DISCUSSION
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Since antiquity, tuberculosis remains a major threat to public health. *M. tb*, the causative agent of the disease has evolved along with human since the dawn of civilization. Meliorated standard of living and improved sanitation reduced the preponderance of the disease even before the advent of chemotherapy. With the introduction of TB chemotherapy and BCG vaccine the prevalence of disease was further reduced. However, even after 80 years of widespread usage of BCG vaccine, tuberculosis still remains a leading infectious cause of death worldwide. The most controversial aspect of this vaccine is its variable efficacy in different clinical trials. Ever since it's early use, the efficacy of the BCG vaccine has been questioned. The scenario is further exasperated with the advent of HIV co-infection and emergence of drug resistance due to non-compliance to long term anti-tuberculosis drug therapy. Keeping in view this devolving situation, World Health Organization (WHO) declared TB a global emergency in 1993 (World Health Forum; 1993).

Although TB can be effectively cured with the current chemotherapy, the six months tenure for treatment of the disease is too long, and often leads to significant toxicity. These facts, drive us to an apparently daunting task, to find a better way to cure TB and to replace age old therapy with a better substitute. Another promising new way to effectively treat tuberculosis is to supplement the current anti-TB drug treatment with a new strategy that could induce a strong immune response with the potential for developing long-lasting immunity against re-infection by *M. tb*.

The initial wave of protective immunity against *M. tb* involves the generation of T helper type 1 (Th1) CD4+ T cells, which secrete interleukin 2 (IL-2) and gamma interferon (IFN-γ) (Barnes, Abrams et al. 1993; Orme, Andersen et al. 1993).

Reduction in the number of activated T cell and their suppressed proliferation in response to *M. tb* infection cells might be one of the potent reasons for prevention of immune cells to attain microbicidal action. Th1 cell have been reported to be suppressed by cells like regulatory T cells (Treg) and mesenchymal stem cells (MSCs) (Thornton and Shevach 1998; Bartholomew, Sturgeon et al. 2002; Di Nicola, Carlo-Stella et al. 2002.

MSCs are multi-potent stromal cells that are generally found in the bone marrow. These cells possess remarkable immunosuppressive properties and can inhibit the proliferation and function of T cell which make them an ideal candidate for clinical application as immunosuppressant. The immunomodulatory effect of MSCs is attributed by a non-specific anti-proliferative action of these cells, that mainly
dependent on cell–cell contact or secreted soluble factors such as indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), nitric oxide (NO), histocompatibility leucocyte antigen-G (HLA-G), transforming growth factor (TGF)-β, interferon gamma (IFN)-γ and interleukin-1β.

One of the initial observations during the study was that the proliferation of T cells varied among healthy contacts and patients. Proliferation of T cells from infected mice when stimulated with Concanavalin A in vitro were selectively and significantly reduced as compared to the healthy control. More importantly, change in the pattern of proliferation of T cells was not an intrinsic property of T cells rather this difference was induced by some accessory cells in the spleen of infected mice. While investigating the identity of these accessory cells, we observed increasingly larger number of Sca1+ cells in infected spleen which is indicative of a different ongoing immune response in the host. Infected mice had higher number of Sca1+ cells compared to healthy subjects. Since activated lymphocytes also express Sca1+ on their surface we gated on Sca-1+B220+CD3+ population of cells and characterized them. They all were CD29; CD44, and Flk-1, but did not express CD34, CD45, CD11b, CD11c, and Gr1. This surface phenotype is characteristic of MSCs. Due to absence of lineage specific markers, MSCs were further trans-differentiated into adipocytes where fat globules deposited within the cells and were detected by oil red staining thus assuring that they are bona fide MSCs.

Recently, it has been documented that the immunomodulatory effect of bone-marrow-derived MSCs suppressed T-cell proliferation. These studies directed our attention towards the characterization of the their immunoregulatory activities.

T cells proliferation involving T cell receptor (TCR) engagement with polyclonal mitogens, is inhibited by MSCs. Numerous studies have demonstrated that MSCs isolated from humans, baboons and mice can suppress the T lymphocyte proliferation. The inhibition in proliferation is induced by alloantigens, mitogens, anti-CD3 and anti-CD28 antibodies in vitro. MSCs have a similar effect on memory and naive T cells, as well as CD4+ and CD8+ T cells. In addition, this suppressive effect did not require major histocompatibility complex (MHC) restriction and could also be mediated by allogeneic MSCs. This might be due to inhibition of cell division, as evidenced by the accumulation of cells in the G0/G1 phase of the cell cycle.

To further assure that unresponsiveness in T cell was due to some accessory cells and not the intrinsic property of T cells, we depleted MSCs from the splenocytes of infected mice and moc treated the splenocytes from uninfected mice that were then stimulated with Con A and subjected to proliferation assay. Moc treatment of splenocyte from uninfected mice was done so as to reduce any error in difference in proliferation which might be due to treatment of splenocytes with LASER in infected animal. The
MSCs negative population of cells from both infected and uninfected mice had comparable proliferation profile. This clearly suggested that MSCs play a crucial role in inhibition of proliferation of T cell in tuberculosis disease model.

Although a large number of previous studies have documented the immunosuppressive activities of MSCs, the underlying mechanisms are only partially known. Contact-dependent mechanisms and soluble factors are thought to collaborate for the induction of MSC-mediated immunosuppression. The first step in the interaction between MSCs and target cells involves cell–cell contact mediated by adhesion molecules, as indicated by studies showing that the inhibition of T-cell proliferation by MSCs requires engagement of the inhibitory molecule PD1 by its ligands. In principle, from a clinical perspective, excessive inhibition of T-cell responses by MSCs would lead to global weakening of host immune response and render the host vulnerable to infectious agents. However, there might be existence of fail-safe mechanisms; for example, TLRs on MSCs on interaction with pathogen-associated ligands, induce their proliferation, differentiation and migration and induce secretion of chemokines and cytokines, and it has been shown that MSCs did not inhibit the T-cell proliferation triggered by TLR3 and TLR4 followed by impaired Notch signaling. Therefore, there is a fair possibility that pathogen associated molecules might reverse the suppressive effects of MSCs on T cells, thereby restoring efficient T-cell responses against pathogens.

MSCs mediated immunoregulation is basically due to release of soluble factors like nitric oxide and indoleamine 2, 3-dioxygenase (IDO), secreted by MSCs in response to IFN-γ produced by target cells. Soluble factors released are either produced constitutively by stem cells or released after contact with target cells. The contact between MSCs and target cells seems to be of foremost importance for such immune suppression. Also, the immunosuppressive activities are mediated by various soluble factors. These soluble factors are nitric oxide, indoleamine 2,3-dioxygenase (IDO), Transforming growth factor-β1 (TGFβ1), hepatocyte growth factor (HGF), IL-10, PGE-2, haemoxigenase-1 (HO-1), IL-6 and soluble human leucocyte antigen-G5, are constitutively produced by MSCs. Hence contact-dependent mechanisms and soluble factors are thought to collaborate for the induction of MSC-mediated immunosuppression. Furthermore, inducible nitric-oxide synthase (iNOS), inhibits T-cell activation through the production of nitric oxide. Cell contact between MSCs and activated T cells induces IL-10 production, which, in turn, has an essential role in stimulating the release of soluble human leucocyte antigen-G5 by MSCs. However, inhibition of any one of these molecules does not result in a complete loss of the immunosuppressive activity of MSCs, and their relative contribution to the immunosuppressive effects varies.
The production of nitric oxide by MSCs has also been implicated as a potential mechanism by which MSCs inhibit T cell proliferation. NO inhibits the proliferation of T cells by suppressing the phosphorylation of signal transducer and activator of transcription-5 (STAT5), a transcription factor crucial for T cell activation and proliferation. Taking these facts into account, NO content in the supernatants of splenocyte cultures from both infected and uninfected mice was analyzed. A dramatic increase in NO content was observed in supernatant from infected mice as compared to control uninfected mice. This lead us to infer that MSCs in the infected organs are induced to produce enhanced amount of nitric oxide after being triggered by IFN-γ producing target cells. To further assure that nitric oxide is playing a crucial role in suppression of T cell proliferation, we performed splenocyte proliferation experiments in the presence of the iNOS inhibitor L-NMMA, which revealed that NO inhibition drastically reversed the proliferative defect of T cells from infected mice clearly indicating that although nitric oxide may not be the exclusive soluble factor responsible for MSCs induced suppression of T cell proliferation but has a major impact on its immunomodulation.

To further strengthen our observation that it is MSCs that are responsible for the observed inhibition of ConA-induced proliferation of splenocytes from *M. tb* infected mice, we removed Sca-1⁺CD29⁺CD44⁺ cells from splenocytes of infected mice. The resultant cells exhibited similar proliferative responses to ConA as splenocytes from uninfected animals. Furthermore, it was observed that MSC-depleted splenocytes produced dramatically lower levels of NO, as comparable to uninfected control mice. Therefore, finally we concluded that MSCs inhibit T cell proliferation by producing NO. Also previously it has been reported that the production of nitric oxide by MSCs is a potential mechanism by which MSCs inhibit T cell proliferation. NO inhibits the proliferation of T cells by suppressing the phosphorylation of signal transducer and activator of transcription-5 (STAT5), a transcription factor crucial for T cell activation and proliferation.

Earlier studies have suggested that NO plays a critical role in the host resistance against tuberculosis. Previous study have documented that iNOS-deficient animals exhibit an aggravated susceptibility to *M. tb* infection. However, these studies were mainly carried out during the early phase of the infection when immune responses are largely confined to components of the innate immune system. Nevertheless, it has been reported that low exposure to NO inhibits bacterial replication, but cannot eliminate the *M. tb* organisms. NO induces apoptosis in T cells and plays a significant role in the immunosuppression during infection by some other microbial pathogens.

From a clinical perspective, excessive inhibition of T-cell responses by MSCs would lead to global weakening of host immune response and therefore render the host vulnerable to infectious agents. *M. tb*
infection induces strong delayed type hyper-responsiveness (DTH) in the periphery in animals as well as in humans. Therefore, we tested the presence of MSCs and *M. tb* in the peripheral lymph nodes (LN). We could not find MSCs or live *M. tb* in the peripheral LNs. This observation helped us to conclude that MSCs are recruited to only those organs where *M. tb* is already present. Next, we tested whether *M. tb* infection results in general immune suppression in the periphery. For this purpose, we immunized infected or uninfected mice with ovalbumin (OVA) emulsified in alum. Draining LNs were harvested and the lymphocytes obtained were challenged *in vitro* with OVA. We did not find any noticeable difference in the antigen-specific immune responses among infected and uninfected mice indicating the absence of global immune suppression. Hence immune suppression takes place only in those organs where *M. tb* has facilitated the recruitment of MSCs which thereby inhibit T cell proliferation. Interestingly, we also found dramatic *M. tb* specific proliferative responses in LNs of infected animals clearly indicating development of robust host immune response. Therefore, *M. tb* induced immunosuppression is limited to the infected organs, and MSCs are responsible for these activities.

Since contact of MSCs with target cells is mandatory for immune suppression it was necessary to investigate the physical location of MSCs. We found that MSCs surround the granuloma-like structures with acid fast bacilli using immunohistochemistry staining anti Sca-1 antibody in mice and anti CD29 antibody in human tissue biopsy samples. To further confirm that MSCs are recruited to the site of infection, we isolated MSCs from *M. tb* infected animals by FACS sorting, labeled with CFSE, and adoptively transferred to infected syngenic animals. Interestingly, we found that MSCs trans-migrated to the periphery of granulomas. Therefore, MSCs create an immunosuppressive barrier around the *M. tb* organisms. Production of NO requires two simultaneous signals: recognition of pathogen-associated molecular patterns (PAMPs) together with signaling mediated by IFN-γ, a product of activated T cells.

The close proximity of MSCs to both live *M. tb* bacteria and IFN-γ producing T cells generates an environment conducive to NO production by these cells. Thus, MSCs create a dynamic equilibrium that inhibits both the pathogen and *M. tb*-reactive T cells. In a scenario where this equilibrium becomes perturbed, such as during HIV infection, IFN-γ-producing T cells might become depleted. In turn, MSCs might fail to produce NO, leading to uncontrolled replication of the *M. tb* organisms and progression towards active tuberculosis.

In response to high concentrations of IFN-γ, MSC are induced to produce indoleamine 2,3-dioxygenase (IDO), the enzyme known to promote the immunosuppressive barrier at the maternal-fetal interface. Also, MSC treatment with IFN-γ *in vitro* has been observed to enhance MSC production of several immunosuppressive cytokines such as TGF-β. Hence it is well documented that IFN-γ alone induces
production of several immunosuppressive molecules by MSCs which inhibits T cell proliferation. To further investigate the role of IFN-γ in the inhibition of T-cell responses by inducing NO production. We observed large amount of IFN-γ in the supernatants of cultures of total mononuclear cells or T cells isolated from M. tb-infected spleens, activated with ConA or plate bound anti-CD3 and anti-CD28, respectively as compared to the uninfected control mice. However, IFN-γ neutralization with anti IFN-γ antibody dramatically inhibited NO production and promoted splenocyte proliferation. Thus, our findings suggested that MSCs create a dynamic equilibrium that inhibits both the pathogen and M. tb-reactive T cells.

As activities of MSCs are exerted by NO, we next determined whether NO has any role in the accumulation of MSCs surrounding the granulomas. MSCs from iNOS2−/− animals which were adoptively transferred to infected wild type animals migrated to the granulomas, but in a significantly lesser extent as compared to migration of MSCs from wild type mice to wild mice. Quantification of MSCs that were recruited to the periphery of granuloma clearly indicated that significant role is played by nitric oxide in localization of MSCs in infected organs. We later also determined the influence of MSCs derived NO in the growth of M. tb in vivo. To do so, MSCs were isolated from wild type or iNOS2−/− mice and adoptively transferred to infected iNOS2−/− or wild type animals respectively. Interestingly, there was no difference in CFUs in spleens in wild type animals that received MSCs from iNOS2−/− animals. In contrast, iNOS2−/− animals that received MSCs from wild type animals exhibited a significant reduction in CFUs. However, the differences in CFUs in these mice were diminished over a period of time. Taken together these observations suggested that a barrier between M. tb and the T cells establishes an equilibrium between host and M. tb, in which MSCs limited the growth of M. tb and simultaneously kept T cells at the bay preventing their access to pathogen within granuloma.

To investigate the relevance of MSCs to the pathogenesis of M. tb we isolated MSCs from infected mice and adoptively transferred these cells to a group of naive syngeneic animals which were then infected with live M. tb organisms. The bacterial loads in the spleens of animals increased significantly during the initial phase of infection when infused with MSCs as compared to the control. However the difference gradually diminished over a period of one month. This finding is consistent with the notion that MSCs assist the host in establishing equilibrium between the pathogenic microorganisms and the immune response. Therefore, depletion of MSCs could represent a potentially novel method for the treatment of tuberculosis. However, this approach is currently limited by the absence of antibodies that can specifically deplete MSCs in vivo.
Previously, it has been shown that Mycobacterium bovis induces production of copious amounts of IL-6 in bone marrow-derived macrophages, which inhibits antigen-specific T-cell proliferation in vitro. However, in M. tb-infected spleen cell cultures very small amounts of IL-6 was produced. Addition of anti-IL-6 was unable to overcome the blockade in splenocyte proliferation. Similarly, purified MSCs failed to induce substantial amounts of IL-6 production in T-cell co-cultures. These findings suggested that immune suppression induced by MSCs during M. tb infection is largely independent of IL-6.

Animal models of infection often do not fully reflect the natural course of infection in humans. Therefore, we investigated the presence of MSCs in surgical biopsies of lesions from patients with active tuberculosis harboring M. tb bacteria. Because Sea-1 is not expressed by human MSCs, we identified these cells using the CD29 marker. We found that cells isolated from M. tb-infected lesions contained large numbers of CD29+ cells. Histological analysis of human granuloma samples revealed the presence of MSCs surrounding the granuloma-like structures. As expected, lymphocytes obtained from the granuloma-containing lymph nodes from tuberculosis patients did not proliferate in response to T cell mitogens. In contrast, LN cells from control patients with suspected lymphadenopathy exhibited normal proliferative responses to the T cell mitogen.

On the basis of the above observations, role of MSCs have been used for the first time in evading immune response in tuberculosis. Systemic infusion of allogeneic MSCs has also led to encouraging results in animals with M. tb infection. In vivo immunosuppressive effect of infused MSCs has been successfully shown. Modulation of immune response by MSCs might led to accelerated bacterial count in infected animals cotransfused with MSCs during initial phase of infection but during later coarse of infection, introduced MSCs has little effect on bacterial load as the difference of CFU diminished when compared to the control.

The immunomodulatory potential of MSCs can be tested for the treatment of tuberculosis, where these cells surround the bacteria entrapped in granuloma or granuloma like structure. It could also contribute to a promising new strategy for treatment of tuberculosis. Treatment strategies that target MSCs should provide several advantages over conventional antibiotic therapy. Firstly, depletion of MSCs should permit the immune system to eradicate the harbored M. tb organisms. Secondly, targeting of MSCs will not involve any physical association of drugs with the organisms, hence avoiding the potential to generate treatment-resistant variants of the bacteria. Thirdly, removal of MSCs might induce a strong immune response with the potential for developing long-lasting immunity against re-infection by M. tb.

Overall, the current data indicate that although MSCs were first proposed for therapeutic purposes in regenerative medicine on the basis of their stem-cell-like qualities, their therapeutic effect can result from
other characteristics, such as their antiproliferative and anti-inflammatory properties. MSCs seem to nonspecifically target cells of the immune system. Ultimately, MSCs provides a tool for inducing peripheral tolerance due to their immunosuppressive activity. This is subsequent to the systemic injection of MSCs and seems to depend on the capacity to “wall off” immune competent cells. They do so by making cells quiescent through the inhibition of cell division, thereby preventing their responsiveess to antigenic triggers. Moreover, the evidence of clinical efficacy of MSCs in different experimental models is almost only during the acute phase of disease. There are limited evidences of transdifferentiation indicating that the therapeutic effectiveness of MSCs relies heavily on their ability to modify the microenvironment of injured tissues. The repair and protection of damaged tissues is supported by the release of anti-inflammatory cytokines, and anti-apoptotic and trophic molecules. So, the plasticity of MSCs in therapeutics might be seen as a recapitulation of the physiological activity of stromal cells in the Hematopoietic Stem Cell (HSC) niche.

The ultimate outcome of the immunomodulatory activity of MSCs seems to be highly influenced by the microenvironmental signals that are encountered following *in vivo* administration of these cells. Indeed, the microenvironmental niche decides the final effect of MSCs on target cells. There are multiple evidences where the opposite outcomes arise due to interaction of MSCs with DCs and NK cells in the presence of different (high or low) concentrations of IFN-γ.

As far as, the clinical perspective is concerned, the most important issue remains to look into the immunogenecity of MSCs. This will address its application in allogeneic environment... The mechanism of how the MSCs administration affect physiological immune response; like activation, proliferation and differentiation of different immune cells in the host. Elucidation of the vital molecular pathways that get activated in MSCs by environmental triggers is yet an important facet yet to be studied. Another important fundamental question involves whether, after *in vivo* administration, MSCs can engraft into tissue inside the tissue whether the MSCs can exert their bystander effects inside ectopic niches, as has been observed for neural stem cells. Previous studies, have already reported the persistence of MSCs *in vivo* in tissues where MSCs effectively evade host immune surveillance. This behavior could be attributed to ‘touch and go’ mechanism where, their rapid migration to the damaged organ and subsequent clearance is followed by the release of stress-induced therapeutic molecules.

Despite our limitations in the existing knowledge of this matter, property of MSCs to exert their therapeutic potential through bystander mechanisms might indicate that persistent engraftment at the site of damage is not a mandatory prerequisite for having an effect on injured cells. The final issue of utmost
importance concerns the safety issue of injected MSCs. An understanding of these concerns will allow for the translation of our basic knowledge of MSCs biology into the design of clinical therapies.