Chapter 7

Discussion
Towards understanding the early events that ensue following infection by *M. tuberculosis* we have been elucidating the interactions of *M. tuberculosis* secretory antigens with DCs and their outcome on host mediated immune responses. Many *M. tuberculosis* secretory antigens are promising candidates for ‘vaccine’ development and in diagnostics (Andersen, 1994; Roberts *et al.*, 1995; Roche *et al.*, 1994; Brandt *et al.*, 1996; Elhay *et al.*, 1998; Roche *et al.*, 1996; Tanghe *et al.*, 2001). However, despite the large volume of data available on these antigens their physiological role(s) at sites of infection have received scant attention. In view of this, using CFP-10 (also known as MTSA-10) as a model antigen, previous work in the lab showed that these antigens activate DCs by inducing their differentiation from bone marrow precursors (Latchumanan *et al.*, 2002). However, CFP10-DCs induced Th0 responses to mycobacteria in a TGF-β and IL-10 dependent mechanism (Balkhi *et al.*, 2004), indicating that antigens such as CFP-10 downregulate pro-inflammatory responses to mycobacteria following initial activation of DCs. In addition, CFP10-DCs modulated the levels and activation status of key intracellular signaling intermediates such as PKC, calcium ion concentration.

It has been reported that a critical balance between PKC and calcium influence the development of Th subsets, such that a strong PKC stimulation (with PMA) of T cells led to the preferential development of Type 2 effectors, while a strong calcium signal (with ionomycin) in contrast led to the development of Type 1 effectors (Noble *et al.*, 2000). Increased intracellular calcium in peripheral blood mononuclear cells along with high Th1 responses have been reported in BCG-immunized and healthy controls as compared to tuberculosis patients (Talreja *et al.*, 2003), indicating the importance of calcium in governing Th1 responses in tuberculosis. Sinha *et al.* (Sinha *et al*. 2006) have shown that CFP-10 induced increased activation of PKC both during DC differentiation and subsequent activation by *M. tuberculosis* WCL and live *M. bovis* BCG, while GM-CSF-DCs induced a poor activation of PKC. On the other hand GM-
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CSF-DCs mounted good intracellular mobilization of calcium to \textit{M. tuberculosis} WCL stimulation, while CFP10-DCs induced a poor calcium influx. Inhibiting PKC in CFP10-DCs induced Th1 responses to WCL, while activating PKC in GM-CSF-DCs with phorbol esters induced Th2 responses. Interestingly, this differential activation of PKC and calcium in the two DCs was regulated by PI 3-K. Inhibiting PI 3-K in CFP10-DCs, prevented PKC activation. In contrast, inhibiting PI 3-K in GM-CSF-DCs increased PKC activation. Similarly, inhibiting PI 3-K in CFP10-DCs induced Th1 responses while inhibiting PI 3-K in GM-CSF-DCs induced Th2 responses.

On similar lines our lab has also demonstrated that CFP10-DCs serve as depots for the survival of mycobacteria by downregulating oxidative burst following infection with mycobacteria (Sinha \textit{et al.}, 2006). This was achieved at two levels. The first was the increase in the levels of a well-known quencher, namely, superoxide dismutase 1 (SOD1) and the second was the poor induction of calcium influx upon infection. SOD1 serves to absorb reactive oxygen species and converts them into hydrogen peroxide. \( \text{H}_2\text{O}_2 \) in turn is scavenged by peroxiredoxins and converted into water and harmless divalent oxygen. In fact \textit{M. tuberculosis} encodes the mammalian homolog of SOD that are active inside infected macrophages (Flynn and Chan, 2001). Addition of \( \text{H}_2\text{O}_2 \) or increasing \( \text{Ca}^{2+} \) inside CFP10-DCs resulted in better clearance of (Sinha \textit{et al.}, 2006). In fact the effects were better upon increasing calcium influx in these DCs.

Mycobacteria are known to interfere with calcium signaling pathways of the host as they inhibit the increase in \text{Ca}^{2+} \text{ normally observed during phagocytosis}. In addition, live \textit{M. tuberculosis} infected macrophages showed a significant reduction in the amounts of \text{Ca}^{2+} \text{ bound calmodulin and phosphorylated CaMKII} that were associated with the cytosolic face of the phagosomal membrane compared with phagosomes containing dead bacteria (Malik \textit{et al.}, 2001). The delivery of lysosomal components to mycobacterial phagosomes could be blocked by using inhibitors of CaMKII or by chelating cytosolic \text{Ca}^{2+}. These data indicate that pathogenic mycobacteria are able to suppress/modulate the increase in cytosolic \text{Ca}^{2+} \text{ that results during host cell interactions}, and thereby inhibit \text{Ca}^{2+} \text{ signaling pathways}, which would otherwise lead to phagosomal maturation. Calcium plays a key role in regulating many of the modulations that are targeted by \textit{M. tuberculosis} for immune evasion. For example,
calcium regulates the activity of calcinuerin that regulates the expression of coronin-1 on phagosomes thus affecting phagosome maturation (Jayachandran et al., 2007). Upon mycobacterial infection of wild-type macrophages, coronin 1 was found to be essential for the activation of the Ca\(^{2+}\)-dependent phosphatase calcineurin, thereby blocking lysosomal delivery of mycobacteria. In the absence of coronin 1, calcineurin was not activated, resulting in lysosomal transfer and death of internalized mycobacteria (Jayachandran et al., 2007). In addition, calcium concentrations and kinetics directly affect the activation of transcription factors such as NF-AT and NF-kB leading to differential cytokine expression and this governs the quantum and quality of immune responses (Feske et al., 2007). As described earlier, calcium influx in mammalian cells is stage dependent, initiating with the depletion of intracellular stores followed by opening up of various channels across the plasma membrane (Berridge, 1993; Lewis, 2007).

Keeping the above in view we investigated how pathogenic mycobacteria modulate intracellular Ca\(^{2+}\) in immune cells. As discussed in the Review of Literature (Chapter 4) there are two major ports of entry of- store-operated Ca\(^{2+}\) channels (SOCs) and Voltage-Gated Calcium Channels (VGCC). Ca\(^{2+}\) entry through store-operated Ca\(^{2+}\) channels (SOCs) have been the subject of extensive research, since store-operated Ca\(^{2+}\) entry (SOCE) or capacitative Ca\(^{2+}\) entry is considered to be the principal route of Ca\(^{2+}\) influx in non-excitable cells (Parekh and Penner, 1997; Parekh and Putney, 2005). The best characterized SOC channels in lymphocytes are known as ‘calcium release–calcium activated channel’ (CRAC channels). However, the literature is relatively silent on the Ca\(^{2+}\) entry pathway via Voltage-Gated Calcium Channels (VGCC), especially in non-excitable cells like DCs. In electrically excitable cells, voltage-gated channels, including long-lasting L-type and R-type Ca\(^{2+}\) channels are activated by membrane depolarization, and serve as the principal route of Ca\(^{2+}\) entry, thereby controlling a variety of crucial physiological processes. It has long been thought that voltage-operated channels are a characteristic feature of excitable cells. To begin with, in this study, we have demonstrated that dendritic cells express the \(\alpha_{1c}\) (Ca\(_{\alpha 1.2}\)) subunit of L-type VGCC and \(\alpha_{1E}\) (Ca\(_{\alpha 2.3}\)) subunit of R-type VGCC on their cell surface.
To further our understanding on the role of calcium in mediating survival of mycobacteria within DCs, in this study we investigated the role of VGCC. Since GMCSF-DCs induced a robust mobilization of calcium following mycobacterial stimulation in contrast to CFP10-DCs, we blocked L-type and R-type VGCC to ascertain if VGCC(s) was responsible for the induction of calcium. Surprisingly, blocking L-type and R-type VGCC further enhanced the levels of calcium influx in GM-CSF-DCs. We, therefore, carried out similar experiments with CFP10-DCs. Here also blocking L-type and R-type VGCC significantly increased calcium influx in CFP10-DCs. This indicated that L-type and R-type VGCC played a negative/inhibitory role in the induction of calcium in DCs. In order to ascertain whether, blocking with antibodies resulted in neutralization and not activation of VGCC, we did experiments with specific siRNAs. Inhibiting VGCC by siRNAs also resulted in increase in calcium influx thus confirming that blocking VGCC with antibodies indeed caused neutralization and not stimulation of L-type and R-type VGCC. A negative role for L-type VGCC has been previously shown when knockdown of the beta subunit resulted in increased frequency of calcium oscillations leading to increased insulin secretion in beta cells (Berggren et al., 2004).

An intriguing fact was that blocking L-type and R-type VGCC increased calcium influx in both GM-CSF-DCs and CFP10-DCs. This indicated that the poor mobilization of calcium by CFP10-DCs was not a result of differential functioning of L-type and R-type VGCC in the two DCs but could be due to differential expression of the same in the two DCs. Indeed both uninfected and BCG infected CFP10-DCs expressed 50 to 100-fold higher levels of L-type and R-type VGCC when compared to BCG infected GMCSF-DCs. This immediately also shed light on the inability of CFP10-DCs to mount an effective oxidative burst and protective Th1 responses (Balkhi et al., 2004; Sinha et al., 2006).

We had earlier demonstrated that lower production of IL-12p40 together with increased production of IL-10 and TGF-β to be one of the reasons for suppressor responses from CFP10-DCs. Blocking IL-10 or TGF-β or conditioning CFP10-DCs with IL-12 induced Th1 responses (Balkhi et al., 2004). In order to investigate the role of L-type and R-type VGCC in programming CFP10-DCs towards differential cytokine expression from DCs, we investigated the expression levels of Th1/Th2
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genes in DCs following blocking L-type and R-type VGCC. Results showed that blocking L-type and R-type VGCC in both CFP10-DCs and also GM-CSF-DCs increased the expression levels of cytokines and transcription factors that favor Th1 responses, perhaps as a result of increased calcium influx, thus attributing a role for L-type and R-type VGCC in modulating the polarization of DCs. In addition blocking these VGCC also increased the levels of CD86 thus equipping DCs to prime T cells in a better way. To confirm the results obtained from microarray we further showed that blocking L-type and R-type VGCC induced increased activation of NF-κB and also resulted in increased transcriptional activation and protein expression of IL-12p40 leading to increased Th1 responses from CFP10-DCs. Several reports indicate a direct or indirect role for VGCC in regulating the activation of transcription factors. For example, L-type VGCC is essential for the CREB phosphorylation (Rajadhyaksha et al., 1999) and also for the activation c-fos during hypoxia (Premkumar et al., 2000).

One of the downstream effects of T cells activated by DCs is the subsequent activation of infected macrophages (Flynn et al., 2004). This leads to mounting of anti-mycobacterial responses by the macrophages (Flynn et al., 2001). Therefore, to extend the functional effects of blocking L-type and R-type VGCC in DCs, we incubated DC-activated T cells with *M. tuberculosis* infected macrophages and monitored the ability of macrophages to kill or restrict the growth of the intracellular pathogen. Results showed that blocking L-type and R-type VGCC in DCs had far reaching effects wherein T cells activated by such DCs mediated effective clearance of *M. tuberculosis* inside macrophages. In fact the extent of clearance/restriction was similar to that obtained by incubating infected macrophages with IFN-γ. This could be attributed to increased IFN-γ production by T cells following their interactions with L-type and R-type VGCC blocked DCs.

To extend the above results, we investigated the role of L-type and R-type VGCC directly in macrophages and PBMCs. Results clearly demonstrated that blocking of L-type and R-type VGCC in macrophages resulted in a similar increase in calcium influx and also mediated effective killing of *M. tuberculosis*. Intriguingly, the killing was 2-fold higher than that obtained by IFN-γ. This effect of L-type and R-type VGCC blockade was also obtained in human PBMCs, whereby a 2-fold higher killing over that by IFN-γ was obtained. These results have important ramifications on the
role of L-type and R-type VGCC in mediating clearance of established M. tuberculosis infection. It has been argued that M. tuberculosis interferes in killing by IFN-γ using various mechanisms. One of the mechanisms is a marked decrease in IFN-γ induced association of STAT1 with the transcriptional co-activators CREB binding protein and p300 in M. tuberculosis-infected macrophages. This indicates that M. tuberculosis directly or indirectly disrupts this protein-protein interaction that is essential for transcriptional responses to IFN-γ (Ting et al., 1999). Other mechanisms include interfering with downstream signaling effects from the IFN-γ receptor (Kincaid et al., 2003) to downregulating the surface levels of IFN-γ receptor itself on infected macrophages and also in PBMCs of patients with active TB (Singhal et al., 2007). In view of the above reports our results have increased significance since blocking or inhibiting the action of L-type and R-type VGCC could bypass the suppressive effects employed by M. tuberculosis in mediating clearance at the level of IFN-γ.

Towards identifying the mechanisms of action of L-type and R-type VGCC, we explored the effect of blocking these VGCC on caspase activation as a measure of apoptosis. It has been demonstrated that M. tuberculosis prevents apoptosis of infected macrophages thereby using macrophages as a niche and also blocking the presentation of antigens to APCs by infected apoptotic macrophages. Velmurugan et al. have demonstrated that inhibition of infection-induced apoptosis of macrophages is controlled by multiple genetic loci in M. tuberculosis. Anti-apoptosis activity is attributable to the type I NADH-dehydrogenase of M. tuberculosis, and is mainly due to the subunit of this multicomponent complex encoded by the nuoG gene (Velmurugan et al., 2007). To look for the apoptotic activity, we monitored the extent of caspase-3 cleavage upon blocking L-type and R-type VGCC in infected macrophages. As shown in Fig. 23, blocking R-type but not L-type VGCC indeed induced increased cleavage of caspase-3. It has been shown that increase in calcium influx induces the caspase-3 activation (Tantral et al., 2004). Tantral and co-workers have shown that IP3R-mediated Ca2+ release plays a critical role in regulating the activity of caspases-3 and -9 independent of Bcl-2 and that increased cytoplasmic Ca2+ levels activate the protein phosphatase, calcineurin, which causes dephosphorylation of the pro-apoptotic protein, Bad, promoting apoptosis. Therefore
our study indicated that one of the mechanisms by which a reduction in bacterial loads upon blocking VGCC could be induction of apoptosis as a result of calcium dependent caspase activation.

The above results so far pointed to a general inhibitory role of L and R-type VGCC in mediating protective responses to *M. tuberculosis* at the level of priming of DCs and effector T cell responses. Therefore it was worthwhile to investigate the levels of L-type and R-type VGCC in human cohorts with different states of *M. tuberculosis* infection -namely PPD^+ asymptomatic healthy/latently infected individuals, patients with active TB disease and patients undergoing chemotherapy. Our results clearly showed that L-type and R-type VGCC are expressed at higher levels in the PBMCs of patients with active TB when compared to healthy/latently-infected individuals. Interestingly, the expression levels of L-type and R-type VGCC in the same patients during follow-ups who became sputum AFB^− following chemotherapy showed a dramatic decrease. These results reaffirm the data obtained thus far towards a negative role of L-type and R-type VGCC in mediating protective responses during *M. tuberculosis* infection. These were further confirmed when PBMCs from patients with active TB induced the expression of genes involved in inducing pro-inflammatory protective responses during innate and adaptive immunity. Further, subjecting the data to binary logit and in-sample dynamic forecast models suggested that the relative levels of these VGCC in PBMCs could serve as a marker for the presence and absence of active TB disease during the various stages of mycobacterial infection.

It remains to be ascertained whether, reduction in bacterial loads resulted in reduced VGCC expression or vice versa. The fact that VGCC levels were expressed at low levels in PPD^+, i.e. latently infected healthy controls, but go up during a disease state and then come down following chemotherapy, indicates that the profile of mycobacterial factors expressed at these three states of infection are different and result in differential upregulation of VGCC in PBMCs and/or infected cells. CFP-10 along with other antigens like 19 kDa lipoprotein known to mount responses that favor the pathogen (Pai *et al.*, 2004; Pennini *et al.*, 2006) could possibly be among those factors. Following chemotherapy, with a reduction in bacterial loads, the expression of factors mediating high VGCC expression is reduced/ altered. This could result in higher mobilization of calcium in infected cells resulting in mounting of pro-
inflammatory responses thus resulting in further clearance of bacteria within cells. In parallel, induction of calcium could also mediate further downregulation of VGCC levels by acting in a negative regulatory loop which could further foster enhanced clearance of bacteria from infected cells. In fact beta subunit of VGCC has been shown to regulate the expression of alpha subunit on the plasma membrane (Bichet et al., 2000).

Blocking VGCC in PBMCs of healthy or TB patient increased the expression levels of granulysin, IFN-γ receptor 2 that are known to mediate killing of \textit{M. tuberculosis} and also downregulated the expression of genes such as CCL2 that promotes Th2 responses pointing to possible downstream mechanisms that would together bring about a reduction in \textit{M. tuberculosis} burden in infected cells. Further, the negative role of L-type and R-type VGCC during \textit{M. tuberculosis} infection was established with our in vivo data, wherein blocking VGCC in \textit{M. tuberculosis} infected mice significantly reduced bacterial loads in infected mice. An injection of antibodies to L-type and R-type VGCC elevates intracellular calcium concentrations in cells \textit{in vivo} during \textit{M. tuberculosis} infection.

It is pertinent to mention here that the role of VGCC in DCs has been controversial. While some groups have reported the presence of active VGCC in DCs (Poggi et al., 1998), others have observed that these channels are inactive in DCs and calcium influx is mainly via calcium release-activated calcium (CRAC) channels (Hsu et al., 2001). There are reports that suggest that L-type-related channels in immune cells are non-voltage-gated, since membrane depolarization by high potassium loading has a minimal effect on \([\text{Ca}^{2+}]_i\); (Sadighi Akha et al., 1996; Grafton et al., 2003; Stokes et al., 2004). However, our data indicate that these channels play a direct role in determining the quantum and quality of immune responses from DCs and macrophages. The role of L-type VGCC in CD4\(^+\) T cells has recently been shown in the context of \textit{Leishmania} infection wherein despite being non-excitable, these T cells express functional L-type VGCC (Hsu et al., 2001). VGCC in these T cells played a major role in inducing calcium influx with their association with the scaffold protein AHNAK-1 (Matza et al., 2008). Therefore, the data on T cells add support to our results, wherein these channels directly influence functional outcomes in non-excitable cells (like DCs). RT-PCR results and staining experiment using anti-L-type
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or anti-R-type VGCC antibodies (Fig. 10) clearly show the presence of these channels on dendritic cell surface. The presence of L-type calcium channel in mast cells is recently demonstrated (Yoshimaru et al., 2009). Further, these channels could be active during an infection, such as HIV (Hsu et al., 2001), Leishmania (Matza et al., 2008) and M. tuberculosis (this study). The fact that CFP10-DCs expressed higher levels of these channels further supports our argument since they were differentiated by a mycobacterial antigen, in contrast to conventional GM-CSF-DCs that indeed expressed very low levels of these channels.

Collectively, our results suggest that L-type and R-type VGCC play important roles in regulating immune responses during M. tuberculosis infection. Inhibition of these channels results in significant increase in calcium mobilization leading to expression of pro-inflammatory genes and the generation of protective immunity to mycobacteria. Significantly, our results on patient samples further indicate that these channels are expressed at high levels during active disease, indicating a negative role played by these VGCC during M. tuberculosis infection. Finally, the reduction of M. tuberculosis infection in mice treated with antibodies to L-type and R-type VGCC indicates their potent roles in determining the course of infection during different stages of M. tuberculosis infection and TB disease.

Finally, M. tuberculosis has been shown to interact differently with DCs and macrophages. These include opposite effects on MHC class II levels (Henderson et al., 1997), IL-12 and IFN-γ secretion and their regulation (Flynn et al., 2001; Wang et al., 2005), the production and downregulation of reactive oxidative species and reactive nitrogen species production and their regulation (Hisert et al., 2004). In this study we have identified a common factor in the form of L-type and R-type VGCC that negatively governs protective responses from both DCs and macrophages that could be targeted for therapeutic intervention. The fact that T cells activated by BCG infected DCs whose L-type and R-type VGCC were blocked could mediate killing of virulent M. tuberculosis inside macrophages opens avenues for combining BCG vaccination with VGCC blocking. This could be accomplished either through immuno-therapy using antibodies or by developing/using specific potent inhibitors providing effective and faster clearance of M. tuberculosis infection not only in patients with active disease but perhaps also in the case of latently infected individuals thereby reducing the global burden of tuberculosis.