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pknE, a serine/threonine kinase of *Mycobacterium tuberculosis* modulates multiple apoptotic paradigms

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ABSTRACT

Mycobacterium tuberculosis, an intracellular pathogen that causes tuberculosis has developed multifactorial mechanisms to evade host signaling responses. Apoptosis, an important innate host immune response that clears the invading pathogen is suppressed by *M. tuberculosis* to gain persistence.

Here, we examined the various apoptotic events suppressed by Protein Kinase E, (*pknE*) a Serine Threonine Protein Kinase (STPK) of *M. tuberculosis* in macrophages infected with Δ*pknE*, a deletion mutant of *pknE* vs. the wild type strain H_{37}Rv using microarray. The data showed increased expression of genes involved in apoptosis and chemokines with suppressed pro-inflammatory cytokines, co-stimulatory molecules, arginase1 and iNOS. The microarray data was validated using qRT-PCR, PCR array, oligoGE array, arginase assay and/or ELISA. Furthermore, we analyzed the phosphorylation of Akt that promotes cell survival using western blotting. Δ*pknE* infected macrophages reduced the phosphorylation of Akt that correlates with the observed apoptotic responses.

Experiments performed using exogenous nitrate donor, sodium nitro prusside to demonstrate the role of *pknE* during nitrate stress showed similar apoptotic responses to that of endogenous nitrate stress in Δ*pknE* infected macrophages. Our data confirms the role of *pknE* in the intra cellular survival of *M. tuberculosis* by suppressing apoptosis during nitrate stress.

1. Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* thwarts the host immune responses by modulating apoptosis. Manipulation of apoptosis creates a specialized niche for *M. tuberculosis* to persist and reactivate (Porcelli and Jacobs, 2008). Apoptosis is mediated by several factors like nitric oxide (NO), tumor necrosis factor (TNF-α) and caspases. TNF-α, a major player in the execution of apoptosis was reported to be influenced by the virulence of *M. tuberculosis* strains (Porcelli and Jacobs, 2008). Furthermore, *M. tuberculosis* induced apoptosis was shown to activate either caspase dependent or independent pathways (O'Sullivan et al., 2007). Since the virulence of *M. tuberculosis* strains influences apoptosis, thereby modulating the intracellular survival, it becomes imperative to understand the role of virulence genes in the pathogenesis of TB.

The signal transduction pathway of *M. tuberculosis* comprises of 11 serine/threonine kinases (STPKs PknA to PknL) that direct the adaptation of tubercle bacilli to environmental cues (Walburger et al., 2004). The genes nuoG, secA2 and *pknE* were shown to aid in the intracellular survival of *M. tuberculosis* by suppressing apoptosis (Briken and Miller, 2008). Besides these genes, *pknG* was reported to block the phagosome lysosome fusion, *pknH* was found to regulate the bacillary load and pknK was found to play a role in adaptive response (Walburger et al., 2004; Papavinasasundaram et al., 2005; Malhotra et al., 2010). Hence STPKs play an important role in the intracellular survival of *M. tuberculosis*. Identifying the mechanisms by which STPKs influence the pathogenesis of TB would help in discovering drug targets.

In our previous report (Jayakumar et al., 2008), we have shown that, the STPK *pknE* gene of *M. tuberculosis* expressed under NO stress contributes to the suppression of apoptosis. The present study examines different mechanisms involved in the inhibition of apoptosis by *pknE* using microarray analysis of Δ*pknE*, a deletion mutant of *pknE* and its wild-type strain infected macrophages. Expression of selective genes from apoptosis signaling, co-stimulatory, cytokines and suppressor of cytokine signaling (SOCS) family, β-chemokines, arginase metabolism and iNOS, Toll-like receptor (TLR), NF-κB and Akt were authenticated using oligoGE array, qRT-PCR, ELISA, arginase assay, PCR array and/or western blotting. The observed differential expression was restored in the complemented strain.

Our study for the first time unveils the role of *pknE* in activating multiple signaling responses that promote the survival of infected macrophages. Our data signifies that the presence of *pknE* could

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alter the interactions with TLRs and influence the suppression of intrinsic pathway of apoptosis. The ensuing suppression, on the other hand increases the expression of arginase1 and Akt promoting macrophage survival. These modulations in cellular signaling elevate pro-inflammatory cytokines that may increase the inflammatory responses. Thus, *pknE* contributes to the survival response of *M. tuberculosis* by regulating the bacilli machinery to resist apoptosis elicited by multiple signals.

2. Materials and methods

2.1. Bacterial strains, culture conditions, and single-cell suspension

The strains *M. tuberculosis* H_{37}Rv, *M. tuberculosis* H_{37}RvΔ*pknE* and complemented *M. tuberculosis* H_{37}RvΔ*pknE* were grown in Middlebrook complete 7H9 broth as reported earlier (O'Sullivan et al., 2007) with 50 μg/ml of hygromycin and 20 μg/ml of kanamycin when required. A single-cell suspension was made from mid-exponential phase cultures by syringing in a 26-gauge needle 7–8 times. The suspension was centrifuged at 200*g*, the supernatant was aliquoted and stored at −80 °C. The viability of the cells were determined using Middlebrook 7H10 agar plates using representative samples.

2.2. Cell culture and infection

The THP-1 human monocytic cell line was induced to differentiate into adherent macrophage-like cells by overnight exposure to 5 nM phorbol myristate acetate. The differentiated cell line was infected with the respective bacterial strains at a multiplicity of infection 1:10 for 4 h and washed with serum-free media twice. The experiments were carried out on day 1, day 5, nitrate stress and the cells were lysed for RNA extraction or western blotting. During the NO stress response, post-infection, the cells were treated with 10 mM sodium nitro prusside (SNP) nitrate donor suspended in complete DMEM for 3 h and the cells were lysed for RNA extraction. The supernatants were stored at −20 °C for cytokine analysis.

2.3. Microarray and Quantitative Real-time -PCR

The cells were harvested on day 5 post infection based on our previous observation (Jayakumar et al., 2008) and total RNA was isolated using Rneasykit (Qiagen, USA). From total RNA, cRNA was prepared and hybridized on agilent 22 k human microarray. Agilent's scan control software (v.6.3) was used for scanning and capturing the images, and agilent feature extraction software (v.8.1) was used for data processing. The data analysis was done using GeneSpring GX version 7.2 and Microsoft Excel. Briefly, the data normalization was done by recommended Per Spot and Per Chip: Intensity dependent (Lowess) normalization and differentially regulated genes were filtered by assigning values for upregulation of genes (ratio above 2) and down-regulation of genes (ratio less than 0.5).

Quantitative Real-time PCR (q-RTPCR) was performed for the samples on day 1, day 5 and under nitrate stress as biological replicates using gene-specific primers for human interleukins, SOCS and B7 family from TaqMan® Gene Expression Assays (Applied Biosystems, USA) and normalized to 18s RNA.

The arginase metabolism was studied using LUX™ primers (Invitrogen, USA) and the values were normalized to β-actin. SYBR green chemistry-based pathway-focused gene expression profiling RT^{2}Profiler™ PCR Array System (SuperArray Bioscience Corporation, USA) was used for TLR & NF-κB, β-chemokines and analyzed as per the manufacturer instructions. The relative gene expression for the qRT-PCR was calculated based on the ΔΔCT method.

2.4. OligoGEarray

Human apoptosis Oligo GEArrays® (SuperArray Bioscience Corporation) was used to confirm the apoptotic genes on day 1, day 5 and under nitrate stress. The RNA was labeled, hybridized and analyzed as per the manufacturer's instructions.

2.5. ELISA

Cell-free culture supernatants were analyzed for the levels of IL-6 and TNF-α by sandwich ELISA using BD optEIA ELISA kit (BD pharmingen, USA) as per the manufacturer's instructions.

2.6. Arginase enzyme assay

Arginase activity was determined by measuring the total production of urea in culture supernatants as described earlier (Dzik et al., 2004).

2.7. Western blotting

The cell lysates were prepared by lysing 0.5 million cells in 100 μl of 2× lysis buffer (125 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 100 mM DTT, and 0.05% bromophenol blue). The lysates were denatured at 95 °C for 5 min followed by centrifuging at 1500*g* for 15 min. The clear lysates were separated by 12% SDS–polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membrane for 1 h 10 min at 360 mA. Membranes were then blocked for 1 h using 5% nonfat dry milk in tris buffered saline containing 0.1% Tween 20 (TBST). After three washes in TBST, the membrane was incubated overnight at 4 °C with rabbit antihuman polyclonal antibodies (Cell Signaling Technologies, Inc., USA) against phospho and nonphospho Akt (1:1000). Horseradish peroxidase-conjugated goat anti-rabbit antibody (1:300 in TBST) (Amersham Biosciences, USA) was added to the membrane followed by three washes in TBST. The bound antibodies were then detected using chemiluminescent reagent (SuperSignal® West Pico Chemiluminescent Kit, Thermo Scientific, USA) after three washes with TBST by exposure to an X-ray film.

2.8. Statistics

The statistical analysis was carried out using SPSS v.18 for the qRT-PCR, ELISA and arginase activity. Mann Whitney test was used for qRT-PCR analysis and one way ANOVA was used to analyze the data from ELISA and arginase activity. P value < 0.05 was said to be significant and $P < 0.001$ was said to be highly significant in this study.

3. Results

3.1. Microarray analysis

The principal aim of this study was to examine the global apoptotic responses modulated by *pknE*. A 22k human microarray on agilent platform was used to compare the profiles of gene expression in THP-1 cells infected with wild-type H_{37}Rv (Rv), deletion mutant of *pknE* (Δ*pknE*) and complemented *pknE* (CΔE) strains on day 5, post infection. In order to compensate dye specific effects, and to insure statistically relevant data, a color swap dye reversal was performed. The data was filtered, analyzed using the agilent software as described in Section 2.3.

The data revealed Control vs Rv to up-regulate 409 genes with a down-regulation of 166 genes. While Control vs Δ*pknE* up-regulated 1544 genes and down regulated 1227 genes. CΔE was able to partially restore wild-type expression with 285 genes being up-regulated and 85 genes down-regulated. The summary of data is represented as Venn diagram (Fig. 1A and B) and the complete gene list can be accessed from Gene Expression Omnibus repository (Accession No. GSE20026).

Analysis of microarray data revealed that Δ*pknE* infected macrophages has reduced inflammatory responses with down regulation of vital metabolic pathways namely amino acid metabolism. The blunted immune response and down regulation of metabolic pathways supports the triggering of apoptosis by Δ*pknE*. In order to confirm the varied apoptotic responses by high throughput techniques we grouped the differentially expressed genes into eight functional categories with known function in *M. tuberculosis* pathogenesis namely apoptosis signaling, co-stimulatory molecules, cytokines and suppressor of cytokine signaling (SOCS) family, chemokines and their receptors, arginase metabolism and iNOS, Toll-like receptor (TLR) and NF-κB (Fig. 2). The dynamics of gene expression was examined on day 1 and day 5. Although Akt, a pro-survival kinase was not observed in microarray, this was included in our study to confirm the apoptotic responses.

3.2. *ΔpknE induces the expression of p53 and intrinsic mode of apoptosis*

Microarray analysis showed 42 genes to be differentially expressed by Δ*pknE* compared to Rv. These genes belong to varied apoptotic families namely TNFR family, caspases, NLR (nod like receptor) family, *Nol*3, mitochondria family and TP53. The differential expression was confirmed using oligoGE array and represented as heat map (Fig. 1C). The fold change generated by comparing Δ*pknE* vs. Rv from oligoGE array is shown in Table 1. The data confirmed that, Δ*pknE* infected macrophages undergo apoptosis by increasing the expression of mitochondrial genes. The expression

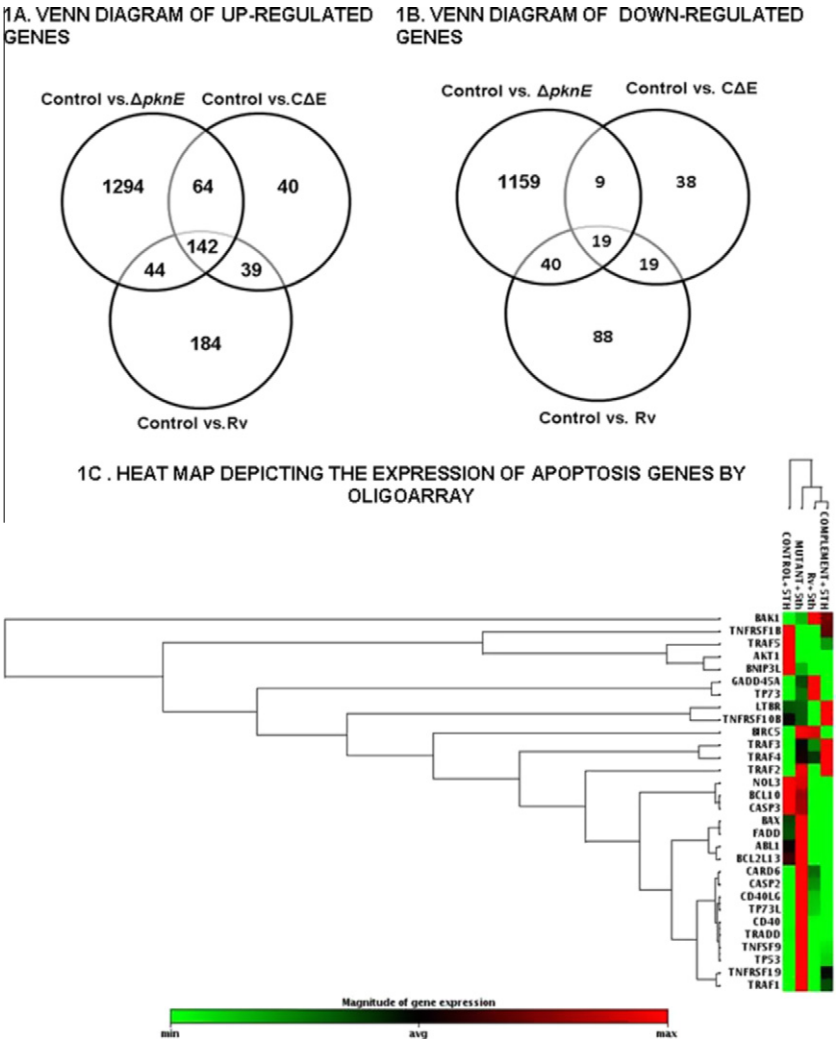

Fig. 1. Microarray and oligo array analysis of gene expression observed on day 5. (A) Venn diagram of up-regulated genes from microarray, 142 Genes up-regulated in all experiments, 1294 Genes up-regulated in experiments Control vs. Δ*pknE*, 44 Genes up-regulated in experiments Control vs. Δ*pknE* and Control vs. Rv, 184 Genes up-regulated in experiments Control vs. Rv, 39 Genes up-regulated in experiments Control vs. Rv and Control vs. CΔE, 40 Genes up-regulated in experiments Control vs. CΔE, 64 Genes up-regulated in experiments Control vs. Δ*pknE* and Control vs. CΔE. (B) Venn diagram of down-regulated genes from microarray, 19 Genes down-regulated in all experiments, 1159 Genes down-regulated in experiments Control vs. Δ*pknE*, 40 Genes down-regulated in experiments Control vs. Δ*pknE* and Control vs. Rv, 88 Genes down-regulated in experiments Control vs. Rv, 19 Genes down-regulated in experiments Control vs. Rv and Control vs. CΔE, 38 Genes down-regulated in experiments Control vs. CΔE, 9 Genes down-regulated in experiments Control vs. Δ*pknE* and Control vs. CΔE. (C) Heat map depicting the expression of apoptosis genes by oligoarray. The RNA used in microarray experiments was used to confirm the genes involved in apoptosis by human oligoGE array. The genes were identified by chemiluminescence on an X-ray sheet and analyzed by GEArray® Expression Analysis Suite. The gene normalization was done using inbuilt controls. The data is from two independent experiments.

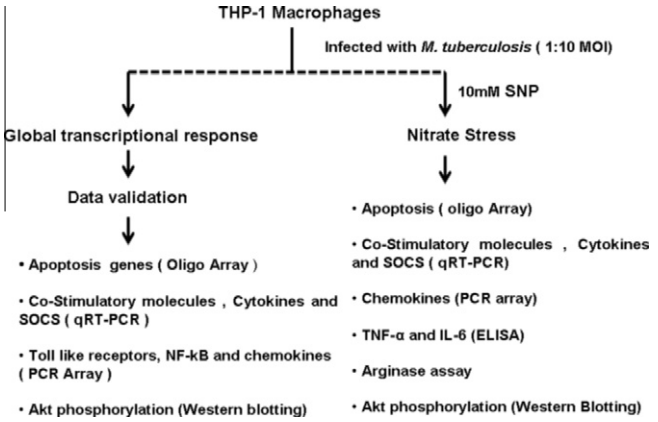

Fig. 2. Schematic representation of data validation by microarray and nitrate stress.

Table 1
Δ*pknE* increases the expression of apoptotic genes. The RNA from microarray experiments on day 5, RNA preparations from day 1 and nitrate stress were analyzed for the expression of apoptotic genes by human oligo GE array. The genes were identified by chemiluminescence on an X-ray sheet and analyzed by GEArray® Expression Analysis Suite. The gene normalization was done using inbuilt controls and the values were generated as fold change using the software provided by the manufacturer. The data is from two independent experiments.

Fold change of Δ*pknE* vs. H_{37}Rv by Oligo array					
Gene list day 1		Gene list day 5		Gene list during nitrate stress	
Bax	103	*Bax*	194.87	*Bag3*	11.91
TNFRSF1B	222	*BCL10*	45.75	*Bax*	57.87
		Bid	56.97	*BCL2L13*	18.39
		CASP3	29.59	*BIRC5*	12.38
		CASP14	40.67	*CARD9*	37.46
		CD70	220.92	*CASP9*	13.82
		FADD	172.93	*CD70*	12.54
		Mcl-1	0.01	*DAPK2*	12.15
		NOL3	82.58	*Mcl-1*	0.63
		TNFSF8	232.18	*TNFSF9*	10.10
		TNFSF9	236.48	*TNFRSF12A*	14.25
		TNFRSF10B	58.94	*TRAF4*	15.81
		TNFRSF14	55.13	*TRAF5*	34.69
		TP53	238.95		
		TRADD	159.98		
		TRAF1	214.21		
		TRAF2	61.24		

of *Bax* (103-fold) was increased on day 1 and further increased to 194.87-fold on day 5. *Bid* another mitochondrial gene showed its expression of 56.97-fold on day 5. The resultant apoptosis in Δ*pknE* infected macrophages reduced the expression of anti apoptotic gene Mcl-1 (0.63-fold) (Fig. 1C). The other apoptotic molecules TP53 (238.95-fold), *Nol3* (82.5-fold) were also increased (Table 1 and Fig. 1C). oligo array highlighted the expression of *CD70* (220.92-fold) as highest expressing gene next to *TP53* and *Bax* (Table 1 and Fig. 1C).

CΔE was able to restore the phenotypes observed to that of Rv infected macrophages. This clearly proves that *pknE* contributes to the suppression of apoptosis by modulating varied cell death pathways.

3.3. ΔpknE suppresses the expression of co-stimulatory molecules

The microarray data showed 15 genes to be differentially regulated by Δ*pknE* compared to Rv infected macrophages. The expression of CD80 and CD86 that belong to the B7/CD28 pathway was further confirmed using qRT PCR due to their role in initiating and regulating T-cell responses. Δ*pknE* infected macrophages reduced the expression of CD80 and CD86 compared to Rv on day 1 (Fig. 3), similar trend was observed on day 5 (data not shown). The CΔE strain was able to restore the expression to that of Rv infected macrophages. This shows that *pknE* plays a role in cellular activation during pathogenesis.

3.4. ΔpknE suppresses the expression of cytokines by increasing SOCS expression

Microarray analysis of Δ*pknE* compared to Rv infected macrophages showed 37 genes to be differentially modulated in the cytokines and SOCS-related pathway.

Among the 37 genes selective genes from pro-inflammatory (IL-1β,IL-8, IL-12p40 (IL-12B), IL-18, IL-23A, and TNF-α), anti-inflammatory (IL-6, IL-10, IL-13 and TGF-β) and SOCS family of proteins SOCS 1–7 and CISH that have a suppressive role on cytokines were validated by qRT-PCR on day 1 and day 5. Although only SOCS-1, -3 and CISH showed differential expression from the microarray data, we were interested to investigate the other genes SOCS 2–7, which have not been reported. Macrophages infected with Δ*pknE* dampen the expression of pro-inflammatory molecules on day 1 (data not shown) and a similar trend was observed on day 5 (Fig. 4A). Interestingly, the anti-inflammatory cytokines were also suppressed on day 1 (data not shown), while TGF-β alone was elevated on day 5 (Fig. 4B).

Among the SOCS family of genes studied, SOCS-1 (2.78-fold) was highly expressed on day 1 (Fig. 4C) which gradually decreased along with SOCS-3, -5 in Δ*pknE* compared to Rv infected macrophages on day 5 (Fig. 4D). Day 5 showed increase in the expression of SOCS-6, -7 and CISH genes in Rv while their expression was reduced in Δ*pknE* infected macrophages (Fig. 4D). CΔE was able to partially restore the expression of SOCS genes to that of Rv infected macrophages. The suppression of inflammatory mediators by Δ*pknE* occurs by regulating the SOCS genes. Our data shows that, *pknE* contributes to increased inflammatory responses.

3.5. ΔpknE elevates the expression of chemokines that signals apoptosis

Since the expression of interleukins was impaired in Δ*pknE* infected macrophages, the expression of chemokines which is also involved in inflammatory responses was analyzed. Microarray data showed increased chemokine expression in Δ*pknE* infected macrophages compared to Rv. Δ*pknE* infected macrophages showed 33 genes from CXC or alpha, CC or beta family as major chemokine response.

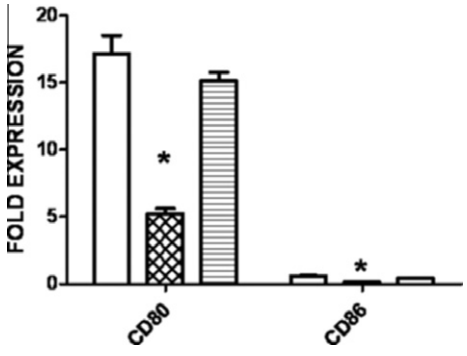

Fig. 3. Expression Analysis of co-stimulatory genes on day 1. The expression was analyzed by qRT-PCR and the values in the figure represent the fold change generated from biological replicates normalized using18s rRNA. The symbol * indicates $p < 0.05$ when Rv vs control was compared with Δ*pknE* vs control. The histograms with the different shades denote Rv vs Control, Δ*pknE* vs Control, CΔE vs Control.

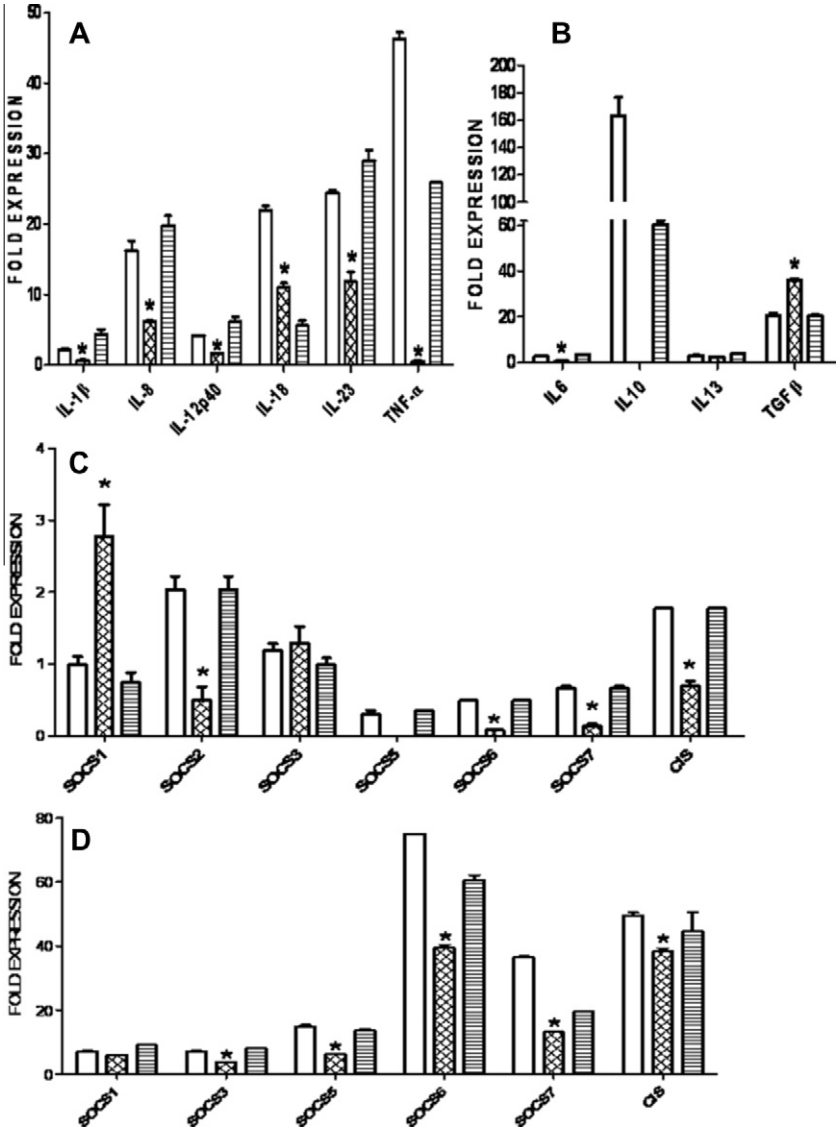

Fig. 4. Expression analysis of cytokines and SOCS genes. The expression was analyzed by qRT-PCR and the values in the figure represent the fold change generated from biological replicates normalized using18s rRNA. The symbol * indicates $p < 0.05$ when Rv vs control was compared with Δ*pknE* vs control. The histograms with the different shades denote ▭ Rv vs Control, ▩ Δ*pknE* vs Control, ▤ CΔE vs Control. (A) Pro-inflammatory cytokines on day 5. (B) Anti-Inflammatory cytokines on day 5. (C) SOCS expression on day 1. (D) SOCS expression on day 5.

The β-chemokine family that was reported to play a protective role against *M. tuberculosis* (Saukkonen et al., 2002) was chosen and confirmed using PCR array. The results confirmed the increase in β-chemokine expression by Δ*pknE* infected macrophages (Table 2). Hence increase in chemokines could have reduced the survival of Δ*pknE* inside the macrophages. *pknE* is able to orchestrate the inflammatory events needed for the survival of *M. tuberculosis.*

3.6. ΔpknE increases the expression of arginase2 and modulates iNOS

The expression of NO is influenced by cytokines and plays a prominent role in host innate immunity. It is produced by iNOS mediated catalysis of L-arginine to L-citrulline. The recycling of citrulline to arginine is catalyzed by two enzymes, argininosuccinatesynthase (*Ass*) and argininosuccinatelyase (*Asl*). Our observation of Δ*pknE* dampening the pro-inflammatory responses prompted us to study the regulation of iNOS (Wu and Morris, 1998).

The microarray analysis revealed four genes to be differentially expressed by Δ*pknE* compared to Rv infected macrophages, namely NOS2A (iNOS), arginase2 (*Arg*2), *Asl*, and *Ass*. The expression was confirmed by qRT-PCR. Δ*pknE* infected macrophages suppressed iNOS, *Ass*, *Asl* and *Arg*1 expression on day 1 and day 5 (Fig. 5). Interestingly Δ*pknE* infected macrophages increased the expression of *Arg*2 on day 5 compared to Rv infected macrophages (Fig. 5). The expression levels of genes in arginase family was restored to that of Rv infected macrophages by CΔE. Our data shows that *pknE* modulates the arginase pathway to increase the intra - cellular survival of *M. tuberculosis*.

3.7. ΔpknE increases the expression of TLRs

Seventeen genes related to TLR pathways showed differential expression by microarray data. The activation of TLR is an early event which signals the downstream pathways for cellular activation. Hence the expression was confirmed on day 1 post-infection using PCR array. The data showed highest expression of TLR recep-

Table 2
Δ*pknE* increases the expression of β-chemokines. The RNA from microarray experiments on day 5, RNA preparations from day 1 and nitrate stress was subjected to Sybr green chemistry based RT^{2}Profiler™ PCR Array System. The values were generated as fold change using the software provided by the manufacturer. The symbol ND$ indicate transcript not determined in both Δ*pknE* and Rv infected macrophages. The data is from two independent experiments.

Fold change of Δ*pknE* vs. H_{37}Rv				
S.no	Gene symbol	Day 1	Day 5	Nitrate stress
1	*CCL1*	28.8	2.3	ND$
2	*CCL3*	0.82	1.5	0.28
3	*CCL4*	2.42	1.32	0.86
4	*CCL5*	2.43	2.29	1.61
5	*CCL8*	2.56	1.88	1.43
6	*CCL15*	1.28	1.54	ND$
7	*CCL16*	5.37	1.58	2.8
8	*CCL18*	3.02	1.69	1.23
9	*CCL19*	3.17	0.93	2.3
10	*CCR1*	2.7	1.08	1.14
11	*CCR2*	2.1	0.95	ND$
12	*CCR3*	4.1	1.45	1.18
13	*CCR4*	4.13	1.34	1.64
15	*CCR6*	3.75	1.89	2.02
16	*CCR7*	2.72	1.78	0.35
17	*CCR8*	1.38	1.21	1.7
18	*CCR10*	1.3	1.43	2.18
19	*CCRL1*	0.94	76.1	ND$
20	*CCRL2*	4.71	1.59	0.53
21	*CCBP2*	6.9	0.97	ND$
22	*MYD88*	1.48	1.2	1.5

tors 2, 4, 6, 8, 9, CD180, LY86 and the adapter molecule, MyD88. While CD14 receptor was down-regulated (Table 3).

3.8. ΔpknE reduces the activation of Akt

Akt, a downstream kinase of phosphoinositide-3-kinase (PI3K), plays a role in activating anti-apoptotic genes. In order to show that Δ*pknE* infected macrophages undergo apoptosis due to defective survival response, we studied the phosphorylation kinetics of Akt. Δ*pknE* infected macrophages reduced the phosphorylation of Akt (Fig. 6A.) compared to Rv. CΔE was able to complement the phosphorylation event observed in Rv infected macrophages. This shows that *pknE* induced survival signal is dependent on the phosphorylation of Akt.

3.9. ΔpknE might suppress the activation of NF-κB

The final target for cellular activation is the expression of transcription factors and we studied the role of NF-κB which is a master regulator of multiple immune responses including genes that promote cell survival. Nine genes of this family showed differential expression from microarray data. The expression was validated on day 1 using PCR array (Table 3). The data showed that Δ*pknE* infected macrophages suppress the activation of NF-κB by parallel expression of inhibitors to NF-κB. Another important observation was the increased expression of TNFRSF9 by Δ*pknE* (236.48-fold) on day 5 (Table 1) which was reported to inhibit NF-κB (Wang et al., 2008). Hence the modulation of NF-κB also contributes to the survival response initiated by *pknE*.

3.10. Nitrate stress response

Previously we have reported that *pknE* responds to NO stress of the host thereby suppressing apoptosis (Jayakumar et al., 2008). In order to demonstrate the suppression of apoptosis by *pknE* during NO stress, we studied the various apoptotic phenotypes using nitric oxide donor SNP (sodium nitro prusside).

The Δ*pknE* infected macrophages down regulated the expression of Mcl-1(0.63) with increase in *Bax* (57.87-fold) (Fig. 7A and Table 1) compared to Rv infected macrophages. Δ*pknE* infected macrophages also reduced the expression of CD80 and CD86 (Fig. 7B). The expression of pro –inflammatory cytokines IL-1β, TNF-α and TGF-β levels were augmented in Δ*pknE* infected macrophages compared to Rv (Fig. 7C). The augmented expression of TNF-α was further confirmed using ELISA (data not shown). Another important observation from our data was the reduced expression of IL-6 by Δ*pknE* (0.4-fold) (Fig. 7D) which was further confirmed by ELISA (data not shown). Among the SOCS family of genes that negatively regulate cytokine synthesis Δ*pknE* increased the expression of SOCS-2 and SOCS-7 alone compared to Rv (Fig. 8A) infected macrophages. The expression of β-chemokines also increased similarly to that observed in the absence of NO donor (Table 2).

The genes of arginase metabolism *Arg2*, *Asl* and *Ass* were elevated in Δ*pknE* infected macrophages compared to Rv (Fig. 8B). Surprisingly, the expression of iNOS transcripts were not observed in infected macrophages. Since arginase can compete with iNOS, we sought to estimate the arginase activity in cell culture supernatants to assess the impact of iNOS. As expected, Δ*pknE* had less arginase activity compared to Rv, CΔE, and controls (Fig. 8C). The survival kinase, Akt also showed reduced phosphorylation in Δ*pknE* infected macrophages compared to Rv infected macrophages (Fig. 6B). The CΔE strain showed similar gene expression responses to that of Rv infected macrophages. Overall, in the presence of nitrate stress Δ*pknE* infected macrophages reproduced similar events observed in the endogenous NO host response. This

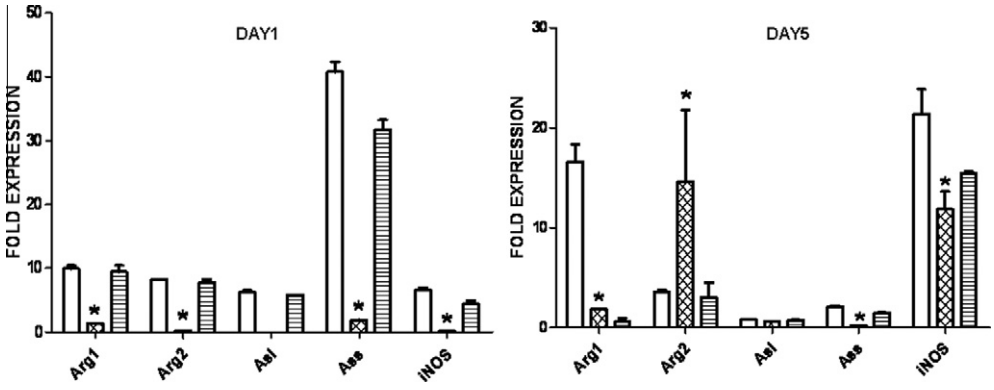

Fig. 5. Expression analysis of genes from arginase metabolism. The expression was analyzed by qRT-PCR and the values in the figure represent the fold change generated from biological replicates normalized using β-actin. The symbol * indicates $p < 0.05$ when Rv vs control was compared with Δ*pknE* vs control. The histograms with the different shades denote Rv vs Control, Δ*pknE* vs Control, CΔE vs Control.

Table 3
Δ*pknE* increases the expression of TLR genes and suppresses NF-κB. The RNA preparation on day 1 was subjected to Sybr green chemistry based RT^{2}Profiler™ PCR Array System. The values were generated as fold change using the software provided by the manufacturer. The data is from two independent experiments.

S.no	Gene symbol	Fold change of Δ*pknE* vs. H_{37}Rv
Toll like receptor family		
1	*CD14*	0.65
2	*TLR2*	2.3
3	*TLR3*	3.6
4	*TLR4*	2.5
5	*TLR5*	0.16
6	*TLR6*	5.44
7	*TLR7*	2.3
8	*TLR8*	7.3
9	*TLR9*	4.6
10	*TLR10*	5.9
11	*CD180*	6.08
12	*LY86*	6.4
13	*TIRAP*	3.45
14	*TICAM1*	4.19
15	*TICAM2*	3.62
NF-κB family		
16	*NFKB1*	5.53
17	*NFKB2*	3.1
18	*NFKBIA*	2.3
19	*NFKBIL1*	3.02
20	*RELA*	1.35
21	*CHUK*	4.2
22	*IKBKB*	4.30

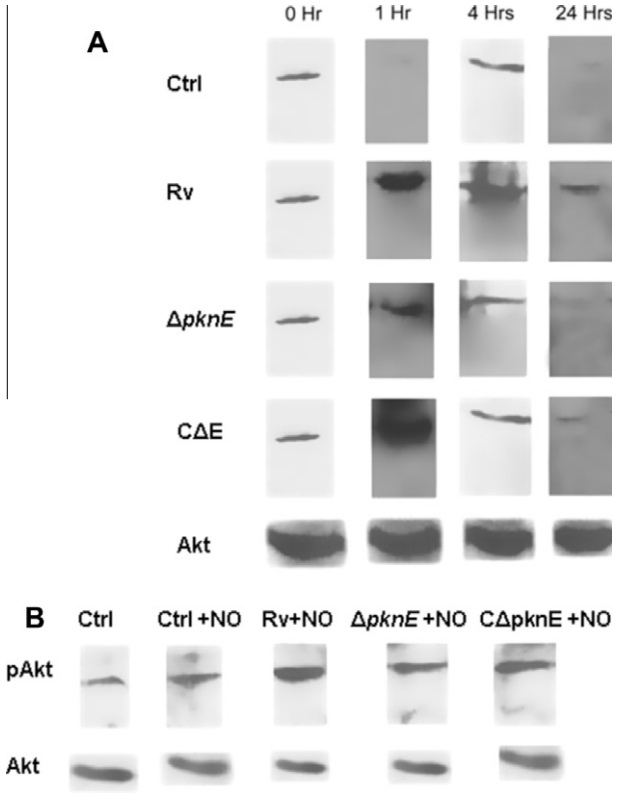

Fig. 6. Phosphorylation analysis of Akt. The western blots denote the phosphorylation event observed in the macrophages infected with strains that were probed with phosphorylated and non phosphorylated Akt antibodies. The data are representative of three independent experiments. (A) Phosphorylation kinetics. (B) Phosphorylation in the presence of nitrate stress.

clearly proves that *pknE* responds to the NO stress of the host thereby contributes to decreased apoptosis by modulating multiple pathways enabling the intracellular survival of *M. tuberculosis*.

4. Discussion

Apoptosis is considered as a defense strategy to limit the growth of intracellular pathogens. The virulence exhibited by *M. tuberculosis* prevents apoptosis, thereby restricting the adaptive immune responses to enable continued intracellular parasitism (Porcelli and Jacobs, 2008). Previously we have shown that *pknE* has a role in reducing the apoptosis of *M. tuberculosis* infected macrophages using a deletion mutant of *pknE* which showed reduced intracellular growth with increased apoptosis (Jayakumar et al., 2008). In the present investigation we have analyzed the mechanism by which *pknE* inhibits apoptosis using the global transcriptional analysis of Δ*pknE* and wild-type infected macrophages.

Analysis of macrophages infected with Δ*pknE* showed increased expression of *Bax*, *Bid*, TP53 and caspases involved in the intrinsic mode of apoptosis with decreased Mcl-1 expression. This shows that *pknE* suppresses the intrinsic mode of apoptosis to facilitate intracellular survival of *M. tuberculosis* by increasing Mcl-1 expression. Similar to our observations, Mycobacterium was shown to increase Mcl-1 to prevent apoptosis (Sly et al., 2003; Hasan et al., 2006). Likewise azurin, a redox protein from *Pseudomonas aeruginosa* induced apoptosis was shown to parallely increase *p53* and *Bax* (Yamada et al., 2002).

The ensuing apoptosis in Δ*pknE* infected macrophages reduced the expression of co-stimulatory molecules (CD80 and CD86) and pro-inflammatory cytokines. Interestingly the increased apoptosis elevated the levels of TGF-β leading to dampened immune response observed in Δ*pknE* infected macrophages. Our observation coincides with other reports that showed similar responses in varied systems (Fadok et al., 1998; Hathaway et al., 2002; Kusunoki et al., 2004; Rajavelu et al., 2008). This finding suggests that by suppressing apoptosis, *pknE* contributes to alter the immune responses elicited by the host.

Although Δ*pknE* infected macrophages failed to induce a pro-inflammatory response, increase in β-chemokine expression was observed. This increase in β-chemokine expression could have reduced the intracellular survival of Δ*pknE* leading to apoptosis. Similar to our study β-chemokines were reported to reduce the intracellular survival of *M. tuberculosis* (Saukkonen et al., 2002). We also studied the expression of another chemokine IL-8, which was shown to be influenced by TNF-α and the cell wall components of *M. tuberculosis* (Zhang et al., 1995; Muhl et al., 1999). Interestingly the IL-8 expression was also reduced that correlated with reduced TNF-α in Δ*pknE* infected macrophages. Our data indicates that *pknE* modulates multiple immune responses by altering the apoptosis and chemokine expression.

The reduced expression of pro and anti inflammatory cytokines prompted us to assess the influence of *pknE* on SOCS expression. SOCS was shown to negatively regulate the cytokine mediated signal transduction (Yoshimura et al., 2007). Among the various SOCS family members, the expression of SOCS1 was increased in Δ*pknE* infected macrophages. Our data supports the facts that, increase in apoptosis would lower pro-inflammatory responses (Kinjyo et al., 2002). Apoptosis suppressed by *pknE* could alter the expression of SOCS affecting the innate immune responses of the host.

Our observation of Δ*pknE* infected macrophages to dampen the pro-inflammatory responses tempted us to examine the expression of iNOS and its regulation by exploring the arginase pathway. As expected, the expression of iNOS was reduced in Δ*pknE* infected macrophages. This is in line with a previous report where pro-inflammatory cytokines were shown to induce iNOS (Das et al.,

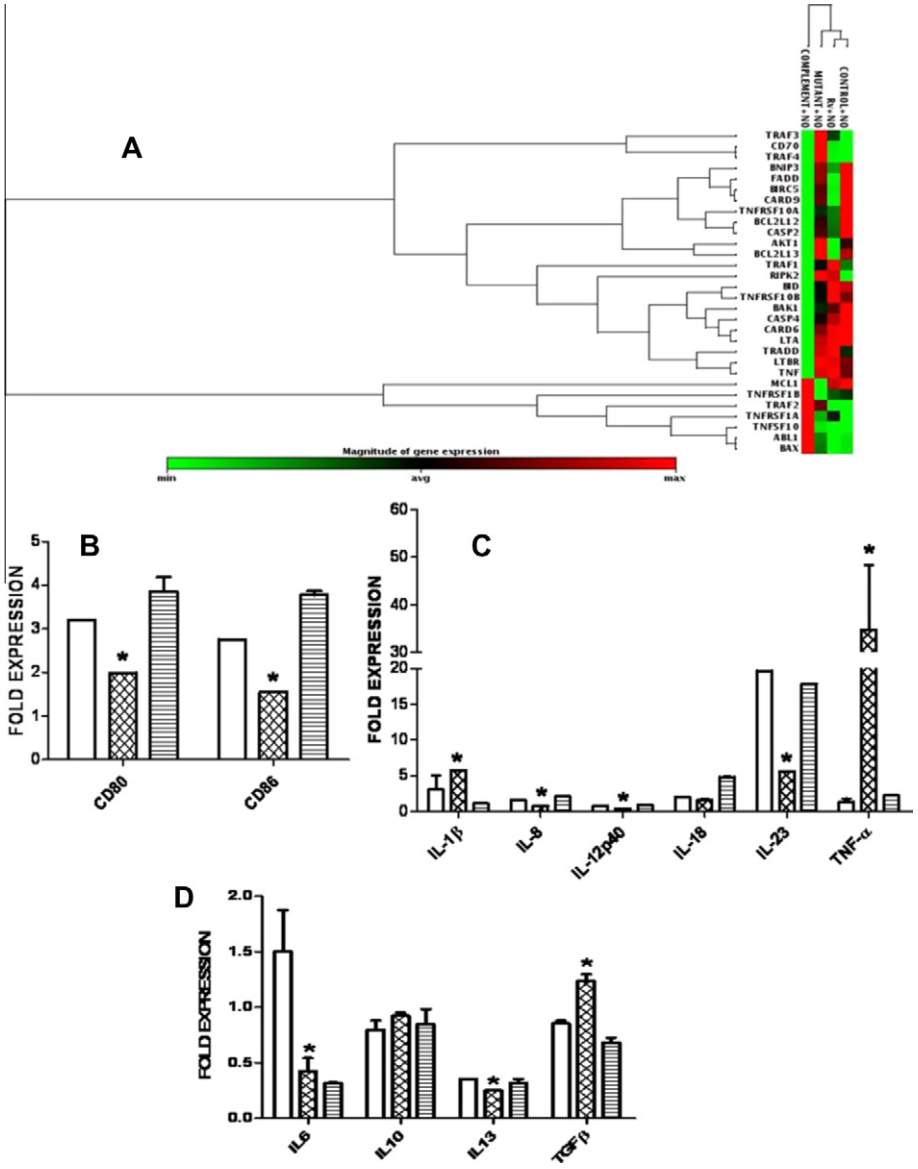

Fig. 7. Expression profiling during nitrate stress response -1. (A) Heat map depicting the expression of apoptosis genes by oligo array. THP-1 differentiated macrophages were infected with respective mycobacterial strains followed by nitrate stress with10 Mm SNP for 3 h. The total RNA population was isolated and cDNA was prepared, hybridized to human oligo GE array to identify the apoptotic gene expression. The genes were identified by chemiluminescence on an X-ray sheet and analyzed by GEArray® Expression Analysis Suite. The gene normalization was done using inbuilt controls and the data is representative of two independent experiments. (B) Expression analysis of co-stimulatory genes. (C) Pro-inflammatory cytokines. (D) Anti-inflammatory cytokines (Fig. 6B–D). The expression for these genes was analyzed by qRT-PCR and the values in the figure represent the fold change generated from biological replicates normalized using 18s rRNA. The symbol * indicates $p < 0.05$ when Rv vs control was compared with Δ*pknE* vs control. The histograms with the different shades denote ▭ Rv vs Control, ▩ Δ*pknE* vs Control, ▤ CΔE vs Control.

2010). Furthermore the expression of *Ass* and *Asl* genes were reduced confirming the failure to induce iNOS. Our data for the first time clearly demonstrates that Δ*pknE* infected macrophages could not undergo NO and TNF-α dependent apoptosis. The Δ*pknE* induced apoptosis is dependent on the intrinsic mode of apoptosis with elevation of β-chemokines and TGF-β.

The observation of impaired iNOS expression by Δ*pknE* infected macrophages compelled us to study the expression of arginase 1&2. Δ*pknE* infected macrophages reduced the expression of arginase1 with reduced intra cellular survival. This finding is similar to an earlier report in *M. tuberculosis* that showed increased arginase1 to support intra-cellular survival (El Kasmi et al., 2008). In contrast to arginase1, the expression of arginase2 was increased in Δ*pknE* infected macrophages. The reduced expression of arginase1, iNOS and increased arginase2 expression correlates to an earlier report in *Helicobacter pylori* where the apoptosis was shown to be dependent on arginase2 and independent of iNOS and pro-inflammatory responses (Gobert et al., 2002). Our data provides evidence that *pknE* modulates the arginase pathway for the intra cellular survival of *M. tuberculosis*.

TLRs play an important role in the pathogen recognition and initiation of both innate and adaptive immunity. TLRs act through MyD88 dependent and independent pathways to induce pro-inflammatory cytokine expression. The role of TLRs in *M. tuberculosis* remains a debate due to the fact that *M. tuberculosis* has plethora of ligands to activate wide range of receptors for its entry inside the host. However studies on *M. tuberculosis* have reported their role in apoptosis and inflammatory responses (Quesniaux et al., 2004; Korbel et al., 2008). Our analysis of Δ*pknE* infected macrophages showed increased expression of TLRs 2, 4, 6, 8 and 9 includ-

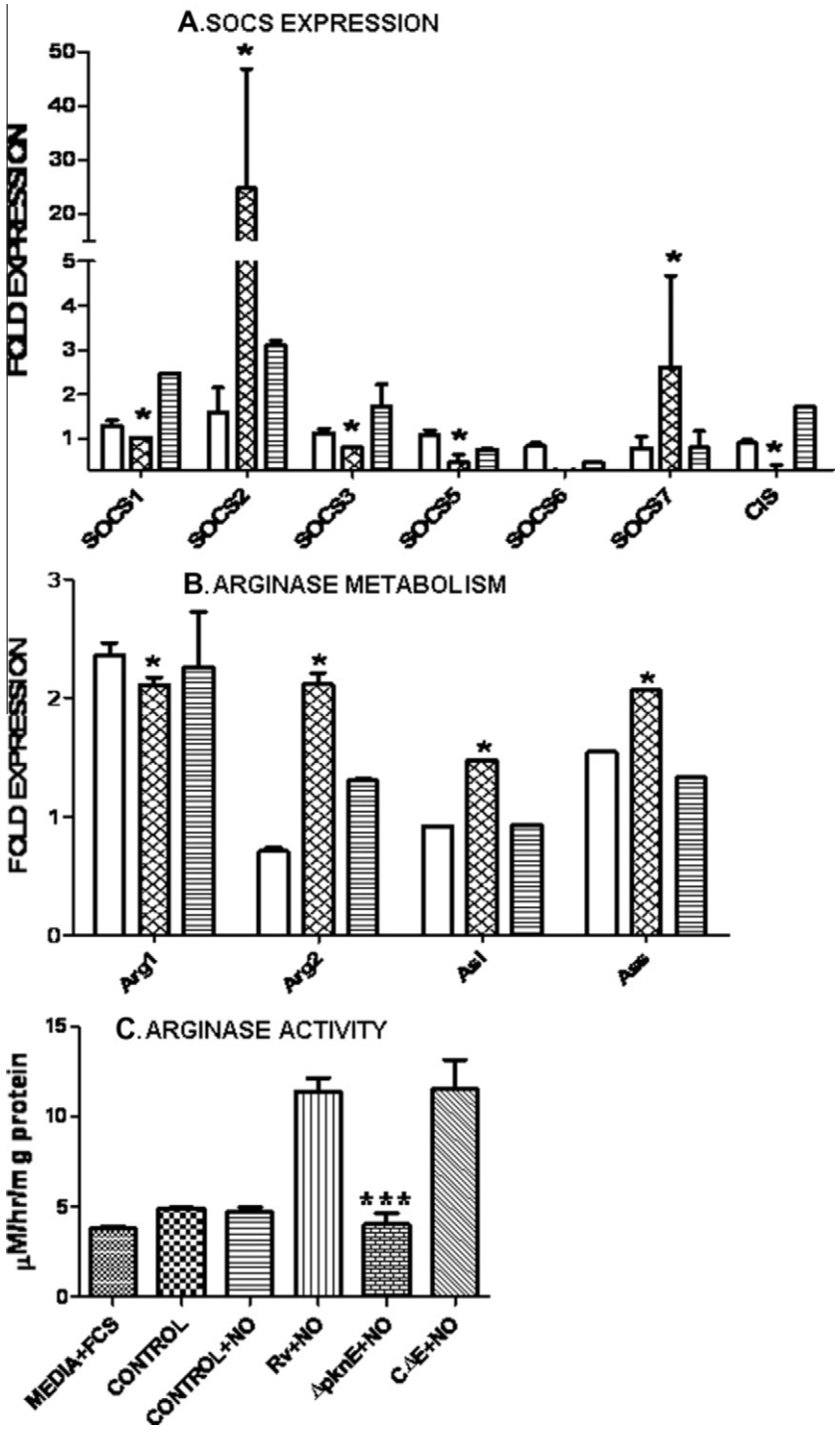

Fig. 8. Expression profiling during nitrate stress response -2. The expression was analyzed by qRT-PCR and the values in the figure represent the fold change generated from biological replicates normalized using 18s rRNA for SOCS expression and β-actin for arginase metabolism. The symbol * indicates $p < 0.05$ when Rv vs control was compared with Δ*pknE* vs control. The histograms with the different shades denote Rv vs Control, Δ*pknE* vs Control, CΔE vs Control. (A) SOCS expression. (B) Arginase metabolism. (C) Arginase activity in cell culture supernatants. Supernatants collected from nitrate stress experiment were subjected to the activity assay. The data represent values from three independent experiments. The symbol *** indicates $P < 0.001$ when Rv + NO was compared with Δ*pknE* + NO. Media + FCS – media with10% FCS alone, control – uninfected macrophages, control + NO – uninfected macrophages treated with NO donor, Rv + NO – Rv infected macrophages treated with NO donor, Δ*pknE* + NO – Δ*pknE* infected macrophages treated with NO donor, CΔE + NO – complemented strain infected macrophages treated with NO donor.

ing MyD88. This suggests that Δ*pknE* increases the expression of TLRs adding *pknE* as another ligand for possible TLR interactions. The downstream activation of TLR by the adapter molecule MyD88 culminates in NF-κB expression which is a master regulator for inflammatory mediators and pro survival targets (Jo et al., 2007; Zhang and Ghosh, 2001). NF-κB plays an important role in apoptosis dependent on the expression of two receptors TNFRSF8 and TNFRSF9. The increase in the receptor TNFRSF8 activates, while TNFRSF9 blocks NF-κB thereby promoting apoptosis (Wang et al., 2008). Our observation suggests that NF-κB activity could be blocked in Δ*pknE* infected macrophages due to the increased expression of TNFRSF9 and other suppressors of NF-κB. Hence

our observation of reduced pro-inflammatory cytokines coincides with the suppression of NF-κB. *pknE* thus shares the function of modulating the innate immune responses at different levels promoting intra-cellular survival of *M. tuberculosis*.

Finally to demonstrate the suppression of apoptosis by *pknE*, we performed the phosphorylation kinetics of Akt. Akt, a downstream kinase to PI3K was shown to increase cellular survival by various mechanisms like phosphorylation of *Bad*, increase in IL-6 and modulation of caspases (Berra et al., 1998). Here we show that the Δ*pknE* infected macrophages have reduced phosphorylation of Akt. Since increase in Akt was reported to suppress TP53 (Zhou et al., 2009), our observation of suppressed Akt and increased TP53, *Bax* expression in Δ*pknE* infected macrophages correlates with increased apoptosis. *pknE* enables intra-cellular survival of *M. tuberculosis* by modulation of arginase1, IL-6, TNF-α, chemokines and Akt.

The direct role of *pknE* in NO stress response suppressing apoptosis is demonstrated in our study using SNP, a classical NO donor that was proved to mimic in vivo situations of NO stress (Blond et al., 2000). As expected there was a similar apoptotic response. Addition of NO donor elevated the levels of TNF-α and iNOS in Δ*pknE* infected macrophages. This is in agreement with a previous study that showed addition of NO donor to increase TNF-α (Wang et al., 1997). Another interesting observation was severe impairment of IL-6 secretion by Δ*pknE* infected macrophages. *pknE* mediated increase in IL-6 could be a reason for inhibition of IFN-γ observed in TB infection (Nagabhushanam et al., 2003). Our data with NO donor and a microarray study (Voskuil et al., 2003) clearly shows that *pknE* responds to NO in the cellular environment. NO sensed by *pknE* contribute to the suppression of apoptosis as modeled (Fig. 9), facilitating intracellular survival of *M. tuberculosis*.

In the pursuit for analyzing molecular snapshots of pathogenesis many microarray studies have documented the gene expression profiles by using *M. tuberculosis* strains on various model systems (kahnert et al., 2006; Gonzalez-Juarrero et al., 2009; Silver et al., 2009; Thuong et al., 2008). Our study is unique in addressing the suppression of cell death influenced by anti-apoptotic gene *pknE*. We found that the expression levels of most of the host genes when infected with wild-type strain H_{37}Rv correlated with studies published earlier using different cell types (Gonzalez-Juarrero et al., 2009; Silver et al., 2009). Moreover the partial complementation of certain phenotypes by the complemented strain in our study is due to the expression of *pknE* gene from heterologous promoter and many studies have documented similar phenomenon

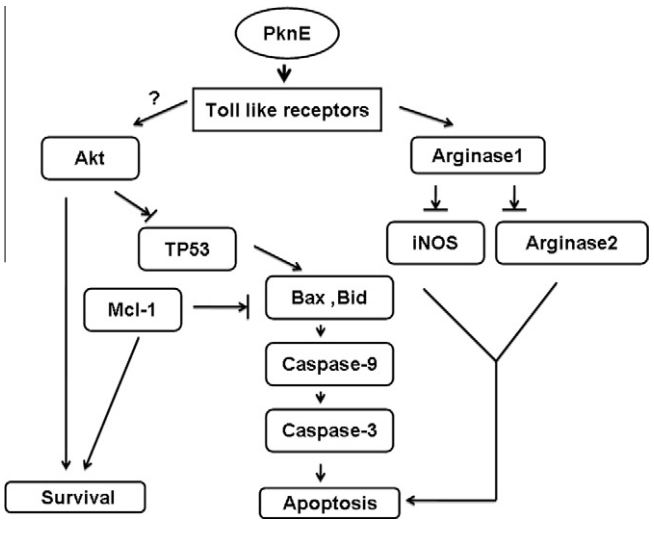

Fig. 9. Proposed model of cell death pathway inhibited by *pknE*.

(Tufariello et al., 2006; Domenech et al., 2009; Dhar and Mckinney, 2010). Analoguous to our study, nuoG gene of *M. tuberculosis* was shown to modulate the phagosomal NOX2 to inhibit apoptosis (Miller et al., 2010). Hence it can be inferred that *M. tuberculosis* has varied genes like *pknE* for NO stress and nuoG for oxidative stress to inhibit the immunity established by the host. Our study reveals that *pknE* influences the inhibition of apoptosis by various pathways contributing to the survival of *M. tuberculosis*.

5. Conclusion

In summary, our data demonstrates that *pknE* responds to NO stress and contributes to defective apoptosis. The defective apoptosis occurs by altering the expression of TLRs that leads to suppressed intrinsic mode of apoptosis. This impaired apoptosis elevates the pro-inflammatory responses and modulates the co-stimulatory molecule expression. Furthermore, increased arginase1 and Akt contributes to the suppression of apoptosis. The defective apoptosis enabled by *pknE* by varied pathways facilitates *M. tuberculosis* survival.

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