Tuberculosis is a chronic infectious disease that continues to kill some 3 million people annually. About 2 billion people worldwide are infected with *M. tuberculosis*, the causative agent of the disease (WHO 2002). The emergence of AIDS is expected to reactivate tuberculosis in millions of dormant individuals, causing a sharp rise in the number of diseased patients and mortality. *M. tuberculosis* is therefore responsible for the highest morbidity rate among all the infectious agents. The very first attempt using subunit therapeutic vaccine devised by Robert Koch failed utterly in providing protection against the pathogen. Ten years later, French scientists Calmette and Guerin, used as a vaccine an attenuated strain of *M. bovis* BCG. Although BCG prevented disseminated TB in newborns, but it failed considerably to protect against re-infection. The efficacy of BCG vaccine is both doubtful and controversial in providing protection. The reason might be that either the BCG-induced immune response was quantitatively too weak to protect against *M. tuberculosis* or it was qualitatively insufficient; that is, it did not stimulated the combination of effector and memory T cells that were required for the protection (Kaufmann 2001). The antigenic preparations such as PPD could bias the response towards Th1 type; however, these preparations would not provide as sustained or as strong a stimulus as an active mycobacterial infection (Sacco et al. 2002). The doubtful efficacy of BCG vaccine, the emergence of multi-drug resistant strains of mycobacteria, AIDS pandemic and the relative inefficiency of subunit vaccines; point towards the urgent need for the development of an effective vaccine for tuberculosis.

Vaccination is considered to be the most cost-effective and preventive measure in protection against any disease. Today, we have vaccines for a variety of bacterial and viral diseases. These vaccines have played a major role in decreasing the morbidity and mortality. Live attenuated vaccines permit the efficient presentation of antigens in context of both MHC-I and MHC-II molecules that could stimulate CD8+ and CD4+ T cell responses. But the ability of attenuated vaccine to induce Th1 specific responses in humans, however, was less characterized and varies from pathogen to pathogen. Though live vaccines have an advantage of antigen persistence but the attenuated whole organism vaccines raise several issues that could preclude their widespread applications for certain diseases like AIDS, malaria etc. DNA vaccines could induce both humoral and broad cellular responses (Ulmer et al. 1993) in small animals. The optimism of using DNA vaccine in humans was tampered by the fact that the constitutive exposure to the antigen induced spontaneous apoptosis in Th1 subset of T cells. Moreover, it has been suggested that the persistence of antigen and/or the amount of antigen also affects the qualitative
aspects of CD4\(^+\) T cell and CD8\(^+\) T cell memory and effector responses (Ochsenbein et al. 1999, Opferman et al. 1999). Swain et al. (1999) have shown class II independent generation of CD4 memory T cells, which reflected that constant exposure or presence of the antigen was unnecessary for the maintenance of memory cells. Further, Murali-Krishna et al. showed that after naïve CD8 T cells differentiate into memory cells, they evolved an MHC class I-independent "life-style" and did not required further stimulation with specific or cross-reactive antigen for their maintenance (Murali-Krishna et al. 1999).

A potential shortcoming of the non-live vaccines like fusion proteins, antigens in the adjuvants was their relative inefficiency in generating Th1 or CD8\(^+\) T cell response. Thus limiting their application for those diseases requiring cellular immunity. Moreover, their immunogenicity also depends on the type of adjuvants. Though some adjuvants had been shown to induce cell-mediated immune responses in mice but till date no appropriate adjuvant was available for human (Bennett et al. 1992). Currently, the only widely used adjuvant in human is alum, which improved antibody production but had little effect on the generation of Th1 or CD8\(^+\) T cell response (Seder and Hill 2000).

CD4\(^+\) and CD8\(^+\) T lymphocytes form the major components of the acquired immune response against any intracellular pathogen. These lymphocytes cannot act in isolation but they need the antigen to be processed and presented in association with MHC-I and MHC-II molecules on the surface of APCs. The complex displayed on the surface is recognized by the T cells to mount an immunological defense. Among all the known APCs, DCs are considered to be the most potent APCs for the activation of naïve T cells because they constitutively express the optimum level of MHC-I, MHC class II and accessory molecules. Immature DCs are highly phagocytic but have low T cell costimulatory capability. They mature by phagocytosing the pathogens or by GM-CSF and IL-4 which up regulates the expression of MHC and costimulatory molecules (Austyn 1996, Sallusto and Lanzavecchia 1994). Mature DCs lose the capability to capture and process the antigen but acquire the capacity to activate the naïve T cells. It has been recognized for many years that encounter of a host with antigen could result in either cell mediated or humoral classes of immune response (Parish CR, 1972). During the 1970s, it was suggested that these different types of responses might be attributable to heterogeneity of CD4\(^+\) T cells. This concept was strengthened during the 1980s by the observation that mouse CD4\(^+\) T cell could be sub divided on the basis of cytokines profile (Mosmann and Coffman 1989b). Th1 cells mainly produce IL-2, IFN-γ and lymphotoxin.
and were responsible for cell-mediated immunity. In contrast, Th2 cells chiefly secrete IL-4, IL-5, IL-10, IL-9 and IL-13 and were responsible for humoral immunity (Mosmann et. al. 1989b, Abbas et. al. 1996).

On analysis of the lymphokine profile of the vaccinated animals, we observed significant enhancement in the production of IFN-γ and IL-2 but not IL-4. This pattern was noticed irrespective of immunization with three different vaccine preparations viz. SMTV, AMTV and XMTV used in the study. This indicated that in all the three groups of mice, the vaccination strategy evoked predominant level of Th1-immune response. It has been very well established that the healthy tuberculin reactors showed protective immunity against tuberculosis and their peripheral blood mononuclear cells (PBMC) stimulated with *Mycobacterium tuberculosis* produced high concentration of IFN-γ (Sanchez et al. 1994, Zhang et al. 1995). HIV-infected tuberculosis patients had the most severe disease, with frequent extra-pulmonary dissemination and substantial depression in IFN-γ production (Gong et al. 1996). In many experimental rodent models of intracellular infection, which included malaria, listeria and mycobacteria, mice made deficient in genes for IFN-γ showed an increased susceptibility to infection with accelerated mortality (Heinzel et al. 1993, Sypek et al. 1993). Further it had been shown by Tascon et al., that IFN-γ production was essential for protective immunity against *Mycobacterium tuberculosis* (Tascon et al. 1998). The production of cytokines in response to mycobacterial products was vital for the resolution of disease (Vanham et al. 1997). Demangel et al. had used BCG-infected DC to trigger a protective immune response and they also noticed high IFN-γ secretion in the groups immunized with BCG-infected DC (Demangel et al. 1999). CD4+ clones from individuals with strong CMI produced predominantly IFN-γ, whereas those clones that enhanced antibody formation produced IL-4. CD8+ cytotoxic T cells secreted IFN-γ. Immunologically unresponsive individuals with leprosy produced interleukin-4 (Salgame et al. 1991). BALB/c mice had a propensity to develop a type 2 immune response against intracellular pathogens like *Chlamydia trachomatis*, *Leishmania major*, and *Mycobacterium bovis* BCG (Wakeham et al. 2000, Shibuya et al. 1998, Yoshida et al. 1995). However, our vaccine, even in BALB/c mice skewed the response towards Th1.

The differentiation of Th cell precursor into Th1 or Th2 cells have important biological implications in terms of susceptibility or resistance to a particular disease (Swain and Bradley 1992, Ullman et al. 1990). A number of studies had focused on the
role of Th1 and Th2 cells in resistance to infection. The development of immunity to Leishmania, Listeria, Mycobacterium, Schistosoma, Trypanosoma and HIV was associated with pronounced expansion of Th1 lymphocytes and increased production of cytokines typical of that subset (Boom et al. 1990, Heinzel et al. 1989, Nabors and Tarleton 1991, Pearce et al. 1991, Scott et al. 1988, Mosmann and Moore 1991). The infection of mice with intracellular protozoan parasite L. major provided the cleanest example of the different consequences of a Th1 or Th2 response to a pathogen. Susceptibility to infection correlated with Th2 activation and resistance with Th1 stimulation (Heinzel et al. 1989). Similarly in Schistosoma mansoni, experimental infection leads to a non-protective Th2 response, while vaccination induced a protective Th1 response (Scott et al. 1989). Further, protection by listeria infection was correlated with Th1 type of response and with production of IFN-γ and IL-2, without any evidence of IL-4 cytokine. However, the production of IL-10, a Th2 cell cytokine, by listeria-infected macrophages inhibited Th1 development and was the cause of progression of the disease. Further, a study of cytokine gene expression in leprosy indicated that the enhancement of Th1 function was associated with a decrease in Th2 activity and resolution of the disease (Yamamura et al. 1991). Orme et al. showed that mycobacteria specific CD4+ T cells were typically Th1 type, in that they were potent IFN-γ producers. IFN-γ was a central cytokine in the activation of anti-mycobacterial activities of macrophages (Orme et al. 1993). The early cytokine milieu and costimulatory molecules promoted the differentiation of CD4+ T cells into Th1 cells (Flynn and Chan 2001). These studies strongly suggested that the Th1 group of lymphocytes mediated the protective immune response against the intracellular pathogens.

To further substantiate our results on the generation of mainly Th1 response by the vaccines, we monitored the production of IgG1 and IgG2a type of antibodies. It has been reported that when B cells interacted with Th1 cells, they chiefly produced IgG2a type of antibodies. Further, when B cells interacted with Th2 cells they mainly secreted IgG1 type of antibodies (Stevens et. al., 1988). Since IgG1 (Th2) and IgG2a (Th1) were also considered as the markers for the type of immune response generated, the IgG2a/IgG1 ratio could help in defining the T cell phenotype induced by vaccination (Coffman et al. 1988, Morris et al. 2000). We obtained the sera from the different groups of vaccinated and control animals. The generation of PPD specific IgG1 and IgG2a antibodies were monitored. We observed profound augmentation in the production of
IgG2a type of antibodies. Further, the increase was noted in all the vaccinated groups (AMTV, SMTV and XMTV) but not in the case of mice immunized with isoniazid-treated *M. tuberculosis*. Not much enhancement in the release of IgG1 was measured. These experiments further authenticated our findings on the generation of predominant Th1 immune response by our vaccines. Furthermore, it indicated that the immune system could be favorably skewed towards Th1 response on immunization with the bacteria encapsulated in the macrophages.

T cell-dependent B cell activation and the induction of isotype switching required cognate interaction between B cell and Th cell. After the activation and differentiation of B cells, depending on the presence of lymphokines secreted by Th cells, IgM to IgG or IgE or IgA were produced. Interleukin-4 acted on B cell and induced the secretion of IgG1 and IgE. In contrast, when IFN-γ acted on B cell, it enhanced the production of IgG2a (Snapper and Paul 1987). Berton et al. suggested that the enhanced expression of IgG1 required direct contact between Th2 cell and B cell (Berton and Vitetta 1992).

The role of IgG1 and IgG2a had been established in the susceptibility and resistance to diseases. It had been shown in the case of leishmaniasis that anti-OX40L mAb treatment substantially reduced the production of anti-*L. major* immunoglobulin IgG1 and there was regression in the disease (Akiba et al. 2000). Experimental infection of BALB/c mice with a high number of *L. major* parasites resulted in a predominant Th2 response, leading to rapidly progressing and non-healing lesions. Further, administration of anti-IL-4 antibody resulted in the regression of such lesions; cured mice displayed a dominant Th1 response with increased *L. major*-specific IgG2a antibodies (Uzonna and Bretscher 2001). Cavinato et al. showed that when the malaria parasite infected mice were treated with IgG1 or IgG2a purified from hyper-immune serum obtained from mice infected with *P. chabaudi*, IgG2a showed a stronger protective activity (Cavinato et al. 2001). Koyama et al. carried out comparative studies on the levels of serum IgG1 and IgG2a in susceptible B10. BR mice infected with different strains of *Trichuris muris*. They found that the level of serum IgG2a was useful marker for IFN-γ production and protection from *T. muris* infection (Koyama and Ito 2001). It has been reported in the case of tuberculosis that the Ag85 DNA-primed-protein-boosted animals, preferentially produced IgG2a isotype of antibodies and showed protection against intravenous *M. tuberculosis* H37Rv infection (Tanghe et al. 2001). DNA vaccination encoding MPT-63 induced prevailing IgG2a response and these animals also secreted high levels of IFN-γ.
Our results showed that the vaccination strategy used by us induced the enhanced production of mycobacteria-specific IgG2a type of antibodies as compared to IgG1 type.

We observed enhanced lymphoproliferation of pathogen-reactive cells in all the three groups of the mice that were immunized with different preparations of vaccine (AMTV, SMTV and XMTV). This indicated that the vaccination strategy subjugated in this study evoked significant proliferation of the pathogen reactive lymphocytes in all the three groups. It is a well established fact that CD4+ Th1 T cells play an important role in defense against intracellular diseases like tuberculosis, leishmaniasis, salmonellosis etc. (Moser et al. 1998). Athymic mice and mice whose T cells had been depleted were much more susceptible to infection with mycobacteria as compared to wild type (Tascon et al. 1998, Colston and Hilson 1976, Orme et al. 1992). HIV-infected patients that lacked CD4+ T cells were prone to tuberculosis and had the most severe form of disease. Hence, the generation of mycobacterium-reactive cells in vaccinated animals suggested that these animals should show better protection against subsequent infection with mycobacterium.

CD4+ T cells required two distinct signals to proliferate and subsequently differentiate into an armed effector cells that mediate adaptive immunity. First signal determines which CD4+ T cells would respond to a particular antigen, was delivered via TCR, in the form of peptide fragments bound to MHC II molecules. The second signal, which was not delivered via TCR and was not antigen specific, had been termed as a "costimulatory signal" because, while essential, it did not by itself induced any response in T cells. However, when a T cell had its receptor ligated and received a costimulatory signal, the T cell would proliferate and differentiate into an armed effector cell. T cells that bind antigen but did not received costimulatory signals were thought to die or to become anergic, a state in which the cell could not be activated even if it received both the signals required for activating them. Thus an encounter with antigens could lead to two quite distinct outcomes, proliferation and differentiation into effector cells; or inactivation or death. Which outcome occurred was determined by the appropriate delivery of the costimulatory signals. Thus, it seems that the regulation of the expression of costimulatory molecules on the surface of the APC was an important event. It had been reported that several parasites could utilize an evasion strategy by modulating the expression of costimulatory molecules on the host cells and thus paralyzing CMI, the effector arm of the immunity. We therefore, became very curious to evaluate the impact of our vaccines in modulating the expression of costimulatory molecules on the surface of
the host cells. The expression of costimulatory molecules examined was B7.1, B7.2, CD40, ICAM-1 and CD44. Interestingly, on vaccination, heightened expression of all the costimulatory molecules was observed on the host cells.

Clinical studies conducted by Agrewala et al., on leprosy, also a mycobacterial disease, showed that down regulation of B7.1 and CD28 might be responsible for defective T cell signaling by B7.1/CD28 pathway caused by *M. leprae*. This might lead to clonal inactivation of *M. leprae*-reactive T cells; consequently the bacilli grow without restriction in macrophage (Agrewala et al. 1998). In contrast, our vaccination strategy boosted the expression of B7.1 (200-400%) and B7.2 (255-377%). We could observe significant increase in the groups of mice vaccinated with either SMTV or AMTV or XMTV. Both B7.1 and B7.2 costimulate T cells through their interaction with CD28 and CTLA-4. The expression of one of these ligands on a cell that lacks costimulatory molecules could convert a nonfunctional APC into a functional one. This had important implications in the upregulation of immune responses. In fact, CD28/B7-mediated costimulation had provided a new approach to cancer therapy, because the inability of some tumors to induce immune responses had, in some cases, been correlated with deficiency in providing costimulatory signals (Lenschow et al. 1996). The introduction of B7.1 or B7.2 into tumor cells in several models enhanced the anti-tumor response (Lenschow et al. 1996). Since B7.1 and B7.2 were considered to be the most potent costimulatory molecules and induction of enhancement of the expression of these molecules might be viewed as an important parameter in the elicitation of the pathogen reactive protective effector cells. Blocking B7.1 interactions during T cell activation induced functional inactivation of Th1 cells, leading to a state of hyporesponsiveness or anergy (Schwartz 1992, Chen and Nabavi 1994). Further, B7.1 knockout mice demonstrated significantly reduced immune responses (Freeman et al. 1993a).

Kuchroo et al., reported that B7.1 might be involved in inducing T cells to become IFN-γ producers, while B7.2 enhanced the production of IL-4 (Kuchroo et al. 1995). Freeman et al. demonstrated that B7.1 and B7.2 costimulation mediated distinct outcomes, since B7.2 provided initial signal to induce naïve T cells to become IL-4 producers, thereby directing the immune response more towards Th0/Th2, whereas B7.1 was a more neutral differentiation signal (Freeman et al. 1995). We found that enhanced B7.1 or B7.2 expression induced by AMTV, SMTV and XMTV immunization did not interfere with IFN-γ secretion. Demangel et al. demonstrated similar results when they
immunized the animals with BCG-infected DC. This effect was also reported in the case of another intracellular pathogen, *Toxoplasma gondii*. Both B7.1 and B7.2 were upregulated upon infection of human monocytes with *T. gondii*, but an early production of IFN-γ was observed (Subauste et al. 1998). Cheadle et al. observed that blocking the costimulation through B7.1 and B7.2, using blocking antibodies, significantly reduced the secretion of IFN-γ by the T cells co-cultured with BCG exposed DC (Cheadle et al. 2003). Hence, the augmented expression of B7.1 and B7.2 in the vaccinated groups of mice suggested a robust Th1 kind of immune response.

We also studied the expression of CD40 on the splenocytes isolated from vaccinated and control animals. We demonstrated high expression of CD40 in vivo (44.5% in case of splenocytes from AMTV group), which further increased upon in vitro stimulation with PPD (102.91%, an increase of 131%). Interaction between the CD40 receptor on APC and its ligand (CD40L) on activated T cells played a critical role in immunity to intracellular pathogens by up-regulating the production of IL-12 (Grewal et al. 1997). CD40- or CD40L-deficient mice showed an increased susceptibility to leishmanial infection and also an impaired priming of Th1-type cells, correlating with a lack of activation of the macrophage effector functions required for parasite clearance (Campbell et al. 1996, Kamanaka et al. 1996, Soong et al. 1996). Demangel et al. demonstrated that CD40-stimulated BCG-infected DC displayed increased capacity to release bioactive IL-12 and to activate IFN-γ producing T cells in vitro (Demangel et al. 2001). Therefore, the augmented CD40 expression in vivo in our study suggested enhanced immune responses to mycobacteria.

Recent studies by Leemans et al. identified CD44 as a new macrophage-binding site for *M. tuberculosis* that mediated mycobacterial phagocytosis, macrophage recruitment and protective immunity against pulmonary tuberculosis (Leemans et al. 2003). CD44−/− macrophages reflected reduced binding and internalization of the mycobacteria. Further, CD44−/− mice displayed a decreased survival and an enhanced mycobacterial outgrowth in lungs and liver during pulmonary tuberculosis (Leemans et al. 2003). We also looked into the status of expression of CD44 on the splenocytes isolated from the vaccinated and control animals. We observed augmented expression of CD44 on the splenocytes from the vaccinated animals. We further gated the splenocytes based on their forward and side scatter heights and checked the expression on the monocytes zone of cells and found that number of cells expressing high levels of CD44
were increased upon *in vitro* stimulation with PPD. Our study suggested a potential role of CD44 in anti-mycobacterial immunity.

CD44 is a member of the hyaluronate receptor family of cell adhesion molecules, which had been shown to play a selective role in controlling lymphocyte migration (DeGrendele et al. 1997, Camp et al. 1993). CD44 was expressed on hematopoietic cells and was linked to cytoskeletal elements like hyaluronic acid, collagen, fibronectin, and osteopontin (Goodison et al. 1999). It was necessary for the extravasation of activated T cells into inflammatory sites (DeGrendele et al. 1997), but it was not required for normal leukocyte circulation (Camp et al. 1993). In vitro experiments suggested that CD44 was also involved in cytoskeleton-dependent *phagocytosis* of heat-killed *Staphylococcus aureus* by polymorphonuclear cells (PMNs) (Moffat et al. 1996) and in phagocytosis of apoptotic PMNs by human monocyte-derived macrophages (Hart et al. 1997). A potential role for CD44 in the immune response to *M. tuberculosis* was suggested by the observation that CD44\textsuperscript{high}-expressing T cells (memory T cells) accumulated in the lungs of mice during infection with this pathogen (Laydova et al. 1998, Feng et al. 1999, Feng et al. 2000). CD44 expression was high on activated cells, as its expression on the cell surface was activation dependent. Its augmented expression on the surface of cells of vaccinated mice indicated that effector cells were generated *in vivo* upon vaccination. The effect was further increased upon *in vitro* stimulation with PPD. This suggested that our vaccine effectively generated the pathogen reactive effector cells *in vivo*, such that they could get into action as soon as they come across the pathogen or its derived antigen.

It was interesting to note very high expression of ICAM-1 on macrophages as a result of vaccination. Further, not only the expression was enhanced but also the percentage of cells that expressed ICAM-1 was increased upon stimulation with PPD. Studies by Igietseme et al. using ICAM-1 knockout (ICAM-1\textsuperscript{−/−}) mice showed that ICAM-1 was crucial for rapid T-cell activation, early recruitment and control of genitally acquired *Chlamydia trachomatis* (Igietseme et al. 1999). Chirathaworn et al. have indicated that ICAM-1 could act like a second signal for T cell activation and induce secretion of Th1 cytokines (Chirathaworn et al. 2002). Moreover, Saunders et al. proposed that ICAM-1 might be playing an important role in containing the infection, formation of granuloma and dissemination of the bacteria (Saunders et al. 1999). The increase in ICAM-1 expression on macrophages could be a response of the host to the invasion by mycobacterium or it could be induced by mycobacterium for its own benefit. We proposed that ICAM-1 was enhanced by the mycobacterium on the surface of
macrophages to use it as an anchoring residue to attach to the surface of the macrophage and might utilize it for invasion. The reason for our belief was that theoretical modeling showed binding affinity of ICAM-1 with prolly lipopeptide of \textit{M tuberculosis}. The function of prolly lipopeptide was still unknown. Further, the ligand of ICAM-1, LFA-1 had sequence homology with p450 haem thiolate protein of \textit{M tuberculosis}. Thus our finding of high expression of ICAM-1 induced by mycobacterial infection might be very well correlated with the invasion strategy adopted by the pathogen. However, the increase in ICAM-1 expression post-immunization and upon \textit{in vitro} stimulation of the splenocytes by PPD as well suggested that it might be a response of the host to induce a protective immune response against the pathogen. It could be suggested that ‘activated’ macrophages might be using ICAM-1 to capture mycobacteria and eradicating it from the system. The role of increased ICAM-1 expression however, still needs to be critically elucidated.

We started the study with the expectation that vaccine comprising of bacteria cultured in allogeneic or xenogeneic macrophages would show more strong induction of Th1 immune response as compared to syngeneic vaccine. But contrary to our belief, we found that all the three vaccination strategies exhibited nearly similar strength of immune response. To unravel the reason behind this, we stained the costimulatory molecules expressed on the infected cells before injecting them into the animals. To our surprise we found that both AMTV and SMTV expressed very high levels of B7.1, B7.2 and ICAM-1 molecules. Probably, \textit{in vivo} SMTV besides being phagocytosed by DC might also be interacting with the syngeneic T cells and this high expression of costimulatory molecules augmented the activation of antigen specific T cells. Thus generating an equally strong immune response. Demangel et al. had used syngeneic BCG-infected DC for immunizing the animals and they also demonstrated that the infected DC expressed high levels of B7.1 and B7.2 molecules (Demangel et al. 1999). They also noted Th1-like immune response after administration of BCG-infected DC.

We could generate the Th1-oriented immune response by immunizing the animals with our vaccines (irradiated infected-macrophages). We further tested whether the Th1-oriented immune response generated by our vaccine would render protection against the mycobacterial infection? To address this question, the vaccinated and control animals were infected intraperitoneally with H37Rv. It had been shown earlier that in mice and rat model, even if route of infection was non-pulmonary, mycobacterium could still reach lungs where they multiplied preferentially. Further, through intraperitoneal route most of
the mycobacterium gets lodged in the spleen and relatively few reached the lungs (Kubica and Wayne 1984). Thus, the animals were sacrificed and the spleen was taken for organ homogenate plating to enumerate the bacterial load. We found significant (p<0.001) decrease in the number of mycobacteria in the case of vaccinated animals. Intriguingly, even the group immunized with isoniazid treated mycobacterium showed significant decrease in the bacterial load in the spleen. The disparity could be elucidated on the basis that in case of natural infection there was initial increase in mycobacterium specific Th1 cells as was also evident by lympho-proliferation data (Fig 5) and increased secretion of IFN-γ in response to PPD (Fig 6). As reported by Dalton et al. and Das et al. that the increased release of IFN-γ induced apoptosis of mycobacterium reactive Th1 cells (Dalton et al. 2000, Das et al. 1999). Further Baldwin et al. in their work demonstrated that short-term reduction in bacterial counts could not, in fact, be the most important criterion and pathology data of the study model might in fact gave a better picture of the long-term effectiveness of the vaccine (Baldwin et al. 1998). Therefore we also processed the lung and spleen tissue for histopathological studies. The histopathological studies established that animals immunized with isoniazid treated mycobacteria had granulomatous reactions in their lungs and spleen. In other words, mycobacterial infection had caused lesions and tissue destruction as in the case of unimmunized animals. On the contrary, the vaccinated animals had profound lymphoid infiltration surrounding the airways, blood vessels and even in alveolar septa without any destruction of tissue to restrict the growth of mycobacterium. This confirmed that our vaccine were effective in generating an immune response strong enough to keep the mycobacteria from multiplying in vivo.

To further substantiate the effectiveness of our vaccine, we tested it in another model using Salmonella typhimurium. We used irradiated salmonella infected-macrophages to immunize the animals. Then we challenged them with lethal dose of S. typhimurium and monitored the survival of the animals. Interestingly we noticed significantly reduced mortality in the case of vaccinated animals. This further supported our hypothesis, that this approach could also be used for any intracellular pathogen requiring the cell mediated immune response.

DCs comprise a family of APCs that coordinate signals derived from different parts of the immune system. DCs could uptake an array of antigens including microorganisms, extra cellular fluids and apoptotic bodies released by dying cells, which
they process and present in the form of peptides bound to both MHC-I and MHC-II molecules (Mellman and Steinman 2001). After engulfing microbes or apoptotic bodies, DCs undergo a process of maturation and migrate to secondary lymphoid organs where they could stimulate naive T cells. Dendritic cells were known to control the outcome of the immune response by determining which class of effector T cells was induced (reviewed by Palucka and Banchereau 2002). Dendritic cells could drive Th1 responses against intracellular pathogens and viruses or Th2 dominated response to extra cellular pathogens, including parasites (Mellman and Steinman 2001). Thus targeting antigens directly to DCs might generate appropriate immune response leading to a breakthrough in the development of vaccine.

Several studies have been published using DC-based immunotherapy, mostly in cancer patients (Fong and Engelman 2000) with melanoma, myeloma, lymphoma, prostate, renal cell, ovarian, breast or colon cancer. Evidence of clinical improvement (e.g. regression of metastases) and enhanced T cell immunity (antigen specific proliferation and delayed type hypersensitive reactions) were obtained in some cases, even in late stage of disease. Demangel et al. infected DC with live BCG and used them for immunization of mice (Demangel et al. 1999). They found encouraging results regarding protection against *M. tuberculosis* H37Rv in aerosol model of infection. Pombo et al. used *Plasmodium falciparum* infected RBCs for immunizing the patients and found induction of strong cell-mediated immune response (Pombo et al. 2002). It was reported that DC preferably and efficiently phagocytose cells undergoing apoptosis and cross present the internalized antigens in context with MHC-I as well as MHC-II molecules (Morrissette et al. 1999). Zanten et al. showed that DC efficiently phagocytosed viral protein pp65 transfected cells induced to undergo apoptosis by ultra-violet B (UVB) irradiation, and induced a CD4+ and CD8+ T cell response specific for the pp65 protein (Zanten et al. 2002).

Keeping in view of the above mentioned facts we hypothesized a model (Fig 17) where the animals were immunized with irradiated allo-macrophage tuberculosis vaccine (AMTV), comprising of H37Rv cultivated in MHC-mismatched-macrophages to induce desired Th1 kind of cell mediated immune response specific against mycobacterium. The induction of protective immune response could be elucidated on the basis, that the irradiated cells undergo apoptosis (Zitvogel et al. 1996). The apoptotic cells were phagocytosed by DC in vivo, which then evoked antigen specific CD4+ Th1 cells. Allogeneic immunization also activated alloreactive T cells that produced high level of
Allogeneic vaccine

1. *In vitro* 

Immunize

Macrophage infected with bacteria

Allo-MHC

Gamma-irradiated cells undergoing apoptosis

TCR-MHC-Ag peptide complex

IL-2, IFN-γ

2. *In vivo*

a) Allo-stimulation

b) Apoptotic cells phagocytosed

Dendritic cell

CD8+ T cells

Ag Depots

IFN-γ IL-2

Ag-specific naive T cell

Dendritic cells also secrete IL-12 and are the only potent APC that can activate naive T cells and IL-12 skews them towards Th1 polarity.

3. Generation of effector cells

Clonal proliferation of Ag-specific Th1 cells

Fig 17: The model explaining the induction of protective immune response upon immunization with the irradiated preparation of allogeneic vaccine i.e. bacteria infected allogeneic macrophages. 1. The figure shows that the mice were immunized with the bacteria cultured in allogeneic macrophage. 2. In vivo it can stimulate the immune system in two ways: a) Allogeneic immunization activates alloreactive T cells that produce high levels of IL-2 and IFN-γ, these cytokines help in growth and proliferation of effector T cells. b) The irradiated cells that are injected undergo apoptosis. Dendritic cells engulf the apoptotic cells, which then evokes antigen specific CD4+ and CD8+ T cell response. Dendritic cells also secrete IL-12 and are the only potent APC that can activate naive T cells and IL-12 skews them towards Th1 polarity. 3. Pathogen specific cell mediated immune response is induced.
IL-2 and IFN-γ (Mozzanica et al. 1995). IL-2 helped in engineering the growth of antigen specific T cells (Mozzanica et al. 1995).

The rationale behind using infected macrophage was derived from the fact that almost one third of the world’s population harbor mycobacterium in their body but only 5-10% developed active TB. Nearly 90% of the individual develop effective and long-lasting immunity against the pathogen. There might be a possibility that mycobacterium residing in the host macrophages were secreting unique antigens, which were the effective inducers of long lasting protective immunity in 90% of the infected population. Thus by using infected macrophages; we would be using the unique repertoire of the antigens that had been expressed in the host cells which otherwise might not be secreted by the *M. tuberculosis* when cultured *in vitro*. The basis for using irradiated allogeneic macrophages was that these allo-cells would function as a unique system for delivering antigens secreted by live mycobacterium within the macrophage to DC. Moreover, no adjuvant was required because the allo-cells themselves acted as an adjuvant for eliciting the secretion of cytokines. The alloreactive T cells were known to produce high amount of IL-2, which was a growth factor for promoting the proliferation of T cells.

This study could generate enough proof to accept our hypothesis of directly targeting the DCs *in vivo* with the unique repertoire of the antigens by using infected macrophages, which were induced to undergo apoptosis by gamma-irradiation. Gamma-irradiation was known to induce apoptosis in the cells (Zitvogel et al. 1996). The novel approach of using infected allogeneic macrophages, which were induced to undergo apoptosis, as vaccine fulfilled the requirements necessary for generating a favorable Th1-oriented immune response against intracellular pathogens like mycobacterium and salmonella. As explained in Fig 17, the infected allo-macrophages activate alloreactive T cells *in vivo* that produced high amount of IFN-γ and IL-2. Apoptotic bodies from these infected macrophages were engulfed by DCs and were processed and presented to naïve T cells. Thus generating an environment favorable for the desired Th1 kind of immune response. It was thus anticipated that such preparation should work as an effective vaccine against intracellular pathogens like mycobacterium, salmonella etc. Moreover, allogeneic vaccine was a promiscuous vaccine, since it did not followed the rules of MHC-restriction because it was based on allo-stimulation and engulfment of foreign apoptotic cells by dendritic cells. The vaccine should thus also work in the case of human volunteers.