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Regulation of Interferon-γ receptor (IFN-γR) expression in macrophages during *Mycobacterium tuberculosis* infection

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Abstract: Interferon-gamma (IFN-γ) is a key cytokine that mediates immunity to tuberculosis (TB). *Mycobacterium tuberculosis* (*M. tb*) is known to downregulate the surface expression of IFN-γ receptor (IFN-γR) on macrophages and peripheral blood mononuclear cells (PBMCs) of patients with active TB disease. Many *M. tb* antigens also downmodulate IFN-γR levels in macrophages when compared with healthy controls. In the current study, we aimed at deciphering key factors involved in *M. tb* mediated downregulation of IFN-γR levels on macrophage surface. Our data showed that both *M. tb* H37Rv and *M. bovis* BCG infections mediate downmodulation of IFN-γR on human macrophages. This downmodulation is regulated at the level of TLR signaling pathway, second messengers such as calcium and cellular kinases i.e. PKC and ERK-MAPK, indicating that fine tuning of calcium response is critical to maintaining IFN-γR levels on macrophage surface. In addition, genes in the calcium and cysteine protease pathways which were previously identified by us to play a negative role during *M. tb* infection, also regulated IFN-γR expression. Thus, modulations in IFN-γR levels by utilizing host machinery may be a key immune suppressive strategy adopted by the TB pathogen to ensure its persistence and thwart host defense.

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Introduction

Tuberculosis is a global health issue. The lack of an effective vaccine is a major impediment in the path of TB control. Consequently, a deeper understanding of host-pathogen interaction remains a key factor in tackling this major health menace and may also contribute toward discovery of an effective vaccine [1]. IFN-γ is a key cytokine that mediates immunity to TB and many other infections. Proper functioning of the cytokine and its downstream effector functions depend upon its interaction with its cognate receptor i.e. IFN-γR, which is a heterodimeric protein complex. The downstream pro-inflammatory responses ensue from an external cytokine stimuli and receptor binding. Consequently, IFN-γR gene knock-out mice or mice having genetic defects in IFN-γR are extremely susceptible to *M. tb* infection [2]. This phenotype is further exemplified in HIV^+ individuals wherein low $CD4^+$ T cell counts translate into lower production of IFN-γ; thus eliciting poor immune responses to intracellular pathogens [3].

Abrogating IFN-γR mediated signaling is an important immune evasion strategy employed by many pathogens including *M. tb* [4, 5]. Consequently, the TB pathogen has devised numerous strategies to thwart the protective effects of this pivotal cytokine. Downregulation of the surface levels of IFN-γR is observed in infected macrophages and PBMCs of TB patients [5]. Many *M. tb* antigens also downmodulate IFN-γR levels when compared with healthy controls [6]. Although, *M. tb* infection is known to downregulate surface levels of IFN-γR on macrophages, the key molecular players involved in this immuno-suppressive process have not been identified. Concomitant with these immune evasive mechanisms employed by *M. tb*, arise the intriguing

observations that patients with active TB disease secrete high levels of IFN-γ making IFN-γ as a poor correlate of disease susceptibility [7]. However, this can partially be attributed to the ability of *M. tb* to potently downregulate IFN-γR levels on macrophages, thus making infected cells unresponsive to an external pro-inflammatory stimulus.

In the light of these observations, we aimed at investigating the host factors that may be involved in *M. tb* mediated downregulation of IFN-γR levels. Our results demonstrated that several factors such as signaling intermediates or cellular kinases of key signaling pathways and second messengers played a role in orchestrating this process.

Our data showed that both *M. tb* H37Rv and *M. bovis* BCG infections mediate downmodulation of IFN-γR on human macrophages with *M. tb* H37Rv being more potent in causing this phenomenon. Receptor downmodulation is regulated in a TLR dependent manner and also involves second messengers such as calcium. Cellular kinases i.e. PKC and ERK-MAPK negatively regulated IFN-γR levels on macrophages. This indicated that fine tuning of calcium response is critical in maintaining IFN-γR levels on macrophage surface. In addition, genes in the calcium and cysteine protease pathways which were previously identified by us to play a negative role during *M. tb* infection, also seem to regulate IFN-γR expression in response to *M. tb* infection [8]. Previous work from our lab also implicated these factors in *M. tb* mediated manipulation of host immune system which included upregulation of L-type Voltage Gated Calcium Channels (VGCCs), suppression of protective immunity in dendritic cells and inhibition of apoptosis [9, 10].

Methods

Cell culture, bacteria and infection

Monocytic cell line THP-1 and mouse bone marrow derived macrophages were used in this study. THP-1 cell line was acquired as a gift from Dr. Pawan Sharma from the International Centre for Genetic Engineering and Biotechnology, New Delhi. Cells were maintained in RPMI-1640 (GIBCO, Grand Island, NY) medium supplemented with inactivated 10% FBS (HyClone), 2mmol/L L-glutamine and 1% penicillin-streptomycin. PMA was used to differentiate cells into macrophages at 50 ng/ml for 16h in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and maintained in a standard tissue culture incubator at 37 °C with air conditions of 5% CO_2 and 95% air. Prior to infection, *M. bovis* BCG or *M. tb* H37Rv were grown in Middlebrook 7H9 (DIFCO) liquid medium supplemented with OADC (oleic acid/albumin/dextrose/catalase) with 0.05% Tween-80.

Flow Cytometry

IFN-γR staining: After the requisite treatment, to minimize the non-specific binding of the antibody, CD16/CD32 Fc receptors were first masked using Fc Block (BD Biosciences, USA) and thereafter stained with PE-tagged anti-CD119 (IFN-γR) (BD Biosciences, #558937 Clone GIR-94) monoclonal antibody to stain for the surface levels of IFN-γR. Following thorough washes; acquisition and analyses of cells were performed on FACS Calibur (Becton & Dickinson). The data were analyzed and plotted using CellQuest-Pro software. Staining on mouse BMDMs was carried out using biotinylated anti-IFN-γR monoclonal antibody (BD Biosciences #550582, Clone 2E2) and secondary staining was performed with PE labeled streptavidin. Specificity of staining for IFN-γR was ascertained using relevant isotype. (Supplementary Fig. 1)

Transfection of macrophages with siRNAs and infection

2 x 10^6/ml macrophage cells were transfected with 60 pmol siRNAs in HiPerFect transfection reagent (Qiagen) using OPTIMEM medium (Invitrogen) as described previously [8]. The cells were supplemented with complete medium after 5h and further incubated for 36h. Knockdown efficiency was checked using western blotting. After the desired infection, cells were processed for further experimentation.

Stimulation of cells and infection

PMA treated THP-1 cells were infected with *M. bovis* BCG or *M. tb* at 2MOI for indicated times. Various biopharmacological inhibitors were used; such as 3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB-8) (Sigma) at a concentration of 100 μM to inhibit internal calcium burst by blocking IP_3R, EGTA (Sigma) at 3 mM to inhibit external calcium influx, 1,4-diamino-2,3-dicyano-1,4-bis (2-aminophenylthio) butadiene (U0126) (Sigma) at 10 μM for inhibiting MAPK-ERK and Calphostin C (Sigma) at 0.1 μM to inhibit PKC in macrophages. Unless mentioned otherwise, cells were incubated with the above

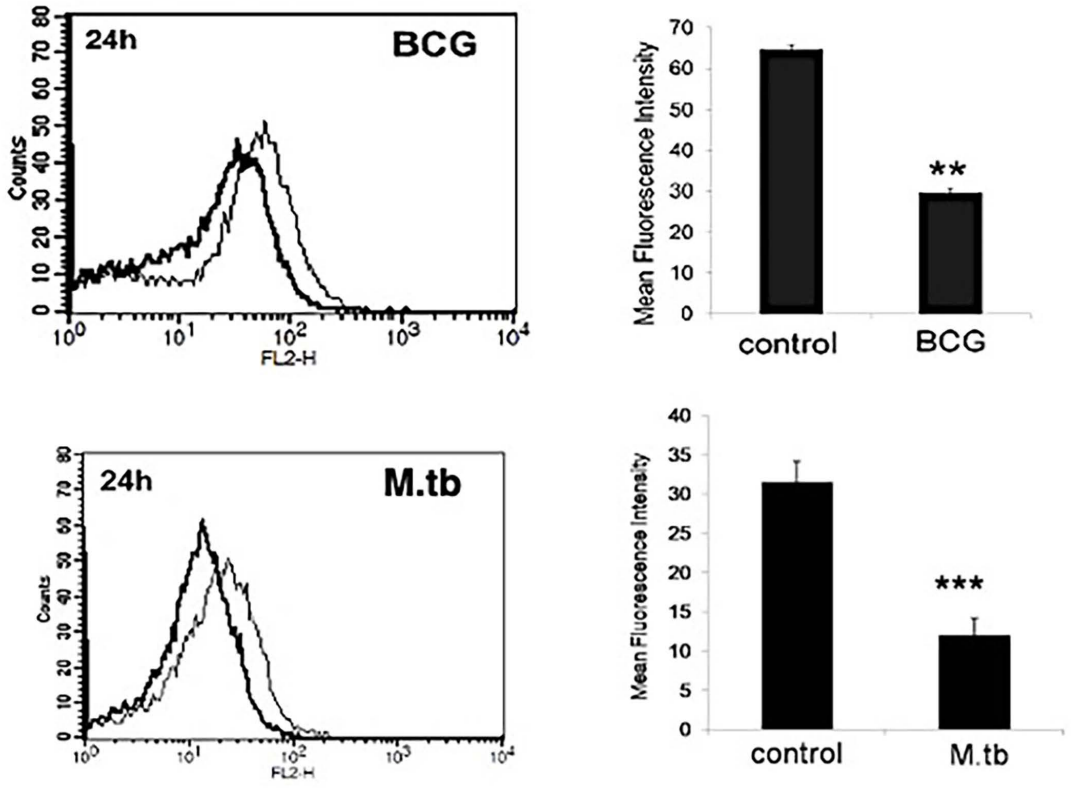

Figure 1: *M. bovis* BCG and *M. tb* H37Rv downregulate IFN-γR expression on macrophages. Monocytic THP-1 cells were incubated with 50ng/ml PMA for 16h for differentiation into macrophages. Following this, cells were infected with either *M. bovis* BCG or *M. tb* H37Rv at 2MOI for 24h and IFN-γR was monitored using flow cytometry. Thin lines represent expression levels in uninfected controls whereas bold lines in the histograms represent level of expression after infection. Adjacent bar graphs summarize the mean fluorescence intensity values (MFI). p values, *p<0.05 and **p<0.01 and ***p<0.001, were ascertained using Student's *t*-test. Data are expressed as mean ± S.D., n=3.

reagents for 1h before the desired infection. Cells were thereafter processed for flow cytometry.

Differentiation of mouse bone marrow derived macrophages

For some experiments, macrophages derived from mouse bone marrow (BM) precursor cells were used. 6-8 week old female Balb/c mice were used for all experiments. Briefly, BM from the tibias and femurs of mice were flushed out. Cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 40 ng/ml Macrophage-Colony stimulating factor (M-CSF) for 5 days for full differentiation into mature macrophages with media change every alternate day. Differentiated macrophages were infected with *M. tb* or *M. bovis* BCG and processed further for flow cytometry or microscopy.

Confocal microscopy

For monitoring IFN-γR levels in BMDMs, BM precursor cells were seeded at a density of $1x10^6$/ml cells. After differentiation to mature macrophages, cells were infected with *M. bovis* BCG at 2MOI for 24h. Thereafter, cells were washed thoroughly and fixed with 4% paraformaldehyde. Following washes, cells were permeabilized with 0.1% Triton-X and subsequently blocked with 5% BSA for 1h. Following this, cells were labeled with biotimylated anti-IFN-γR. This was followed by addition of PE tagged streptavidin. Confocal microscopy was done using Nikon A1 laser microscope with 60X magnification, numerical aperture 1.4 and refractive index 1.5, Plan Apo Optics equipped with Argon laser. The images were analyzed using NIS Elements AR software.

Ethical approval: The research related to animals use has been complied with all the relevant national regulations and institutional policies for the care and use of animals. All the experiments involving animals

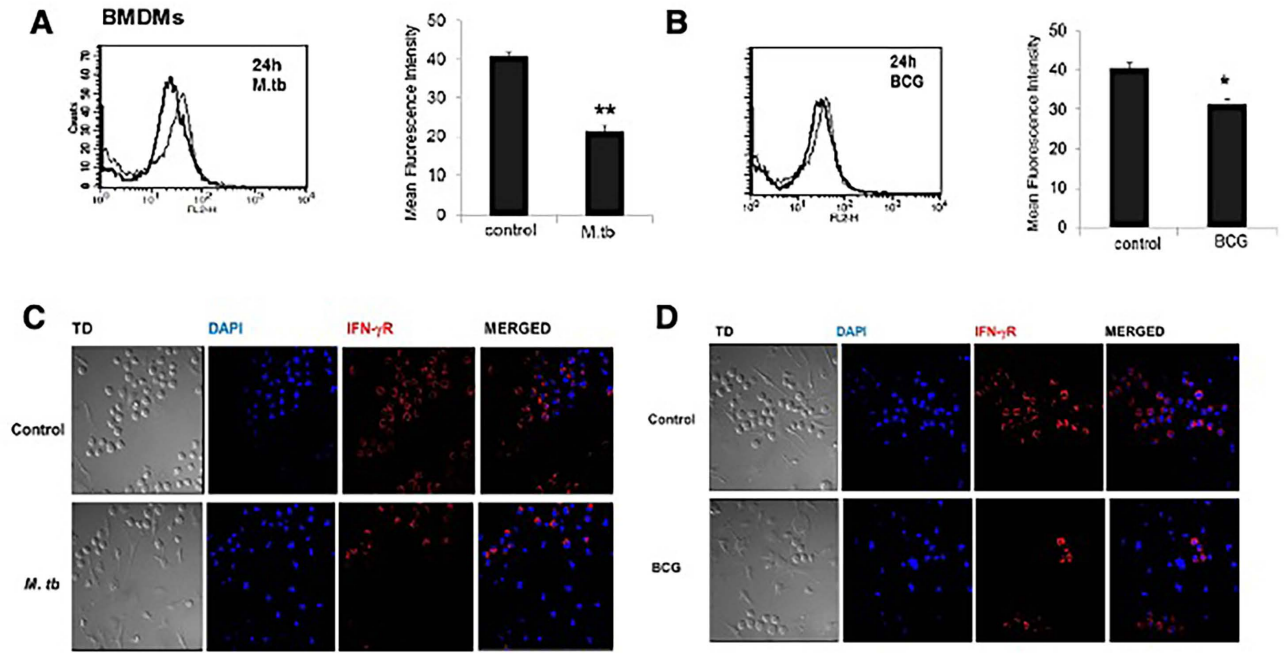

Figure 2: Mycobacteria downregulate IFN-γR expression on murine macrophages. Mouse BMDMs were infected with either (A) *M. tb* H37Rv or (B) *M. bovis* BCG at 2MOI for indicated times. IFN-γR levels were monitored by flow cytometry. Thin lines indicate receptor levels in uninfected cells whereas bold lines represent receptor levels in infected cells. Bars adjacent to histogram depict the summarized MFI values. *p<0.05 and **p<0.01, using Student's *t*-test between control and infected groups. Data expressed as mean ± S.D (n=3). Confocal images of murine BMDMs showing surface levels of IFN-γR (red) infected with 2MOI *M. tb* H37Rv (C) or *M. bovis* BCG (D) for 24h. Cells were incubated with biotinylated anti-IFN-γR antibody and probed with Streptavidin PE. Nucleus was stained with DAPI (blue) and microscopy was performed using Nikon A1 laser microscope and analyzed using NIS Elements AR software.

and human samples were approved by the Institutional Animal Ethics and Institutional Human Ethics Committees of Dr. B R Ambedkar Center for Biomedical Research, University of Delhi. All experimental protocols adhered to the guidelines of these committees.

Results

Mycobacteria downregulate IFN-γR levels in macrophages

Previous work has already established that pathogenic microorganisms including mycobacteria like *M. tb* and *M. avium intracellulare* downregulate IFN-γR levels on macrophages [5, 11]. Our study aimed at gaining further insights into this phenomenon.

To begin with, we scored for IFN-γ levels during mycobacterial infection. PMA treated human monocytic THP-1 cells were infected with *M. bovis* BCG or *M. tb* H37Rv at 2 MOI for 24h and 48h and receptor levels were scored by FACS. As shown in Figure 1, both *M. bovis* BCG and *M. tb* H37Rv down-regulated IFN-γR levels on macrophages at 24h, thus confirming the observations made earlier. However, reduction in IFN- γR levels caused by *M. tb* H37Rv was more significant compared with *M. bovis* BCG. Reduction in receptor levels on macrophages at 24h indicated that this process was essentially an early event during the course of infection. No significant change in IFN-γR levels was seen at later time points i.e. at 48h (Supplementary Fig. 1).

To further confirm these results, we extended these observations to primary mouse macrophages (BMDMs). Similar observations were obtained for BMDMs wherein *M. tb* H37Rv was able to significantly downregulate IFN-γR levels compared to BCG at the same time point (Figure 2A and B). These results were also confirmed using confocal microscopy (Figure 2C and D). Consistent with the observations made earlier by Singhal et al., *M. tb* infection was able to significantly reduce IFN-γR levels in human PBMCs also. [5] (Supplementary Fig. 2).

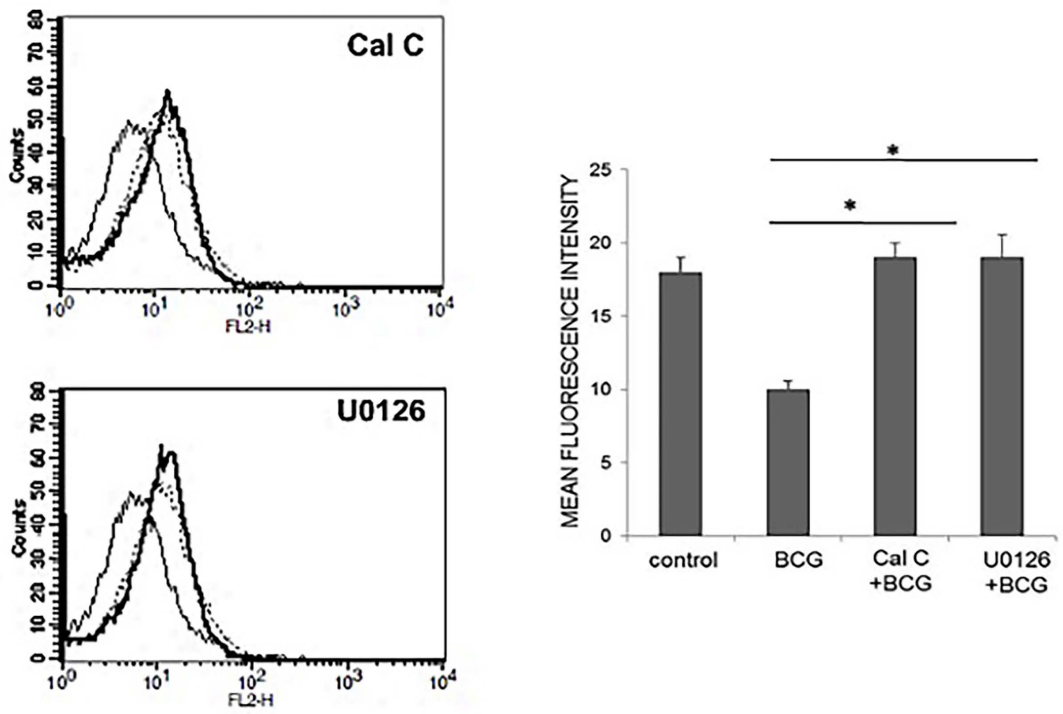

Figure 3: PKC and MAPK-ERK regulate *M. bovis* BCG mediated IFN-γR expression on macrophages. THP-1 derived macrophages were treated with specific bio-pharmacological inhibitors to PKC (Calphostin C; Cal C, 0.1μM) and ERK-MAPK (U0126, 10μM) for 1h prior to infection with *M. bovis* BCG at 2MOI for 24h. IFN-γR levels were monitored by flow cytometry. *p<0.05, using one-way ANOVA with Tukey's correction to calculate statistical significance between different groups. Data are represented as mean fluorescence intensities and expressed as mean ± S.D. (n=3). Dotted lines represent unstimulated controls, thin lines represent BCG infected groups and bold black lines represent infected groups treated with specific inhibitor. Inhibiting either PKC or ERK-MAPK reversed the down-modulation of IFN-γR by *M. bovis* BCG.

PKC and MAPK-ERK positively regulate *M. bovis* BCG mediated IFN-γR expression in human macrophages

Next, we aimed at dissecting the pathways involved in mycobacteria induced IFN-γR downregulation. It is established that MAPK pathway plays an important role in regulating several immunological processes [12, 13]. We further aimed at deciphering the role of MAPK pathway in regulating IFN-γR levels. To this end, we inhibited important signaling intermediates such as PKC and MAPK-ERK in THP-1 derived macrophages using their specific bio-pharmacological inhibitors (Calphostin C-0.1μM; U0126-10μM) for one hour prior to *M. bovis* BCG infection. IFN-γR levels were thereafter probed by flow cytometry (Figure 3). As shown in Figure 3, inhibiting either PKC or ERK-MAPK reversed the down-modulation of IFN-γR by *M. bovis* BCG. This pointed toward the fact that mycobacteria down-modulate IFN-γR levels on macrophage surface via PKC and MAPK pathways.

Calcium homeostasis regulates *M. bovis* BCG mediated IFN-γR expression in human macrophages

Our earlier work indicated an important role of calcium in exacerbating *M. tb* pathogenesis. We demonstrated that patients with active TB showed an increased L-type VGCC expression in macrophages, which in turn correlated with dampened macrophage-mediated protective responses [14]. Therefore, we next explored the role of calcium and its entry from various routes in mediating IFN-γR modulation upon infection. To that end, THP-1 derived macrophages were treated with either EGTA (3mM) to chelate extracellular calcium or TMB8 (100μM) to prevent calcium release from intracellular stores such as ER. In addition, we also used Verapamil (20μM) to block L-type VGCCs. As shown in Figure 4, calcium homeostasis played a significant role in shaping IFN-γR modulation. While inhibiting calcium influx from external medium brought back the downmodulated IFN-γR to levels comparable with uninfected cells at 24h, inhibiting calcium release from internal stores also reversed the downmodulation

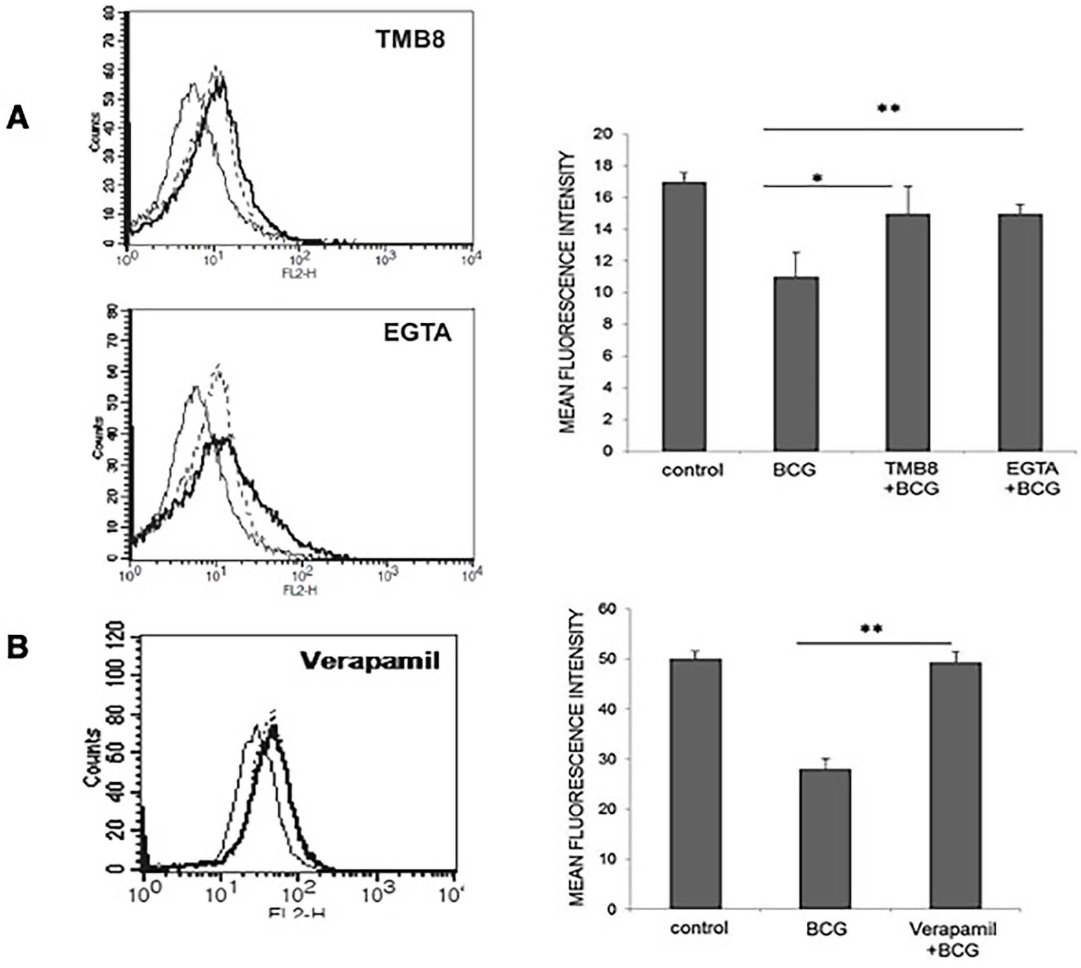

Figure 4: Calcium homeostasis regulates *M. bovis* BCG mediated IFN-γR expression on macrophages. THP-1 derived macrophages were treated with specific bio-pharmacological inhibitors to (A) calcium influx from external medium (EGTA, 3mM) and calcium release from intracellular stores (TMB8, 100μM) or (B) voltage gated calcium channels (Verapamil, 20μM) for 1h prior to infection with *M. bovis* BCG at 2MOI for 24h. IFN-γR levels were monitored by flow cytometry. In all the panels, dotted lines represent receptor levels in control groups, thin lines represent receptor levels in BCG infected groups and bold black lines represent receptor levels in specific inhibitor treated infected groups. *p<0.05 and **p<0.01, using one-way ANOVA with Tukey's correction to calculate statistical significance between different groups. Data are represented as mean fluorescence intensities and expressed as mean ± S.D. (n=3). While inhibiting calcium influx from external medium brought back the down-modulated IFN-γR to levels comparable with uninfected cells at 24h.

of IFN-γR at the same time point. This indicated a critical role for calcium homeostasis in the regulation of IFN-γR at the cell surface. Blocking voltage-gated calcium channels also reversed the downmodulation of IFN-γR by BCG, thus once again indicating a negative role for this channel in mediating protective responses (Figure 4B).

We also attempted to dissect the role of calcium sensing machinery (STIM1, STIM2, and ORAI1) in influencing the expression of IFN-γR upon infection. However, a marginal, almost insignificant restoration of IFN-γR levels on macrophages upon knockdown of these molecules was observed. (Supplementary Fig. 3).

M. bovis BCG mediates IFN-γR down modulation in a TLR dependent manner.

Owing to the pivotal role of TLR signaling pathway in shaping immune response, we next investigated the role of TLR pathway intermediates in mediating IFN-γR downregulation by *M. bovis* BCG. To that end, we individually knocked down TLR pathway intermediates such as MyD88 and TRAF6 in differentiated THP-1 cells and monitored IFN-γR surface expression. As shown in Figure 5, knockdown of TRAF6 but not MyD88 restored the BCG downmodulated IFN-γR levels indicating a

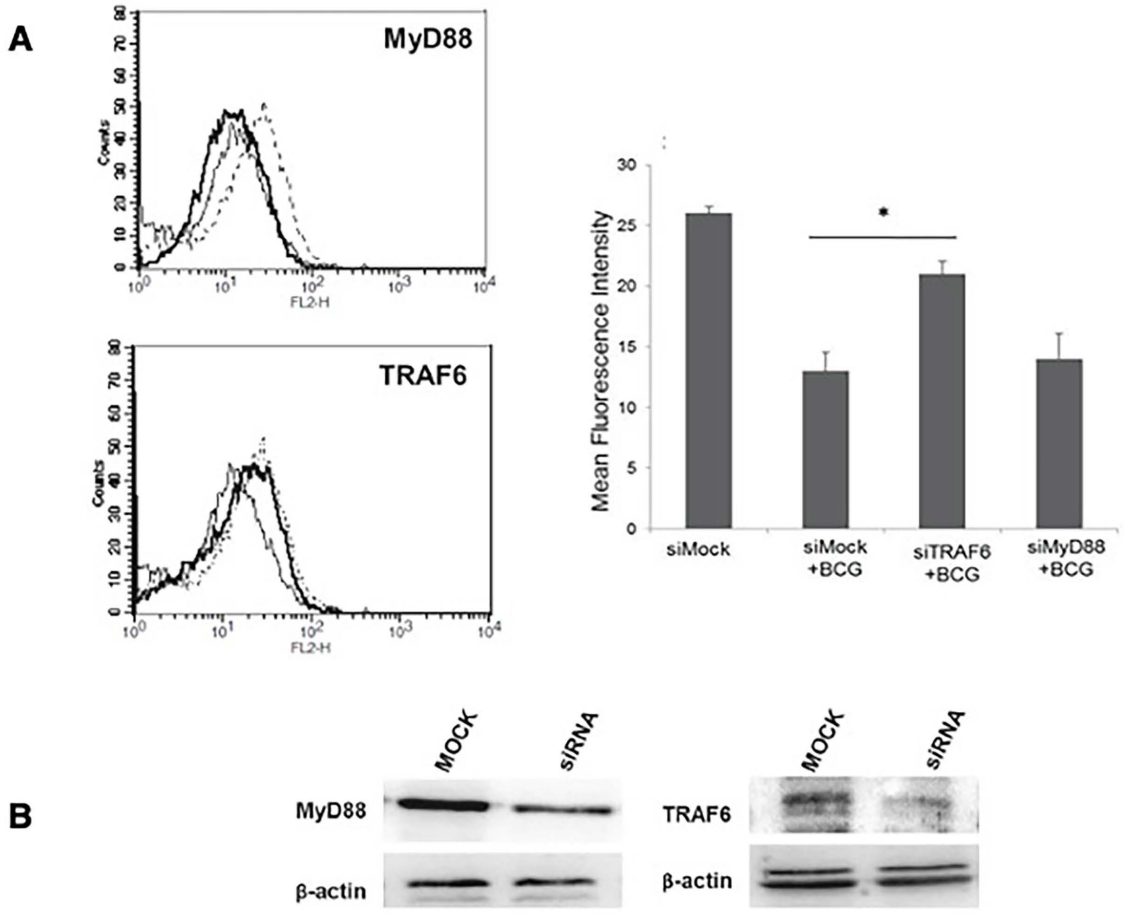

Figure 5: TLR pathway regulates BCG mediated IFN-γR expression on macrophages. (A) THP-1 derived macrophages were transfected with specific siRNAs against indicated molecules for 36h followed by infection with *M. bovis* BCG for 24h. IFN-γR levels were monitored by flow cytometry. In all the panels, *p<0.05 and **p<0.01, using one-way ANOVA with Tukey's correction to calculate statistical significance between different groups. Data are represented as mean fluorescence intensities and expressed as mean ± S.D. (n=3). Dotted lines represent receptor levels in groups transfected with control siRNAs (MOCK), thin lines represent receptor levels in *M. bovis* BCG infected groups transfected with control siRNAs and bold black lines represent receptor levels in *M. bovis* BCG infected groups transfected with specific siRNAs against indicated molecules. (B) Western blots to depict the knockdown efficiency of the siRNA treatment for the indicated molecule.

role for the TLR pathway in the process. However, TLRs themselves had no siginifacnt role to play in regulating IFN-γR levels on macrophages in response to *M. bovis* BCG infection. (Supplementary Fig. 4).

Specific genes in the calcium-cysteine protease pathways negatively regulate *M. bovis* BCG mediated IFN-γR expression in human macrophages

Previous work from our lab had established the role of specific genes of calcium cysteine protease or calcium/calmodulin pathways in negatively regulating protective immune responses from dendritic cells. siRNA library screening revealed the involvement of these pathways in attenuating protective responses since knockdown of select genes significantly reduced bacterial burden and increased the level of pro-inflammatory responses. Further, knockdown also promoted autophagy, which has now emerged as a potent anti-microbial effector function [8]. Therefore, using siRNA mediated knockdown, we specifically knockdown these genes in order to investigate their role in regulating IFN-γR levels. As shown in Figure 6, knockdown of *M. bovis* BCG indicating a negative role these genes in regulating IFN-γR levels. It provided an evidence of an additional mechanism of negative effects emanating from these genes.

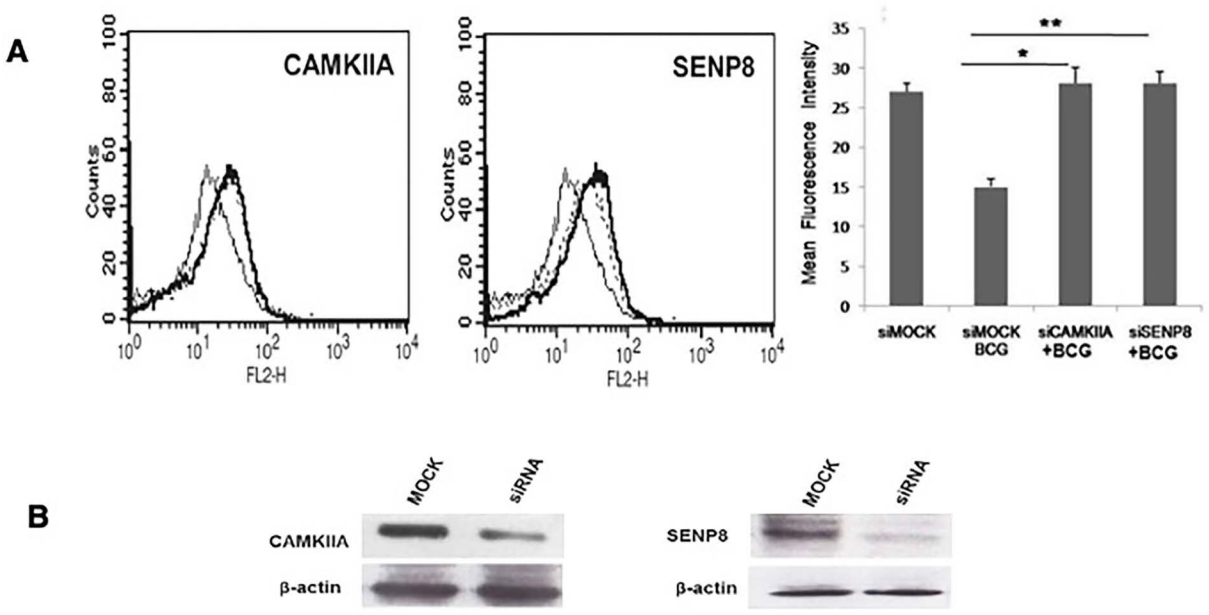

Figure 6: Calcium cysteine protease pathway genes regulate *M. bovis* BCG mediated IFN-γR expression on macrophages. (A) THP-1 derived macrophages were transfected with specific siRNAs against indicated molecules for 36h followed by infection with *M. bovis* BCG for 24h. IFN-γR levels were monitored by flow cytometry. In all the panels, dotted lines represent receptor levels in groups transfected with control siRNAs (MOCK), thin lines represent receptor levels in *M. bovis* BCG infected groups transfected with control siRNAs and bold black lines represent receptor levels in *M. bovis* BCG infected groups transfected with specific siRNAs against indicated molecules. *p<0.05 and **p<0.01, using one-way ANOVA with Tukey's correction to calculate statistical significance between different groups. Data are represented as mean fluorescence intensities and expressed as mean ± S.D. (n=3). (B) Western blots to depict the knockdown efficiency of the siRNA treatment for the indicated molecule.

Discussion

Among the cytokines, IFN-γ plays a determining role in containing *M. tb* infection [15]. It primes macrophages and augments their protective effector functions [16]. IFN-γ can directly or indirectly regulate many pathogen inhibitory processes like apoptosis, autophagy and ROS generation. [17] These mechanisms have emerged as potent anti-bacterial mechanisms and many of their effector molecules are directly under the influence of IFN-γ stimulation. As a consequence, *M. tb* and several other intracellular pathogens have devised numerous strategic ways to subvert its protective effects. Blocking IFN-γ signaling limits the impact of host pro-inflammatory milieu and significantly dampens the quality and quantum of protective responses. Consequently, as an evasive strategy, several pathogens possesses the ability to hijack this pathway. Pathogens such as *Leishmania*, Mycobacteria etc. inhibit downstream signaling stemming from IFN-γ-IFN-γR interaction [11, 18].

Lower surface levels of IFN-γR in in *M. tb* infected cells is was attributed to decreased transcription of IFN-γR mRNA. This was directly linked to the impaired expression of transcription factor Sp1, critical for aiding transcription of IFN-γR gene. The resulting phenotype made macrophages refractory to the protective effects of IFN-γ, despite its presence at optimum levels. This observation led to an important explanation for *M. tb*'s ability to successfully evade host immunity even amidst a robust TH1 response ensuing from infection. Previous work from our lab has also suggested a role of several *M. tb* antigens in mediating IFN-γR downregulation on APCs [6].

In the current study, we aimed at gathering a deeper insight into this phenomenon and investigate the key host players regulating receptor downmodulation. Involvement of various signaling pathways, molecular kinases, and second messengers was implicated in bringing down the surface levels of IFN- γR. To this end, we employed flow cytometry and microscopy to monitor receptor levels on macrophages following *M. bovis* BCG or *M. tb* infection.

Our results were consistent with the earlier findings that *M. tb* brings down the surface level of IFN-γRs on macrophages. This event essentially occurred in a TLR pathway dependent manner. Although, TLR pathway comprises the innate immune sensing machinery and

generates pro-inflammatory responses, it is a common observation that many pathogenic micro-organisms manipulate this pathway or utilize its components to mediate suppressor responses. For example, TLR2 is an essential factor enabling *M. tb* to subvert IFN-γ mediated protective responses. [19, 20]. Likewise, pathogens like *Leishmania* and *Yersinia* also hijack the TLR pathway to ensure their survival and skew host responses towards a more suppressive type [21, 22]. Therefore, in the backdrop of these observations, we investigated the role of TLRs in *M. tb* mediated modulation of IFN-γR. We observed that IFN-γR levels were MyD88 independent but TRAF6 dependent. Signals from TLR intermediates such as TRAF6 merge into various downstream pathways [23]. Our results pointed towards a role of MAPK-ERK and PKC in mediating regulation of IFN-γ and IFN-γR levels. Thus, signals fed from TRAF6 activation in the TLR pathway to downstream signaling molecules (ERK1/2) or associated kinases such as PKC regulated IFN-γR levels.

Previous results from our lab revealed an inverse correlation between L-type VGCCs expression and immunity to TB [14]. Our data revealed that differential routing of calcium upon *M. tb* and HIV co-infection plays a dominant role in regulating apoptosis thereby strengthening pathogen persistence [10]. Keeping in view the above, we also extended these observations to our studies. We observed, while inhibiting calcium influx from external medium brought back the downmodulated IFN-γR to levels comparable with uninfected cells at the given time point, inhibiting calcium release from internal stores also reversed the down-modulation of IFN-γR at 24h. This indicated a critical role for calcium homeostasis in the regulation of IFN-γR at the cell surface. Blocking voltage-gated calcium channel also reversed the down-modulation of IFN-γR by BCG, thus once again indicating a negative role for this channel in mediating protective responses. Lastly, select genes of calcium cysteine and protease pathways in the host previously found out by us to play a negative role in mediating immunity against TB, also played a role in regulating IFN-γR in macrophages. Knockdown of these genes restored receptor levels upon infection further reiterating their suppressive role. Thus, aiding IFN-γR downregulation may provide an additional evidence of how these genes may negatively regulate host immunity.

In summary, IFN-γR downregulation on the macrophage surface favors the pathogen by limiting the role of extrinsic IFN-γ. This may be an important immune evasive mechanism to thwart the very many protective benefits of this pleiotropic cytokine [24]. A deeper understanding of the specific signals and processes that regulate this and the mechanisms thereof, such as the role of second messengers and small RNAs would increase our understanding of this host-pathogen interaction more critically.

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Author Contributions: Experimental design and conception were done by GK, YS and KN. GK and BKT performed the experiments. Data analyses were carried out by KN, GK and BKT. Contribution of reagents/ buffers/ materials/ analysis tools was from YS lab. GK and KN wrote the manuscript.

Conflict of interest: Authors state no conflict of interest

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