uninfected cells. Percent positive cells have been indicated in individual histograms.
Results

Mac1, CD54 and IFNR expression on H37Ra positive and negative mouse peritoneal cells after infection with DIL C18 stained *M. tuberculosis*

Mac1, CD54 and IFNR expression on control and H37Ra infected peritoneal cells have been compared earlier. In order to study whether peritoneal cells which actively phagocytose H37Ra differ in expression of Mac1, CD54 and IFNR from cells with out intracellular bacteria, mice were infected intraperitoneally by DIL C18 stained H37Ra. Counter staining of the peritoneal cells after *in vivo* infection with DIL C18 stained bacteria by anti mouse Mac1 antibody indicated that DIL C18 stained *M. tuberculosis* was essentially present in Mac1+ peritoneal cells at 8 and 24 hours post infection. Results in Fig 28 also show that a substantial population of Mac1+ cells was DIL C18− implying that all Mac1+ cells did not engulf *M. tuberculosis*. The proportion of Mac1+ cells which had not engulfed the bacteria (Mac1+ / DIL C18−) to those which had engulfed (Mac1+ / DIL C18+) increased with time. Results in Fig 29 reveal that mean channel fluorescence (MCF) intensity of Mac1 expression increased in Mac1+ / DIL C18− as well as Mac1+ / DIL C18+ cells with respect to control. However, the upregulation of Mac1 expression is greater in DIL C18+ cells, both at 8 hours and 24 hours after infection. In case of CD54 expression, at 8 hours post infection CD54+ / DIL C18− cells have a lower level of CD54 expression than on CD54+ / DIL C18+, as depicted in Fig.31, but at 24 hours post infection CD54 expression increased in both types of CD54 expressing cells and became almost equal. On the other hand, the IFNR expression was almost similar in the cells which had engulfed the bacteria and which had not, both at 8 and 24 hours post infection (Fig.33).
FIG 28. Expression of Mac1 on H37Ra positive and negative population of peritoneal cells

50x10^6 DIL C18 stained bacteria (H37Ra) were injected intra-peritoneally in each mouse. After 8 and 24 hours, peritoneal cells were harvested, stained with anti mouse Mac1 FITC antibody and analyzed by flow cytometry.
50x10^6 DIL C18 stained bacteria (H37Ra) were injected intra-peritoneally in each mouse. After 8 and 24 hours, peritoneal cells were harvested, stained with anti mouse Mac1 FITC antibody and analyzed by flow cytometry. All the comparisons were done by unpaired t-test (*, p<0.05). Each bar represents mean of three observations ± SD.
FIG 30. Expression of CD 54 on H37Ra positive and negative population of peritoneal cells

50×10⁶ DIL C18 stained bacteria (H37Ra) were injected intra-peritoneally in each mouse. After 8 and 24 hours, peritoneal cells were harvested, stained with anti mouse CD54 FITC antibody and analyzed by flow cytometry.
50x10^6 DIL C18 stained bacteria (H37Ra) were injected intra-peritoneally in each mouse. After 8 and 24 hours, peritoneal cells were harvested, stained with anti mouse CD54 FITC antibody and analyzed by flow cytometry as described in Materials and Methods. All the comparisons were done by unpaired t-test (*, p<0.05). Each bar represents mean of three observations ± SD.

FIG 31. CD 54 expression on H37Ra positive and negative populations of peritoneal cells of mice infected DIL C18 stained *M. tuberculosis*.
FIG 32. Expression of IFNR on H37Ra positive and negative population of peritoneal cells

50x10^6 DIL C18 stained bacteria (H37Ra) were injected intra-peritoneally in each mouse. After 8 and 24 hours, peritoneal cells were harvested, stained with biotinylated anti mouse IFNR antibody, SA-FITC and analyzed by flow cytometry.
FIG 33. IFNR expression on H37Ra positive and negative populations of peritoneal cells of mice infected DIL C18 stained \textit{M. tuberculosis}

50x10^6 DIL C18 stained bacteria (H37Ra) were injected intra-peritoneally in each mouse. After 8 and 24 hours, peritoneal cells were harvested, stained with biotinylated anti mouse IFNR antibody, SA-FITC and analyzed by flow cytometry as described in Materials and Methods. All the comparisons were done by unpaired t-test (*, p<0.05). Each bar represents mean of three observations ± SD.
Results

Mac1, CD 54 AND IFNR expressions of H37Ra positive and negative adherent mouse peritoneal cells after *in vitro* infection with DIL C18 stained *M. tuberculosis*

In tissue cultured adherent mouse peritoneal cells infected with DIL C18 stained H37Ra *in vitro*, the proportion of Mac1+ cells which had taken up the bacteria (DIL C18+) was 64.3% and 74.2% respectively at 8 and 24 hours post infection (Fig 34). Results in Fig 35 show that the average Mac1 expression (MCF) was significantly greater in DIL C18+ cells as compared to DIL C18− (Fig 35 B). Thus, it appears that upregulation of Mac1 was greater in cells which had actually taken up the bacteria.

Results in Fig 36 and 37 show a similar pattern when expression of CD54 was analyzed on DIL C18+ and DIL C18− populations of adherent peritoneal cells. In this case also, average expression of CD54 was significantly greater in DIL C18+ cells as compared to DIL C18− cells.

Similar studies with IFNR expression however indicate that the mean IFNR expression was not significantly different in DIL C18+ and DIL C18− cells (Fig 38 and 39).

Studies so far were concentrated on the modulation of various receptors on peritoneal leukocytes in response to *M. tuberculosis*. *M. tuberculosis* induced changes in some functional parameters were also studied. Nitric oxide (NO) produced by the macrophages in response to different stimuli is one of the important parameters of macrophage functions. Standardization for studying this parameter was done by using mouse macrophage cell line, RAW 264.7.
FIG 34. Expression of Mac 1 on H37Ra positive and negative population of adherent mouse peritoneal cells infected *in vitro*

Peritoneal cells harvested from C57Bl/6 mouse were cultured in a 24 well plate (2x10^6 cells/ml/well) for 2 hours. Adherent cells were washed and infected with DIL C 18 stained *M. tuberculosis* at a MOI of 1:10. After 8 and 24 hours, cells were harvested from wells, stained with anti mouse Mac1 FITC antibody and analyzed by flow cytometry.
FIG 35. Mac1 expression on H37Ra 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 (2x10^6 cells/ml well) for 2 hours. Adherent cells were washed and infected with DIL C18 stained *M. tuberculosis* at a MOI of 1:10. After 8 and 24 hours, adherent cells were harvested from wells, stained with anti mouse Mac1FITC antibody and analyzed by flow cytometry as described in Materials and Methods. (A) Percentage of Mac1 positive cells, and (B) changes in mean fluorescence (MCF) of Mac1 expression as percentage of control. All the comparisons were done by unpaired t-test (*, p<0.05). Each bar represents mean of three observations ± SD.
FIG 36. Expression of CD54 on H37Ra positive and negative population of adherent mouse peritoneal cells infected in vitro

Peritoneal cells harvested from C57 Bl/6 mouse were cultured in a 24 well plate (2x10⁶ cells / ml / well) for 2 hours. Adherent cells were washed and infected with DIL C18 stained M. tuberculosis at a MOI of 1:10. After 8 and 24 hours, cells were harvested from wells, stained with anti mouse CD54 FITC antibody and analyzed by flow cytometry.
Peritoneal cells were harvested from C57Bl/6 mouse and cultured in a 24 well culture plate (2x10⁶ cells/ml/well) for 2 hours. Adherent cells were washed and infected with DIL C18 stained *M. tuberculosis* at a MOI of 1:10. After 8 and 24 hours, adherent cells were harvested from wells, stained with anti mouse CD 54 FITC antibody and analyzed by flow cytometry as described in Materials and Methods. (A) Percentage of CD54 positive cells, (B) changes in mean fluorescence (MCF) of CD54 expression as percentage of control. All the comparisons were done by unpaired t-test (*, p<0.05). Each bar represents mean of three observations ± SD.
FIG 38. Expression of IFNR on H37Ra positive and negative population of adherent mouse peritoneal cells infected \textit{in vitro}.

Peritoneal cells harvested from C57Bl/6 mouse were cultured in a 24 well plate (2x10^6 cells / ml / well) for 2 hours. Adherent cells were washed and infected with DIL C 18 stained \textit{M. tuberculosis} at a MOI of 1:10. After 8 and 24 hours, cells were harvested from wells, stained with biotinylated anti mouse IFNR antibody, SA- FITC and analyzed by flow cytometry.
FIG 39. IFNR expression on H37Ra 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 (2x10⁶ cells / ml / well) for 2 hours. Adherent cells were washed and infected with DIL C18 stained *M. tuberculosis* at a MOI of 1:10. After 8 and 24 hours, adherent cells were harvested from wells, stained with biotinylated anti mouse IFNR antibody, SA-FITC and analyzed by flow cytometry as described in Materials and Methods. (A) Percentage of IFNR positive cells, and (B) changes in mean fluorescence (MCF) of IFNR expression as percentage of control. All the comparisons were done by unpaired t-test (*, p<0.05). Each bar represents mean of three observations ± SD.
Results

Production of nitric oxide by RAW 264.7 macrophage cell line in response to IFN γ and TNF α

RAW 264.7 cells were treated with different doses of IFN γ and TNF α separately or together for 24, 48 and 72 hours and the amount of NO secreted was estimated in culture supernatants. Results in Fig 40 show that there was a dose and time dependent induction of NO in RAW 264.7 cells in response to IFNγ. TNFα was far less effective as compared to IFN γ, and induced NO production only at 40 ng /ml dose. IFN γ and TNF α appear to have a synergistic effect on NO production at 72 hours time point.

Production of nitric oxide by M. tuberculosis infected RAW 264.7 cells in presence and absence of IFN γ and TNF α

We then attempted to see if infection with M. tuberculosis had any effect on the ability of RAW 264.7 cells to release NO. Results in Fig 41 show that RAW 264.7 infected with H37Ra (MOI=10) released significant amount of NO. There was a marked boosting effect of IFN γ on NO release by infected RAW 264.7 cells. TNFα was not very active in this respect. Similar results were obtained when JNU 7, a clinical isolate of M. tuberculosis, was used for infection (Fig 42). Interestingly, secretory antigens of H37Ra do not induce NO production even in the presence of IFNγ (Fig 43).
FIG 40. Changes in nitric oxide production by RAW 264.7 cells in response to IFNγ and TNFα.

RAW 264.7 cells cultured in a 24 well plate (0.2 x 10^6 cells / ml / well) were treated with different doses of recombinant murine IFNγ and TNFα for varied time periods. At different time points supernatants were collected. The amount of nitric oxide in the supernatant was estimated as described in Materials and Methods. Nitric oxide production by treated and untreated cell at each time point was compared by unpaired t-test (*, p<0.05). Each bar represents mean of three independent experiments carried out in triplicates ± SD.
FIG 41. Changes in nitric oxide production by *M. tuberculosis* (H37Ra) infected RAW 264.7 cells in presence and absence of IFNγ and TNFα

RAW264.7 cells cultured in a 24 well plate (0.2 x10⁶ cells / ml / well) were infected with *M. tuberculosis* (H37Ra) in presence or absence of different doses of recombinant murine IFNγ and TNFα for varied time periods. At different time points supernatants were collected. The amount of nitric oxide in the supernatant was estimated as described in Materials and Methods. Nitric oxide production by treated and untreated cell at each time point was compared by unpaired t-test (*, p<0.05). Each bar represents mean of three independent experiments carried out in triplicates ± SD.
FIG 42. Changes in nitric oxide production by *M. tuberculosis* (JNU7) infected RAW 264.7 cells in presence and absence of IFNγ and TNFα.

RAW264.7 cells cultured in a 24 well plate (0.2 x10^6 cells / ml / well ) were infected with *M. tuberculosis* (JNU7) in presence or absence of different doses of recombinant murine IFNγ and TNFα for varied time periods. At different time point supernatants were collected. The amount of nitric oxide in the supernatant was estimated as described in Materials and Methods. Nitric oxide production by treated and untreated cell at each time point was compared by unpaired t -test (*, p<0.05). Each bar represents mean of three independent experiments carried out in triplicates ± SD.
FIG 43. Changes in nitric oxide production by RAW 264.7 cells in response to culture filtrate proteins (CFPs) of *M. tuberculosis*

RAW 264.7 cells were cultured in a 24 well plate in RPMI 1640 with 10% FCS (0.2 x 10⁶ cells / ml / well) and treated with 100 ng of culture filtrate proteins (CFPs) of different isolates of *M. tuberculosis* or LPS for varied time periods. At different time points supernatants were collected. The amount of nitric oxide in the supernatant was estimated by Griess reagent as described in Materials and Methods. Nitric oxide production by treated and untreated cell at each time point was compared by unpaired t -test (*, p<0.05). Each bar represents mean of three independent experiments carried out in triplicates ± SD.
FIG 44. Changes in arginase activity of *M. tuberculosis* infected adherent peritoneal cells

Peritoneal cells were harvested from C57Bl/6 mouse. Harvested cells were cultured in a 24 well culture plate in RPMI 1640 with 10% FCS (2x10^6 cells / ml / well) and infected with *M. tuberculosis* (H37Ra) at a MOI of 1:10 for varying time periods. At different time points adherent cells were harvested. Arginase activity in the cells was assayed as described in Materials and Methods. Arginase activity by treated and untreated cell at each time point was compared by unpaired t -test (*, p<0.05). Each bar represents mean of three independent experiments carried out in triplicates ± SD.
Results

Changes in arginase activity of *M. tuberculosis* infected adherent peritoneal cells

Activated murine macrophages and dendritic cells metabolize L-arginine by two alternative pathways involving either inducible NO synthase or arginase. Inducible NO synthase catalyzes the conversion of L-arginine into NO and citrulline whereas, arginase hydrolizes arginine to ornithine and urea. Results in Fig.44 show that *M. tuberculosis* infection of peritoneal macrophages activated arginase enzymes at early time points (4 and 8 hours) of infection. After 8 hours of infection, arginase activity declined markedly. This decline may be responsible for building up of a pool of arginine in the cell as a substrate for NO production. Our earlier results showing a late activation of NO production in response to *M. tuberculosis* supports this suggestion.