GENERAL DISCUSSION
Recently, it has been established that the interaction between the immune cells and the cancer cells at the tumor microenvironment is an important determinant for tumor progression in a host. Among the different tumor microenvironment residing immune cells, macrophages and T regulatory cells (Tregs) are found to be crucial regulators of malignant progression for most of the solid tumors. Therefore, in this study, we have first shown the mechanism of alternative polarization of tumor associated macrophages (TAM). In addition, we have determined how Tregs influence malignant progression of solid tumors and finally we have designed a novel immunotherapeutic strategy where established tumors can be eliminated by modulation of both the TAM and Tregs at the tumor microenvironment. All the studies were carried out in B16F10 melanoma model since; it is a highly metastatic, poorly immunogenic murine tumor model and very difficult to treat once it is established. Therefore, we performed our studies in this tumor model with an aim that any therapy that works against this highly metastatic tumor may be successfully employed against other less aggressive tumors.

The first section of our study was aimed to determine the molecular mechanism of alternative activation of TAM at the tumor microenvironment. It was observed that TAM produced high level of anti-inflammatory mediators (IL-10 and TGF-β) instead of producing pro-inflammatory mediators (IL-12, IFN-γ and nitric oxide) following stimulation with their classical activator, LPS. LPS is a TLR-4 ligand, therefore, it was hypothesized that TAM might exhibit an alternative TLR signaling pathway. All TLRs elicit their signal in one of the two different intracellular pathways – MyD88 dependent pathway or TRIF dependent pathway. Interestingly, it was found that TAM exhibited a defective MyD88 dependent pathway and the expressions of the MyD88 downstream signaling molecules (IRAK-1 and TRAF-6) were found to be severely down-regulated in TAM. Moreover, a specific negative regulator (IRAK-M) of the MyD88 dependent pathway was found to be very much augmented in TAM. However, TAM exhibited an alternative
TRIF dependent pathway to enhance ERK-1/2 MAP kinase activation which led to high level of anti-inflammatory mediator (IL-10 and TGF-β) production and elevated IRAK-M expression.

The next section of our study was aimed to examine the involvement of Tregs during tumor metastasis initiation. Tregs can be broadly classified into thymic or natural Tregs (nTregs) and adaptive or induced Tregs (iTregs) depending upon their site of origin and they can be differentiated with respect to neuropilin1(Nrp1) surface expression. The nTregs express high level of Nrp1 whereas; iTregs exhibit lower level of Nrp1 expression. It was found that Nrp1\textsuperscript{low} iTreg frequency was markedly upregulated in the metastatic lung due to enhanced iTreg differentiation at the mediastinal lymph node (mln) of metastatic tumor bearing mice. Moreover, it was found that mln macrophages from metastatic tumor bearing host predominantly induced iTreg differentiation in a B7-H4 dependent and MHC-II restricted manner. These iTregs exhibited enhanced CCR4 expression and inhibition of CCR4 with a recombinant immunotoxin (TARC-PE38) resulted marked reduction in lung metastasis. However, the recombinant immunotoxin affected both the iTregs as well as nTregs. Therefore, it was essential to specifically block the generation of the iTregs for examining their involvement in the metastasis initiation process. In this regard, inhibition of B7-H4 expression in mln macrophages of metastatic tumor bearing mice could be helpful. Interestingly, IL-10 and TGF-β were found to be the crucial regulators of B7-H4 expression on macrophages and accordingly blocking both of these cytokines with their respective neutralizing antibodies inhibited lung metastasis.

Finally, our study was aimed to develop an immunotherapeutic strategy against established tumors by modulating both the TAM and Tregs at the tumor microenvironment. Thus, two immunomodulators were selected for our study– the heat-killed \textit{Mycobacterium indicus pranii} (\textit{Mw}) was chosen for its profound immunomodulatory action on macrophages and the GITR agonistic antibody, DTA-1, was considered due to its well-known Treg suppressing function. Initially, the immunomodulatory potential of \textit{Mw} was utilized to repolarize TAM back to their
normal pro-inflammatory function within the tumor microenvironment. Although, \textit{Mw} induced functional reprogramming of TAM \textit{in vitro} it failed to do so \textit{in vivo}. Later, it was found that \textit{Mw} failed to induce TAM repolarization \textit{in vivo} due to the presence of high number of Tregs at the tumor microenvironment. However, use of \textit{Mw} in combination with DTA-1 induced TAM repolarization and eradicated established tumor. However, only DTA-1 treatment failed to provide efficient protection against established tumor since; it was unable to induce TAM repolarization within the tumor microenvironment.

Thus, the present study for the first time has characterized how immune cell-tumor cell interaction at the tumor microenvironment regulates the growth and immune evasion mechanism of growing solid tumors. Moreover, this study has depicted a mechanism of effective anti-cancer immunotherapy by modulating two important components of the tumor immune microenvironment namely, TAM and Tregs. Therefore, this study has shown a way for development of anti-tumor immunotherapeutic treatment strategies against advanced stage solid tumors by repolarization of one or more immune effector cells at the tumor immune microenvironment.