Chapter 5

Voltage Gated Calcium Channels Play an Inhibitory Role in Priming of T-cell Response by Dendritic Cells
Calcium ion is a universal signaling intermediate, which is known to control various biological processes. Calcium and calcium influx play pivotal roles in orchestrating innate immune responses. Voltage gated calcium channels (VGCCs) are the major route of calcium entry and regulate multiple functions including gene transcription. Towards characterization of the interactions of DCs with *M. tuberculosis* secretory antigens, previous work in the lab has shown that antigens such as CFP-10 induce differentiation of mouse bone marrow precursors into immature DCs (CFP10-DCs) (Latchumanan *et al.*, 2005). These DCs are phenotypically (based on surface marker expression) and morphologically similar to DCs differentiated conventionally with GM-CSF. However, functional characterization of CFP10-DCs showed that, unlike DCs differentiated conventionally with GM-CSF (GM-CSF-DCs) that induce Th1 responses, CFP10-DCs induced suppressor responses to mycobacteria in an IL-10 and TGF-β dependent mechanism (Balkhi *et al.*, 2004). Furthermore, CFP10-DCs also modulated key intracellular signaling molecules such as PKC and PI3K. In addition not only do CFP10-DCs induce suppressor responses but also serve as depots for the survival of mycobacteria. Investigations into the mechanisms of bacterial survival in CFP10-DCs pointed to a low oxidative burst (Sinha *et al.*, 2006). On the other hand GM-CSF-DCs displayed a robust oxidative burst and reduced bacterial loads. This indicated that antigens such as CFP-10 are secreted to downregulate protective responses to mycobacteria via initial activation of DCs.

One of the regulating factors for the generation of suppressor responses from CFP10-DCs and also reduced oxidative burst that led to increased bacterial burden in these DCs was the influx of calcium in following stimulation by mycobacteria. While mycobacterial stimulation of GM-CSF-DCs induced a profound increase in intracellular calcium uptake, a weak increase in intracellular Ca\(^{2+}\) was observed in the case of CFP10-DCs. There are two ports of calcium entry in cells. One is via CRAC (calcium release-activated Ca\(^{2+}\) channels) channels which operate once intracellular
calcium stores are exhausted, the other route of calcium entry is via the voltage gated calcium channels (VGCC). While the organization and functional domains of CRAC channels are not fully worked out including the lack of specific reagents to study them, the role of VGCC has been extensively studied in physiological processes (especially nerve physiology) in mammalian cells. However, not much data exist on their role in governing immune responses to pathogens. Nevertheless, we decided to study the role of VGCC in mediating calcium influx in the two DCs.

**Antibodies to L-type and R-type calcium channels bind to DCs**

Although bio-pharmacological inhibitors to L-type and R-type VGCC are available (Cattaruzza et al., 2000), in our hands these inhibitors were toxic to cells even at 0.5 x IC50 concentrations and hence could not be used. Therefore, we used specific antibodies to L-type and R-type VGCC in our experiments. At the onset, we ensured that the above antibodies showed binding to DCs by FACS (Fig. 1). Our results clearly show that antibodies to both L-type (panels a and d) and R-type VGCC (panels b and e) showed binding to VGCC on CFP10-DCs (panels a-c) and GM-CSF-DCs (panels d-f), while incubation with non-specific antibody (panels c and f) showed insignificant binding.
Figure 1. Antibodies to L-type and R-type VGCC bind DCs.

Antibodies to L-type Ca\(^{2+}\) α1C (cat # sc-25686) and R-type Ca\(^{2+}\) α1E (cat # sc-16225) VGCC and NF-κB p65 subunit (cat # sc-7151) were biotinylated using NHS biotin as per standard protocols. Cells were washed and counter stained with streptavidin-PE. FACS was performed using FACSCalibur (Beckton & Dickinson) and the data were analyzed employing the CellQuest Pro software. Histograms depict surface levels of L-type (a & d) and R-type (b & e) VGCC on CFP10-DCs (a-c) and GM-CSF-DCs (d-f). Histograms (c & f) depict binding of anti-NF-κB p65 (used as non-specific control). The thin lines depict staining with streptavidin-PE, while the thick lines depict staining with specific antibody. One of three independent experiments is shown.

Blocking L-type or R-type VGCC in DCs does not affect maturation

Since we have observed that antibodies to L-type and R-type VGCCs bind to dendritic cells, it was important to look for the effects of the binding antibodies on the maturation of DCs. Blocking L-type or R-type VGCC did not have any effect on BCG induced maturation of either CFP10-DCs or GM-CSF-DCs as seen by the surface level expression of various markers such as CD80 (Fig. 2A), H-2d (Fig. 2B) and ICAM (Fig. 2C).
Figure 2. Antibodies to L-type and R-type VGCC does not affect DC maturation. CD80 (Panel A), H-2d (Panel B) and ICAM (Panel C) surface level expression in CFP10-DCs (upper row) and GM-CSF-DCs (bottom row). Thin line depicts DCs stimulated with BCG while thick line shows DCs incubated with the indicated antibodies and then stimulated with BCG. Data from one of three independent experiments is shown. anti-NS is antibody to NF-κB p65 (used as non-specific control).

Inhibiting L-type and R-type VGCC in DCs increases calcium influx upon stimulation with BCG

As mycobacteria induced calcium influx was superior in GM-CSF-DCs than in CFP10-DCs (Sinha et al., 2006, Salam et al., 2008), to begin with we looked at the roles played by L-type and R-type VGCC in mediating the increase in calcium influx in GM-CSF-DCs. As shown in Fig. 3, stimulation of GM-CSFDCs with BCG (panel a) resulted in a robust (164 µM) influx of calcium, while stimulation of CFP10-DCs with BCG (panel e) induced weak (78 µM) calcium mobilization. Surprisingly, incubation with either anti-L-type (panel c & g) or anti-R-type (panel d & h) VGCC resulted in a significant increase in calcium influx in GM-CSF-DCs (536 and 598 µM). A similar increase in calcium influx was observed in CFP10-DCs (240 and 259
μM). Incubation with a non-specific antibody (panel b & f) had no significant effect. This indicated that L-type and R-type VGCC played an inhibitory role in mediating calcium influx in DCs, in the context of mycobacterial infection. Further, blocking L-type or R-type VGCC did not have any significant effect on BCG induced maturation of either CFP10-DCs or GM-CSF-DCs.

![Graphs showing calcium influx](image)

**Figure 3. Neutralizing L-type and R-type VGCC in DCs induces calcium influx upon M. bovis BCG stimulation.**

Real time increase in calcium influx over 5 min in DCs stimulated with 1 MOI BCG.GM-CSF-DCs (a-d) or CFP10-DCs (e-h). Prior to stimulation, DCs were blocked with anti-L-type antibody (c & g) or anti-R-type antibody (d & h) or non-specific antibody (b & f). Data are representative of three to five independent experiments.
Inhibiting L-type and R-type VGCC in DCs increases calcium influx upon stimulation with \textit{M. tuberculosis} H37Rv WCL

We repeated the above experiment with whole cell lysate of \textit{M. tuberculosis} H37Rv (representing virulent mycobacterial components). Previous work has shown that responses from DCs pulsed with \textit{M. tuberculosis} WCL are the similar to those obtained by live intact \textit{M. tuberculosis} (Sinha et al. 2006, Salam et al 2008). Similar to BCG, an increase in calcium influx upon blocking L-type (panel c & g) or R-type (panel d & h) VGCC in DCs was observed, while blocking with a non-specific antibody (panel b & f) had no significant effect (Fig. 4). In fact, the calcium mobilization in CFP10-DCs was doubled on inhibition with anti-L-type (panel g) or anti-R-type (panel h) VGCC.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4.png}
\caption{Neutralizing L-type and R-type VGCC in DCs induces calcium influx upon \textit{M. tuberculosis} H37Rv WCL stimulation. Real time increase in calcium influx over 5 min in DCs stimulated with \textit{M. tuberculosis} H37Rv WCL. GM-CSF-DCs (a-d) or CFP10-DCs (e-h). Prior to stimulation, DCs were blocked with anti-L-type antibody (c & g) or anti-R-type antibody (d & h) or non-specific antibody (b & f). Data are representative of three to five independent experiments.}
\end{figure}
Calcium influx in L-type and R-type VGCC blocked Dimer-DCs is increased on stimulation with *M. tuberculosis* H37Rv WCL

CFP-10 is co-transcribed with Early Secretory Antigenic Target (ESAT-6) from RD1 region of *M. tuberculosis* genome and forms a tight 1:1 complex with ESAT-6 (Renshaw *et al*., 2002). Structure function studies of the heterodimer have identified moieties that play critical roles in the induction of immune responses (Brodin *et al*., 2005). In addition, CFP-10 and CFP10:ESAT6 heterodimer induce similar immune responses (Marei *et al*., 2004). Therefore, we looked for calcium influx in DCs differentiated with CFP10:ESAT6 heterodimer (Dimer-DCs) upon stimulation with *M. tuberculosis* H37Rv WCL (Fig. 5). We observed that similar to CFP10-DCs, Dimer-DCs induced weak calcium mobilization on stimulation with *M. tuberculosis* H37Rv WCL (panel a). However, incubation with either anti-L-type (panel b) or anti-R-type (panel c) VGCC resulted in a significant increase in calcium influx in Dimer-DCs.

![Figure 5. Neutralizing L-type and R-type VGCC in Dimer-DCs induces calcium influx upon *M. tuberculosis* H37Rv WCL stimulation.](image)

Real time increase in calcium influx over 5 min in Dimer-DCs stimulated with *M. tuberculosis* H37Rv WCL (a). Prior to stimulation, Dimer-DCs were blocked with anti-L-type antibody (b) or anti-R-type antibody (c) or non-specific antibody (b & f). Data are representative of three independent experiments.

L-type and R-type VGCC silenced DCs show increased calcium influx

Since, blocking VGCC with antibodies resulted in further increase in calcium influx, in order to see if incubation with antibodies resulted in either blocking or stimulation of VGCC, we did a similar experiment employing siRNAs against L-type and R-type VGCC. Fig. 6 shows that siRNA treatment indeed decreased the mRNA levels of L-
type and R-type VGCC in CFP10-DCs and GM-CSF-DCs while treatment with non-specific siRNA had no significant effect with respect to the respective controls. Next, as shown in Fig. 7, inhibiting L-type (panels c and g) or R-type (panels d and h) VGCC with specific siRNA resulted in a similar increase in calcium mobilization, while non-specific siRNA had no effect (panels b and f). This indicated that the observed effects with antibody incubation were a result of blocking of the VGCC. The collective results in figure 6 and figure 7 confirmed that L-type and R-type VGCC played an inhibitory role in mobilizing calcium influx in DCs.

Figure 6. siRNA mediated silencing of L-type and R-type VGCC.

5 x 10⁶/ml bone marrow precursors were transfected with 60 pmoles of siRNA against L-type and R-type VGCC for 72h using the Hiperfect transfection reagent (Qiagen) in OPTIMEM medium (Invitrogen). 5h following transfection either CFP-10 (Panel A) or GM-CSF (Panel B) was added and the incubation continued for 72h for DC differentiation. Subsequently, RNA was enriched using TRIZOL reagent and levels of VGCC were monitored by RT-PCR. C, control untransfected DCs. NS, DCs transfected with siRNA against firefly luciferase (used as non-specific control). L, DCs transfected with siRNA against L-type VGCC. R, DCs transfected with siRNA against R-type VGCC. Lower panel represents β-actin as loading controls.
Figure 7. Silencing L-type and R-type VGCC in DCs induces calcium influx.
Real time increase in calcium influx over 5 min in DCs stimulated with 1 MOI BCG. Calcium influx in BCG stimulated GM-CSF-DCs (a-d) and CFP10-DCs (e-h) following siRNA mediated silencing of L-type (c & g), R-type (d & h). siRNA against firefly luciferase was used as non-specific control (b & f). Data are representative of three to five independent experiments.

Calcium mobilization in CFP10-DCs real time
We also looked at calcium influx using live cell imaging by time-lapse confocal video-microscopy. However, over here, experiments were conducted with M. tuberculosis H37Rv whole cell lysate (WCL) instead of live mycobacteria. Stimulation of CFP10-DCs with M. tuberculosis H37Rv WCL induced a weak increase in calcium influx in the cells (panel A, Fig. 8). Blocking L-type (panel B, Fig. 8) or R-type (panel c, Fig. 8) VGCC increased calcium influx as seen from increased fluorescence of a number of cells and appearance of new fluorescing cells. Figure 9 graphically represents the data obtained by video microscopy. Blocking L-type (red line) or R-type (green line) VGCC in CFP10-DCs induced a significant increase in calcium influx upon M. tuberculosis H37Rv WCL stimulation.
Figure 8. Blocking L-type and R-type VGCC in CFP10-DCs induces strong influx of calcium.

CFP10-DCs were loaded with FLUO-3-AM (panel A). CFP10-DCs were incubated with antibody to L-type VGCC (panel B) or R-type VGCC (panel C) and subsequently loaded with FLUO-3-AM. Following acquisition of 15 frames as baseline, DCs were stimulated with 10 µg/ml M. tuberculosis H37Rv WCL. A total of 90 frames were recorded. The movie depicts frames # 7-55.

Figure 9. Blocking L-type and R-type VGCC in CFP10-DCs increases calcium upon M. tuberculosis H37Rv WCL stimulation.

Increase in intracellular calcium levels in CFP10-DCs upon 10 µg/ml M. tuberculosis H37Rv WCL stimulation measured by live cell imaging using time-lapse video confocal microscopy is shown. DCs were stimulated at frame # 15 and data on a total of 90 frames were collected and analyzed using the Image-Pro AMS6.0 software. The values were normalized to unity in order to represent all groups in a single graph. CFP10-DCs (Blue), CFP10-DCs + L-type VGCC blocking (Red), CFP10-DCs + R-type VGCC blocking (Green). Data are representative of three independent experiments.
CFP10-DCs express higher levels of L-type and R-type VGCC

Since blocking L-type and R-type VGCC increased calcium influx in both GM-CSF-DCs and CFP10-DCs, we investigated whether the differential calcium mobilization observed in CFP10-DCs and GM-CSF-DCs was a result of differential expression of L-type and R-type VGCC in the two DCs. The binding data in Fig. 1 indicated that uninfected CFP10-DCs expressed higher surface levels of both L-type and R-type VGCC when compared with uninfected GM-CSF-DCs. This was also reflected in the transcript levels of the two DCs (Fig. 6). We extended these observations by looking at their levels following infection of DCs with BCG. As shown in Fig. 10A, both L-type (panel a) and R-type (panel b) VGCC levels in BCG infected CFP10-DCs were significantly higher when compared with BCG infected GM-CSF-DCs. We further confirmed this by looking at their transcript levels by qPCR. As shown in Fig. 10B, BCG infected CFP10-DCs expressed significantly higher levels of L-type and R-type VGCC mRNA (P < 0.04 and P < 0.05, respectively), when compared with BCG infected GM-CSF-DCs. These results indicated that poor calcium influx in CFP10-DCs in response to mycobacterial stimulation could be a result of increased expression of L-type and R-type VGCC.
Figure 10. CFP10-DCs express higher levels of L-type and R-type VGCC following BCG infection.

Histograms in Panel A show surface levels of L-type (panel a) and R-type (panel b) in BCG infected CFP10-DCs (thick lines) and BCG infected GM-CSF-DCs (thin lines). Panel B shows, results from qPCR of L-type and R-type VGCC transcripts in BCG infected DCs. The Bars represent \(2^{-ΔΔCt}\) values in BCG infected CFP10-DCs and GM-CSF-DCs. Data are the mean of three independent experiments. Error bars represent mean ± S.D. \(P < 0.008\) (for L-type levels between CFP10-DCs and GM-CSF-DCs); \(P < 0.02\) (for R-type levels between CFP10-DCs and GM-CSF-DCs). Two-tailed Student’s t-test was employed for \(P\) values.

### Blocking VGCC results in increased release of calcium from intracellular stores

Intriguingly, since blocking calcium-inducing channels (L-type and R-type) resulted in further increasing calcium influx, it was important to identify the source of this calcium. To this end, we suspended L-type and R-type VGCC-blocked-CFP10-DCs in calcium sufficient (culture medium) or calcium deficient medium (PBS) and measured calcium influx upon BCG stimulation. As shown, under calcium sufficient conditions, both phases of calcium influx, i.e. the intracellular release followed by import from the extracellular medium (as a result of activation of CRAC channels following depletion of intracellular stores (Lewis, 2007; Catterall, 2000), could be
observed (Figure 11, panels b and c). In contrast, in calcium deficient medium, i.e. in the absence of extracellular calcium, one could only observe increased release of calcium from intracellular stores (Figure 11, panels e and f). The subsequent phase of calcium influx from the extracellular medium was not observed. This indicated that blocking L-type and R-type VGCC resulted in increased release of calcium from intracellular stores followed by activation of CRAC channels that together resulted in higher mobilization of calcium in CFP10-DCs. This was further confirmed when the release from intracellular stores was inhibited using TMB-8, wherein the observed increase of both phases was blocked (Figure 11, panels h and i).

Figure 11. Blocking VGCC increases calcium release from intracellular stores.
Real time increase in calcium influx over 5 min in CFP10-DCs stimulated with 1 MOI BCG. Prior to stimulation, DCs were blocked with anti-L-type antibody (b, e & h) or anti-R-type antibody (c, f & i). Subpanels a, d and g represent BCG stimulation in the absence of VGCC blocking. For panels A and B, DCs were suspended in calcium sufficient (RPMI) medium and calcium (PBS) deficient medium, respectively. For panel C, DCs were incubated with 100 μM TMB-8 prior to blocking with L-type or R-type VGCC and calcium measurements were carried out in calcium sufficient (RPMI) medium. Data from one of three independent experiments are shown.
One of the intracellular enzymes involved in the generation of IP3 is Phospholipase Cγ (PLCγ) (Lewis, 2007; Catterall, 2000). PLCγ acts on phosphoinositol 2 phosphate and converts it into IP3 and diacylglycerol. While IP3 binds to IP3 receptors on the endoplasmic reticulum to release calcium from intracellular stores, diacylglycerol activates protein kinase C. We therefore, specifically blocked PLCγ using a biopharmaceutical inhibitor and looked at calcium induction following blocking VGCC and BCG stimulation. As shown in Figure 12, inhibiting PLCγ completely blocked increase in calcium influx upon blocking VGCC (panels b and c). Put together, the above results clearly indicate the role of PLCγ in calcium induction following blocking L-type and R-type VGCC.

![Figure 12. Inhibiting PLCγ inhibits calcium induction following blocking of VGCC.](image)

Real time increase in calcium influx over 5 min in CFP10-DCs stimulated with 1 MOI BCG. Prior to stimulation, DCs were incubated with specific PLCγ inhibitor U73122 for 30 min followed by incubation with antibodies to L-type and R-type antibody. Panel a, CFP10-DCs treated with U73122, panel b and c, U73122 treated CFP10-DCs incubated with anti-L-type and anti-R-type antibodies, respectively.

Blocking L-type and R-type VGCC in DCs increases expression of Th1 promoting genes

Next, we investigated modulation in the expression of genes in DCs following blocking of L-type and R-type VGCC. To this end CFP10-DCs and GM-CSF-DCs were incubated with antibodies to L-type and R-type VGCC and subsequently infected with BCG. RNA was enriched, processed and probed against a pathway specific Th1/Th2/Th3 array. As shown in Fig. 13, blocking L-type and/or R-type VGCC in BCG-infected CFP10-DCs (lower panels) induced the expression of genes promoting Th1 and pro-inflammatory responses. This included Fosl1 (spot # B4), CD80 (spot B3), CD86 (spot C3), CCAAT/enhancer binding protein (C/EBP) beta.
(Spot # D3), Fosl2 (spot # C4), IL-12p40 (spot # F5), IL-15 (spot # C6), IL-18 (spot # D6), IL-6 (spot # G7), Jak1 (spot # E8), NF-kB1 (spot # F10), TNF (spot # D13). Increase in the levels of CD86 upon L-type and R-type VGCC blocking indicated that these DCs would be better equipped to prime T cells. In addition message levels of IL-10 (spot # E5) were also upregulated. However, increased IL-10 protein levels were not observed in these groups by ELISA, indicating regulation at the post-transcriptional level. An essentially similar pattern was observed in BCG infected GM-CSF-DCs (upper panels) following blocking of VGCC. The expression of SOCS1 (spot # B11) and SOCS3 (spot # D11) were also increased in addition to IL-10 (spot # E5). These results indicate that high expression of L-type and R-type VGCC in CFP10-DCs conditioned DCs to induce suppressor responses via attenuated calcium influx.

Figure 13. Blocking L-type and R-type VGCC in DCs induce expression of Th1 promoting genes.

Expression levels of Th1/Th2/Th3 pathway specific gene array in GM-CSF-DCs (upper panels) or CFP10-DCs (lower panels) infected with 1 MOI BCG for 24h in the presence or absence of anti-L-type or anti-R-type antibody blocking. Spot # A1, GAPDH control; spot # C15, # D15 and # E15 are negative controls. Data from one of two independent experiments are shown.
Table 1. Genes upregulated following L-type and R-type VGCC blocking in BCG infected DCs.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Position in blot</th>
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<tbody>
<tr>
<td>Fosl1</td>
<td>Spot # B4</td>
</tr>
<tr>
<td>CD86</td>
<td>Spot C3</td>
</tr>
<tr>
<td>CCAAT/enhancer binding protein (C/EBP) beta</td>
<td>Spot # D3</td>
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<tr>
<td>Fosl2</td>
<td>Spot # C4</td>
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<td>IL-12p40</td>
<td>Spot # F5</td>
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<td>Spot # G7</td>
</tr>
<tr>
<td>Jak1</td>
<td>Spot # E8</td>
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<tr>
<td>NF-κB1</td>
<td>Spot # F10</td>
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<tr>
<td>TNF</td>
<td>Spot # D13</td>
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Inhibiting L-type and R-type VGCC increased activation of NF-κB

Although a number of genes were upregulated following VGCC blocking, we chose to functionally validate two key genes, namely, IL-12p40 and NF-κB. This was because IL-12p40 plays a dominant role in regulating pro-inflammatory responses from DCs that are crucial for mediating protection against *M. tuberculosis* infection (Banchereau *et al.*, 1998; Reis e Sousa *et al.*, 2001). On the other hand, NF-κB positively regulates the expression of many genes that were upregulated including TNF, IL-15 and IL-18 (Verhasselt *et al.*, 1999). Compared to GM-CSF-DCs, CFP10-DCs showed reduced activation of NF-κB when stimulated with *M. bovis* BCG (Fig. 14) or *M. tuberculosis* WCL (Fig. 15) as ascertained by EMSA (P < 0.009). However, blocking L-type and R-type VGCC in CFP10-DCs (Fig. 14B) (and also in GM-CSF-DCs; Fig. 14C) resulted in increased activation of NF-κB. The increase in the activation was more evident with R-type blocking when compared with L-type blocking.
Figure 14. Blocking L-type and R-type VGCC induces increased activation of NF-κB.

A, GM-CSF-DCs or CFP10-DCs were infected with 1 MOI BCG for indicated times. EMSA for NF-κB was carried out with 10-14 μg of nuclear extracts. Arrow points to the specific band. GM-CSF-DCs (B) or CFP10-DCs (C) DCs were incubated with blocking antibody to L-type or R-type VGCC prior to infection with BCG. Data are representative of three to five independent experiments.
Figure 15. Blocking L-type and R-type VGCC induces increased activation of NF-κB.

EMSA were performed as described recently (Salam et al., 2008) by incubating nuclear extract from DCs infected with M. tuberculosis WCL with 32P-end-labeled 19-mer double stranded consensus NF-κB oligonucleotide sequence. The DNA-protein complex of CFP10-DCs or GM-CSF-DCs was separated from free oligonucleotide on a 5% native polyacrylamide gel. Arrow points to the specific band. Data are representative of three independent experiments.
Inhibiting L-type and R-type VGCC does not change the composition of the NF-κB complex

The NF-κB family of transcription factors exist as homodimers or heterodimers of 5 distinct proteins (p50, p52, RelA, RelB and cRel), and play an important roles in regulating inflammatory and immune-response genes (Ghosh et al., 1998). Latent NF-κB transcription factors are typically present in the cytoplasm in a complex with inhibitory IκB proteins (Ghosh et al., 1998). Stimulation of cells with NF-κB activators, including LPS, TNF and CD40L, results in phosphorylation of IκB proteins by the IKKβ (IκB kinase) complex (Karin and Ben-Neriah, 2000). Phosphorylated IκB proteins are rapidly degraded, allowing translocation of NF-κB complexes to the nucleus. Supershift analyses of GM-CSF-DCs and CFP10-DCs showed that the bound complex consisted of c-Rel and p65 subunits (Fig. 16, panel A). Moreover, blocking L-type and R-type VGCC in CFP10-DCs (Fig. 16, panel C) and in GM-CSF-DCs (Fig. 16, panel B) show no change in the subunit composition of NF-κB complex.
Figure 16. Subunit composition of the NF-κB complex remains same in L-type and R-type VGCC blocked DCs.

EMSA were performed as described above. Nuclear extract was incubated with the indicated antibody and then EMSA performed. Panel A shows supershift in CFP10-DCs and GM-CSF-DCs. GM-CSF-DCs (Panel B) and CFP10-DCs (Panel C) were blocked with anti-L-type and anti-R-type VGCC antibodies. C represents CFP10-DCs and G represents GM-CSF-DCs. Data are representative of three independent experiments.
Blocking L-type and R-type VGCC induces high IL-12 expression

We next investigated whether blocking L-type and R-type VGCC results in increased expression of IL-12p40. To this end, we monitored the acetylation of histone H3 at the IL-12p40 promoter between position -121 to -131 (a region that has been functionally characterized to be important for IL-12 expression) (Ma et al., 1997), by chromatin immunoprecipitation (ChiP) and protein expression by ELISA. As shown in Fig. 17A, ChiP analyses showed increased pull down of acetylated histone H3 in BCG infected GM-CSF-DCs when compared with CFP10-DCs, as evident by increased levels of the PCR amplified product. In fact, IL-12p40 levels in BCG infected CFP10-DCs were lower than uninfected DCs, thus confirming our earlier observations (Sinha et al., 2006) at the transcriptional level. Specificity of ChiP was ascertained when no amplification was obtained in the group where the antibody against histone H3 was omitted. This indicated increased transcriptional activity at the IL-12p40 promoter in GM-CSF-DCs. However, blocking L-type and R-type VGCC in CFP10-DCs showed increased pull down of acetylated histone H3 resulting in increased levels of the PCR product. This indicated a direct role of blocking L-type and R-type VGCC in mediating increased IL-12p40 transcriptional activity. The reduced levels of PCR product in GM-CSF-DCs in the presence of L-type and R-type VGCC blocking could be a result of feedback regulation, since the levels were quite high upon BCG infection itself. The ChiP data corroborated very well with the protein levels of IL-12p40. Blocking L-type and R-type VGCC in BCG infected CFP10-DCs (Fig. 17B) significantly increased IL-12p40 levels.
Figure 17. Blocking L-type and R-type VGCC induces high expression of IL-12p40.

A, ChIP analysis of acetylated histone H3 (AcH3) from the IL-12p40 promoter in uninfected (U) or BCG infected CFP10-DCs or GM-CSF-DCs in the presence or absence of anti-L-type (L) or anti-R-type (R) antibody. Nab, no antibody in BCG infected GMCSF-DCs. Data from one of three independent experiments are shown. B, IL-12p40 levels in culture supernatants of CFP10-DCs processed as in A. Data from one of three independent experiments are shown.
Inhibiting CFP10-DCs with L-type and R-type VGCC induces Th1 recall response to BCG

It was worthwhile to investigate the effect of L-type & R-type calcium channels on the quality of immune responses induced by CFP10 differentiated DCs against BCG. So, we investigated if inhibiting CFP10-DCs with L-type and R-type VGCC would induce recall Th1 responses to \textit{M. bovis} BCG To this end, we incubated CFP10-DCs and GM-CSF-DCs with blocking antibodies against L-type and R-type VGCC, prior to BCG stimulation. DCs were then extensively washed and co-cultured with BCG-specific T cells for 48h. Intracellular levels of IFN-\(\gamma\) and IL-10 in CD4\(^+\) T cells were analyzed by flow cytometry. Figures 18(A and B) depict the results of the above experiment. Consistent with our earlier studies (Balkhi \textit{et al.}, 2004, Salam \textit{et al.}, 2008), stimulation of GM-CSF-DCs with BCG gave a Th1 response with a higher percentage of CD4\(^+\) T cells secreting IFN-\(\gamma\) as compared to IL-10 levels (Fig. 18), while stimulation of CFP10-DCs with BCG gave a Th2 response with higher percentage of IL-10 secreting CD4\(^+\) T cells than IFN-\(\gamma\) secreting CD4\(^+\) T cells (Fig. 18). However, blocking of L-type and R-type VGCC in CFP10-DCs gave a Th1 response with a significant increase in IFN-\(\gamma\) secreting CD4\(^+\) T cells (Fig. 18). Also, inhibiting L-type and R-type VGCC in CFP10-DCs significantly decreased the percentage of IL-10 secreting CD4\(^+\) T cells. Incubation with isotype matched non-specific antibody had no effect on T cell responses with GM-CSF-DCs.

An increase of 50% and 60% in percentage of IFN-\(\gamma\) secreting CD4\(^+\) T cells was seen when L-type and R-type VGCC were blocked in T cells, respectively. While only L-type VGCC blocked T cells show a slight increase in IFN-\(\gamma\) secreting CD4\(^+\) T cells. Blocking L-type and R-type VGCC in CFP10-DCs and GM-CSF-DCs significantly decreased the percentage of IL-10 secreting CD4\(^+\) T cells.
Figure 18. Blocking CFP10-DCs with L-type and R-type VGCC induces Th1 recall response to BCG.

Percentage of antigen specific CD4+ T-cells secreting IFN-γ (A) and IL-10 (B) when co-cultured with CFP10-DCs and GMCSF-DCs in the upper right quadrant. L & R-type channels were blocked prior to BCG stimulation and then washed and cultured with the two types of DCs. For blocking channels in T-cells, cells were incubated with anti-L-type and anti-R-type antibodies and then cocultured with DCs.
Chapter 5 - Results

T cells activated by VGCC-blocked-DCs mediate killing of M. tuberculosis in macrophages

To investigate the functional relevance of the results obtained so far, we next tested the ability of T cells activated by VGCC-blocked DCs to kill mycobacteria inside macrophages as recently carried out with chemokine and cytokines conditioned CFP10-DCs (Salam et al., 2008). We first ensured that blocking L-type and R-type VGCC in CFP10-DCs and GM-CSF-DCs activated T cells that secreted high levels of IFN-γ and low levels of IL-10. Next, BCG infected DCs were co-cultured with T cells enriched from BCG immunized mice. Form the DC-T cell co-culture, T cells were separated by MACS and incubated with M. tuberculosis H37Rv infected macrophages. Control groups included M. tuberculosis infection of resting and IFN-γ activated macrophages in the absence of any T cell addition. CFU in macrophages incubated with T cells that were activated by L-type and R-type VGCC-blocked-BCG-infected CFP10-DCs, showed a significant decrease when compared to CFU from either M. tuberculosis infected resting or activated macrophages or when compared with T cells activated by CFP10-DCs in the absence of VGCC blocking (Fig. 19). A similar decrease in CFU were seen when T cells activated by L-type and R-type VGCC-blocked-BCG-infected GM-CSF-DCs were employed. These results indicate that blocking L-type and R-type VGCC in DCs activated T cells that subsequently mediated effective killing of virulent M. tuberculosis inside macrophages.
L-type or R-type VGCC-blocked-BCG-infected GM-CSF-DCs (GMCSF) or CFP10-DCs (CFP10) were co-cultured with T cells from BCG immunized mice. From the coculture, T cells were enriched and incubated with M. tuberculosis H37Rv infected macrophages. Cells were lysed and CFU was determined. As controls, infected macrophages without incubation with T cells and macrophages treated with 2 ng/ml IFN-γ prior to infection with M. tuberculosis in the absence of T cell addition were also included. Data are the mean of three independent experiments. Error bars represent mean ± s.d. $P < 0.01$ for Mph+IFN-g vs CFP10+anti-Ltype; $P < 0.01$ for Mph+IFN-g vs CFP10+anti-Rtype and $P < 0.006$ for Mph vs GMCSF+anti-Ltype. Two-tailed Student’s t-test was employed for $P$ values.

Figure 19. T cells activated by L-type and R-type VGCC-blocked-DCs kill M. tuberculosis inside macrophages.