Chapter 5

Results
5. Results

5.1 Virulent *M. tb* strain H37Rv induces Th17 cell responses in the lung

It is well accepted that Th1 cell responses are indispensable for host protection against TB (Flynn et al., 1993). The vaccine strain BCG induces robust Th1 cell responses, yet it is not an effective vaccine against adult pulmonary TB in many individuals (Brewer, 2000; Fine, 1989; Fine, 1995). Therefore, additional immune response(s) are required for optimal vaccine efficacy. Recently, Th17 cells have been implicated in protective immunity against TB (Khader et al., 2007). Previous studies have demonstrated that RD1, which is absent in BCG, plays a dominant role in protective immune responses and bacterial virulence (Lewis et al., 2003). Thus, an RD1 deletion mutant of H37Rv resembles BCG in its infectivity (Simeone et al., 2009). Therefore, we tested the virulence and cytokine production by virulent strain H37Rv and vaccine strain BCG. We challenged C57BL/6 mice with a low dose (~110 CFU) of H37Rv or BCG by the aerosol route. We found that H37Rv and BCG replicated to a similar extent during the initial phase of the infection (*Figure 4*). However, at later time points, growth of BCG was gradually diminished (p<0.032) (*Figure 4*), suggesting that adaptive immune responses play an important role in clearing BCG. After 21 days of infection, only a few bacilli were found in the lungs of animals infected with BCG (*Figure 4*). These kinetics of BCG and H37Rv infection are in agreement with the published literature (Flynn et al., 1993; Glickman and Jacobs, 2001; Goter-Robinson et al., 2006; Kelly et al., 1996).
Results

![Graph of CFU in Lung](image)

**Figure 4.** CFU from the lung homogenate of C57BL/6 mice that were infected with H37Rv, BCG, H37RvΔRD1 or BCG::RD1 strains.

Our observations were further supported by the finding that BCG induced dramatically fewer granuloma-like lesions in the lung compared with H37Rv (**Figure 5**).
Figure 5. Histology of the lung tissue sections after 21 days (stained with H&E). H37Rv and BCG::RD1 induced more granuloma like lesions, whereas BCG and H37RvΔRD1 induced fewer granuloma like lesions compared to H37Rv or BCG::RD1.

Interestingly, we observed significantly higher numbers of IL-17-producing CD4+ T cells in the lungs of animals infected with H37Rv, as compared with BCG (p<0.0001) (Figure 6).
Figure 6. Infection with H37Rv or BCG::RD1 induces both Th1 and Th17 immunity, whereas BCG and H37Rv::AR1 selectively induce Th1 cell responses in the lung. Intracellular staining for IFN-γ and IL-17 of CD4⁺ T lymphocytes isolated from the lungs of C57BL/6 mice infected with H37Rv, BCG, H37Rv::AR1 or BCG::RD1.

In sharp contrast, both H37Rv and BCG induced IFN-γ in the bronchoalveolar lavage (BAL) fluid (Figure 6 & Figure 7). This is further supported by the increased amounts of IL-17 produced in the BAL fluid of mice infected with H37Rv but not BCG (p<0.0001) (Figure 6 & Figure 7). Although significantly lower, BCG-infected animals produced some IL-17 in the BAL fluid (Figure 7). However, we were unable
to detect significant IL-17-producing CD4\(^+\) cells in the lung of BCG-infected animals (Figure 6), suggesting that the source of IL-17 in BCG-infected animals is not Th17 cells. It has been previously shown that γδ T cells are the primary source of IL-17 in the lung during BCG infection (Umemura et al., 2007).

Consistent with the observation that H37Rv induces higher Th17 responses in the lung, we found significant levels of IL-6 (p<0.001) and TGF-β (p<0.001), two key cytokines required for the differentiation of Th17 cells (Bettelli et al., 2006; Mangan et al., 2006), in the BAL fluid of animals infected with H37Rv (Figure 7). In contrast, both H37Rv and BCG produced similar amounts of IL-12p40 (Figure 7), a cytokine that supports Th1 cell differentiation. These observations suggested that H37Rv creates an environment that is conducive to the differentiation of both Th1 and Th17 cells, whereas BCG promotes Th1 cell differentiation only. These data indicated that H37Rv promotes the differentiation of both Th1 and Th17 cell responses, whereas BCG induces Th1 responses but fails to support Th17 cell differentiation in infected cells. Nonetheless, a recent study indicated that BCG is unable to induce IL-17-producing cells during primary challenge but can do so after several rounds of challenge (Cruz et al., 2010). Therefore, it is highly likely that repeated immunization with BCG induces IL-17 production by innate-like cells or recruits IL-17-producing cells to the lung due to the local chemokine milieu.
Figure 7. H37Rv or BCG::RD1 induces both Th1 and Th17 immunity, whereas BCG and H37RvΔRD1 selectively induce Th1 cell responses. IL-6, IL-12p40, TGF-β, IFN-γ and IL-17 in BAL fluids of infected mice were measured by a Luminex microbead-based multiplex assay using Bio-Plex (Biorad) kits.

Compared to virulent H37Rv, BCG possesses multiple deletion mutations. These mutations are called regions of difference (RD). Among these RD regions, RD1 is the most dominant and plays an important role in virulence (Ganguly et al., 2008b). Thus, an RD1 deletion mutant of H37Rv resembles BCG in its infectivity (Lewis et al., 2003). Within the RD1 region, the proteins ESAT-6 and CFP-10 have been shown to form a complex and participate in a type VII secretion system (Brodin et al., 2004; Guinn et al., 2004). Therefore, we tested the virulence and cytokine production induced by H37RvΔRD1. Consistent with the results obtained for BCG, we observed that H37RvΔRD1 initially grew to a similar extent as the parental H37Rv strain, but at later time points its growth gradually diminished (Figure 4). Akin to BCG, H37RvΔRD1 induced fewer granuloma-like lesions (Figure 5). BCG and H37RvΔRD1 induced very low IL-17 production in the lung (Figure 6 and Figure 7). However, both strains induced similar quantities of IFN-γ (Figure 6 and Figure 7). Furthermore, IL-12 production was comparable between these strains, whereas IL-6
and TGF-β production was dramatically reduced as compared with H37Rv (Figure 7). To provide further support for these findings, we performed similar experiments with the BCG recombinant strain in which the RD1 region was reintroduced (BCG::RD1). In agreement with previous reports (Pym et al., 2002), BCG::RD1 showed a dramatically higher virulence as compared with the parental BCG strain, but was comparable to H37Rv (Figure 4 and Figure 5). Consistent with this finding, BCG::RD1 induced both IFN-γ and IL-17 (Figure 6 and Figure 7), as well as the Th1- and Th17-differentiating cytokines IL-12p40, IL-6, and TGF-β (Figure 7) in the lungs. These observations suggested that the RD1 region is responsible for the induction of Th17 cell responses.
5.2 IL-17 induced by H37Rv or BCG::RD1 mediates improved vaccine efficacy

Previously, it has been shown that IL-17 plays an important role in the secondary immune response following vaccination with virulent H37Rv (Khader et al., 2007). Thus, we examined whether IL-17 induced by virulent strains contributes to improved vaccine efficacy. For this purpose, we infected animals with H37Rv, BCG, H37RvΔRD1, or BCG::RD1 for 30 days. These animals were subsequently treated with antibiotics for four weeks, and then rested for an additional month. We could not find any detectable *M. tb* organisms in these animals. These mice were then challenged with H37Rv through aerosol infection. We found that animals previously infected with BCG or H37RvΔRD1 exhibited robust protective immunity compared with primary infection (p<0.01) (Figure 8). Interestingly, we found that animals that were previously infected with H37Rv produced dramatically enhanced protective immune responses (Figure 8).

![Graph showing CFU in Lung vs Days After Infection](image)

**Figure 8.** Clearance of H37Rv and BCG::RD1 induces improved protective immune responses compared with BCG and H37RvΔRD1. Animals were infected with the indicated bacterial strains for 30 days and treated with antibiotics for an additional 28 days. Animals were rested for one month and re-challenged with a low dose of H37Rv (~110 bacilli) by aerosol challenge. Then the bacterial load (CFU) was determined from the lung homogenates of C57BL/6 mice that were previously infected.
with H37Rv, BCG, H37Rv\textDelta RD1 or BCG::RD1 strains.

This is in agreement with previous reports suggesting that virulent strains of M. tb H37Rv induce superior protective immune responses (Jung et al., 2005; Kamath and Behar, 2005). However, the kinetics of host protective responses in our hand is somewhat different from these studies, which may be due to differential environmental factors in different geographical regions. In fact, it has been well documented that the efficacy of BCG in human vaccine trials dramatically varies depending on the geographical location ((Brewer, 2000; Fine, 1989; Fine, 1995)). Nevertheless, we tested M. tb antigen-specific responses induced by randomly selected animals from our colony. We challenged splenocytes from sixteen animals with M. tb-derived complete soluble antigen (CSA) or the unrelated antigen ovalbumin (OVA) and measured lymphoproliferation. We observed that animals from our colony responded weakly to CSA, whereas no response was detected against OVA. Therefore, these animals were likely exposed to environmental organism(s) that share antigenic similarities with M. tb. As a positive control, we used spleen cells from CSA immunized mice, which showed dramatic proliferation against in vitro rechallenge with CSA (Figure 9). These observations might also be relevant to the variable vaccine response of BCG.
Results

Figure 9. Proliferation of splenocytes in response to CSA. Splenocytes were isolated from sixteen randomly selected animals from our colony or from CSA-immunized mice. Proliferation of splenocytes in response to CSA was measured by $^1$H-thymidine incorporation assay. As a negative control, spleen cells were stimulated with OVA, and as a positive control, we measured the response against CSA by splenocytes from CSA-immunized mice.

In either case, our findings suggested that, while BCG and H37RvΔRD1 induced significant protective immunity against TB, this was not sufficient to confer complete protection against disease pathology. In contrast, H37Rv and BCG::RD1 induced improved protective immune responses.

Histological studies indicate that re-challenge with H37Rv induced granuloma-like lesions in animals that were previously infected with BCG or H37RvΔRD1 (Figure 10). However, these lesions involved smaller areas of the lung than those of unvaccinated animals (compare Figure 10 and Figure 5).
Figure 10. Histological analysis of lung tissue sections derived from the H37Rv re-challenged animals, which were previously infected with the indicated mycobacterial strains.

Collectively, these observations suggested that the RD1 region enhances protective immune responses.

We found that animals that were previously infected with H37Rv or BCG::RD1 induced significantly higher numbers of Th17 cells in the lungs than animals infected with BCG or H37RvΔRD1 (p<0.001) (Figure 11).
Results

Figure 11. H37Rv or BCG::RD1 induced significantly higher numbers of Th17 cells in the lungs than animals infected with BCG or H37RvΔRD1. Intracellular staining of CD4+ T lymphocytes in the lung of mice re-challenged with H37Rv.

We tested whether IL-17 was responsible for the improved vaccine efficacy of H37Rv or BCG::RD1. For this purpose, we injected animals with anti-IL-17 or control mouse IgG antibodies every 72 hours during re-challenge with H37Rv or BCG::RD1. Treatment with anti-IL-17 abrogated the observed enhancement in protective immune responses induced by H37Rv or BCG::RD1 (Figure 12).
Figure 12. Treatment with anti-IL-17 abrogated the enhancement in protective immune responses induced by H37Rv or BCG::RD1. Animals were with anti-IL-17 or control mouse IgG antibodies every 72 hours during re-challenge with H37Rv or BCG::RD1. CFU was from lung homogenates of mice that received anti-IL-17 or control antibodies.

Therefore, these observations suggested that H37Rv induced Th17 cell responses, which complemented Th1 cell responses for improved protection against TB.
5.3 Dendritic cells infected with H37Rv or BCG::RD1 direct Th1 and Th17 cell responses, whereas BCG and H37RvΔRD1 selectively induce Th1 cell responses.

Our *in vivo* experiments demonstrated that H37Rv induces Th17 cell differentiation. Therefore, to provide insight into the mechanism whereby H37Rv promotes Th1 and Th17 cell differentiation, we compared the cytokines induced by DCs (characterized with CD11c, CD11b, CD80, CD86, and MHC Class II markers—*Figure 13*) infected with H37Rv, BCG::RD1, BCG, and H37RvΔRD1.

*Figure 13. Characterization of dendritic cells (DCs) by flow cytometric analysis using anti-CD11c, -CD11b, -CD80, -CD86, -MHC class II, and -IgG2a (isotype control).*

54
We found that H37Rv- or BCG::RD1-infected DCs produced substantial amounts of IL-12p40, IL-6, and TGF-β (Figure 14). However, BCG and H37RvΔRD1 induced dramatically reduced amounts of IL-6 (p<0.001) and TGF-β (p<0.001) by DCs than H37Rv and BCG::RD1 (Figure 14). Nevertheless, IL-12p40 was induced at comparable levels by all bacterial strains. Interestingly, we found that IL-6 and TGF-β production was dependent on TLR-2 and MyD88, whereas IL-12p40 production was independent of TLR-2 but required MyD88 (Figure 14).

**Figure 14.** Production of IL-12p40, IL-6, and TGF-β in DCs infected with H37Rv, BCG, H37RvΔRD1 or BCG::RD1. H37Rv- or BCG::RD1-infected DCs, but not BCG or H37RvΔRD1-infected DCs, produced substantial amounts of IL-6, and TGF-β.

To determine whether these cytokines play a role in Th cell differentiation, we co-cultured ovalbumin (OVA)-specific CD4+ T cells from OT-II T cell receptor
(TCR) transgenic (Tg) animals with infected DCs in the presence of OVA peptide and collected supernatant to determine the production of IFN-γ and IL-17 (Figure 15).

![Graphs showing cytokine production](image)

**Figure 15.** Dendritic cells infected with H37Rv or BCG::RD1 direct differentiation of both Th1 and Th17 cells whereas BCG and H37RvΔRD1 selectively induce Th1 cell responses. Production of IFN-γ and IL-17 in the culture supernatants of OT-II TCR Tg CD4⁺ T cells co-cultured with infected DCs were measured by Bio-Plex assay.

The results, which were supported by intracellular cytokine staining, indicated that H37Rv-infected DCs directed the differentiation of both IL-17- and IFN-γ-producing cells (Figure 15 and Figure 16).
**Figure 16. Intracellular cytokine staining for IFN-γ and IL-17 in OT-II TCR Tg CD4+ T cells co-cultured with DCs infected with H37Rv, BCG::RD1, BCG or H37RvΔRD1.**

In sharp contrast, DCs infected with BCG or H37RvΔRD1 supported only Th1 cell differentiation. While the levels of IFN-γ production were similar for DCs infected with H37Rv, BCG, H37RvΔRD1 or BCG::RD, production of IL-17 was significantly higher in DCs infected with H37Rv or BCG::RD1 as compared with cells infected with BCG or H37RvΔRD1 (p<0.001). Furthermore, it is known that IL-22 is also secreted by IL-17 producing Th cells and recent study suggested that IL-22 was upregulated during *M. tb* infection in rhesus macaques and protective in function (Zeng et al., 2011). Therefore, we have also checked the IL-22 mRNA transcript level in our DC-T cells co-culture experiments and found that IL-22 mRNA transcript was
5-8 fold upregulated in H37RV or BCG::RD virulent strains compared to BCG and H37RvΔRD1 (*Figure 17*).

![IL-22/GAPDH fold expression](image)

*Figure 17. IL-22 mRNA expression profile from OT-II TCR Tg CD4⁺ T cells co-cultured with DCs after infection of DCs with H37Rv, BCG, BCG::RD1 or H37RvΔRD1.*

Therefore, these data indicated that the RD1 locus plays an important role in directing Th17 cell responses during *M. tb* infection.
5.4 ESAT-6 drives Th17 cell differentiation by inducing IL-6 and TGF-β production in DCs

ESAT-6-reactive T cells are prevalent in TB patients and in animals infected with *M. tb* (Brandt et al., 1996; Ravn et al., 1999; Ulrichs et al., 1998). Furthermore, it has been shown that ESAT-6-specific T cells provide substantial protection against TB (Gallegos et al., 2008). Therefore, it has been assumed that ESAT-6 is a good candidate for development of a TB vaccine (Aagaard et al., 2009). From the preceding section, it was clear that the RD1 region plays an important role in directing Th17 cell differentiation, which in turn contributes to protective immune responses against TB. Differentiation of Th17 cells requires IL-6 and TGF-β simultaneously (Bettelli et al., 2006; Mangan et al., 2006). Therefore, we determined whether ESAT-6 induces these cytokines in DCs. We found that DCs treated with ESAT-6 produced both IL-6 and TGF-β (*Figure 18*). However, ESAT-6 did not induce IL-12 in DCs (*Figure 18*).

![Graph showing cytokine production](image)

*Figure 18. ESAT-6 induces IL-6 and TGF-β in DCs in a TLR-2- and MyD88-dependent manner. DCs from C57BL/6, TLR-2−/−, or MyD88−/− mice were treated with*
ESAT-6 protein, LPS, or denatured ESAT-6 protein, or untreated. Supernatants were harvested at different time points and IL-12p40, IL-6, and TGF-β cytokines were measured.

CD4⁺ T cells from OT-II TCR Tg mice co-cultured with DCs in the presence of ESAT-6 and OVA differentiated into IL-17-producing cells (Figure 19).

**Figure 19.** Production of IFN-γ and IL-17 by OT-II TCR Tg CD4⁺ T cells co-cultured with DCs that were treated with ESAT-6 protein and LPS.
5.5 TLR-2 is required for the induction of IL-6 and TGF-β in DCs by ESAT-6

Our findings indicated that ESAT-6 induces IL-6 and TGF-β production in DCs, which drives Th17 cell differentiation. Previous reports have suggested that ESAT-6 binds to TLR-2 (Pathak et al., 2007). Therefore, we tested whether TLR-2 is required for the capacity of ESAT-6 to induce IL-6 and TGF-β production. For this purpose, we compared cytokine production by DCs derived from wild type and TLR-2<sup>−/−</sup> mice. We found that DCs from TLR-2<sup>−/−</sup> mice were unable to produce IL-6 and TGF-β (Figure 18). To confirm that innate immune signalling is required for the capacity of ESAT-6 to induce IL-6 and TGF-β production in DCs, we performed experiments with DCs isolated from MyD88<sup>−/−</sup> mice. As expected, DCs from MyD88<sup>−/−</sup> mice were also unable to produce IL-6 and TGF-β in response to ESAT-6 stimulation (Figure 18).

Taken together, our findings indicated that IL-6 and TGF-β induced by ESAT-6 in DCs generate an environment that promotes the differentiation of Th17 cells.

We have already determined the capacity of DCs treated with H37RvΔRD1 to produce IL-6 and TGF-β. Our results clearly showed that neither H37RvΔRD1 nor BCG were able to induce IL-6 and TGF-β in DCs derived from either wild type, TLR2<sup>−/−</sup>, or MyD88<sup>−/−</sup> mice (Figure 14). Interestingly, we found that the parental strain H37Rv, BCG::RD1, H37RvΔRD1 and BCG induced IL-12p40 production in DCs isolated from both wild type and TLR-2<sup>−/−</sup> mice. However, none of these strains induced IL-12p40 production in DCs derived from MyD88<sup>−/−</sup> mice. Therefore, taken together, these observations suggested that ESAT-6 induces IL-6 and TGF-β production in a TLR-2-dependent manner. In contrast, production of IL-12p40 by DCs following infection with mycobacteria is independent of ESAT-6 and TLR-2 expression. However, IL-12p40 production induced by mycobacteria is dependent on MyD88 signalling.
5.6 TLR-2\(^{-}\) animals fail to induce \textit{M. tb}-mediated Th17 cell responses and show enhanced susceptibility to \textit{M. tb} infection

From the preceding section it is clear that interaction of ESAT-6 with TLR-2 creates an environment that is conducive to the differentiation of Th17 cells, which in turn results in protective immunity against TB. Therefore, to confirm that TLR-2 signalling is important for the observed Th17 cell responses and improved vaccine efficacy, we performed vaccination experiments in TLR-2\(^{-}\) animals. These animals were infected with H37Rv for one month and subsequently treated with antibiotics for four weeks as described in the materials and methods, and then rested for an additional one month. These animals were then challenged with virulent strain H37Rv. H37Rv-immunized TLR-2\(^{-}\) mice generated protective immunity against H37Rv at a level similar to wild-type mice immunized with BCG during primary challenge (\textit{Figure 20}). However, TLR-2\(^{-}\) mice exhibited reduced protective immune responses as compared with wild type littermates (\textit{Figure 20}). Consistent with this finding, from histological analyses, we observed more granuloma-like lesions in the lungs of TLR-2\(^{-}\) mice (\textit{Figure 21}). H37Rv\(\Delta RD1\) and BCG::RD1 also showed reduced protection in TLR-2\(^{-}\) mice and induced more granuloma-like structures (\textit{Figure 20} and \textit{Figure 21}) similar to BCG and H37Rv. Other mycobacterial components, such as LAM and lipoproteins, can also activate TLR-2 (Srivastava et al., 2009). Therefore, the observed differences in protective immune responses in TLR-2\(^{-}\) animals could be caused by multiple TLR-2-dependent agonists. However, most of the \textit{M. tb}-derived TLR-2 ligands induce only suppressive immune responses (Srivastava et al., 2009). Therefore, the observed protective responses are most likely contributed by RD1-derived proteins. Furthermore, these differences are comparable with responses induced by H37Rv versus BCG in wild-type animals.
Figure 20. TLR-2-deficient animals induce reduced levels of protective immune responses. For in vivo experiments, C57BL/6 and TLR-2\(^{+/−}\) mice were infected with H37Rv for 30 days, and subsequently treated with antibiotics for 4 weeks as described in the materials and methods. Mice were then re-challenged with H37Rv. CFUs were quantified from the lung homogenates of mice re-challenged with H37Rv.
Figure 21. Histology of lung tissue sections of TLR-2<sup>−/−</sup> mice infected with the indicated strains of mycobacteria.

Next, we analyzed effector T cells in the lungs. We found that TLR-2<sup>−/−</sup> animals generated IFN-γ-producing cells comparable to wild type littermates. However, these animals produced significantly fewer numbers of IL-17-producing cells in their lungs (p<0.001) (Figure 22). This finding is further strengthened by a recently published report, suggesting that TLR-2 is indispensable for the generation of Th17 responses during *M. tb* infection (Teixeira-Coelho et al., 2010). Therefore, these observations suggested that TLR-2 plays an important role in mounting Th17 cell responses to H37Rv, which in turn confers protective immunity to TB.
Results

Figure 22. TLR-2−/− mice generated IFN-γ-producing cells comparable to wild type but produced significantly fewer numbers of IL-17-producing cells in their lungs, 30 days after infection with H37Rv, BCG, BCG::RD1 or H37RvΔRD1. Intracellular staining was done for IFN-γ and IL-17 in CD4+ T lymphocytes derived from the lung and detected in the flow cytometer.
5.7 ESAT-6 down regulates miR146a expression in *M. tb* infected DCs, which results in increased production of IL-6 that promotes Th17 cell differentiation

Recent studies in our group (Singh, Y. et al., unpublished data) indicate that H37Rv bacteria can modulate the host immune response by interfering primary target cells - the macrophage or dendritic cells - functions and cytokines expression by modulating the expressions of various miRNAs. Our miRNAs microarray analysis in murine model suggested that various miRNA expressions are modulated after *M. tb* infection (*Figure 23*). Our miRCURY LNA miRNAs microarray data revealed that the expressions of 10 miRNAs (miR712, 23a, 710, 881, 882, 877, 146a, 125a-5p, 99b and 222) are modulated in the *M. tb* infected DCs (*Figure 23*). We have further focussed on miR146a since it is a negative regulator of innate immune components like IL-6, and it is upregulated by *M. tb* infection as well as lipopolysaccharide (LPS) stimulation.
Figure 23. Differential expression of miRNA in M. tb infected dendritic cells. The hit map diagram shows the result of the two-way hierarchal clustering of genes and samples. Each row represents a miRNA and each column represents a sample. The colour scale shown at the bottom illustrates the relative expression level of a miRNA across all samples: red colour represents an expression level above mean, blue colour represents expression lower than the mean. 8 different miRNAs were upregulated after M. tb infection compared to uninfected control and 2 miRNAs were down regulated with control in C57BL/6 mice (Adapted from Singh, Y. et al, unpublished data).
To investigate the molecular basis for the capacity of H37Rv to induce high levels of IL-6, the major cytokine required for Th17 differentiation, we compared induction of microRNA-146a (miR146a), a negative regulator of innate immune components such as IL-6, in infected DCs (Starczynowski et al., 2010; Taganov et al., 2006). Interestingly, we found that BCG or H37RvΔRD1 significantly upregulated miR146a in DCs as compared with H37Rv or BCG::RD1-infected DCs (p<0.01) (Figure 24). These observations suggest that the presence of that RD1 region is involved in the down-regulation of miR146a.

![miR146a/5S RNA fold expression](image)

**Figure 24. RD1 region is involved in the down-regulation of miR146a.** miR146a expression profile of DCs infected with H37Rv, BCG, BCG::RD1 or H37RvΔRD1. miR146a expression was normalized with 5S rRNA control primer.

Furthermore, specific knock-down of miR146a expression dramatically upregulate both mRNA and protein level of IL-6 in BCG or H37RvΔRD1-infected DCs (Figure 25) compared to H37Rv or BCG::RD1-infected DCs (p<0.02). These data indicated that H37Rv or BCG::RD1 promotes the differentiation of Th17 cell responses through the down regulation of miR146a, whereas BCG or H37RvΔRD1
fails to support Th17 cell differentiation due to its induction of miR146a, the negative regulator of IL-6, in infected cells.

![Graph A](image1.png) ![Graph B](image2.png)

**Figure 25.** Knock-down of miR146a expression dramatically upregulate both mRNA and protein level of IL-6 in BCG and H37RvΔRD1 infected DCs. (A) IL-6 mRNA expression profile after infection of DCs with H37Rv, BCG, BCG::RD1 or H37RvΔRD1 and compared after knock-down of miR146a with anti-miR146a miRCURY LNA™ knock-down probes. IL-6 mRNA expression increases after knock-down of miR146a increases. (B) IL-6 cytokine production increases after knock-down of miR146a increases.

Interestingly, we observed that ESAT-6 dramatically inhibited miR146a expression in both LPS- and BCG-treated DCs (Figure 26). Thus, ESAT-6 allows IL-6 production in DCs by inhibiting the induction of miR146a.
Figure 26. ESAT-6 dramatically inhibited miR146a expression in both LPS- and BCG-treated DCs. Quantitative expression of miR146a profile after LPS and ESAT-6 treatment in DCs.

Taken together, all these observations suggest that ESAT-6 down regulates miR146a expression in M. tb infected DCs, which results in increased production of IL-6 that promotes Th17 cell differentiation.