Expression of co-stimulatory molecules B7.1 & B7.2 on macrophages infected with various strains of *Mycobacterium tuberculosis* & its influence on T-cell apoptosis

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Received November 15, 2006

**Background & objectives:** Activation of T cells is mediated through two critical signals provided by activated macrophages. The first signal is triggered when T cell receptor (TCR) binds to the major histocompatibility antigen (MHC/Ag) complex. The second signal is the interaction of co-stimulatory molecules with their respective ligands on T cells for their activation and proliferation. We undertook this study to observe the modulation in B7.1 (CD80) and B7.2 (CD86) co-stimulatory molecules on *Mycobacterium tuberculosis* infected monocyte derived macrophages (MDM) and their role in T helper (Th1) cell apoptosis.

**Methods:** *M. tuberculosis* clinical strains (S7 and S10) and laboratory strains (H37Ra and H37Rv) were used to infect the MDMs. The modulation of apoptosis was assessed by treating T cells with anti-CD3 and anti-CD28 antibodies. The infected MDMs were co-cultured with autologous PPD pulsed T cells to ascertain the role of co-stimulatory molecules during infection.

**Results:** In infected MDMs, all strains on day 1 but only S7 on day 2 showed significant decrease (*P*<0.05) in B7.1 expression compared to uninfected. The expression levels of B7.2 were also low on day 1 in S7, S10 and H37Ra infected MDMs. The anit-CD3 induced apoptosis in PPD pulsed T-cells showed further reduction with anti-CD28 antibodies. However, the modulation observed in B7.1 expression in infected MDMs was not reflected in T cell apoptosis in co-culture experiments.

**Interpretation & conclusions:** Our results confirmed the role of B7.1 in rescuing the activated T-cells from undergoing apoptosis. During infection when the expression of B7.1 is downregulated, other co-stimulatory molecules may take over its crucial role to confer protective immune response against *M. tuberculosis*.

**Key words** Apoptosis - B7.1 - B7.2 - co-stimulation - *Mycobacterium tuberculosis*

*Mycobacterium tuberculosis* is a highly successful intracellular pathogen known for its guileful evading mechanisms from the protective immune response and cause chronic disease in human. Importantly, *M. tuberculosis* has the ability to disrupt the normal functioning of phagosomes1-3 thereby reducing the
capacity of mycobacterial antigens to be processed and presented on the major histocompatibility (MHC) class-II molecules. T-cells recognize the antigens on MHC-I or -II for activation to further modulate macrophage functions resulting in either inhibition or augmentation of intracellular *M. tuberculosis* growth.

Activation of T-cells is mediated through two critical signals provided by activated macrophages that play an important role in prompting the protective immune response of Th-1 type against *M. tuberculosis*. The first signal is antigen specific and requires T-cell receptor (TCR) binding to the MHC/antigen complex presented on the macrophage. The second signal is antigen independent and involves the interaction of co-stimulatory molecules and/or adhesion molecules that bind to their respective ligands on T-cells. In the absence of second signal, lymphocytes fail to respond effectively and are rendered anergic. Therefore, expression of co-stimulatory molecules on macrophage is essential for activation and proliferation of antigen specific T-cells.

In our earlier epidemiological studies, we showed the predominance of IS6110 single copy strains of *M. tuberculosis* and their involvement in active transmission of the disease in BCG trial area of Tiruvallur district, Tamil Nadu. The immunological studies carried out with these strains (especially in two clinical strains namely S7 and S10) exhibited differential protective immune responses in healthy volunteers. The sonicate antigen prepared from S7 induced the proliferation of predominantly Th-2-type cells secreting interleukin-4 (IL-4), which in turn suppressed Th1 response in purified protein derivative (PPD) positive healthy volunteers. This observation prompted us to speculate that there might be a differential or preferential induction of Th-1 cell apoptosis and that the modulated expression of co-stimulatory molecules could play the essential or significant role in this phenomenon. Also, the down-regulation of these co-stimulatory molecules on macrophages can be one of the virulence associated mechanisms in *M. tuberculosis* infection.

To test this hypothesis, in this study, live *M. tuberculosis* clinical strains S7 and S10 were used along with the laboratory strains to infect monocyte derived macrophages (MDMs) and to study the modulation in B7.1 (CD80) and B7.2 (CD86) co-stimulatory molecules. Infected MDMs were also co-cultured with autologous Th-1 type of cells to understand the role of B7.1 and B7.2 in Th-1 cell apoptosis.

### Material & Methods

**Bacterial strains**: The *M. tuberculosis* clinical strains S7 and S10 were obtained during the Model DOTS study conducted at the BCG trial area of Tiruvallur District. These strains had single copy of IS6110 and were prevalent in the community based on our restriction fragment length polymorphism (RFLP) studies. These two strains were chosen for this study as they showed differential immune response and differential mode of infection in our previous studies. The laboratory strains H37Ra and H37Rv were included for comparison.

**Mycobacterial culture**: The mycobacterial strains were grown as stationary cultures in Sauton’s liquid medium until mid-log phase of 4-6 wk. The bacterial cells were harvested, washed and bacterial clumps were dispersed by passing through 26-gauge needle ten times. The cell suspension was centrifuged at low speed to remove the remaining clumps. The bacilli were adjusted to 10 x 10^6 cells/ml as single cell suspension in sterile 1x PBS, and stored as aliquots at -70°C until use. The viability was checked before the start of the infection studies.

**MDM culture and infection**: The heparinized venous blood was collected from healthy laboratory volunteers. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation and re-suspended in RPMI 1640 supplemented with 10 per cent autologous plasma. Monocytes were isolated from PBMC by magnetic activated cell sorting (MACS) beads according to the manufacturer’s instructions (Miltenyi Biotec Inc. CA, USA). The MACS separated population contained at least 98 per cent monocytes as determined by flowcytometry analysis using CD14-FITC labelled monoclonal antibodies (Becton Dickinson, CA, USA). A total of 1 x 10^6 monocytes were seeded in 24 well tissue culture plates and allowed to mature and differentiate into macrophages up to 6 days for infection studies. The differentiated MDMs were infected with S7, S10, H37Rv or H37Ra at a multiplicity of infection (MOI) of 1:10 for 3 h. The supernatants and cells were collected to be used for zero (0 day) time point. For other time points, the infected cells were washed three times with plain RPMI 1640 to remove the extracellular bacilli and replenished with fresh medium. The infected cells were incubated until required time points (1, 2, 5, 7 days) and supernatants were collected for the assays. Uninfected cells were used as a negative control.
Expression of B7.1 and B7.2 by fluorescent activated cell sorter (FACS): The infected MDMs were harvested at various time points using trypsin-EDTA and stained for the surface expression of CD14, CD80 and CD86 using fluorescein isothiocyanate (FITC)-conjugated anti-CD14, PE-conjugated anti-CD80 and PE-Cy5-conjugated anti-CD86 antibodies (Becton Dickinson, CA, USA) for 20-30 min as described by Gonzalez et al. with minor modification for human macrophages. After washing with Dulbecco’s modified phosphate buffered saline (DPBS) containing 1 per cent FCS, the cells were fixed with 4 per cent paraformaldehyde in PBS for 15 min on ice. The labelled cells were acquired using BD FACSCalibur and analyzed using CellQuest software.

Effect of co-stimulation on T-cell apoptosis: The PBMCs from normal subjects (n=8) were used to study the T-cell apoptosis. Briefly, 100 µl of 1 µg/ml anti-CD3 in PBS was coated on a 48 - well plate for overnight at 4°C and then the unbound antibodies were washed twice in PBS. The T-cells from PBMCs were isolated using CD2 MACS beads, according to the manufacturer’s instructions. Purified T-cells at a density of 1x10^6 were either treated with 0.5 µg of anti-CD28 (1 mg/ml) or left untreated. These cells were seeded to anti-CD3 coated plates and incubated overnight.

The PBMCs with cell density of 1x10^6, supplemented with 10 per cent autologous plasma in RPMI 1640, were cultured in the presence of PPD (10 µg/ml) for 5 days in 24-well plates. The supernatants were collected to check the cytokine levels for confirming the Th cell type. The CD2 T-cells were purified from these PPD stimulated PBMCs using MACS beads as given above. These purified and PPD activated T-cells were also treated in a similar way with anti-CD3 and anti-CD28 antibodies. The apoptosis in both the conditions was detected using annexin V-FITC by FACS.

Annexin V-FITC assay for T cell apoptosis: The T-cell apoptosis was estimated by annexin V-FITC staining kit (R&D Systems, MN, USA) as per the manufacturer’s instructions. Briefly, the T-cells were pelleted at 1200 rpm for 10 min and washed twice in 1xPBS. The reaction mixture containing annexin V-FITC was prepared in 100 µl of binding buffer as per the instruction and was added to the samples and vortexed gently. The cells with reaction mixture were incubated at dark for 15-30 min for labelling and fixed in 4 per cent paraformaldehyde. An additional 400 µl of binding buffer was added prior to acquiring. The labelled cells were then acquired using BD FACSCalibur and analyzed using CellQuest software.

Co-culture of MDMs with T-cells: The autologous MDMs were infected with various strains of M. tuberculosis for two days when modulation in co-stimulatory molecule B7.1 was observed. The CD2 T-cells were purified from the PPD stimulated PBMCs using MACS beads as given above. These purified and PPD activated T-cells were added to infected MDMs in the ratio of 1:10 (macrophage: CD2 T-cells) and incubated for 18 h to detect Th-1 cell apoptosis as described above.

Statistical analysis: The results were statistically analyzed using GraphPad Prism software (version 4.02, 2005, San Diego, CA). The data were compared by two-way ANOVA. A statistical significance was shown by P<0.05.

Results

Expression of co-stimulatory molecules B7.1 and B7.2 on MDMs: A spontaneous and significantly (P<0.05) decrease in the expression of B7.1 was observed on day 1 in all the infected MDMs. The expression of B7.1 though increased on day 2 in infected MDMs, it was still significantly low in S7 compared to control. On day 3, there was no change in expression of B7.1 on MDMs infected with different strains as compared to uninfected cells (Fig. 1).

A spontaneous decrease in the expression of B7.2 was observed on day 1 in all infected MDMs but was significant in S7, S10 and H37Rv (P<0.05). On day 2, only H37Ra infected MDMs showed increase in the expression which was significant compared to S7 infected MDMs (P<0.05). On day 3, B7.2 expression levels further increased in control, S7 and H37Rv infected MDMs but none showed any significance (Fig 2).

Th-1 cell apoptosis: The pre-activated T-cells spontaneously underwent apoptosis on anti-CD3 treatment. The addition of anti-CD28 to anti-CD3 treated cells showed lower levels of apoptosis comparable to control T-cells. The PPD pulsed T-cells showed maximum apoptosis which decreased on treatment with anti-CD3. There was further decrease in PPD pulsed T-cell apoptosis on addition of anti-CD28, this decrease was very minimal (Fig. 3 A, B).

Co-culture of MDMs - Th-1 cell apoptosis: There was reduction in PPD pulsed T-cell apoptosis in all the
infected MDMs compared to control but not statistically significant. Also, there was no strain specific modulation of Th-1 cell apoptosis (Fig. 4).

**Discussion**

It is well established that expression of co-stimulatory molecules on macrophage acts as second signal and is essential for activation and proliferation of antigen specific T-cells. The aim of this study was to know whether the clinical strains modulate the expression of co-stimulatory molecules on macrophages to further downregulate the T-cell response for their advantage.

In our earlier study, we observed strain dependent induction of apoptosis in infected THP-1 cells and its magnitude was a function of the number of internalized mycobacteria. These results indicated differential mode of infection by clinical strains and their adaptation to different survival strategies that may lead to immune suppression and pathogenesis of the disease.

To further explore the role of co-stimulatory molecules on apoptosis and their modulation by various strains, initial experiments were done on infected THP-1 cells. We observed increase in B7.1 but no change in B7.2 expression after infection. Infected THP-1 cells with clinical strains showed lower levels of B7.1 expression than the laboratory strains (data not shown). This observation gave us a clue that the modulation of B7.1 might be strain dependent. Hence, it was decided to look for modulation of these co-stimulatory molecules in infected MDMs. The results showed reduced expression of B7.1 and B7.2 in all infected MDMs at early time point of 1 day after infection. Any direct supportive evidence for this observation was not found in the literature. However, in one study it has been shown that the MTSA-10 transfected J774
macrophages had lower levels of B7.1 while the levels in expression of B7.2 and ICAM-1 were not affected.

From earlier reports, it was observed that B7.1 was responsible for development of Th-1 like cells and B7.2 promotes mainly the expansion of Th2 lymphocytes. In this study, we did not find any significant increase in the levels of B7.1 and B7.2 at any time point in various infected MDMs compared to uninfected and hence exact contribution of various strains in polarization of T-cells into Th1/Th2 type is not clear.

The interaction of co-stimulatory molecules B7.1 and B7.2 on antigen-presenting cells with CD28 on T cell is necessary for optimal activation of antigen-recognizing T-cells. Therefore, expression of co-stimulatory molecules that in turn mirror the activated state of immune response is essential in promoting the

Fig. 3. Effect of co-stimulation with anti-CD28 on pre-activated T-cell apoptosis (A) and PPD pulsed T-cell apoptosis (B) was assessed. The results are represented as mean ± SEM from eight different experiments.

expansion of specific T-cells particularly the Th-1 type cells which confer protection in tuberculosis infection. It is established that activation of T-cells using anti-CD3 alone without any co-stimulation induces apoptosis of Th-1 cells but not Th2 cells. It can be speculated that *M. tuberculosis* clinical strains might have evolved mechanisms to downregulate monocyte/macrophage co-stimulatory molecules and induce selective Th-1 cell apoptosis which it exploits for its survival.

In this study, it was observed that non-activated T-cells spontaneously underwent apoptosis on anti-CD3 treatment and were rescued from apoptosis when the co-stimulation signal anti-CD28 was given indicating the role of co-stimulatory molecules in protecting the cells from apoptosis. The study by Schmitz and Krappmann indicated that the naïve T-cells undergo anergy while pre-activated T-cells undergo activation induced cell death (AICD) on triggering with anti-CD3 alone, further confirming our results. The possible mechanism involved may be the lack of phosphatidylinositol 3'-kinase (PI 3'-K) activity following CD3 ligation leading to death. The co-stimulation with anti-CD28 possibly initiates the PI 3'-K activity which abrogates apoptosis. This mechanistic approach has been established for Th-1 cell apoptosis in the lack of co-stimulation in mouse model. One can speculate that the same mechanism could be involved for both non-activated and activated T-cells. Here, the observed decrease in apoptosis of

Fig. 4. Effect of modulated B7.1/B7.2 during MDMs infection on PPD pulsed T-cell apoptosis. The co-culture of various strains infected MDMs with autologous PPD pulsed purified T-cells to understand the role of co-stimulatory molecules in Th1 cell apoptosis. The results represented are mean ± SEM from six experiments.
PPD pulsed T-cells (like Th-1 type cells) on addition of anti-CD28 co-stimulatory molecule compared to control and anti-CD3 alone further imparts the importance of co-stimulation in the survival of protective T-cells.

These findings were further expanded by MDMs and T-cell co-culture experiments. Surprisingly, there was no significant decrease in the apoptosis of PPD pulsed T-cells when co-cultured with infected MDMs as compared to uninfected MDMs. It can be speculated that some compensatory co-stimulatory molecules along with B7.1 and B7.2 on infected MDMs may be required in protecting the activated T-cells from undergoing apoptosis. The role of such additional co-stimulatory molecule was shown in one study where CD40 present on infected MDMs interacted with CD40L present on activated T-cells namely PPD pulsed T-cells. In this study, in addition to B7.1 and B7.2, other compensatory mechanism may be required in infected MDMs which will allow proliferation rather than rescuing the cells from undergoing apoptosis. Such compensatory mechanism may be absent in the uninfected MDMs.

In conclusion, these results indicated the crucial role of second signal in rescuing T-cells from undergoing apoptosis. However, in this study, there was no prominent effect of strain related modulation of co-stimulatory molecules in rescuing the T-cell apoptosis. The role of CD40, another co-stimulatory molecule as a substitute for the function of B7.1 and B7.2 need to be tested to understand the communication of these molecules with their ligands on T-cells.

Acknowledgment

The first author (PR) thanks the Indian Council of Medical Research, New Delhi for providing the Senior Research fellowship. Authors acknowledge partial support received from the National Institute of Health (NIH), USA, through NIAID/TRC ICER programme.

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