Intracellular killing of *Mycobacterium avium* by activated macrophages: The role of nitric oxide

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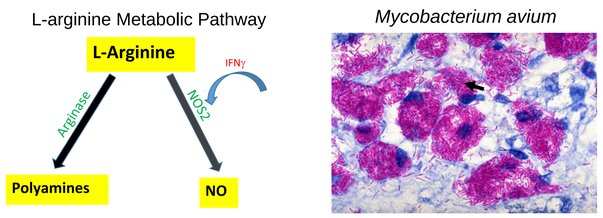
Introduction

Non-Tuberculosis Mycobacteria (NTM), specifically *Mycobacterium avium* (*M. avium*) is a rod-shaped microorganism that causes lung infections in patients with weakened immune systems or underlying lung diseases such as cystic fibrosis, COPD, and HIV. Previous in vitro studies in our laboratory have shown that nitric oxide (NO) plays a critical role in controlling *M. tuberculosis* growth inside macrophages. Others have reported that *Mycobacterium avium* can survive and multiply within non-activated cultured macrophages. Still, the antimicrobial mechanism of NO within activated macrophages to control *M. avium* growth remains to be clarified. This study is aimed to investigate whether the production of nitrogen-reactive species (NO) is a critical factor in the control of *M. avium* infection in murine-activated macrophages.

Our *hypothesis* is "that activation of *M. avium* infected macrophages with IFNγ will significantly increase NO production, then improving infection clearance."

To test our hypothesis, we used the murine macrophage cell line RAW 267-4. One million RAW cells were infected with *M avium* at MOI 10:1 for 4 hours and rinsed twice with PBS to eliminate non-adhered mycobacteria. The infected macrophages were cultured in the presence or absence of 100 U/ML of IFNγ for 24 and 48 hours. Greiss Assay and colony-forming units (CFU) respectively monitored NO levels and intracellular mycobacteria growth at each time point. After protein quantitation of 50 μg of cellular extracts were tested for arginase activity by an enzymatic assay.



Mycobacterium avium

Hypothesis & Methods

Hypothesis:
"Activation of *M. avium* infected macrophages with IFNγ will significantly increase NO production, then improving infection clearance."

Methods:

1. One million RAW cells were plated into 6 well plates and then infected with 10:1 MOI of *M. avium* in the presence and absence of IFNγ for 24 and 48 hrs.
2. At each time point supernatants were collected to determine nitrite production using the Greiss Reagent. Cellular extract were prepared to test for mycobacteria growth by CFU, NOS2 protein by Western blot and arginase activity by enzymatic assay.
3. Statistical analysis: The results are expressed as the mean ± SEM from 3 different experiment for each condition. Statistical analysis were performed by two-tailed *t* test using the PRIZM software.

Results

Arginase expression in *M. avium* infected macrophages

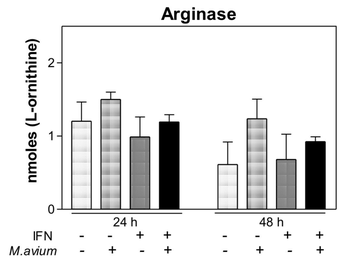


Figure 2. Arginase activity in cell lysates from infected Raw cells with *M. avium* in presence or absence of IFNγ was assessed by enzymatic assay. The assays were performed at 24 and 48 hrs. Arginase levels are measured indirectly by the conversion of L-arginine to L-ornithine by the catabolizing enzyme arginase. Under the conditions established in the experiments, there was not any significant production of arginase in macrophages infected with *M. avium.*

Nitrites production & NOS2 protein expression

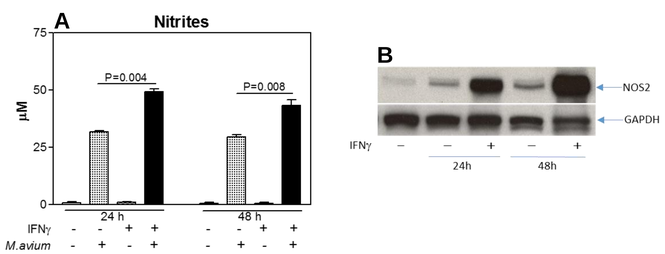


Figure 3. **A.** After 24 and 48 hours, culture supernatants of RAW cells infected with *M. avium* at MOI of 10:1, showed a significant increase in nitrite levels (P=0.004 and P=0.008) respectively in the presence of IFNγ. No nitrites production was observed in uninfected cells. **B**. The increased production of nitrites paralleled with an increase in NOS2 expression when stimulated with IFNγ.

Mycobacterium growth in agar

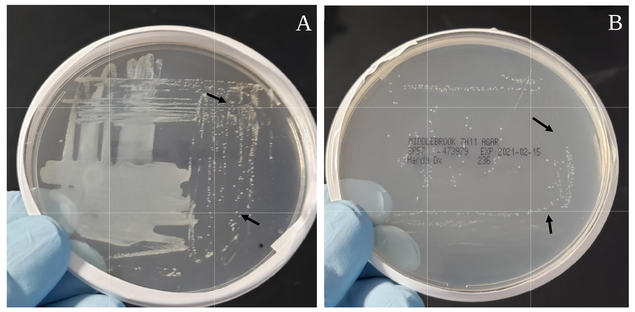
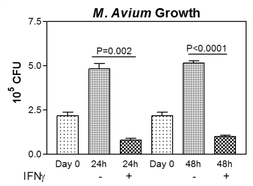


Figure 4. The survival of *M. Avium* in RAW infected cells, was determined by colony-forming units. ***A***. *M. avium* growth in absence of IFNγ and ***B.*** in presence of IFNγ. The results Shown are representative of three different experiments at 48 h in culture. Mycobacterial growth was similar at 24 hr time period

Nitrites production & NOS2 protein expression

		10^5 CFU[b]		
M. Avium	IFNγ (50U/ml)	Day 0	Day 1	Day 2
+	-	2.05	4.87	5.13
+	-	1.93	4.32	4.93
+	-	2.58	5.31	5.43
+	+	2.05	1.01	1.01
+	+	1.93	0.98	1.23
+	+	2.58	1.41	1.31



[a] Intracellular growth of *M.avium* during the 2-day period was measured by CFU

[b] Day 0: 2.18 ± 0.19
Day 2: 4.83 ± 0.28 (P=0.016)
Day 2: 1.13 ± 0.13 (P=0.003)
Day 4: 5.16 ± 0.14 (P=0.012)
Day 4: 1.18 ± 0.08 (P=0.102)

Table1. Susceptibility of *M. avium to* IFNγ-stimulated murine macrophages[a] Results are mean ± SEM from three separate experiments for each condition. Harvested cells were lysed and plated into agar plates. Bacterial growth was visualized after 14 days.

Conclusions

1. **We conclude that the increased resistance of *M. avium* was associated with and dependent on IFNγ stimulation that kills several mycobacteria in a dependent generation of reactive nitrogen intermediates (NO).**
2. **These data also illustrate that NO can promote or inhibit mycobacterial growth and that there is a delicate equilibrium that underlies its production.**
3. **The opposite effects of NO on the resistance to *M. avium*, emphasize the distinct nature of the strategies used by *M. avium* to survive the host's antimicrobial machinery.**
4. **The effect of NO and mycobacterial growth in other strains of the *M. avium* complex and its effect, needs to be further evaluated.**

Future Plans

1. **While this hypothesis worked for the *M. avium* strain, determine if there is a significant inhibitory effect of NO on intracellular growth of other NTM strains (*M. abscessus* and *M. intracellulare)* commonly found in Louisiana.**
2. **Since L-arginine is the substrate for the activation of NOS2 we will like to assess the role of L-arginine-rich environment in facilitating the extracellular spreading of NTM strains.**

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