Originally published as:

Kumar, A., Lewin, A., Rani, P.S., Qureshi, I.A., Devi, S., Majid, M., Kamal, E., Marek, S., Hasnain, S.E., Ahmed, N.
Dormancy Associated Translation Inhibitor (DATIN/Rv0079) of Mycobacterium tuberculosis interacts with TLR2 and induces proinflammatory cytokine expression
(2013) Cytokine, 64 (1), pp. 258-264.

Author version:

Dormancy Associated Translation Inhibitor (DATIN/Rv0079) of *Mycobacterium tuberculosis* induces proinflammatory cytokine expression and interacts with TLR2

Author names and affiliations:

Ashutosh Kumar[a], Astrid Lewin[b], Insaf A. Qureshi[c], Pittu Sandhya Rani[a], Savita Devi[a], Mohammad Majid[1], Elisabeth Kamal[b], Stefanie Banik[b], Seyed E. Hasnain[d], Niyaz Ahmed[a*]

[a] Pathogen Biology Laboratory, Department of Biotechnology and Bioinformatics, University of Hyderabad, Hyderabad, India

[b] Division 16 Mycotic and Parasitic Agents and Mycobacteria, Robert Koch Institute, Nordufer 20, 13353 Berlin, Germany

[c] Department of Biotechnology and Bioinformatics, University of Hyderabad, Hyderabad, India

[d] Kusuma School of Biological Sciences, Indian Institute of Technology Delhi, New Delhi, India

Corresponding author:

Dr Niyaz Ahmed, Dept. of Biotechnology and Bioinformatics, University of Hyderabad, School of Life Sciences @ South Campus, HCU Post, Hyderabad 500046, India
Telefax +91 40 66794585, Email: niyaz.ahmed@uohyd.ac.in

Abstract

Mycobacterium tuberculosis, the cause of tuberculosis in humans, is present approximately in one third of the world's population, mostly in a dormant state. The dosR regulon proteins are mainly responsible for its dormancy survival. To maintain latency, mycobacteria achieve a balanced interplay of different cytokines secreted by immune cells during the granulomatous stage. The function of most of the dosR regulon proteins of *M. tuberculosis* is unknown. In this study, we have shown that one of the dosR regulon proteins, DATIN, encoded by the gene *Rv0079*, can stimulate macrophages and peripheral blood mononuclear cells (PBMC) to secrete important cytokines that may be significant in granuloma formation and its maintenance. The expression level of DATIN in *Mycobacterium bovis* BCG was found to be upregulated in pH stress and microaerobic conditions. Computational modeling, docking and simulation study suggested that DATIN might interact with TLR2. This was further confirmed through the interaction of recombinant DATIN with TLR2 expressed by HEK293 cells. When *in vitro* differentiated THP-1 cells were treated with recombinant DATIN, increased secretion of TNF-α, IL-1β and IL-8 was observed in a dose dependent manner. When differentiated THP-1 cells were infected with a modified BCG strain overexpressing DATIN, augmented secretions of TNF-α, IL-1β and IL-8 were observed in comparison to a reference strain containing empty vector. Similarly, in human PBMCs, *M. bovis* BCG overexpressing DATIN upregulated the secretion of IFN-γ, TNF-α, IL-1β and IL-8. The cytokine profiles dissected herein point to a possible role of DATIN in maintenance of latency with the help of the proinflammatory responses.

Key words: *Mycobacterium tuberculosis*, dormancy antigens, dosR regulon proteins, proinflammatory cytokines, TLR2, Rv0079, granuloma

1. Introduction

Tuberculosis is one of the deadly diseases that result in heavy morbidity and mortality. *M. tuberculosis* persists in a sizeable portion of the world's population, probably in a latent form. It infects alveolar macrophages and multiplies therein. During latency, these infected macrophages are arranged in an ordered structure in lungs and form the granuloma that signifies a microenvironment characterized by low oxygen and high nitric oxide. A set of 48 genes of dos regulon is up-regulated in stress conditions and presumably play important roles in survival of mycobacteria within the granuloma, under latency. The expression of these genes are controlled by a regulated gene cluster consisting of three genes (Rv3134c/3133c/3132c), among them, DosR (Rv3133c) functions as a cognate response regulator [1-8]. It has been shown that the cellular composition of granuloma is in a dynamic state with respect to oxygen tension [9]. *M. tuberculosis* should be able to switch its metabolic activity from aerobic respiration to nitrate respiration in time with change in the oxygen concentration within the granuloma. dosR regulon plays an important role in recovery from anaerobiosis [10] and therefore it is essential for survival. It has been shown that dosR antigens induce stronger humoral immune response in individuals with latent infection when compared to acutely infected individuals [11]. This suggests stronger expression of dormancy survival regulon genes during latency. Apart from this, not many studies are carried out to identify the functions and interactions of the proteins encoded by dosR regulated genes. During infection, *M. tuberculosis* is recognized *via* TLRs and gets phagocytosed by alveolar macrophages. TLR2 in combination with TLR1/TLR6 and TLR4 [12, 13] together recognize mycobacterial components [14, 15]. It has been shown that inflammatory responses are necessary for granuloma formation and long term survival of *M. tuberculosis*; TNF-α, IFN-γ, IL-1β, IL-12, reactive oxygen (ROIs) and nitrogen intermediates (RNIs) are important for the maintenance of the granuloma structure [16, 17]. In addition, the chemokine IL-8 attracts circulating leukocytes to infiltrate inflamed tissues [18].

DATIN encoded by ORF *Rv0079* is one of the strongly up-regulated genes of the dosR regulon [8]. It has been demonstrated that DATIN may significantly act as regulator of dormancy functions such as translation control and stabilization of translational apparatus of mycobacteria [19, 20]. DATIN was originally found to be up-regulated under different *in vitro* and *in vivo* induced stress conditions such as hypoxia [21] and treatment with nitric oxide [7]. It was also

shown to be recognized by PBMCs from *M. tuberculosis*-infected individuals, as well as by most of the *M. tuberculosis* specific T-cell lines which were generated against lysate or culture filtrate of *M. tuberculosis* grown under low oxygen conditions or standard aerated condition. These observations support the contention that DATIN could express during infection and under stress conditions [11]. In this study, we show the up-regulation of DATIN under pH stress and microaerobic conditions and we demonstrate the interaction of DATIN with TLR2 leading to the secretion of proinflammatory cytokines such as TNF-α, IL-1β and IL-8 by the cultured macrophages and IFN-γ, TNF-α, IL-1β and IL-8 by human PBMCs. These results, as presented, might be significant in understanding the role of dormancy related proteins in the maintenance of latent state of tuberculosis.

2. Materials and methods

2.1. Recombinant strain generation and purification of DATIN:

The detailed methodology of the construction of recombinant strains [*E. coli* BL21 (pRSETA+Rv0079) and BCG (pMV261+Rv0079)] and purification of recombinant DATIN has been described previously (19, 22-24).

2.2. qRT-PCR to quantify the relative expression of DATIN:

M. bovis BCG (pMV261) and *M. bovis* BCG (pMV261+Rv0079) were grown up to cell density (OD_{600}) of 2.0 in Middlebrook broth with 10% OADC containing 25 μg/ml Kanamycin at 37 °C. They were then kept for 1 week at 37 °C in Middlebrook 7H9 broth of pH 5.3 supplemented with 10% OADC containing 25 μg/ml Kanamycin for pH stress or they were kept in microaerobic condition for oxygen stress. Microaerobic condition (6.2-13.2% oxygen) was achieved using the GENbox system from BioMérieux. RNA and cDNA were prepared from the BCG cultures grown in stress-free condition (neutral pH, atmospheric oxygen concentration), pH stress and microaerobic condition according to the protocol described by Khattak *et al.* [25]. Primers used for amplifications were (i) for gene *Rv0079*: 5´-GTA TTT CCC CCA GGA TGG TCA-3´ and 5´-GTC GTA GCG GGA GTA GAG CAG-3´ and (ii) for 16S rRNA gene: 5´-GAC CCC GTG AAG TCG GAG TCG -3´and 5´-TTC ATG ACG TGA CGG GCG GT -3´. Relative transcription level of DATIN in pH stress and microaerobic conditions was calculated in

comparison to stress-free condition by the ΔΔCT method. Relative expression of DATIN in the overexpressing strain [BCG (pMV261+Rv0079)] was also calculated in comparison to the BCG strain containing the empty vector [BCG (pMV261)]. Expression of 16S rRNA gene was used as reference gene for normalization.

2.3. Differentiated macrophages and human blood cells:

The human macrophage cell line THP-1 (DSMZ GmbH, Braunschweig, Germany) and recombinant HEK293 cell line (HEK-Blue™-hTLR2, InvivoGen, San Diego, US) were used for treatment with DATIN to measure cytokines in the culture supernatants and TLR2 activation. THP-1 and PBMC were used for infection with BCG. The THP-1 was maintained in RPMI 1640 (GIBCO® Invitrogen, Darmstadt, Germany) supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD, USA) by passaging twice weekly.

Aliquots of human blood were obtained from German Red Cross and PBMCs were isolated by Ficoll-Paque^{TM} Plus (GE Healthcare, USA) gradient centrifugation according to the manufacturer's recommendations and as described in Sharbati *et al*. [26]. Cells were resuspended in ACK-lysis buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$ and 0.1 mM Na_2EDTA, pH 7.3) to remove remaining erythrocytes from the cell pellets and then incubated for 5 min on ice. PBS was added to the lysis buffer, the cells were washed with PBS twice and then resuspended in IMDM medium with L-Glutamine (PAA, Pasching, Austria) and 3% human AB serum (PAA, Pasching, Austria).

HEK-Blue™-hTLR2 cells were grown in HEK-Blue™ selection medium that contained several selective antibiotics to ensure persistent expression of various transgenes introduced in HEK-Blue™-hTLR2 cells. Normocin was added to protect HEK-Blue™-hTLR2 cells from any possible microbial contamination. The cells were passaged (when 75-85% confluent) using PBS by gentle pipetting. Growth media and detection media for engineered HEK293 cell line were prepared according to the manufacturer's (InvivoGen, San Diego, USA) protocol. All cell lines and human PBMC were maintained at 37 °C with 5% CO_2.

2.4. Cytokine analysis of culture supernatants of THP-1 cells and PBMC:

In 24 well plates (TPP, Trasadingen, Switzerland), approximately 0.2 million THP-1 cells per well were differentiated into adherent macrophage-like phenotype with an overnight treatment of Phorbol 12-myristate 13-acetate (PMA) at a concentration of 10 ng/ml. The cells were washed twice with RPMI1640. THP-1 cells were then either treated with different concentrations of DATIN (1.0 μg, 2.5 μg and 5.0 μg) or infected at a multiplicity of infection (MOI) of 50 with BCG (pMV261) or BCG (pMV261+Rv0079) for 24 h. THP-1 cells treated with 100 U/ml of human IFN-γ (Invitrogen, Darmstadt, Germany) and 10 ng/ml of LPS, were used as positive control. Untreated cells were used as negative control. One million isolated PBMCs were seeded in 24 well plates and after 24 h, cells were infected by a MOI of 2 with BCG (pMV261) or BCG (pMV261+Rv0079). Uninfected cells were used as negative control while cells used as positive control were treated with 10 ng/ml of LPS. All culture plates were incubated at 37 °C with 5% CO_2. Culture supernatants were collected after 24 hrs and were stored at -80^{o}C until assayed. The cytokine levels of IFN-γ, TNF-α, IL-1β and IL-8 were measured with ELISA Kits (Ready-SET-Go) from eBioscience (Natutec, Frankfurt, Germany) by following the manufacturer's instructions.

2.5. DATIN –TLR-2 docking:

Prediction of protein-protein interaction site was done using MetaPPISP [27] while protein-protein docking was performed with the web version of PatchDock [28] as decribed earlier [29]. The crystal structure of TLR1-TLR2 heterodimer (PDB ID: 2Z82) retrieved from Protein Data Bank (receptor) was used for docking study with DATIN model (ligand) under default complex-type settings. Molecular visualization and general analysis were done using the program PyMOL [30]. HBOND program was used to identify hydrogen bonds at the molecular interface [31].

2.6. DATIN-TLR2 interaction assay:

In order to identify the interaction of DATIN with TLR2, a cell-based colorimetric assay was performed with engineered HEK293 cells containing multiple genes from the TLR2 pathway (that include TLR2 and genes participating in the signaling cascade). HEK-Blue™-hTLR2 cells also express an optimized alkaline phosphatase gene engineered to be secreted (sAP) and controlled by several transcription factors such NF-κB and activator protein-1. To measure the

interaction of DATIN with TLR2, different concentrations of DATIN (8 ng, 40 ng, 0.2 µg, and1µg) per aliquot used to treat approximately 50000 HEK-Blue™-hTLR2 cells. Treated cells were incubated at 37 °C in a CO_2 incubator overnight in HEK-Blue™ detection medium. In the presence of any ligand interacting with TLR2, HEK-Blue™-hTLR2 cells would secrete sAP in the HEK-Blue™ detection medium and result in a color change from pink to purple/blue. Detection of color change was done with a spectrophotometer at 620 nm. We used another His-tagged recombinant protein $rADI_{C424A}$ as negative control. $rADI_{C424A}$ is a mutant arginine deiminase (ADI) protein of *Giardia duodenalis,* which was expressed and purified from the similar expression system as used for DATIN. TLR2 deficient HEK-Blue cells [(which express TLR4 instead of TLR2 on the cell surface), namely the HEK-Blue™-hTLR4 (InvivoGen, USA)] were used as negative control.

3. Results

3.1. DATIN is up-regulated under pH stress and microaerobic conditions: We wanted to determine the effect of stress such as low oxygen pressure or low pH on the expression of DATIN. Furthermore, we were interested in knowing the level of up-regulation of DATIN in a BCG derivative overexpressing *Rv0079* [strain BCG (pMV261+Rv0079)]. The strain *M. bovis* BCG with the empty vector pMV261 displays a physiologic expression of *Rv0079* that was represented as relative expression level 1 in Figure 1 and served as reference to determine the relative expression in the over-expressing derivative. We observed a15 fold expression of *Rv0079* in BCG (p MV261+Rv0079) as compared to the control BCG strain [BCG (pMV261)] (Figure 1). It has been reported that Rv0079 is upregulated in different stress conditions as compared to stress-free conditions [7, 8]. We also determined relative expression of *Rv0079* in different stress conditions in comparison to stress-free conditions using ΔΔCT method and the expression of the 16S rDNA for normalization. As compared to stress-free growth conditions, *Rv0079* was expressed approximately 22-fold in pH stress and approximately 2.5-fold in microaerobic conditions, respectively (Figure 2).

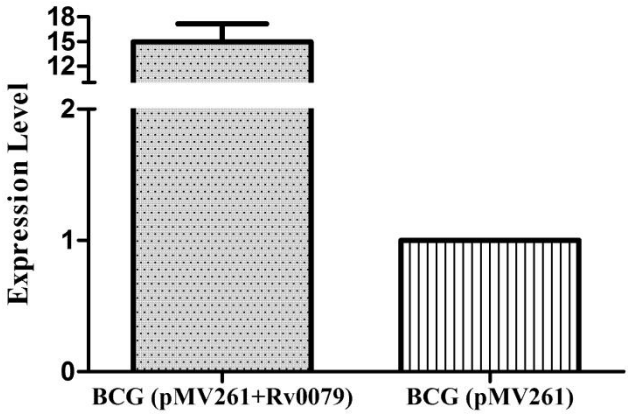

Figure 1: **DATIN expression in BCG strain overexpressing Rv0079.** RNA was isolated from *M. bovis* BCG (pMV261) and *M. bovis* BCG (pMV261+Rv0079) grown under normal condition. Reverse transcription followed by real time PCR was done to quantify the expression levels of DATIN. Upregulation of DATIN was observed in *M. bovis* BCG (pMV261+ Rv0079) as compared to *M. bovis* BCG (pMV261).

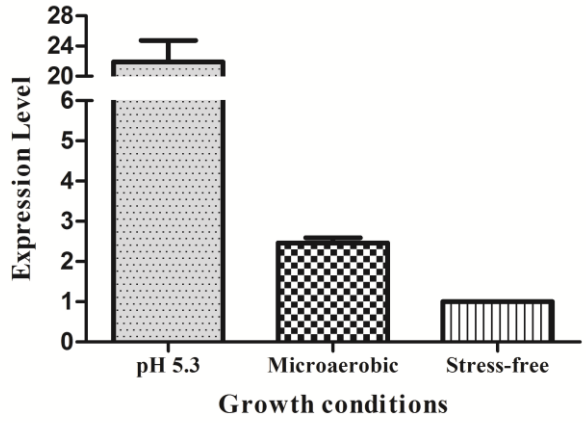

Figure 2: **Impact of stress on DATIN expression.** RNA was isolated from *M. bovis* BCG grown under different conditions. Reverse transcription followed by real time PCR was done to quantify the expression levels of DATIN. Upregulation of DATIN was observed under pH stress and microaerobic conditions.

3.2. DATIN induces proinflammatory cytokines: Recombinant purified DATIN induced proinflammatory response in PMA differentiated THP-1 cells. A significant increase in secretion of TNF-α, IL-1β and IL-8 was observed as compared to untreated cells (Figure 3). Further, cultured THP-1 cells were infected with BCG strains. The amount of TNF-α, IL-1β and IL-8 secreted by the THP-1 varied as a function of the type of BCG strain used [BCG (pMV261) or BCG (p MV261+Rv0079)]. The over expressing strain induced higher secretion of TNF-α, IL-1β

and IL-8 (Figure 4). IFN-γ is the most important cytokine for inhibit intracellular mycobacteria and we therefore wanted to include this cytokine in our studies. So, we treated human PBMCs with BCG strains for measuring IFN-γ together with TNF-α, IL-1β and IL-8. The amount of IFN-γ, TNF-α, IL-1β and IL-8 secreted by the PBMCs also varied as a function of the BCG strains [BCG (pMV261) or BCG (pMV261+Rv0079)] used for infection. The BCG strain over-expressing DATIN induced higher secretion of IFN-γ, TNF-α, IL-1β and IL-8 (Figure 5). In conclusion, our experiments with differentiated THP-1 cells and human PBMCs indicate that DATIN activates immune cells to secrete cytokines relevant to formation and maintenance of granuloma.

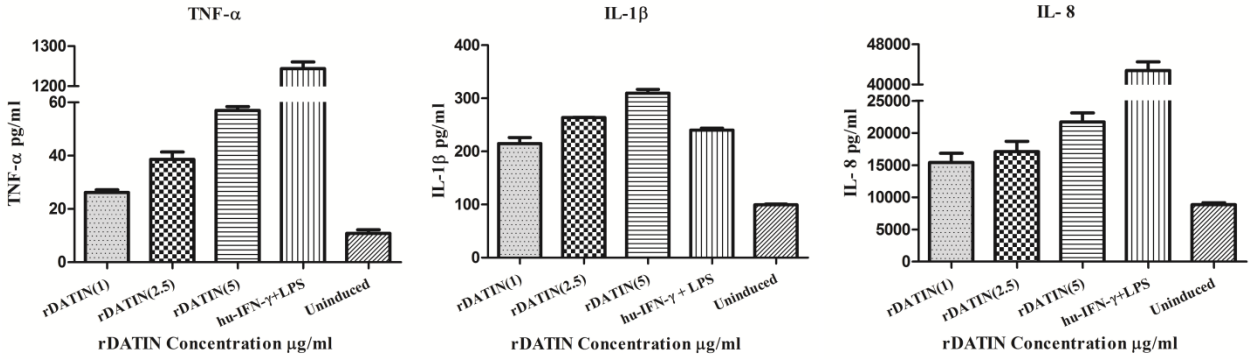

Figure 3: **rDATIN stimulates the secretion of proinflammatory cytokines by THP-1 cells.** Bar diagrams portraying the dose dependent release of proinflamatory cytokines (TNF-α, IL-1β and IL-8) consequent to treatment by rDATIN (1 µg, 2.5 µg, 5 µg) for 24 hr. Data represent the means±SDs of three technical replicates.

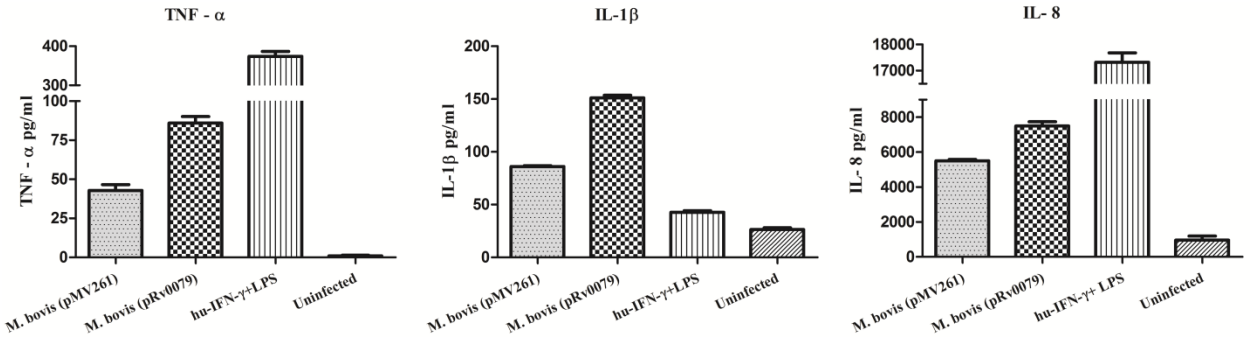

Figure 4: **Overexpression of DATIN by *M. bovis* BCG stimulates the secretion of proinflammatory cytokines by THP-1cells.** Secretion of the cytokines TNF-α, IL-1β and IL-8 by THP-1 cells infected with BCG (pMV261) and BCG (pRv0079) for 24 h. Data represent the means±SDs of the results of three technical replicates.

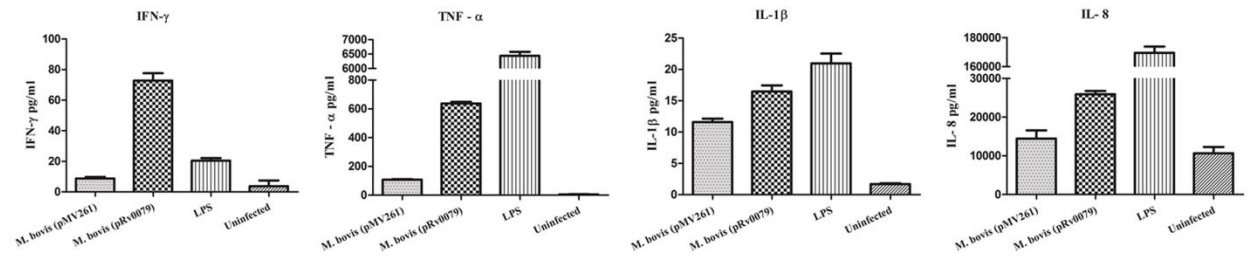

Figure 5: ***M. bovis* BCG overexpressing DATIN stimulates the secretion of proinflammatory cytokines by PBMCs.** Secretion of the cytokines IFN-γ, TNF-α, IL-1β and IL-8 by human PBMCs infected by BCG (pMV261) and BCG (pRv0079) for 24 h. Data represent the means±SDs of the results of three technical replicates.

3.3. DATIN interacts with TLR2: Our data suggest that DATIN induces the secretion of pro-inflammatory cytokines. Further, we were interested to identify the receptors on the cells through which DATIN triggers and up-regulates cytokine expression. In order to identify possible interacting domains of DATIN as juxtaposed to TLR2, several programs such as HEX [32], GRAMM-X [33] and PatchDock were employed for unbound protein-protein docking with TLR2 as a receptor and DATIN as a ligand. Approximately 100 predictions were generated using PatchDock. Ten best solutions were selected on the basis of geometric shape and complementarity score and were further analyzed to identify one that buried maximum surface area upon complex formation. Since there were no biological data available to identify the binding interface, MetaPPISP [27] was used to generate possible interacting residues with respect to both DATIN and TLR2. This profile was harnessed to analyse the 10 docked complexes for the presence of such residues in the interface. Several of the lowest energy docking models emerging from this exercise showed DATIN on the side of the TLR2. Among ten docked complexes, complex 9 was identified as the most plausible one on the basis of minimum energy score and binding interface residues. A docking model of TLR2-DATIN is shown in Figure 6A, in which loop 8 region of DATIN is docked into a domain formed by loop 2 region of TLR2 (Figure 6A). There was no covalent bond observed in docking studies while interaction is dependent on electrostatic forces. To confirm DATIN-TLR2 interaction, the recombinant HEK-Blue™-2 cell line was used which expressed TLR2 on the cell surface.

Generally, if any ligand interacts with TLR2, it should activate downstream signaling through NFkB activation resulting in transcription and secretion of alkaline phosphatase (sAP) engineered in the cell line; it should then react with detection media and result in colour change. Accordingly, we compared the interaction of TLR2 and DATIN with its interaction with the positive control provided in the detection kit (InvivoGen). DATIN showed significant interaction with TLR2 (Figure 6B). The activation of TLR2 induced by the application of 1 µg of DATIN achieved 76.5% of the positive control reading. When we applied $rADI_{C424A}$ protein (instead of DATIN) as a negative control, no TLR2 activation was observed. We also used the HEK-TLR4 cells which express TLR4 on the cell surface to see if there is any interaction with TLR4. These cells were the same as the HEK-TLR2 cells (only the expressed TLR type was different). There was no reaction with the HEK-TLR4 cells confirming the observed signaling was through TLR2. The negative reaction with TLR4 also ruled out the possibility of the presence of any lipopolysaccharide fractions in our protein preparation. Apart from these critical controls, other experiment level controls such as selection media control, positive control, growth media control, and fetal bovine serum control were included in the test. In sum, these observations clearly support the interaction of DATIN with TLR2.

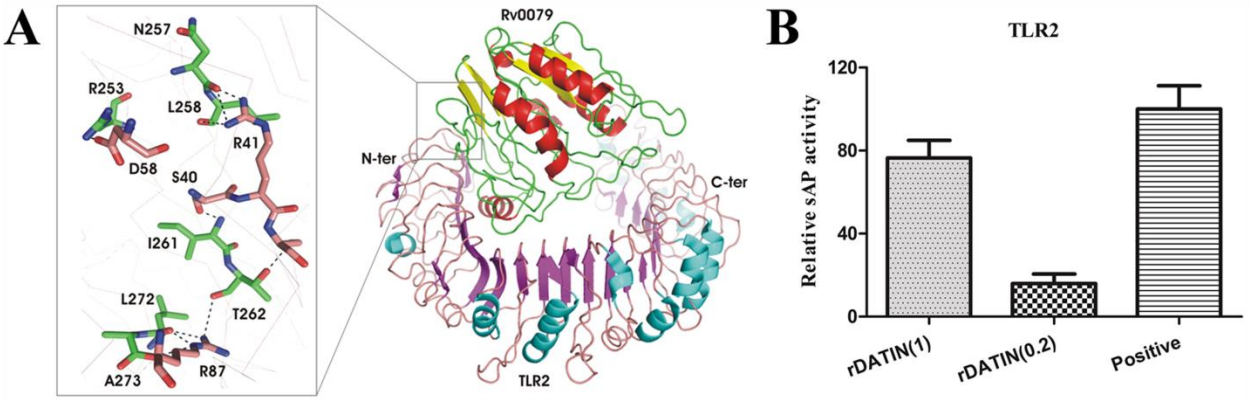

Figure 6: **DATIN interacts with TLR2.** A. Interaction of DATIN with TLR2 using PatchDock. The residues of DATIN and TLR2 are colored in cyan and green respectively. The residues showing interaction among both proteins are labelled and shown as stick model in element colors (carbon colored green/pink, nitrogen colored blue, and oxygen colored red). Hydrogen bonds are annotated by black dashed lines. B. Comparative activation of TLR2 by rDATIN with respect to positive control. HEK-Blue™-2 cells expressing TLR2 were treated with different amounts (1 µg and 0.2 µg) of rDATIN and interaction of TLR2 with rDATIN was quantified by measuring the activity of the secreted alkaline phosphatase (sAP) read as color change which was documented as absorption measurement at 620nm. The sAP activity caused by the positive control was set to 100%.

4. Discussion

One of the most baffling aspects of tuberculosis infection is that only a small fraction of the infected or exposed individuals develop active disease. In the remaining cases, *M. tuberculosis* might persists in a dormant, non-replicative form and a state of drug indifference for a prolonged time with an unbattered potential to resuscitate whenever a conducive *milieu* is available. Understanding the latent or dormant form of *M. tuberculosis* has been a tough endeavour because no significant animal models are available and the *in vitro* models are not quite successful or remain impractical. Further, the micro-environment of the granuloma, where the dormant *M. tuberculosis* lodges, is impermeable to the present day anti-TB drugs or there is less effect of antibiotics on dormant tubercle bacilli due to their non-replicating state [34]. Given this, the fundamental challenge in the control of tuberculosis could be due to the frustrating lack of state of art concerning molecular mechanisms triggering the onset of latency, the maintenance of the latent state and the reactivation of dormant bacilli. In this study, we attempted to explore this problem at the level of dosR regulon gene functions by a combination of predicted protein-protein interaction followed by validation through cell signaling and *in vitro* cytokine profiling using recombinant protein as well as transformed BCG strains. The use of recombinant BCG strains for the analysis of genes from the *M. tuberculosis* complex is a mainstream and accepted approach and it is especially suited because a majority of BCG and *M. tuberculosis* genes are 100% homologous and many of the dosR regulon genes have been already analyzed in BCG.

It has been shown that dosR regulon genes are up-regulated during dormancy and that they have important immunological roles [11, 35] to play including their newly identified dormancy functions such as stabilization of ribosomes [19, 36]. In order to find a new way to cure tuberculosis it is important to know the effect of virulence associated genes of the dosR regulon and their interaction with the host immune system. DATIN is one of the strongly up-regulated genes in the dosR [8], the latter encompasses an essential set of genes needed for adaptation to stress conditions. While DATIN has a established role in translation control [19, 36], its amino acid sequence presented with high antigenic index prompted us to look into its possible immunological/antigenic potential. Further, DATIN was earlier listed as a candidate to induce human T-cell responses [11]. While pursuing these upstream observations, and when we treated

differentiated human macrophages (THP-1) with DATIN, an increased secretion of proinflammatory cytokines was observed (Figure 3) revealing that DATIN activates macrophages and could likely play a significant role in innate immune functions that govern the microenvironment of tuberculous lesions. As observed, the over-expressing recombinant BCG strain induced higher levels of IFN-γ, TNF-α, IL-1β and IL-8 secretion (Figure 4 and 5) supportive of a role for DATIN in mounting a proinflammatory response. It is noteworthy that TNF-α plays a significant role in granuloma formation, macrophage activation and also immune regulatory functions [16, 17]. The studies in mouse models suggested that IFN-γ and TNF-α were produced in the lungs of mice with a persistent *M. tuberculosis* infection [37]. We interpret that immune regulation by the secretion of proinflammatory cytokines IFN-γ, TNF-α, IL-1β and IL-8 may be significant in the formation of granuloma, in maintenance of its microenvironment and also for the persistence of latent mycobacteria.

M. tuberculosis is specifically recognized by toll like receptors such as TLR2, whose activation triggers transcription of proinflammatory cytokines such as IL1β, TNFα, and IL8, which are all essential for the recruitment of the cells of inflammation to the site of infection and also to contain the infection. It is known that ligands from mycobacteria can induce secretion of pro-inflammatory [38-40] and anti-inflammatory [41] molecules *via* TLR2 signaling. Our data specifically suggest the interaction of DATIN with TLR2 which we see as an interesting possibility that has the potential to enrich the library of bacterial factors that influence host responses.

Our computational modeling could generate with a high degree of confidence a model with binding sites on DATIN corresponding to ribosomal 30S [19] and the TLR2 (this study). *In silico* docking analyses indicated that DATIN specifically interacts with the loop 2 region of TLR2 (Figure 6). Although technically highly demanding, it will be possible in future to further confirm the uniqueness of the binding pockets, or otherwise, on both the ligand and receptor(s) sites through mutagenesis. Nevertheless, the present set of experiments and the data generated were of sufficiently high quality and were robust enough to approach the hypothesis of TLR2 interaction. We could demonstrate that DATIN interacts with TLR2 in a dose-dependent manner. Another protein ($rADI_{C424A}$) used as control and applied in the same concentrations did not bind to TLR2. The DATIN concentrations reached within a granuloma are not known and as a

consequence we do not know the physiological relevance of the amounts of DATIN applied in our experiments. But it is an often applied and accepted method to express proteins and then add them to cell cultures to monitor cellular response towards the protein. In all these studies the relevance with respect to physiological concentrations is difficult to espouse or predict. It is also to be considered that the local concentration of molecules within a tissue or aggregation of cells is not uniform, leading to the possibility of relatively high local concentrations of ligands. Having this said, our results strongly support a role of DATIN in cell signaling *via* TLR2.

Given these observations interpreted in the light of our previous study [19] that described DATIN to be a translation inhibitor, it is tempting to espouse that the two phenomena, namely the ribosome binding/stabilization and interaction with TLR2 to trigger proinflammatory signaling could be interrelated as they both favor latency. However, it is not plausible from our data that both the processes are interdependent although this possibility cannot be negated. Nonetheless, in the event of DATIN limiting its own translation by ribosome binding, as previously suggested [19], reduced DATIN availability might affect proinflammatory activity needed to maintain granuloma. But, the granuloma microenvironment (cytokine mediated oxidative stress) could again serve as a trigger for DosR and DATIN expression; given this, the existence of a finely negotiated homeostasis of DATIN expression and function at the level of granuloma cannot be ruled out. Further the exact mode of secretion of DATIN and its presentation to TLR2 would remain matter of future research although its presence in mycobacterial culture supernatants hints at its being in the extracellular compartment (11).

Finally, we believe that our study provides a much needed snapshot of dos regulon protein functions with DATIN being an interesting candidate at the cross roads of two different latency functions. We believe that the combination of computational, cell culture based and recombinant BCG based approaches was the uniqueness of the study. In view of the predicted interaction model, experiments carried out using recombinant DATIN and the corroboration of DATIN functions using the BCG environment made the study further interesting. Future discovery and vaccine development efforts would likely benefit from this knowhow.

Acknowledgements

We thank Christoph Ufermann for his help with the TLR2 assay. This work was supported by a Centre of Excellence Grant from the Department of Biotechnology (DBT) of the Indian Government entitled 'Multidisciplinary approaches aimed at interventions against Mycobacterium tuberculosis' (BT/01/C0E/07/02; component B2b) and another grant from the Council for Scentific and Industrial Research (CSIR) India under the OSDD program to NA. The authors would like to also acknowledge support from the German Research Foundation (DFG) sponsored international research training group (IRTG) entitled 'Internationales Graduiertenkolleg - functional molecular infection epidemiology - GRK1673 (Berlin-Hyderabad)'. SEH is a J. C. Bose National Fellow (India) and a Robert Koch Fellow of the Robert Koch Institute, Berlin, Germany. PSR and AK would like to acknowledge DBT and the CSIR, respectively, for the award of Senior Research Fellowships.

References

[1] C. Boon, T. Dick, Mycobacterium bovis BCG response regulator essential for hypoxic dormancy, Journal of bacteriology. 184 (2002) 6760-6767.

[2] M. A. Florczyk, L. A. McCue, A. Purkayastha, E. Currenti, M. J. Wolin, K. A. McDonough, A family of acr-coregulated Mycobacterium tuberculosis genes shares a common DNA motif and requires Rv3133c (dosR or devR) for expression, Infection and immunity 71 (2003) 5332-5343.

[3] R. W. Honaker, R. L. Leistikow, I. L. Bartek, M. I. Voskuil, Unique roles of DosT and DosS in DosR regulon induction and Mycobacterium tuberculosis dormancy, Infection and immunity. 77 (2009) 3258-3263.

[4] S. L. Kendall, F. Movahedzadeh, S. C. Rison, L. Wernisch, T. Parish, K. Duncan, J. C Betts, N. G. Stoker, The Mycobacterium tuberculosis dosRS two-component system is induced by multiple stresses, Tuberculosis. 84 (2004) 247-255.

[5] M. J. Kim, K. J. Park, I. J. Ko, Y. M. Kim, J. I. Oh, Different roles of DosS and DosT in the hypoxic adaptation of Mycobacteria, Journal of bacteriology. 192 (2010) 4868-4875.

[6] H. D. Park, K. M. Guinn, M. I. Harrell, R. Liao, M. I. Voskuil, M. Tompa, G. K. Schoolnik, D. R. Sherman, Rv3133c/dosR is a transcription factor that mediates the hypoxic response of Mycobacterium tuberculosis, Molecular microbiology 48 (2003) 833-843.
[7] M. I. Voskuil, D. Schnappinger, K. C. Visconti, M. I. Harrell, G. M. Dolganov, D. R. Sherman, G. K. Schoolnik, Inhibition of respiration by nitric oxide induces a Mycobacterium tuberculosis dormancy program, The Journal of experimental medicine. 198 (2003) 705-713.
[8] M. I. Voskuil, K. C. Visconti, G. K. Schoolnik, Mycobacterium tuberculosis gene expression during adaptation to stationary phase and low-oxygen dormancy, Tuberculosis. 84 (2004) 218-227.
[9] C. L. Cosma, O. Humbert, D. R. Sherman, L. Ramakrishnan, Trafficking of superinfecting Mycobacterium organisms into established granulomas occurs in mammals and is independent of the Erp and ESX-1 mycobacterial virulence loci, The Journal of infectious diseases. 198 (2008) 1851-1855.
[10] R. L. Leistikow, R. A. Morton, I. L. Bartek, I. Frimpong, K. Wagner, M. I. Voskuil, The Mycobacterium tuberculosis DosR regulon assists in metabolic homeostasis and enables rapid recovery from nonrespiring dormancy, Journal of bacteriology. 192 (2010) 1662-1670.
[11] E. M. Leyten, M. Y. Lin, K. L. Franken, A. H. Friggen, C. Prins, K. E. van Meijgaarden, M. I. Voskuil, K. Weldingh, P. Andersen, G. K. Schoolnik, S. M. Arend, T. H. Ottenhoff, M. R. Klein, Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of Mycobacterium tuberculosis, Microbes infection 8 (2006) 2052-2060.
[12] A. Ozinsky, D. M. Underhill, J. D. Fontenot, A. M. Hajjar, K. D. Smith, C. B. Wilson, L. Schroeder, A. Aderem, The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors, Proceedings of the National Academy of Sciences of the United States of America. 97 (2000) 13766-13771.
[13] S. Stenger, R. L. Modlin, Control of Mycobacterium tuberculosis through mammalian Toll-like receptors, Current opinion in immunology. 14 (2002) 452-457.
[14] H. D. Brightbill, D. H. Libraty, S. R. Krutzik, R. B. Yang, J. T. Belisle, J. R. Bleharski, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, P. J. Brennan, B. R. Bloom, P. J. Godowski, R. L. Modlin, Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors, Science. 285 (1999) 732-736.
[15] Y. Bulut, E. Faure, L. Thomas, O. Equils, M. Arditi, Cooperation of Toll-like receptor 2 and

6 for cellular activation by soluble tuberculosis factor and Borrelia burgdorferi outer surface protein A lipoprotein: role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling, Journal of immunology. 167 (2001) 987-994.
[16] A. M. Cooper, Cell-mediated immune responses in tuberculosis, Annual review of immunology. 27 (2009) 393-422.
[17] S. J. Sasindran, J. B. Torrelles, Mycobacterium Tuberculosis Infection and Inflammation: what is Beneficial for the Host and for the Bacterium? Frontiers in microbiology 2 (2011) 2.
[18] V. C. Broaddus, C. A. Hebert, R. V. Vitangcol, J. M. Hoeffel, M. S. Bernstein, A. M. Boylan, Interleukin-8 is a major neutrophil chemotactic factor in pleural liquid of patients with empyema, The American review of respiratory disease. 146 (1992) 825-830.
[19] A. Kumar, M. Majid, R. Kunisch, P. S. Rani, I. A. Qureshi, A. Lewin, S. E. Hasnain, N. Ahmed, Mycobacterium tuberculosis DosR regulon gene Rv0079 encodes a putative, 'dormancy associated translation inhibitor (DATIN)', PloS one. 7 (2012) e38709.
[20] S. Mishra, Function prediction of Rv0079, a hypothetical Mycobacterium tuberculosis DosR regulon protein, Journal of biomolecular structure & dynamics. 27 (2009) 283-292.
[21] D. R. Sherman, M. Voskuil, D. Schnappinger, R. Liao, M. I. Harrell, G. K. Schoolnik, Regulation of the Mycobacterium tuberculosis hypoxic response gene encoding alpha – crystallin, Proceedings of the National Academy of Sciences of the United States of America. 98 (2001) 7534-7539.
[22] P. Reichelt, C. Schwarz, M. Donzeau, Single step protocol to purify recombinant proteins with low endotoxin contents, Protein expression and purification. 46 (2006) 483-488.
[23] S. Sharbati-Tehrani, B. Meister, B. Appel, A. Lewin, The porin MspA from Mycobacterium smegmatis improves growth of Mycobacterium bovis BCG. International journal of medical microbiology : IJMM 294 (2004) 235-245.
[24] C. K. Stover, V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull, S. B. Snapper, R.G. Barletta, W. R. Jacobs JR, B. R. Bloom, New use of BCG for recombinant vaccines, Nature. 351 (1991) 456-460.
[25] F. A. Khattak, A. Kumar, E. Kamal, R. Kunisch, A. Lewin, Illegitimate recombination: An efficient method for random mutagenesis in Mycobacterium avium subsp. Hominissuis, BMC microbiology. 12 (2012) 204.
[26] J. Sharbati, A. Lewin, B. Kutz-Lohroff, E. Kamal, R. Einspanier, S. Sharbati, Integrated

microRNA-mRNA-analysis of human monocyte derived macrophages upon Mycobacterium avium subsp. hominissuis infection, PloS one. 6 (2011) e20258.
[27] S. Qin, H. X. Zhou, meta-PPISP: a meta web server for protein-protein interaction site prediction, Bioinformatics. 23 (2007) 3386-3387.
[28] D. Schneidman-Duhovny, Y. Inbar, R. Nussinov, H. J. Wolfson, PatchDock and SymmDock: servers for rigid and symmetric docking, Nucleic acids research. 33 (2005) W363-367.
[29] A. Alvi, S. A. Ansari, N. Z. Ehtesham, M. Rizwan, S. Devi, L. A. Sechi, I. A. Qureshi, S. E. Hasnain, N. Ahmed, Concurrent proinflammatory and apoptotic activity of a Helicobacter pylori protein (HP986) points to its role in chronic persistence, PloS one. 6 (2011) e22530.
[30] W. L. Delano, The PyMOL molecular graphics system, Delano Scientific, San Carlos, CA. (2002) http://www.pymol.org/.
[31] K. Mizuguchi, C. M. Deane, T. L. Blundell, M. S. Johnson, J. P. Overington, JOY: protein sequence-structure representation and analysis, Bioinformatics. 14 (1998) 617-623.
[32] G. Macindoe, L. Mavridis, V. Venkatraman, M. D. Devignes, D. W. Ritchie, HexServer: an FFT-based protein docking server powered by graphics processors, Nucleic acids research. 38 (2010) W445-449.
[33] A. Tovchigrechko, I. A. Vakser, GRAMM-X public web server for protein-protein docking, Nucleic acids research. 34 (2006) W310-314.
[34] L. G. Wayne, C. D. Sohaskey, Nonreplicating persistence of mycobacterium tuberculosis, Annual review of microbiology. 55 (2001) 139-163.
[35] Y. Yamamura, K. Tsujimura, S. Seto, M. Uchijima, H. Hozumia, T. Nagatac, Y. Koided, Immunogenicity of latency-associated antigens of Mycobacterium tuberculosis in DNA-vaccinated mice, Procedia in Vaccinology. 3 (2010) 19–26.
[36] A. Trauner, K. E. Lougheed, M. H. Bennett, S. M. Hingley-Wilson, H. D. Williams, The dormancy regulator DosR controls ribosome stability in hypoxic mycobacteria, The Journal of biological chemistry. 287 (2012) 24053-24063.
[37] J. L. Flynn, C. A. Scanga, K. E. Tanaka, J. Chan, Effects of aminoguanidine on latent murine tuberculosis, Journal of immunology. 160 (1998) 1796-1803.
[38] R. N. Aravalli, S. Hu, T. N. Rowen, J. M. Palmquist, J. R. Lokensgard, Cutting edge: TLR2-mediated proinflammatory cytokine and chemokine production by microglial cells in response to

herpes simplex virus, Journal of immunology. 175 (2005) 4189-4193.
[39] S. Basu, S. K. Pathak, A. Banerjee, S. Pathak, A. Bhattacharyya, Z. Yang, S. Talarico, M. Kundu, J. Basu, Execution of macrophage apoptosis by PE_PGRS33 of Mycobacterium tuberculosis is mediated by Toll-like receptor 2-dependent release of tumor necrosis factor-alpha, The Journal of biological chemistry. 282 (2007)1039-1050.
[40] P. S. Rani, N. K. Tulsian, L. A. Sechi, N. Ahmed, In vitro cytokine profiles and viability of different human cells treated with whole cell lysate of Mycobacterium avium subsp. Paratuberculosis, Gut pathogens. 4 (2012) 10.
[41] S. Nair, P. A. Ramaswamy, S. Ghosh, D. C. Joshi, N. Pathak, I. Siddiqui, P. Sharma, S. E. Hasnain, S. C. Mande, S. Mukhopadhyay, The PPE18 of Mycobacterium tuberculosis interacts with TLR2 and activates IL-10 induction in macrophage, Journal of immunology. 183 (2009) 6269-6281.