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Impact of co-signaling on the survival of intracellular pathogens inantigen presenting cells

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Abstract: The "captain of all these men of death", tuberculosis (TB) has been a scourge of thehumankind from time immemorial. TB is a disaster to the medical science due to the multi -faceted dimensions of the survival strategies employed by *Mycobacterium tuberculosis (Mtb)* to escape the immune system of the host. Mycobacteria are ubiquitous, occurring in every habitat and ecosystem of the world and has been one of the most devastating pathogens in the history of humankind. In 2014, the World Health Organization (WHO) estimated that approximately 9.6 million people developed TB globally and about 480,000 were infected with multidrug- resistant TB (MDR-TB) strains. MDR-TB is a form of TB with in-vitro resistance to the two most potent anti-TB drugs, isoniazid, and rifampicin, with or without resistance to any other drugs. One-third of the world's populations are at risk of developing this disease and it is estimated that 2 billion people, or one-fourth of the world's population, are infected with *Mtb.* This pathogen produces nearly 9 million new infections and 1.5 million deaths every year, ranking second, only to HIV, as the leading cause of death from an infectious agent. Hence, there is an urgent need and challenge for scientific community to develop alternative strategies to defeat the problems linked to the reemergence of TB. It is well-known that innate immunity plays an important role in host defense against intracellular pathogens including *M. tuberculosis* the causative agent of TB. Co-stimulatory molecules like CD80/CD86 present on antigen presenting cells provide the major non-cognate stimulatory signals to the T cells for the generation of effective immune response. In the present study we have demonstrated the crucial role played by these co-stimulatory molecules in the induction/up-regulation of co-stimulatory ligands involved in development of innate immune responses. MTB either fail to up-regulate or actually decrease the expression of co- stimulatory ligands and this represents the strategies used by intracellular pathogens to avoid recognition, induce anergy, or cause immunosupression. But our results suggest that the macrophages already stimulated with anti-CD80 and anti-CD86 monoclonal antibodies on infection induces/up- regulates the expression of CD80 and CD86 co-signaling molecules. Further we demonstrated that treatment of macrophages with anti-CD80 and anti-CD86 mAb activate them for higher production of pro-inflammatory cytokines like TNF-a, IL-1, IL-6 and other effector responses such as production of reactive oxygen species and bacterial cytotoxicity. Even

though CD80 and CD86 molecules induce differential responses in T-cells, our findings indicate that in macrophages, however, these molecules probably elicit similar responses, since both anti-CD801 and anti-CD86 mAbs triggered equivalent levels of pro-inflammatory cytokines and ROS in macrophages, it is likely that the downstream- signaling cascades triggered in response to stimulation through CD80 molecules are similar to that of CD86 molecules. Therefore our results suggest that CD80/CD86 molecules are not only essential for induction of adaptive immune responses but also play roles in activation of innate immune responses. These parameters are favorable in controlling the intracellular infection of macrophages. Therefore we examined whether infected macrophages could be induced to kill intracellular pathogens like *M. tuberculosis* and *S. typhimurium* etc. Macrophages activated through anti-CD80 and anti-CD86 mAbs showed enhanced microbicidal activity and reduced the survival of M. tuberculosis. Similar results were observed with other pathogens like *S. typhimurium and M. microti*. It may be conjunctured here that the microbicidal activity demonstrated may be a cumulative effect of augmented release of pro-inflammatory cytokines and nitric oxide by the infected macrophages on engagement of CD80 and CD86 co-signalling molecules.

Keywords: *Mycobacterium tuberculosis, Salmonella typhimurium, Cosignalling, Macrophages; CD80/CD86,Pathogens.*

I. INTRODUCTION: Till date, no other disease in history matches the sheer magnitude of the misery inflicted by TB on the human race in terms of morbidity and mortality. Tuberculosis (TB) is the leading cause of death attributable to a singleinfectious pathogen having significant medical, social, and economic impacts.Tuberculosis (TB) is a major infectious disease and about one third of the world's population is already infected. About 3 million people die and 8 million people develop the active disease each year (WHO report, 2007). In the last decade there is sharp rise in the cases of TB mainly due to emergence of HIV-TB co-infection and emergence of multi-drug resistant strains of Mycobacterium tuberculosis. Moreover the clinical trials of BCG (the only vaccine available and approved against TB) conducted in different parts of the world have shown that this vaccine does not provide consistent protection against TB macrophage (Bannon et. al. 1999) especially in the old age. Hence, there is an urgent need and challenge for scientific community to develop alternative strategies to defeat the problems linked to the reemergence of TB. It is well known that development of immune response, especially in case of naive T cells needs at least two distinct signals for full activation to proliferate and differentiate (Jenkins 1994). The first signal is provided by the specific antigen (MHC-peptide complex) recognition by the T cell receptor (TCR) and the

second signal is provided by the costimulatory molecules (Janeway et. al. 2002). This requirement for second signal explains why adaptive immune responses are stimulated by microbes but not by most self antigens, which are not normally recognized by the innate immune system and therefore do not elicit adaptive immune responses. The second signal, which is not delivered via the TCR and is not antigen specific, has been termed as costimulatory signal because, while essential, it does not by itself induce any response in T cells. However, when a T cell has its receptor ligated and receives a co stimulatory signal, the T cell will proliferate and differentiate into an effector cell. Moreover, T cells that bind antigen but do not receive a costimulatory signal are thought to die or to become anergic (Jenkins et al. 1987), a state in which the cell cannot be activated even if it receives both of the signals required to activate a T cell. Thus, an encounter with antigen can lead to two quite distinct outcomes: proliferation and differentiation into effector cells, or inactivation or death, which outcome occurs is determined by the appropriate delivery of costimulatory signals **(Fig I).** With the continuous discovery of new costimulatory molecules on antigen presenting cells (APCs) and their receptors on T cells, the story is very much an evolving one. But till date, B7-1 (CD80) and B7-2 (CD86) remain the best-defined costimulatory molecules on APCs (Lenschow et al 1996) which provide the second signals for activation of naïve T cells (Harding et. al. 1992, Linsley et. al. 1994). Recently, for the first time Suvaset. al. 2002 gave a concept of bidirectional costimulation and demonstrated that costimulation through CD80 and CD86 not only influence the activation of T cells but by bidirectional costimulation they can also affect the activity of B cells. Since then many studies have shown the significance of CD80 and CD86 in influencing the activity of B cells, stem cells, dendritic cells (Larsen et al 1994) but nothing is known about the role of these molecules in the case of macrophages. How these molecules regulate the activation of macrophages which are the important cells of the immune system and the host for a plethora of intracellular pathogens (Kaufmann 1993) especially the causative agent of Tuberculosis. So what is needed is an integrated picture of how signals delivered through major costimulatory molecules CD80 and CD86 expressed on the surface of macrophages regulate the type of immune response generated during infection. Hence the main aim of the present study is to monitor whether bidirectional costimulation can affect the performance of macrophages. Further, whether costimulatory signals can be selectively delivered to activate and inhibit the function macrophages. Furthermore,whether the delivery of costimulatory signals can curb the growth of intracellular pathogens. The use of agents that block or activate costimulatory pathways will undoubtedly require a better understanding of how the diverse biologic activities of these pathways are orchestrated. Also to find the better biomarkers to predict outcomes of the manipulation of T-cell and APCcostimulation in humans, and preclinical studies with established predictive value. Signaling through CD80 and CD86 can be explored for inducing tolerance/apoptosis in

autoimmune diseases, hypersensitivity reactions, allergies, transplantation and in killing the intracellular pathogens.

II MATERIALS AND METHODS

MICE

Inbred male/female BALB/c, C57BL/6 mice, 6-10 weeks old, were obtained and maintained in the Institute's Animals House Facility. Animals were housed under normal conditions. All experimental procedures were approved by Institutional Review Committee for care and usage of animals.

ANTIGENS, ANTIBODIES, AND LYMPHOKINES

Fetal calf serum was purchased from Harlan Sera Lab (Crawley Down, GB). Fetal calf serum was purchased from Sera Lab (Crawley Down, GB). RPMI 1640 and HBSS from GIBCO (Grand Island, NY) and L-glutamine, L-pyruvate, penicillin and streptomycin were from Serva (Heidelberg, Germany). Recombinant cytokines IL-1, IL-4, IL-10 and IFN- and their capture and biotinylated Abs, streptavidin-HRP, anti-CD80, CD86, CD40, CD28, CTLA-4, CD4, LFA-1 (-chain), I-Ad, and CD40 Abs were purchased from Becton Dickinson (San Diego, CA). LPS (from Salmonella *typhosa*), BSA, SDS, EDTA, sodium acetatae, sodium azide, paraformaldehyde, HEPES, Tween-20, OPD, and Griessreagent (modified), were procured from the Sigma Chemical Co. (St. Louis, MO). BCA protein estimation kit was bought from Pierce (Rockford Illinois). Growth media components were purchased from Hi-media (Chandigarh, India) and Difco (Detroit, MI). Moreover flourochrome-tagged, biotinylated, and coating antibodies like Anti-CD80, CD86, CD40, CD14, MHC-I, MHC-II and their IgGisotypes were purchased from BD Pharmingen, (San Diego, CA). However CD206-RPE, CD11b-RPE and F4/80 ALEXA FLOUR®647 antibodies along with IgGisotype were purchased from AbDSerotec (Kidlington, Oxford, UK).

MEDIUM

Cells were cultured in RPMI 1640 medium supplemented with 10% FCS, L-glutamine (2mM), penicillin (50 g/ml), streptomycin (50 g/ml), HEPES (100mM) and 2-ME (0.05mM). After preparation media was filtered using .02µM filters (MDI Ambala, Punjab, India) in tissue cultures hoods.

ISOLATION OF PERITONEAL MACROPHAGES

Mice were injected intraperitoneally with 2-3 ml of 3% thioglycollate. After 4 days (96hrs), peritoneal cells collected by lavage were seeded onto antibody precoated 24, 48 or 96-well tissue culture plates (Polystyrene coated) in RPMI with 10% calf serum for 2-3 hours to allow the macrophages to adhere to the plates. Non-

adherent cells were subsequently removed by washing with RPMI, and the adherent macrophages were suplemented with RPMI with 10% calf serum. The purity of the cells was analyzed by flow-cytometry by staining them with anti-F4/80-AF®647 and CD11b-RPE Abs. Macrophages were used for experiments immediately following isolation.

CO-STIMULATION OF MACROPHAGES

Peritoneal exudate cells (PECs) from BALB/c mice were harvested by injecting 3% thioglycolate broth as described elsewhere (Mukhopadhyah et al 2002 JI). Macrophage s were maintained in RPMI-1640 media from GIBCO (Grand Island, NY) containing 10% FCS (Sera Labs) and antibiotics. Macrophages after FC blocking for 30′ on ice with FCRγII/III antibody were either added to wells uncoated or coated with anti-CD80 (clone IG10), anti-CD86 (clone GL1), anti-CD40(clone 3/23) mAbs (BD Biosciences PharMingen, San Diego, CA, USA) or IgG2a k isotype match control wells 1x106cells/well for 24 well plate and 2x105 cells for 96 well plate. After 48 h, culture supernatants were harvested and assayed for TNF-a, IL-1, IL-6, IL-12 cytokines or nitric oxide estimation or for flow-cytometry experiments.

NITRIC OXIDE ESTIMATION

Peritoneal exudates cells were harvested from thioglycollate injected mice after 96hrs. The cells were incubated with anti-FcRgIII Ab to block Fc receptors and then added to anti-CD8, anti-CD86 and anti-CD40 antibodies (Abs) and isotype (IgG2a) (BD Biosciences PharMingen) matched antibody coated wells. In control wells, macrophages were cultured with medium alone. After 2h, non-adherent cells were removed and the wells were supplemented with RPMI-1640-FCS 10%. In control wells, macrophages were cultured with medium alone. Cells were cultured for different time points and different concentrations (.001-10µg/ml) of antibodies were also used for stimulation. For infection studies cells were stimulated for 24hrs and then infection with bacteria was given the MOI 1:20 for 4hrs and after 6x washings wells were supplemented with RPMI-1640-FCS 10% containg 50µg/ml of Amikacin. The supernatants were harvested at different time points and nitric oxide (NO) was measured by Griess reagent method. '*' indicates that $p<0.001$ employing students't' test. The data expressed as mead+S.E. is from one of the 3 experiments.

ESTIMATION OF CYTOKINES

The IL-1, IL-6, TNF-a, and IL-12 cytokines in the macrophage culture supernatants after stimulation with IgG2a or anti-CD80/anti-CD86/anti-CD40 antibody for 24 and 48hrs were quantified by sandwich enzyme-linked immunoassay (ELISA). In brief fifty microliter (1 g/ml) of anti-cytokine (IL-1, IL-6, IL-12, and TNF-α) Abs or anti-IgGisotypes (IgG1, IgG2a, IgG2b) Abs diluted in binding solution (0.1M Na2PO4, pH 9.0)

were coated overnight at 4oC. Blocking was done using 1% BSA in PBS and incubated for 1h at room temperature. The standards and the test samples (50 l/well) diluted in dilution buffer (PBS/Tween-20+BSA 1%) were added and incubated overnight at 4oC. Biotinylated anti-cytokine Abs/ anti-IgG-isotype Abs (0.5 g/ml) were added and incubated at room temperature for 1h. Streptavidin-HRP (50 l/well) was added and the plates were incubated at room temperature for 30 min. The substrate, OPD (50 l/well) was added and incubated at room temperature for 15-45 minutes. The reaction was stopped by adding 7% H2SO4 (100 l/well) and absorbance was read at 492 nm. Usual steps of washing using PBS/Tween-20 were followed at each step. Standard curve for the TNF-a/IL-1/IL-6 and IL-12 cytokine was obtained using recombinant standard proteins.

FLOWCYTOMETRY ANALYSIS

For flow cytometry, macrophages were incubated with IgG2a or anti-B7-1/anti-B7-2 antibody for 60 min. Heat-aggregated rabbit sera were used at 1% concentration in the staining buffer to block Fc receptors. After washing in staining buffer, similar incubations were carried out for secondary reagent (anti-rat FITC, Sigma–Aldrich). The cells were incubated 30 min/4oC. Usual steps of washing were followed at each step and cells were fixed in 1% paraformaldehyde. Stained cells were analyzed on a Becton Dickinson flow cytometer (Becton Dickinson,San Jose, CA, USA). The analysis for the mean fluorescence intensity (MFI) was done on histograms where abcissa and ordinate denote log fluorescence and relative cell count, respectively. Post flow cytometrical data analyses were carried out using CellQuest data analysis software (Becton Dickinson).

DATA ANALYSIS

All the data were expressed as mean ± SD/SE. Statistical comparisons were made using students t-test. The significance level was set at $P < 0.05$.

III RESULTS AND DISCUSSION

CD80, CD86 and CD40 expression of costimulatory molecules on the surface of peritoneal macrophages. Onstimulation of APCs, the expression of costimulatory molecules is not constant and varies with time. Hence, the first experiment was conducted to evaluate the phenotypic expression of costimulatory molecules on the surface ofmacrophages to find a suitable time period at which there is optimum expression of these molecules. The expression of CD80, CD86 and CD40 along with MHCII (IAd) was checked by flowcytometry**(Fig. 2**). We observed that at 96h time point after thioglycollate injection, all the three costimulatory molecules i.e., CD80, CD86 and CD40 are differentially expressed on the surface of peritoneal macrophages. The level of CD80 (MFI: 570) expression was comparatively higher than CD86 (MFI: 124) and CD40 (MFI: 73). Expression of MHC class-II molecules (MFI: 257) was also observed. Since after 96h

of thioglycollate injection macrophages expressed all the costimulatory molecules of our interest, we used the same time period for harvesting the cells.

Cosignaling through CD80 and CD86 molecules augmented nitric oxide release by macrophages. Nitric oxide is anindicator of the activation status of macrophages and is also a potent defense mechanism of macrophages against pathogenic microorganisms. Therefore, we wanted to test whether macrophages can be activated by costimulation through CD80 and CD86 to release nitric oxide (**Fig. 3**). We found that as compared to isotype control, treatment with the anti-CD80, anti-CD86 and anti-CD40 Abs significantly ($p< 0.005$) enhanced the secretion of nitric oxide for all regimes. This effect was observed in a dose dependent manner (**Fig. 3A**). For all doses of costimulation, nitric oxide secretion was marginally better for CD80 than CD86 and CD40. We also did experiments to see the effect of costimulation on LPS stimulated macrophages (**Fig. 3B**). The LPS stimulated macrophages showed better secretion of nitric oxide on stimulation through CD80, CD86 and CD40 molecules (**Fig. 3B**). The control cultures comprising of isotype matched control Ab failed to show any change. These results indicate that triggering through CD80, CD86 and CD40 can activate macrophages to secrete nitric oxide.

*Cosignaling through CD80 and CD86 enhanced the production of IL-1, IL-6 and TNF-α.*As signaling throughcostimulatory molecules activates macrophages for nitric oxide production we, next wanted to investigate the influence of signaling through CD80, CD86 and CD40 on the production of IL-1, IL-6 and TNF-α. Macrophages were stimulated with antibodies to CD80, CD86 and CD40 molecules. Then culture supernatants were tested for the secretion of IL-1, IL-6 and TNF-α. We observed that costimulation significantly ($p< 0.005$) enhanced the secretion of IL-1 **(Fig 4A)**, IL-6 **(Fig 4B)** and TNF-α **(Fig 4C)**. The effect was observed in a dose dependent manner. No change was observed in thecontrol culture containing isotype matched control Abs. It is reported that IL-1, IL-6 and TNF-α are the indicators of macrophage activation and also play a key role in defense against pathogens. Hence these results indicate that signals delivered through CD80, CD86 and CD40 can stimulate macrophages and can significantly modulate their activity.

Cosignaling through CD80, CD86 and CD40 augmented the nitric oxide production by infected macrophages. Weknow that macrophages are the host for a plethora of intracellular pathogens. Nitric oxide is the key molecule produced by macrophages for their defense. We had earlier observed that costimulation activates macrophages and also evokes the release of nitric oxide. Subsequently, we next wanted to check whether costimulation via CD80, CD86 and CD40 boosted the release nitric oxide by infected macrophages. As compared to isotype control group, treatment with anti-CD80, anti-CD86 and anti-CD40 Abs

significantly ($p<0.05$ to 0.005) enhanced the secretion of nitric oxide when cells were infected with M. microti**(Fig. 5A)** or with M. tuberculosis H37Rv **(Fig. 5B).** Nitric oxide is a potent bactericidal agent, hence increase in the production of this metabolite may effect the survival of intracellular pathogens.

CD80, CD86 and CD40 co-signaling augmented the secretion of Proinflammatory cytokines by infected macrophages. Wehave already demonstrated that costimulation enhances the secretion of IL-1, IL-6 and TNF-α (**Fig. 6).** Proinflammatory cytokines are crucial in protection against intracellular pathogens. Hence we next monitored the release of these cytokines by macrophages infected by M. tuberculosis and M. microti**(Fig. 6).** It was observed that costimulation through CD80, CD86 and CD40 significantly ($p<0.005$) enhanced the production of IL-1, IL-6 and TNF-by macrophages infected with either M. microti**(Fig 6 A1-C1)** or M. tuberculosis **(Fig 6 A2-C2).** These results indicate that signaling through CD80, CD86 and CD40 activate the macrophages for enhanced release of proinflammatory cytokines.

Cosignaling through CD80, CD86 and CD40 reduced the survival of intracellular pathogens (M. microti and M. tuberculosis) inside the macrophages. Our results signify that costimulation through CD80, CD86 and CD40 modulatesecretion of proinflammatory cytokines and nitric oxide. Hence we wanted to check whether costimulatory signals delivered through CD80, CD86 and CD40 into macrophages infected with intracellular pathogens can inhibit their growth and therefore their survival inside the macrophages **(Fig. 7, 8)**. The macrophages treated with anti-CD80, anti-CD86 and anti-CD40 Ab show significant ($p<0.05$) increased microbicidal activity and decrease in the survival of *M.tuberculosis*and *M. microti*inside the macrophages**(Fig. 7, 8)**. No change was noted in the isotype matched controlcultures. It was interesting to notice that CD80 induced higher microbicidal activity than CD86 and CD40. These results thus indicate that signaling delivered *via*costimulatory molecules can significantly influence the survival of intracellular pathogens.

Since B7-1/B7-2 activates induction of TNF-a as well as reactive oxygen species (ROS) and bactericidal activity in macrophages, anti-B71/anti-B7-2 mAb treatment can be used to tailor immune responses to induce cytotoxicity against intracellular pathogens that reside inside the macrophages. This approach may not be limited to *M. tuberculosis* but also to other infections including AIDS, leishmania, malaria, etc. This novel strategy can also be effectively exploited to develop immunotherapy either using humanized antibodies against B7-1 and B7-2 or CD28 fusinogenic proteins for the treatment of intracellular pathogens since this approach is based on modulating the immune system of the host rather than targeting the pathogen, hence it diminishes the chances of emergence of drug resistant strains of bacteria.

Our results have important implications for the efforts to establish a vaccine against intracellular pathogens, like *Mycobacterium tuberculosis*, *Salmonella typhimurium*etc. since they suggest that vectors which induce/up-regulate theexpression of co-stimulatory ligands on antigen presenting cells like macrophages (which are also the host for *M.tuberculosis*) will help to generate a protective interferon-gamma-dependent immune response which is desired inTuberculosis.

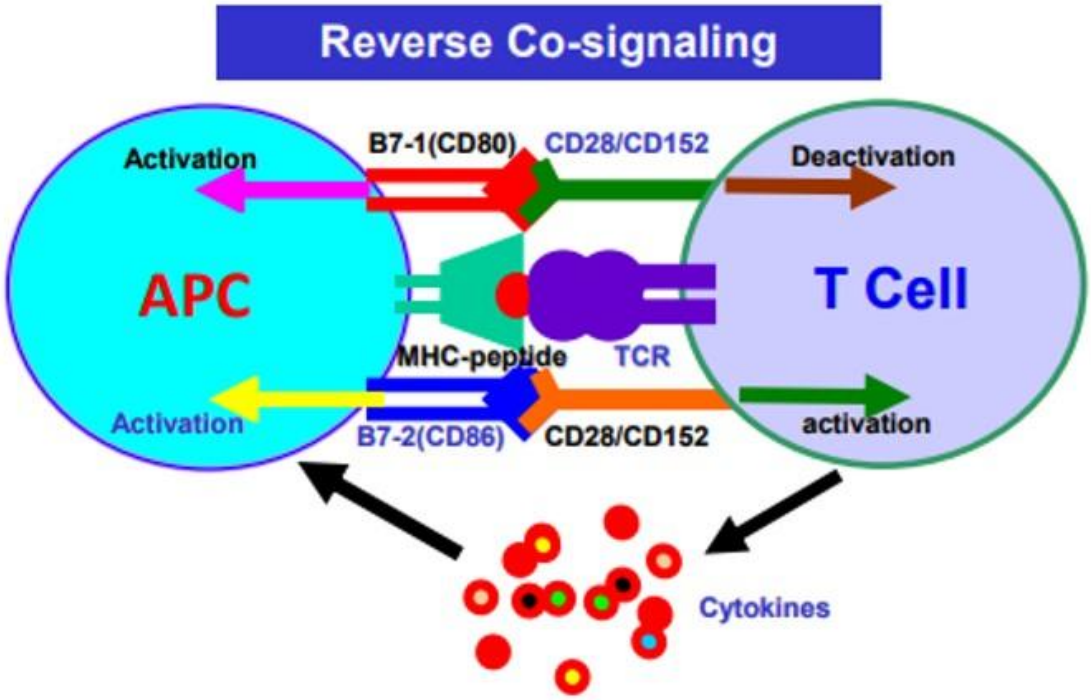

Fig. 1. CD80 and CD86 deliver bi-directional signaling for the activation of T cell and APC. Cognate association between T cell and APC lead to interaction between MHCpeptide with T cell receptor (TcR), and CD80/86 with CD28/CD152. This steers toward the activation and differentiation of T cell. Activated T cell secretes cytokines that ultimately help in the activation and differentiation of APC. The arrows shown in the diagram depict that there occur as well an alternative pathway of bi-directional signaling through costimulatory molecules CD80/CD86 and CD28/CD152 that activates both T cell and APC.

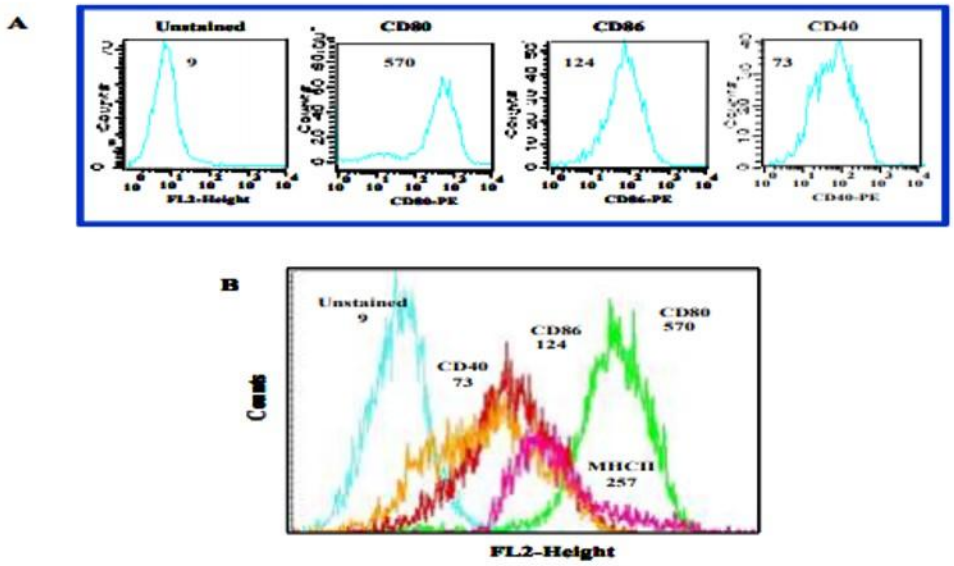

Fig. 2. Expression of CD80, CD86, CD40 and MHCII molecules. Briefly, thioglycollate elicited peritoneal macrophages from BALB/c mice were harvested after 96h and stained for the expression of CD80 (green), CD86 (red), CD40 (orange) and MHCII (IAd) (magenta). The cells were prior incubated with anti-FcRγII/III Ab to block Fc receptors. The cells were gated on macrophage zone and analyzed by CellQuest. The data shown in the inset are the mean fluorescence intensity (MFI) (A). Similar data is also represented in the form of histogram-overlay (B). Results shown are the representative of five similar experiments.

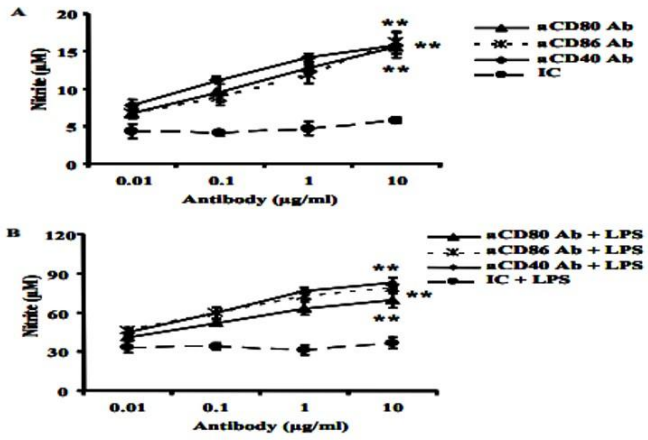

Fig. 3. Signaling through CD80, CD86 and CD40 augments nitric oxide release. Macrophages were stimulated either with different doses of plate bound anti-CD80, anti-CD86, anti-CD40 Abs (A) or Abs + LPS (10μg/ml) (B). Isotype matched Abs (IC) were used in the control cultures. After 48h of culture, SNs were harvested and nitrite production was estimated using Griess reagent. Data are expressed as mean±SEM of triplicate wells and are the representative of two similar experiments. Statistical significance of these data was determined using Students paired t-test by comparison with the respective controls. **indicates $p < 0.005$.

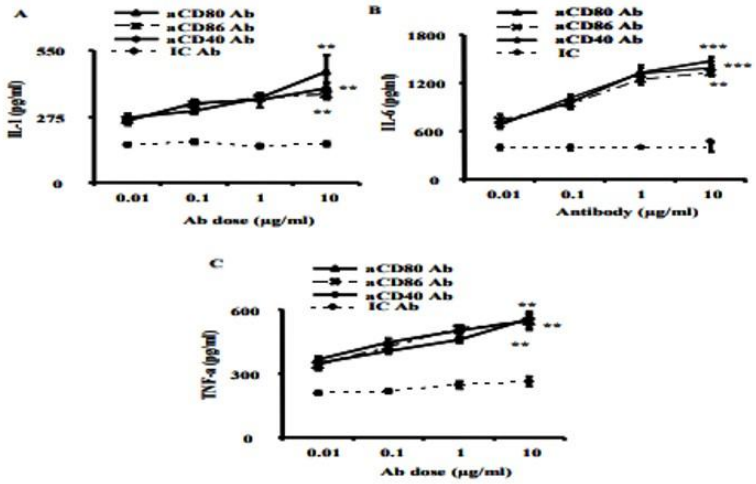

Fig. 4 A-C. Stimulation through CD80, CD86 and CD40 enhances the production of IL-1, IL-6 and TNF-a. Macrophages were stimulated with different doses of plate bound anti-CD80, anti-CD86, anti-CD40 Abs. Isotype matched Abs (IC) were used in the control cultures. After 48h of culture, SNs were harvested and IL-1, IL-6 and TNFa production was estimated by ELISA. Data are expressed as mean±SEM of triplicate wells. Results shown are the representative of three similar experiments. Statistical significance of these data was determined using Students paired t-test by comparison with the respective controls. **indicates p< 0.005

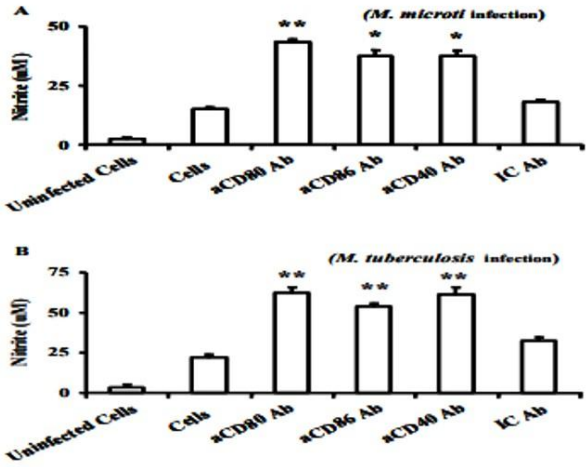

Fig. 5. Signaling through CD80, CD86 and CD40 augments the nitric oxide production by infected macrophages. Macrophages were stimulated with plate bound anti-CD80, anti-CD86, anti-CD40 Abs for 24h. Control cultures were also set using isotype matched control Abs (IC) and medium alone. Later on, cells were either infected with M. microti (A) or M. tb H37Rv (B) in the MOI of 1:20 for 4h. Cells were washed 6x to remove extracellular bacteria and then incubated again for 48h with amikacin (50µg/ml). Nitrite production was measured in culture supernatants using Griess

reagent. Data are expressed as mean±SEM of triplicate wells and are representative of three similar experiments. Statistical significance was determined using Students paired t-test by comparison with the respective controls. * indicates p< 0.05 and ** indicates p< 0.005.

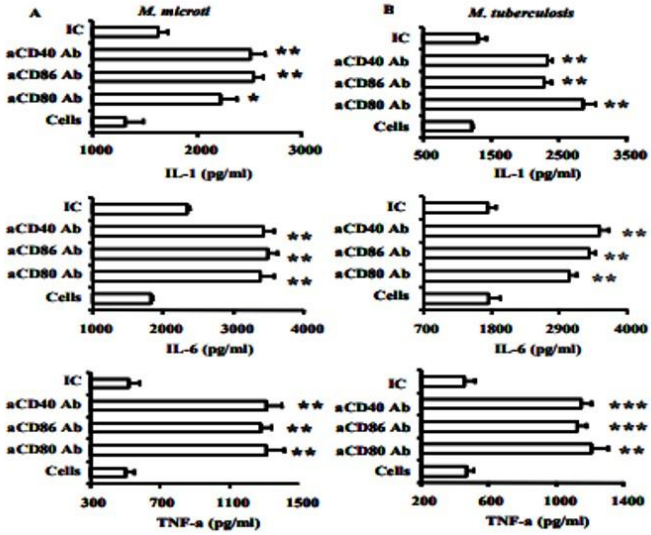

Fig. 6. Signaling through CD80, CD86 and CD40 enhances the secretion of proinflamatory cytokines by infected macrophages. Macrophages were stimulated with plate bound anti-CD80, anti-CD86, anti-CD40 Abs for 24h. Control cultures were also set using isotype matched control Abs (IC) and medium alone. Later on, cells were either infected with M. microti (A) or M. tuberculosis H37Rv (B) in the MOI of 1:20 for 4h. Cells were washed 6x to remove extracellular bacteria and then incubated again for 48h with amikacin (50µg/ml). SNs were harvested and IL-1, IL-6 and TNF-a were estimated by ELISA. Data are expressed as mean±SEM of triplicate wells and are representative of three similar experiments. Statistical significance of data was determined using Students paired t-test by comparison with the respective controls. * indicates p< 0.05, ** indicates p< 0.005 and *** indicates p< 0.0005.

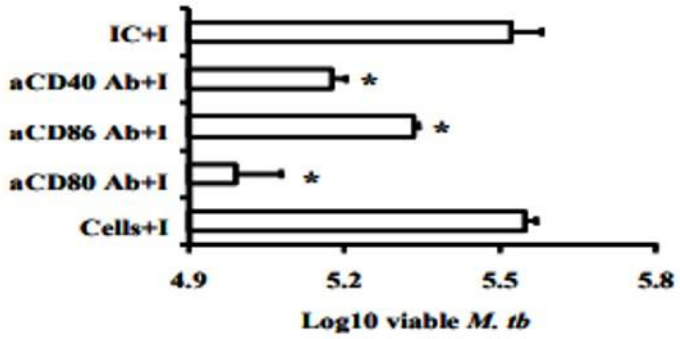

Fig. 7. Costimulation through CD80, CD86 and CD40 regress the intracellular survival of M. tuberculosis. Macrophages were stimulated with plate bound anti-CD80, anti-CD86, anti-CD40 Abs for 24h. Control cultures were also set using isotype matched control Abs (IC) and medium alone. Later on, cells were infected with M. tuberculosis in the MOI of 1:20 for 4h. Cells were washed 6x to remove extracellular bacteria and then incubated again for 48h with

amikacin (50µg/ml). The cells were lysed and the number of viable bacterium for each group was determined by plating the lysates. Data are expressed as mean±SEM of triplicate wells and are the representative of three similar experiments. Statistical significance was determined using Students paired t-test by comparison with the respective controls. * indicates p< 0.05.

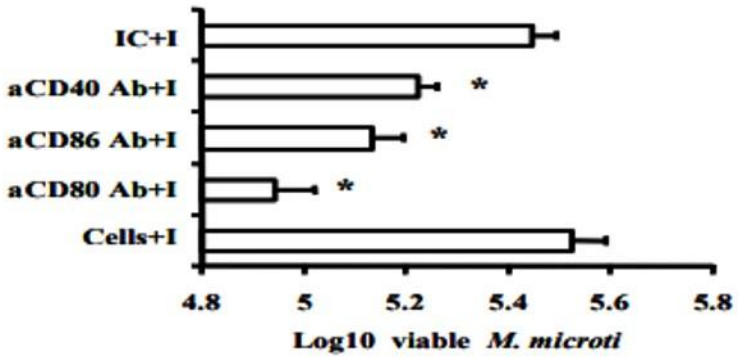

Fig. 8. Costimulation through CD80, CD86 and CD40 checks the survival of M. microti inside the macrophages. Macrophages were stimulated with plate bound anti-CD80, anti-CD86, anti-CD40 Abs for 24h. Control cultures were also set using isotype matched control Abs (IC) and medium alone. Later on, cells were infected with M. microti in the MOI of 1:20 for 4h. Cells were washed 6x to remove extracellular bacteria and then incubated again for 48h with amikacin (50µg/ml). The cells were lysed and the number of viable M. microti for each group was determined by plating the lysates. Data are expressed as mean±SEM of triplicate wells and are representative of three similar experiments. Statistical significance was determined using Students paired t-test by comparison with the respective controls. * indicates p< 0.05 and ** indicates p< 0.005.

V CONCLUSION

Finally, major conclusions drawn from the current study are that signals delivered through costimulatory molecules: i) not only are responsible for optimum activation of T cells but through bidirectional signaling can also influence macrophages; ii) can modulate the secretion of proinflammatory molecules; iii) can regress the growth of intracellular pathogens like *M. tuberculosis, M. microti, etc.;* iv) this novel strategy can be effectively exploited to develop immuno-therapy either using humanized antibodies against CD80, CD86 and CD40 or CD28 fusogenic proteins for thetreatment of intracellular pathogens like *M. tuberculosis, HIV, L. donovani, T. cruzi,* etc.; v) since this approach is based on modulating the immune system of the hosts rather than targeting the pathogen; hence it significantly diminishes chance of emergence of drug resistant strains of bacteria. It may be concluded from the results that macrophages are not only activated and kills intracellular pathogens by cytokines secreted by T cells but also through bidirectional costimulation through signals delivered by costimulatory molecules CD80, CD86 and CD40.

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