OAS1, OAS2 and OAS3 restrict intracellular *M. tb* replication and enhance cytokine secretion

Gina Leisching[a,*], Victoria Cole[a], Aus T. Ali[b], Bienyameen Baker[a]

[a] *NRF-DST Centre of Excellence for Biomedical Tuberculosis Research, South African Medical Research Council Centre for Tuberculosis Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa*
[b] *Department of Pathology, National Health Laboratory Service, Tygerberg Hospital, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa*

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ABSTRACT

The 2′,5′ (OASs) are known as mediators of the antiviral response system through activation of the RNA cleavage pathway. Interestingly, we observe *OAS1*, *OAS2* and *OAS3* upregulation in a number of gene expression signatures which discriminate active TB from latent TB infection, however their biological role during bacterial infection has not yet been elucidated. We observed that the expression of these genes was associated with pathogenicity and virulence of mycobacteria as infection with *Mycobacterium bovis* BCG failed to significantly induce OAS expression. Further, we observed that after silencing of these genes, *M. tb* CFU counts increased significantly 96 h post-infection in comparison to the respective controls. Luminex revealed that OAS silencing significantly decreased IL-1β, TNF-α and MCP-1 and had no effect of IL-10 secretion. We show for the first time that OAS1, 2 and 3 restrict intracellular pathogenic mycobacterial replication and enhance pro-inflammatory cytokine secretion.

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Introduction

Recent evidence suggests largely unifying pathways with regards to sensing and responding to bacterial and viral pathogens. One such pathway is the type I IFN-inducing pathway which is crucial during viral clearance, but detrimental during bacterial infections (Decker et al., 2005), particularly mycobacterial infections (Manca et al., 2001; McNab et al., 2015). During either infection, IFNαβ release induces the transcription of several hundred IFN-stimulated-genes (ISGs) (Ezelle et al., 2016). Amongst these ISGs is the 2′-5′-oligoadenylate synthetases (OAS) gene family.

To date the 2′-5′-oligoadenylate synthetases (OAS), namely OAS1, OAS2 and OAS3 are known for their role in directly enhancing intracellular antiviral mechanisms. Their expression is induced following the release of type I interferons, where they function to polymerize ATP to 2′-5′-linked adenosine oligomers or 2-5A (pppA(2′p5′A)n) in the presence of dsRNA (viral origin) (Kristiansen et al., 2010). The 2–5As then activate the RNase L degradative pathway which functions to cleave the viral RNA and control the infection. Since the OASs are a product of the type I IFN response, it is expected that they may play a role during bacterial

infection, however this has not yet been addressed. Interestingly, *OAS1*, *OAS2* and *OAS3* upregulation has been observed in a number of gene expressions signatures which discriminate active from latent tuberculosis (Berry et al., 2010; Maertzdorf et al., 2012; Ottenhoff et al., 2012). It is known that the activation of all 3 OASs in the absence of viral nucleic acids cleave and degrade host mRNA through RNaseL, which slows down protein synthesis and induces apoptosis (Castelli et al., 1998b), and recently, it was suggested that OAS1 and OAS2 are likely to have alternative roles (Li et al., 2016).

It is unknown whether the expression of the OASs plays a similar role during bacterial infection through enhancing anti-bacterial mechanisms. Here, we characterise *OAS1*, *OAS2* and *OAS3* expression in macrophages during mycobacterial infection by i) determining whether their expression is linked to pathogenicity and/or virulence of mycobacteria, ii) assessing the biological role of OAS1, OAS2 and OAS3 during the infection of macrophages through silencing of each of the genes, and iii) analysing whether OAS silencing affects cytokine and chemokine secretion during infection. Our results reveal for the first time that OAS1, OAS2 and OAS3 restrict intracellular mycobacterial growth, and through an unknown mechanism, appear to enhance cytokine/chemokine secretion. This study suggests that the OASs may assist in priming host-defence mechanisms for both viral and mycobacterial infections and broadens the existing knowledge on host defence mechanisms of the human macrophage.

* Corresponding author.
 *E-mail address:* ginal@sun.ac.za (G. Leisching).

Results

OAS1, OAS2 and OAS3 expression in infected THP-1 cells is associated with mycobacterial pathogenicity and virulence

We first set out to determine whether the expression of *OAS1*, *OAS2* and *OAS3* was associated with mycobacterial pathogenicity and/or virulence through qPCR and immunofluorescence techniques (Figure 1). Interestingly, *OAS1*, *OAS2* and *OAS3* expression was significantly induced 24 h post-infection by the virulent R5527 *M. tb* strain only (Figure 1A), however immunofluorescence indicates that both the hypo- and hypervirulent *M. tb* strains induce significant expression of the protein components of these genes (Figure 1B). Conversely, *Mycobacterium bovis* BCG infection did not stimulate expression of these genes or their proteins 24 h p.i. This data therefore suggests that in infected THP-1 cells, *OAS1*, *OAS2* and *OAS3* expression is dependent on the pathogenicity and virulence of the mycobacterial strain early post-infection.

Silencing of OAS1, OAS2 and OAS3 promotes intracellular mycobacterial replication

To determine whether the absence of these genes affected intracellular mycobacterial replication, each gene was silenced separately and confirmed through qPCR and Western blot (Figure S1) using two siRNAs coding different target sequences (Figure 2). Additionally, the infection time was extended to 96 h to observe whether changes in the standard doubling times of the mycobacteria were affected. Further, host cell viability was assessed throughout the infection/silencing period to determine whether gene silencing affected host cell survival during infection. Finally, a negative siRNA control was included in all experiments to control for the silencing procedure. In these experiments, we included silencing of all 3 OASs at once, as well as in different combinations to determine whether the measured parameters were affected to a greater extent, however no significant differences were observed (data not shown). Figure 2A depicts intracellular CFU counts and host-cell viability during *OAS1* silencing in THP-1s infected with either the pathogenic or non-pathogenic mycobacterial strains. After 24 h, a slight decrease in CFU counts was observed in *M. bovis* BCG and hypovirulent *M. tb* infected THP-1 cells in the infected (denoted *M. bovis* BCG and R1507 respectively) and negative control groups (denoted as 'scr. sequence'). Conversely, this decrease in CFU counts was not observed during infection with the hypervirulent strain (R5527) which illustrates the virulent phenotype of this strain. After silencing with both OAS1 siRNA #1 and #2, little to no significant differences were observed at the 24 h time point, however after 96 h, significant increases in intracellular CFU counts were observed with all strains when comparing the infection without silencing and the negative silencing control (Figure 2A). Further, no significant differences in host cell viability were observed over the 96 h time period. As with *OAS1*, silencing of *OAS2* exhibited similar results for the pathogenic mycobacteria (Figure 2B), however downregulation of OAS2 expression during infection did not affect the intracellular replication of *M. bovis* BCG. Both pathogenic and non-pathogenic mycobacterial CFUs increased significantly 96 h p.i after silencing of OAS3 (Figure 2C) with no significant differences in host cell viability observed. This
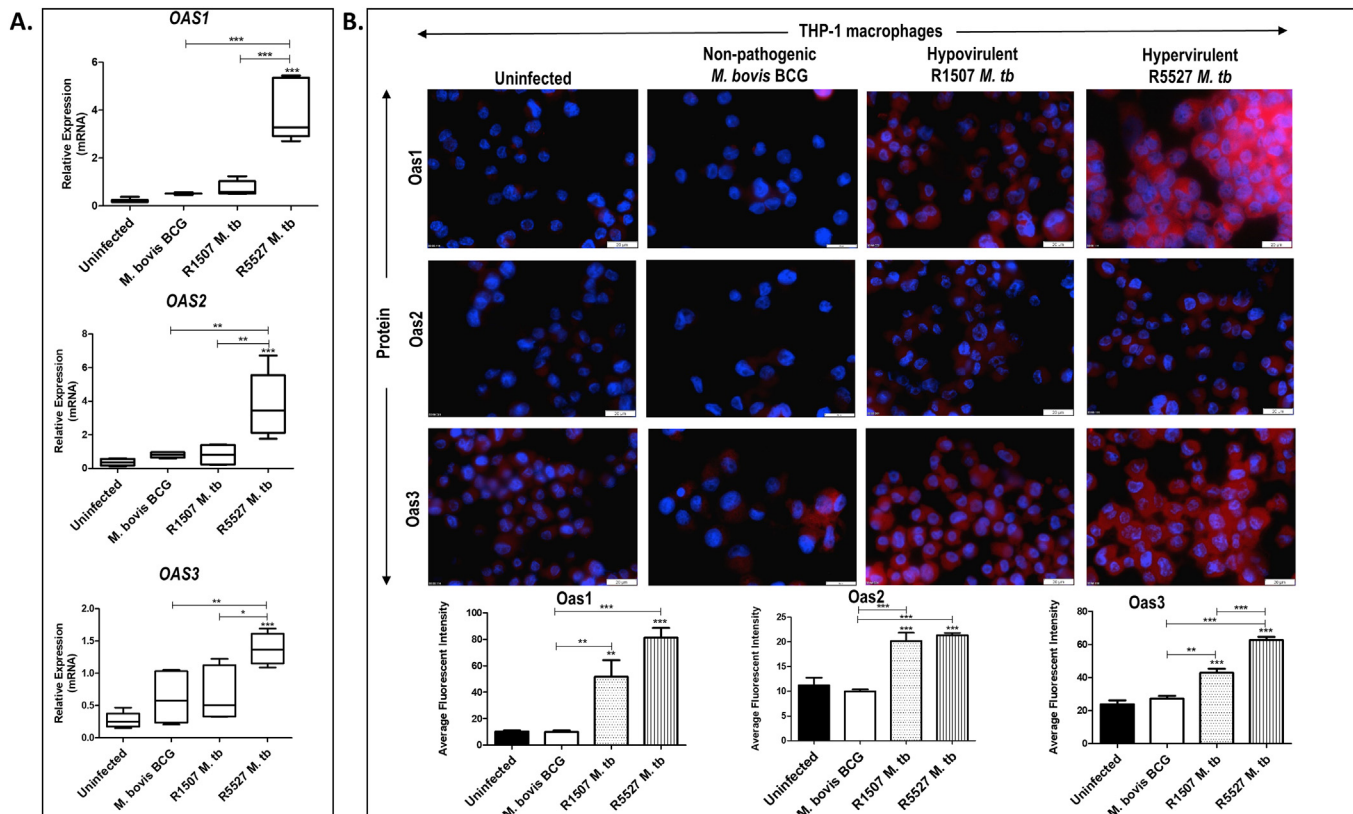
Figure 1. OAS1, 2 and 3 expression in THP-1 cells following infection with *M. bovis* BCG, R1507 *M. tb* (hypovirulent) and R5527 *M. tb* (hypervirulent). A. qPCR analysis of OAS1, 2 and 3 expression 24 h post-infection. *UBC* and *GAPDH* were used as reference genes to calculate the relative expression of the target genes. B. THP-1s were immunostained with OAS1, OAS2 or OAS3 primary antibody and then stained with a Texas Red secondary antibody. Nuclei were counter-stained with Hoechst (blue). Images were acquired on a LSM 780 Elyra S1 Confocal laser-scanning microscope, with a 63X, 1,4NA, oil-immersion objective. Average fluorescent intensity was quantified using Image J of the analysis of 12 images acquired in each experimental condition, carried out in triplicate. Scale bar = 20 μM. ANOVA with a Bonferroni post-hoc test was applied, **p < 0.01, ***p < 0.001 vs. uninfected.
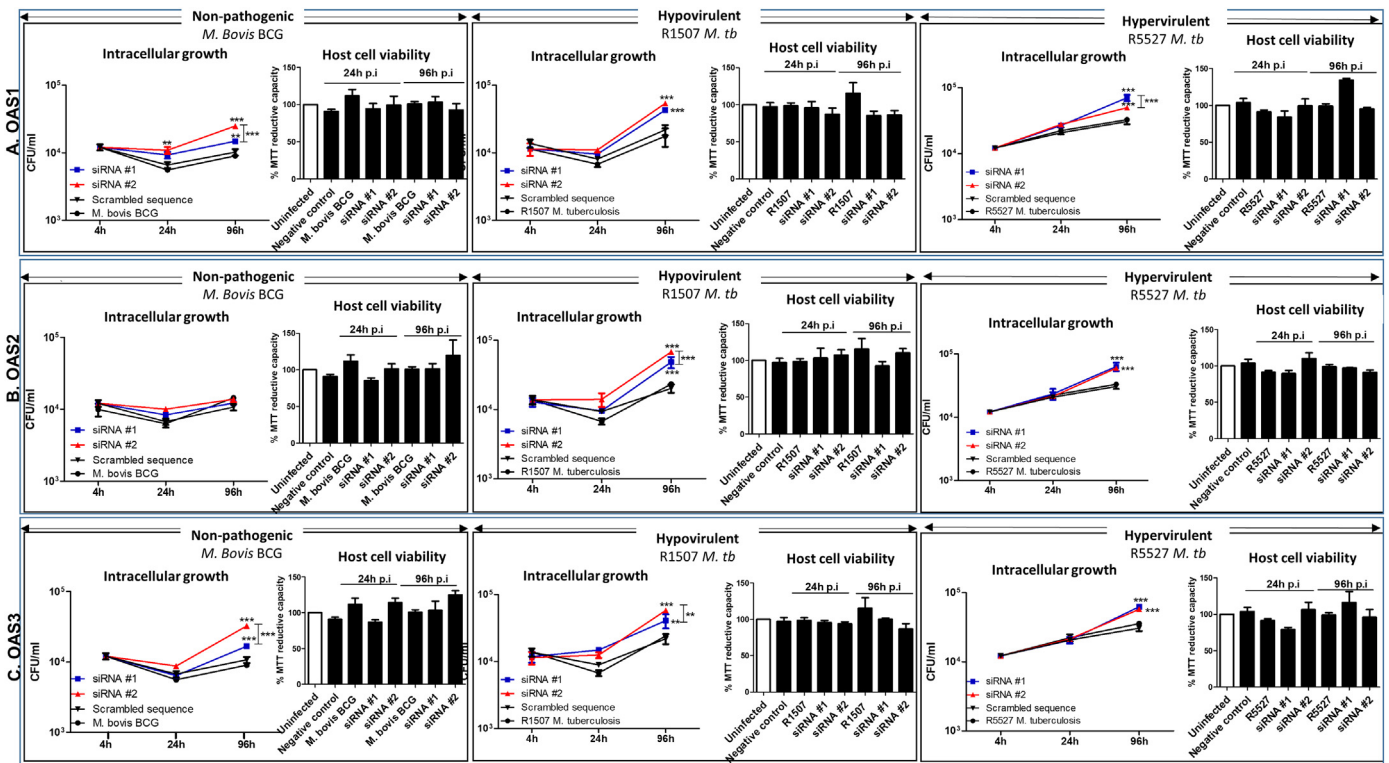
Figure 2. Intracellular growth of pathogenic and non-pathogenic mycobacteria and THP-1 viability after silencing of either OAS1, 2 or 3 over a 96 h time period. Two siRNA molecules (siRNA #1 and siRNA #2) with different targets were used for each gene, as well as scrambled sequence (negative control) A. OAS1 silencing did not affect host-cell viability but significantly increased the intracellular CFU counts of all strains 96 h p.i. B. Silencing of OAS2 had no effect on the intracellular growth of *M. bovis* BCG, however both pathogenic strains had significant increases in their intracellular CFUs 96 h p.i. Host cell viability was not affected by OAS2 silencing. C. Intracellular CFU counts increased significantly 96 h p.i for both pathogenic and non-pathogenic strains after OAS3 silencing. No significant effects on THP-1 viability was observed. Two-way ANOVA with a Bonferroni post-hoc test was applied, **p < 0.01, ***p < 0.001 vs. infected unsilenced and infected silenced (scrambled sequence).

data suggests that when expressed following infection, OAS1, 2 and 3 function to suppress the intracellular replicative capacity of mycobacteria and suggests an antibacterial, host-defence function, which up until now, was previously unknown.

Silencing of OAS1, OAS2 and OAS3 affects cytokine and chemokine expression in THP-1 cells during mycobacterial infection

Mycobacterial infection and replication is controlled through the release of cytokines and chemokines as part of the innate immune response to pathogen invasion. Since the mechanism by which the genes restrain intracellular mycobacterial replication is unknown, and given the fact that OAS1, OAS2 and OAS3 are interferon-induced genes, we sought to determine whether silencing affected cytokine/chemokine expression using Luminex® technology.

Firstly we silenced OAS1 and noted that TNF-α release was significantly reduced at both 24 and 96 h p.i after infection with both pathogenic and non-pathogenic mycobacteria in comparison to the unsilenced infected control and the infected scrambled sequence control (Figure 3A). Similarly, IL-1β release was significantly reduced during infection with all strains, but only at 96 h p.i (Figure 3B). In our model, IL-10 levels were only detectable 96 h p.i by Luminex and we observed that IL-10 release was unaffected by the silencing of *OAS1* (Figure 3C). The analysis of MCP-1 (*CCL2*) indicated that *OAS1* silencing during *M. bovis* BCG infection had no effect on MCP-1 release, however MCP-1 levels were significantly reduced 96 h p.i during infection with both pathogenic mycobacteria (Figure 3D). This data suggests that i) TNF-α and IL-1β secretion is enhanced during mycobacterial infection in the presence of OAS1, ii) IL-10 production is unaffected by the presence/absence of OAS1, and iii)

OAS1 enhances MCP-1 production in response to THP-1 infection by pathogenic mycobacteria only.

Secondly we silenced OAS2 to determine whether its presence affects cytokine production during infection (Figure 4). In comparison to the unsilenced infected control and the infected scrambled sequence control, TNF-α and IL-1β levels were significantly reduced following OAS2 silencing during infection with hypovirulent (24 and 96 h p.i) and hypervirulent (96 h p.i) pathogenic mycobacteria only (Figure 4A and B). IL-10 levels remain unchanged in response to OAS2 silencing (Figure 4C), and MCP-1 secretion was observed to be unaffected in THP-1s infected with pathogenic mycobacteria (Figure 4D). Interestingly, non-pathogenic mycobacterial infection in the absence of OAS2 enhanced MCP-1 secretion. Together these results suggest that i) TNF-α and IL-1β secretion is enhanced during pathogenic *M. tb* infection in the presence of OAS2, ii) IL-10 secretion remains unaffected by OAS2, and iii) MCP-2 is unaffected by OAS2 during pathogenic *M. tb* infection.

Lastly we assessed whether OAS3 affected cytokine secretion during mycobacterial infection (Figure 5). TNF-α levels decreased significantly after *OAS3* silencing in response to infection with pathogenic mycobacteria and remain unchanged in response to non-pathogenic mycobacterial infection (Figure 5A). IL-1β levels were also reduced following silencing, however this was observed during infection with all mycobacterial strains (Figure 5B). As with OAS1 and OAS2, IL-10 secretion was not affected by *OAS3* silencing (Figure 5C), and MCP-1 secretion was only affected by infection with pathogenic mycobacteria only.

The effects of OAS1, 2 and 3 on cytokine secretion during infection with pathogenic and non-pathogenic mycobacteria in THP-1 macrophages may be summarised as follows: TNF-α and IL-
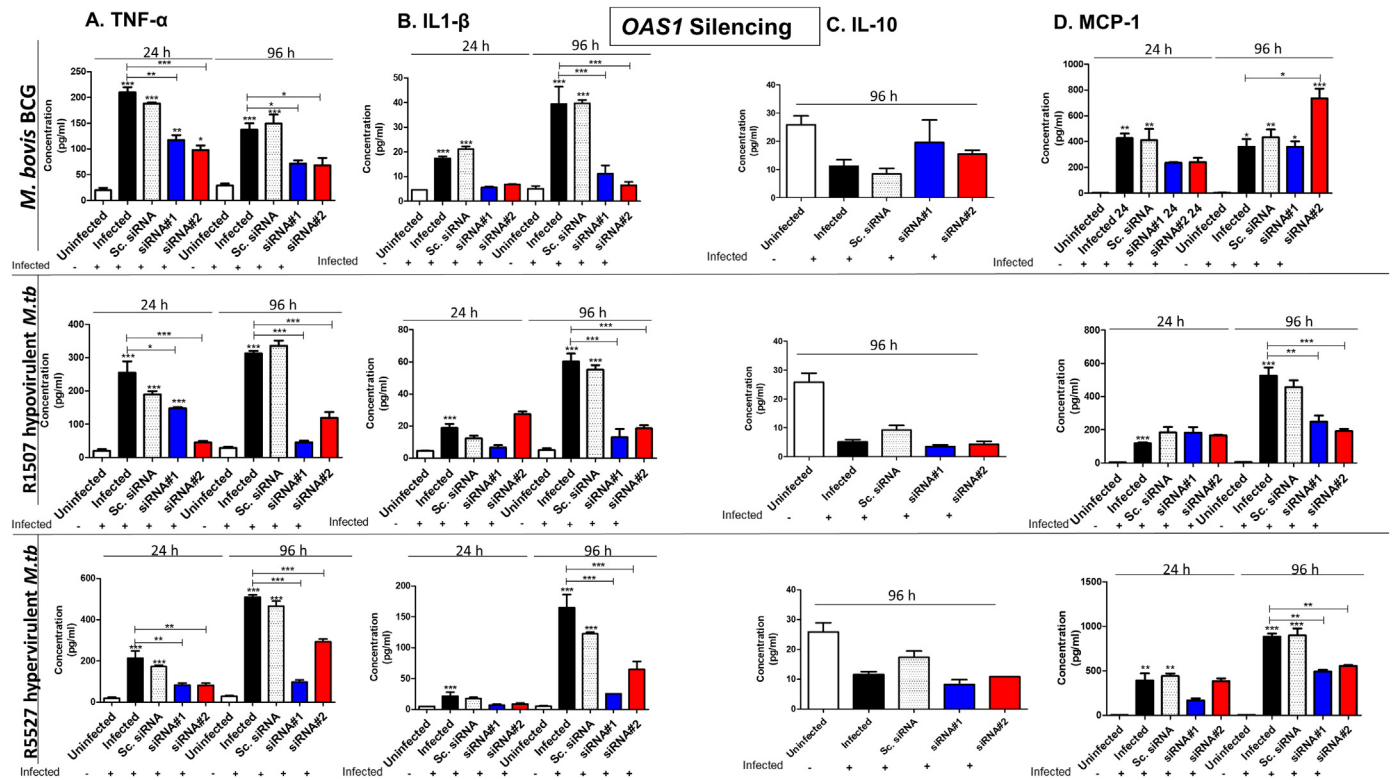
Figure 3. The effect of *OAS1* silencing on TNF-α, IL1-β, IL-10 and MCP-1 secretion in THP-1 cells infected with pathogenic and non-pathogenic mycobacteria as measured by Luminex. Two siRNA molecules (siRNA #1 and siRNA #2) with different targets were used for OAS1, as well as scrambled sequence (Sc. siRNA-negative control) A. TNF-α secretion decreased significantly at both 24 and 96 h p.i after OAS1 silencing in both pathogenic and non-pathogenic infection states. B. Significant differences in IL-1β secretion were only observed 96 h p.i with pathogenic and non-pathogenic mycobacteria. C. Silencing of OAS1 did not affect IL-10 secretion. D. MCP-1 decreased significantly 96 h p.i with pathogenic mycobacteria only. ANOVA with a Bonferroni post-hoc test was applied, **p < 0.01, ***p < 0.001 vs. uninfected.

1β secretion are enhanced by OAS1, 2 and 3 during *M. tb* infection. IL-10 secretion is not dependent on/associated with the presence of the OAS proteins and MCP-1 secretion is enhanced by OAS1 and 3, but not OAS2 during *M. tb* infection.

Discussion

The 2′,5′ oligoadenylate synthetases (OASs) have been documented as playing a role in the antiviral innate immune response during infection and function to produce 2–5A activators of the RNaseL cleavage pathway which ultimately lead to the control of the viral infection (Castelli et al., 1998a; Lohöfener et al., 2015; Li et al., 2016). *OAS1*, *OAS2* and *OAS3* are expressed as a result of IFNαβ and thus form part of the interferon stimulated genes (ISGs) that are associated with the early inflammatory response during infection and are evolutionarily conserved (Justesen et al., 2000; Yao et al., 2018).

Transcriptomics of patients with active tuberculosis reveal the upregulation of *OAS1*, *OAS2* and *OAS3*, which all form part of the TB signature that discriminates active from latent tuberculosis and other diseases (Berry et al., 2010; Maertzdorf et al., 2011; Ottenhoff et al., 2012). This suggests that these genes may play a role during active mycobacterial infection (Leisching et al., 2018), although this is yet to be determined. To answer this question we aimed to characterise the role of these genes in response to pathogenic and non-pathogenic mycobacterial infection and to determine whether their presence affects intracellular mycobacterial survival. Therefore we sought to elucidate whether the OASs play a role in restricting microbial growth during infection. Characterisation of their biological role included i) the analysis of the genes/proteins in response to non-pathogenic and virulent mycobacterial infection, ii) silencing of the genes to determine whether their downregulation

during infection affected intracellular mycobacterial growth over time, and finally iii) since these genes are induced by IFN αβ, whether silencing of OAS1, 2 and 3 affected cytokine expression in THP-1 cells.

We included the use of the non-pathogenic *M. bovis* BCG strain, as well as a hypovirulent (R1507) and hypervirulent (R5527) *Mycobacterium tuberculosis* strain to determine whether pathogenicity and/or virulence affects the expression of OAS1, OAS2 and OAS3 (Figure 1A and B). We observed that OAS expression is directly related to Mycobacterial pathogenicity early post-infection. It cannot be discounted that infection with *M. bovis* BCG does not induce OAS expression at all. It is possible that non-pathogenic strains induce the OASs, but at a later time point post-infection. This early response by infection with pathogenic mycobacteria may be explained by the following: After phagocytosis, most pathogenic intracellular bacteria mediate the phagosome breach which leads to content leakage in the form of dsDNA into the host cytoplasm (Vance et al., 2009; Manzanillo et al., 2012). The ESX-1 secretion system in pathogenic mycobacteria facilitate the perforation of the phagosome (Manzanillo et al., 2012), which leads to the release of extracellular mycobacterial DNA into the host cytosol. The OASs are activated by double stranded nucleic acids (Lohöfener et al., 2015) and are therefore nucleic acid sensors. OAS1 specifically, is structurally homologous to cGAS, the dsDNA sensor (Chen et al., 2016), which when activated, leads to the downstream activation of STING. STING is an essential molecule type I IFN transcription in the presence of foreign nucleic acids (Ishikawa et al., 2009), which then induces *OAS1*, *2* and *3* transcription as part of the ISGs. This may explain the early response of the OASs following infection with the pathogenic strains only.

It is known that cytokines play an important role in controlling mycobacterial infection without promoting uncontrolled and
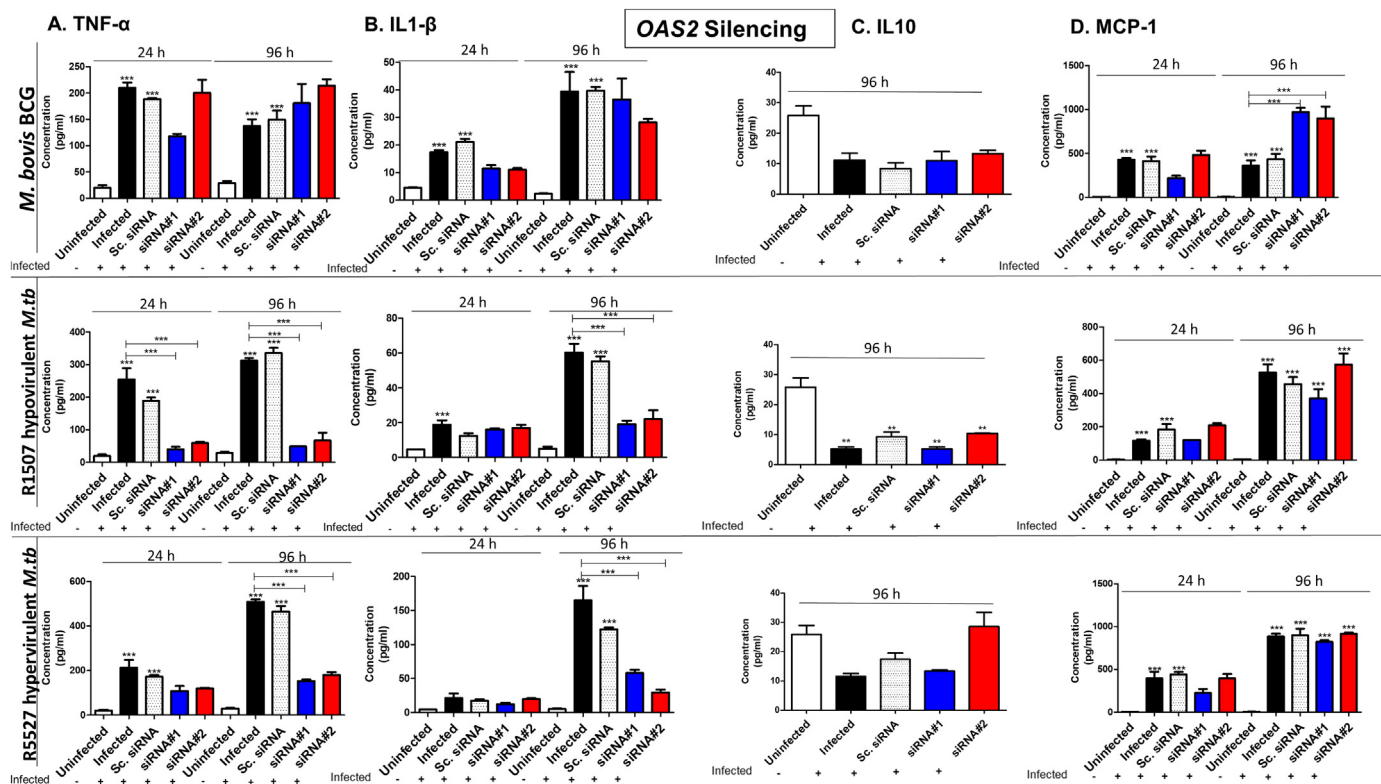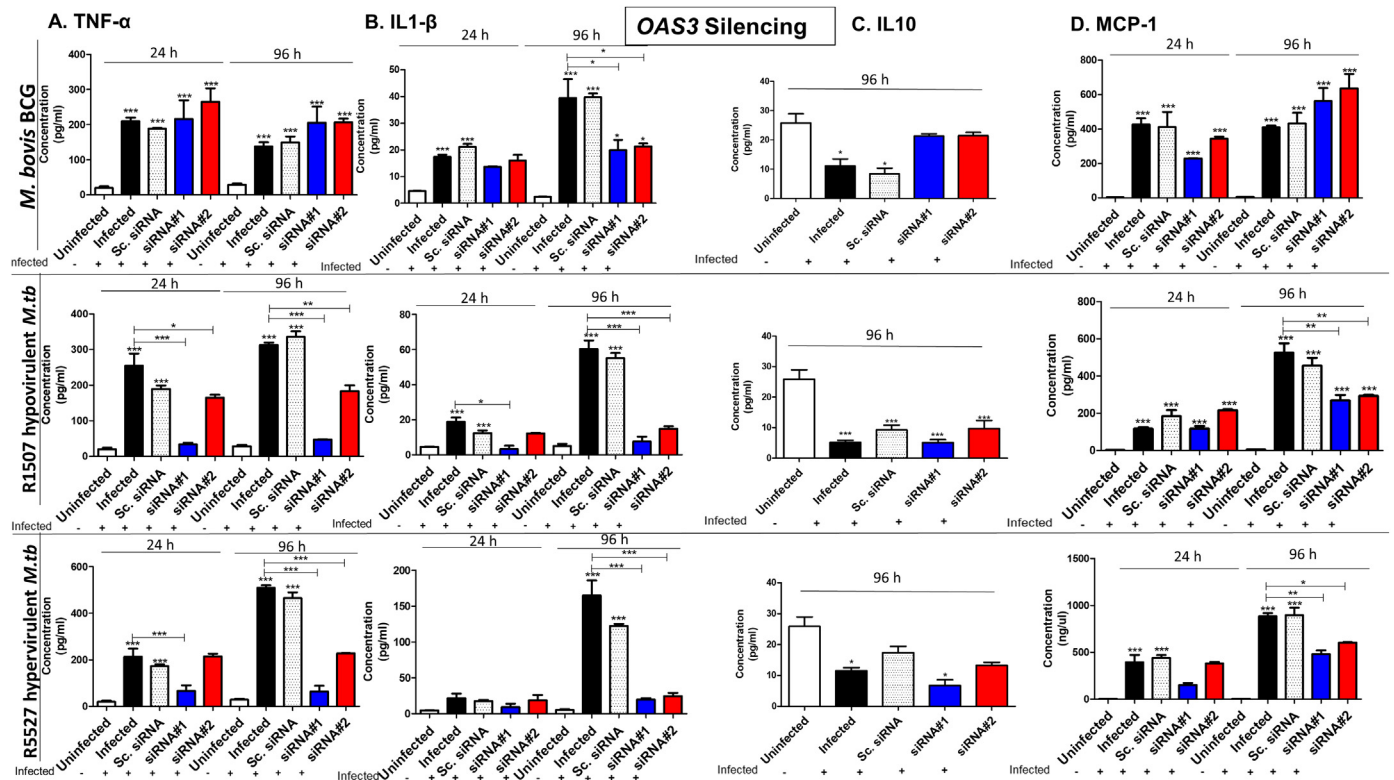
Figure 4. The effect of *OAS2* silencing on TNF-α, IL1-β, IL-10 and MCP-1 secretion in THP-1 cells infected with pathogenic and non-pathogenic mycobacteria as measured by Luminex. Two siRNA molecules (siRNA #1 and siRNA #2) with different targets were used for OAS2, as well as scrambled sequence (Sc. siRNA-negative control) A. TNF-α secretion decreased significantly 96 h p.i after OAS2 silencing in both pathogenic infection states. B. Significant differences in IL-1β secretion were only observed 96 h p.i after infection with pathogenic mycobacteria only. C. Silencing of OAS2 did not affect IL-10 secretion. D. No effect on MCP-1 secretion was observed during pathogenic mycobacterial infection. ANOVA with a Bonferroni post-hoc test was applied, **p < 0.01, ***p < 0.001 vs. uninfected.

damaging inflammatory responses. Since the OASs are induced following the onset of the inflammatory response, we investigated whether the downregulation of *OAS1*, OAS2 and OAS3 affected the intracellular replicative capacity of mycobacteria (Figure 2) and whether the expression of various cytokines were affected (Figures 3–5) as a possible explanation for the unrestricted growth. We observed that at 96 h, both the pathogenic and non-pathogenic mycobacterial CFUs increased significantly when compared to the infected unsilenced and infected silenced control when *OAS1, 2* and *3* were silenced. During viral infection, *OAS1, OAS2* and OAS3 knock-down also promotes the intracellular replication of a number of RNA and DNA viruses (Melchjorsen et al., 2009; Zhao et al., 2012; Wang et al., 2013; Li et al., 2016), which taken together, points to an anti-microbial, rather than just an anti-viral function of these proteins. Interestingly, IL-1β and TNF-α secretion levels are both significantly reduced at this time point post-OAS silencing during infection with both the pathogenic strains in particular. Recently, both IL-1β and TNF-α were described as hallmark cytokines of the innate immune response that contribute to "trained immunity" by macrophages that are essential in controlling mycobacterial growth (Kleinnijenhuis et al., 2014). To date however, there is no work investigating whether the OASs affect the secretion of these cytokines specifically, however the OASs have been documented to enhance IL-12A (Domingo-Gil et al., 2010) and IL-17A (Wang et al., 2013) during viral infection. It is therefore likely that their presence modulates the activation of other signalling pathways responsible for cytokine production.

Further, we observed that *OAS2* silencing did not affect MCP-1 secretion (Figure 4D), but *OAS1* (Figure 3D) and *3* silencing

(Figure 5D) significantly reduced MCP-1 levels. Additionally, intracellular *M. bovis* BCG growth was not significantly affected when *OAS2* was silenced (Figure 2B). Studies assessing the anti-viral roles of the OASs revealed that OAS2 was unable to block Dengue virus (Lin et al., 2009) and hepatitis C virus (Kwon et al., 2013) replication, however this does not explain why OAS2 silencing led to enhanced intracellular replication of *M. tb* in our model. Also, the reason as to why OAS2 does not affect MCP-1 secretion, but affects IL-1β and TNF-α which are all transcribed through NF-κB, is challenging to explain. Two OAS2 isoforms (p69 and p71) exist (Justesen et al., 2000), although very little is known regarding the individual functions of each isoform. It has been proposed recently that since OAS2 is a weak activator of RNaseL in comparison to OAS1 and OAS3, alternative roles for this protein are likely to exist (Li et al., 2016). Therefore OAS2 may function primarily during pathogenic mycobacterial infection, although its precise role, which should include analysis of both isoforms, should be assessed.

IL-10 is a potent anti-inflammatory and immunosuppressive molecule and functions to inhibit a range of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-12 (D'andrea et al., 1993; Moore et al., 2001). It was interesting to note that silencing of the OASs had no effect on IL-10 secretion (Figures 3D, 4D and 5D). This could be explained by the fact that, 1) unlike other cytokines, expression of *IL10* is regulated by the Sp1 and Sp3 transcription factors that are constitutively and ubiquitously expressed (Tone et al., 2000) and function under different elements of regulatory control when compared to the proinflammatory cytokines, and 2) that OASs expression may directly or indirectly suppress IL-10 secretion through unknown mechanisms.

Figure 5. The effect of *OAS3* silencing on TNF-α, IL1-β, IL-10 and MCP-1 secretion in THP-1 cells infected with pathogenic and non-pathogenic mycobacteria as measured by Luminex. Two siRNA molecules (siRNA #1 and siRNA #2) with different targets were used for OAS3, as well as scrambled sequence (Sc. siRNA-negative control) A. TNF-α secretion decreased significantly 96 h p.i after OAS3 silencing in the pathogenic infection state only. B. Significant differences in IL-1β secretion were observed at 96 h p.i after infection with pathogenic and non-pathogenic mycobacteria only. C. Silencing of OAS2 did not affect IL-10 secretion. D. MCP-1 secretion decreased significantly following OAS3 silencing, but was observed during pathogenic mycobacterial infection only. ANOVA with a Bonferroni post-hoc test was applied, *p < 0.05, **p < 0.01, ***p < 0.001 vs. uninfected.

Although the OASs are overexpressed following infection, it may be advantageous to test whether a similar result is observed through a vector-based overexpression system. Additionally, future studies should demonstrate if the induction and function of OAS is dependent on type I IFNs and whether the blockade of type I IFN signaling increases or decreases bacterial loads. Since IL-1β and TNF-α are most greatly affected by OASs knock-down, neutralisation of these two cytokines should greatly affect *M. tb* intracellular replication. Although our study does not uncover the mechanism by which the OASs enhance cytokine secretion during mycobacterial infection, it does uncover for the first time that OAS1, 2 and 3 restrict mycobacterial intracellular replication. Thus the OASs may no longer be associated with enhancing antiviral responses only, but also show an involvement with antimicrobial responses in the macrophage.

Experimental procedures

Cell culture

THP-1 (ATCC-88081201) human macrophage-like cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated foetal calf serum (Biochrome, Germany) and incubated at 37 °C, 5% CO_2. For infection experiments, THP-1 cells were differentiated into macrophage-like cells with Phorbol 12-Myristate 13-Acetate (PMA; Sigma Aldrich, USA) at a final concentration of 100 nM for 24 h prior to infection.

Bacterial strains and infection conditions

M. bovis BCG as well as hyper (R5527) and hypovirulent (R1507) Beijing *M. tuberculosis* clinical isolates (Aguilar et al., 2010) were

used for infection. Mycobacteria were cultured in 7H9 (supplemented with 10% OADC, 0.5% glycerol) without Tween 80 (Leisching et al., 2016a,b). THP-1s were infected with either the hyper-, hypovirulent *M. tb* or *M. bovis* BCG strain at a MOI = 1 using the "syringe settle filtrate" (SSF) method (Leisching et al., 2016a) and allowed 4 h for uptake. The cells were then washed 3 times with phosphate buffered saline (PBS) to remove any extracellular *M. tb*, and incubated for an additional 20 and 92 h in complete medium (for time points of 24 and 96 h). Uninfected THP-1s served as the control/uninfected samples. MOIs as well as intracellular replication p.i was assessed through intracellular CFU counting. Briefly, after each infection time point, cells were lysed with 0.1% sterile Triton X-100 where after bacteria were serially diluted and plated out on 7h11 agar for enumeration.

Gene silencing in THP-1 cells

All three genes were silenced using the FlexiTube siRNA Premix (Qiagen, Cat. # 1027420) for rapid siRNA transfection (Table 1). Addition of the siRNA was done after the 4 h uptake period according to the manufacturer's instructions and allowed 24 and 96 h for silencing. Silencing was determined through qPCR and

Table 1
Target sequences of siRNAs specific to OAS1, OAS2 and OAS3.

Gene	siRNA #1 target sequence	siRNA #2 target sequence
OAS1	CCGCCTAGTCAAGCACTGGTA	ACGGTCTTGGAATTAGTCATA
OAS2	CAACGTGACATCCTCGATAAA	ACAGTCTAAGCTGACCACCAA
OAS3	CTCATCGATACTGCCTGGTAA	TAGGCTTAATGGTCACCCTAA

Western blotting (Supplementary Figure 1) and confirmed for each silencing experiment.

RNA extraction and quantification

After each infection period, RNA was extracted using the RNeasy® Plus Mini Kit (Cat. No. 74134, Qiagen, Limburg, Netherlands) according to the manufacturer's instructions. The extracted RNA was treated with a gDNA eliminator (included in the kit) to remove all traces of genomic DNA for qPCR. RNA quantity and quality was assessed using the Agilent 2100 Bioanalyzer.

cDNA synthesis and qPCR

cDNA was synthesized using 0.5 μg RNA using the Quantitect® Reverse Transcription Kit (Cat. No. 205311, Qiagen, Limburg, Netherlands). Prior to cDNA synthesis, a gDNA wipe-out buffer (included in the kit) was added to the RNA to remove any remaining genomic DNA. qPCRs were run in 96-well plates using the LightCycler® 96 system (Roche, Germany) at a reaction volume of 20 μl. LightCycler® 480 SYBR Green I Master (Cat. No. 04887352001, Roche, Germany) was used to detect amplification of cDNA. The amplification procedure entailed 45 cycles of 95 °C for 10 s followed by 60 °C for 10 s and finally 72 °C for 10s. Gene expression fold-changes were computed using calibrated relative quantities using the equation $N = N0 \times 2Cp$ (Light-Cycler®96 software, Roche). QuantiTect® primer assays (Qiagen, Limburg, Netherlands) were used to assess *OAS1* (Cat.# QT00099134), *OAS2* (Cat.# QT01005249) and *OAS3* (Cat.# QT01005277) expression. *UBC* (Cat.# QT00234430) and *G6PD* (Cat. # QT00071596) were used as reference genes for each run. All qPCRs were done with RNA from three independent experiments, each with 4 technical replicates. Each biological replicate was run in triplicate with a positive control (calibrator) and a non-reverse transcription control in accordance with the MIQE Guidelines (Bustin et al., 2009).

MTT assay

To evaluate whether silencing of the various genes affected host cell viability after infection, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used (Mosmann, 1983). After each infection time period, 0.01 g/ml MTT was added to the cells and incubated for 2 h at 37° C at 5% CO_2. HCl-isopropanol-Triton solution (1% HCl in isopropanol, 0.1% Triton X-100 in a 50:1 ratio) was then added to the cells and gently agitated for 5 min. The optical density (OD) was determined on a plate reader (EL-800, Micro-Tek instruments) at a wavelength of 540 nm and the values expressed as a percentage of the control (uninfected cells).

Immunofluorescence and analysis

Macrophages were seeded on 14 mm glass cover slips and infected with either *M. bovis* BCG, two *M. tb* strains (hypervirulent and hypovirulent) or remained uninfected as described above. Twenty four hours post infection, the cells were heat-fixed at 95 °C for 2 h, followed by permeabilisation with 0.2% Triton X-100 in PBS for 10 min. Cells were immunostained with OAS1 (Sigma Aldrich SAB2101669), OAS2 (Invitrogen PA5-37878) or OAS3 (Sigma Aldrich HPA041253) primary antibodies in 3% BSA for 24 h at 4 °C. Cells were then incubated with Texas Red secondary antibody in 3% BSA in PBS for 90 min and nuclei were counter-stained with Hoechst for 10 min. Images were acquired on a LSM 780 Elyra S1 Confocal laser-scanning microscope, with a 63X, 1,4NA, oil-immersion objective at the Central Analytical Facility (Stellenbosch University, RSA). Hoechst was viewed with a 360 DAPI Filter (Intensity 42%, Gain – 500 ms) and Texas Red Marker with a 572 Texas Red filter (Intensity 100%, Gain – 1000 ms).

Quantitative analysis of immunofluorescence was carried out by analyzing the fluorescent intensity at each pixel across the images using histogram analysis in Image J (Windows version; National Institutes of Health) (Collins, 2007). Background signal was eliminated in the images by using the appropriate thresholding before histogram analysis.

Luminex®

Since OAS expression is interferon-induced during infection, we sought to determine whether silencing of these genes had any effect on cytokine or chemokine secretion in THP-1 cells. We used the Milliplex® map human cytokine/chemokine magnetic bead panel kit (Cat.# HCYTOMAG-60K) to simultaneously quantify the concentrations of TNF-α, IL-1β, IL-10 and MCP-1 in cell culture supernatants at 24 and 96 h post-infection on the Bioplex 200 (Bio-Rad) and used the Bioplex Manager 6.1 software for data analysis (Bio-Rad). Supernatants were collected from three independent experiments, each with 4 technical replicates which were run in duplicate.

Statistical analysis

Statistical significance was performed with GraphPad Prism software. ANOVA was used for comparisons involving 3 or more groups, with a Bonferroni post-hoc correction applied. All values are expressed as means ± SEM with a $p < 0.05$ considered as significant.

Conflict of interest

The authors declare that no conflict of interest exists.

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Ethical approval

Ethical approval was not required.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijid.2019.02.029.

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