"Adjusting to a new home - *Mycobacterium tuberculosis* gene expression in response to an intracellular lifestyle."

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SUMMARY

Mycobacterium tuberculosis is still the most significant single species of bacteria causing disease in mankind. The ability of *M. tuberculosis* to survive and replicate within host macrophages is a pivotal step in its pathogenesis. Understanding the microenvironments that *M. tuberculosis* encounters within the macrophage and the adaptations that the bacterium undergoes to facilitate its survival will lead to insights into possible therapeutic targets for improved treatment of tuberculosis. This is urgently needed with the emergence of multi- and extensively-drug resistant strains of *M. tuberculosis.* Significant advances have been made in understanding the macrophage response on encountering *M. tuberculosis*. Complementary information is also accumulating regarding the counter responses of *M. tuberculosis* during the various stages of its interactions with the host. As such, a picture is emerging delineating the gene expression of intracellular *M. tuberculosis* at different stages of the interaction with macrophages.

KEYWORDS
Mycobacterium tuberculosis; macrophage; microarray; host-pathogen interaction; phagosome; gene expression; innate immunity; adaptive immunity

INTRODUCTION

Mycobacterium tuberculosis (*M.tb*) is the etiological agent of tuberculosis (TB) and is the leading cause of infectious death in adults due to a single bacterial species [1]. It is estimated that every year there are approximately 8 million new cases of TB, and that TB is the cause of around 2-3 million deaths worldwide [301]. Synergy with the human immunodeficiency virus (HIV), along with the emergence of multidrug resistant TB (MDR) and the newly identified extensively drug resistant (XDR) strains [2] threatens to make the disease incurable once again.

M.tb is a facultative intracellular pathogen possessing mechanisms for evading and subverting host immune responses. Only 10% of people infected with *M.tb* develop clinical disease over their lifetime. Thus, there is obviously a struggle between pathogen and host, with the outcome of any infection being greatly affected by variation in host susceptibility [3 4 5 6]and mycobacterial virulence [7 8]. That pathogenic mycobacteria are able to survive within macrophages (MM) and granulomas (both components of the host defence system) demonstrates that they have mechanisms enabling them to inhibit, endure or evade cell-mediated immunity. It is logical to propose that specific mycobacterial genes are critical for the survival and virulence of *M.tb* within the host. This hyopothesis has led to numerous studies, using a wide range of methodologies, to recognise these functionally significant genes.

The production of transposon mutant libraries in mycobacteria [9] [10] has enabled the study of a number of potential virulence factors. Signature tagged mutagenesis has been used to identify mutants that do not survive *in vivo* [11] [12], whereas saturation mutagenesis of *M.tb* has identified mutants that can not survive *in vivo* [13] or in MM [14]. While informative, these studies have identified only the genes that are essential for the survival of *M.tb* in MM and animal models. Obviously this is important information that can be used in the search for new therapeutics but it does not highlight conditionally essential genes required in additional phases of infection, or allow for the identification of non-essential genes that mediate the differences seen when comparing, for instance, virulent and avirulent strains.

Gene expression profiling on a global level has been investigated widely using such methods as microarray analysis, subtractive hybridisation and differential display. Differential display of RNA from two populations of bacteria (e.g. broth grown versus inside a MM) is a sensitive method that requires very small amounts of RNA. However, relatively large numbers of candidate bands must be checked to find a few differential bands and rare mRNA transcripts are very hard to detect without nested priming [15]. Subtractive hybridization has been used to eliminate bacterial genes commonly expressed in both MM and broth culture [16] [17] [18]. The remaining genes are then assumed to be specifically expressed in one or the other environment. Another approach to identifying genes expressed in MM is to construct a library of mycobacterial genomic DNA in a promoter capture vector, with the DNA inserted upstream of a promoterless Green Fluorescent Protein (GFP) gene. Thus, only plasmids containing an active promoter will express GFP. This library is transformed into the mycobacteria of interest which are then used to infect MMs. Any promoter constructs that are more actively expressed in the MMs can be identified using fluorescent readout methodologies [19] [20] [21]. Subtractive hybidization and promoter capture have been used successfully to identify genes expressed by mycobacteria within MMs. However, both methods have drawbacks in that they are technically demanding and are prone to identifying genes that can be found in both pathogenic and non-pathogenic mycobacteria.

Post-genomic studies offer the potential for identifying putative virulence genes by utilising microarray technology to define the changing pattern of *M.tb* gene expression during infection [22]. DNA sequences representing the open reading frames (ORF) for all genes from (a) reference genome(s) are printed or synthesised on a glass slide (or chip) which is then probed with cDNA derived from *M.tb* RNA. The fluorophore-labelled cDNA is co-hybridised in a competitive reaction against cDNA (labelled with an alternative fluorophore) derived from a different condition in a direct comparison, or against a DNA reference that allows multi-way comparisons between RNA samples. The normalized ratio of the fluorophores for each gene reflects the change in RNA abundance between the two conditions. Developments such as the use of genome-directed primers [23], bacterial RNA amplification [24], and high-density microarray platforms have expanded the range of infection models tractable to investigation and the global nature of the data acquired. The application of transcriptome sequencing using next-generation methodologies will clearly improve the genomic resolution of these studies further.

Another approach for studying transcriptomes is the use of Bacterial Artificial Chromosome (BAC) arrays. They have previously been used to compare mycobacterial genomes [25] [26] [27] [28] and, more recently to compare the transcriptomes of intracellular *M.tb* strains [29] [30]. Genomic DNA is cloned into BACs and the clones are positioned on a restriction enzyme map of the genome to produce a set of BACs that cover the genome. Southern blots of BAC digests can be hybridized with cDNA from two transcriptomes and non hybridizing fragments in one or the other sample can be identified. A drawback to this method is that each band produced by the restriction digest of each BAC does not necessarily represent a single gene. The absence of a gene within a band may, therefore be masked by a positive hybridisation with other genes within that same band.

Conversely, the presence of multiple genes in a band can be advantageous in identifying differences in the expression of operons [30].

OVERVIEW OF THE INFECTIOUS PROCESS

M.tb bacilli first encounter host MMs following their inhalation into the alveolar spaces of the lung. It is here where they will encounter the alveolar MM. The first stage of the interaction is the attachment of the bacteria to the MM, followed by ingestion via phagocytosis (see Fig 1). During this process, the bacterium may be exposed to potentially toxic agents such as reactive oxygen intermediates (ROI). They will also trigger the production of cytokines and chemokines by the MM which play pivotal roles in the induction of innate immunity, adaptive immunity and apoptosis. Following ingestion the bacterium resides within an intracellular compartment called a phagosome where they are potentially exposed to additional killing mechanisms such as lysosomal hydrolases, antimicrobial peptides and glutathione. In addition, they must acquire nutrients while residing in this intracellular compartment of the MM usually associated with digestion and recycling of senescent cellular debris. Another important adaptation the TB bacillus must make is the move to a lower oxygen tension within the MM. Obviously some bacteria survive this initial move to the intracellular environment where they can then replicate. One outcome of this replication is the release of bacteria from the initial contact cell to other local host cells such as dendritic cells and newly arriving elicited MMs. Another outcome of bacterial replication is that the host develops adaptive immunity leading to the interferon-γ (IFNγ)-mediated activation of infected MMs. Activated MMs are better able to control bacterial replication through enhanced killing mechanisms such as the production of ROI and reactive nitrogen intermediates (RNI), phagosomal maturation to lysosomes, and apoptosis. The exposure to adaptive immune responses triggers a shift of the bacterial physiology towards a so called dormant or chronic phase. In this state, *M.tb* utilises lipids as a carbon source and the glyoxalate shunt for energy production. The host response to the persisting bacteria is to form a highly organised granuloma that walls off the bacteria in an hypoxic environment where they are commonly found within foamy macrophages. This granuloma may calcify over time or may cavitate and break through to the airways, releasing bacteria to begin the cycle again.

There are a number of critical steps during the cycle of *M.tb* pathogenesis that result in changes to the environment in which the bacterium finds itself. Many of these key phases of infection involve changes to the MΦ-bacterial interplay. Although *M.tb* has been shown to enter neutrophils [31, 32] and epithelial cells [33, 34], the MM is considered to be the host cell in which the bacteria most commonly reside. While the role of neutrophils and epithelial cells in the pathogenesis of *M.tb* is still uncertain, the central role of the MM is generally accepted as a major component in the host/pathogen interaction in TB. Thus, this review will concentrate on the expression of genes by *M.tb* during its interaction with MMs. Additionally, although numerous informative studies have identified expression differences between broth grown *M.tb* and bacteria obtained from the human lung and animal infection models, or by using model organisms like *M. bovis* BCG [35, 36, 37, 38, 39, 40], we have restricted our review to studies utilising *M.tb* in the context of life inside the MM.

BINDING AND UPTAKE (MOVEMENT FROM EXTRACELLULAR TO INTRACELLULAR)

Because mycobacteria are predominantly found within MMs and are known to survive and replicate within that niche, it is generally believed that they have evolved strategies that facilitate their association with phagocytes. It is often assumed that mycobacteria readily enter any MΦ they encounter and it is only then that survival mechanisms of bacterial origin come into play. However, there is evidence that MΦ populations vary in their ability to efficiently associate with *M.tb* [41, 42]. Thus, evidence is accumulating to suggest that pathogenic mycobacteria are not automatically bound and ingested by the first MΦ they encounter on inhalation. On the contrary, *M.tb* bacilli appear to have developed strategies to control their ingestion by MMs, which includes a capsule that acts to regulate the receptor-ligand interactions between *M.tb* and MMs [43].

Host-derived serum opsonins can greatly enhance the uptake of mycobacteria by most MΦ populations [44 45 42], though not by resident alveolar MMs [42]. The serum-independent interaction of *M.tb* with MMs is considered to be important in the early stages of pulmonary infection because the lung is an environment in which serum opsonins are relatively scarce or absent [46 47] and alveolar MΦs have low levels of receptors for serum opsonins [48 42]. Serum-mediated uptake of *M.tb* may be more important at later stages of the infection, when bacteria encounter elicited MΦs [41 42]. Thus, in the absence of significant levels of serum (a situation encountered by *M.tb* on entering the lung) *M.tb* appears to have developed strategies to limit its association with MΦs and to direct its entry into certain sub-populations of MΦs. Entry of *M.tb* into the MΦ appears to rely on bacterial adherence to MΦ receptors/surface molecules followed by phagocytosis, with no substantial evidence for active mechanisms of bacterial invasion. Numerous receptors have been implicated in mediating the serum-independent entry of *M.tb* into MΦs including the mannose receptor [49], the lipopolysaccharide receptor (CD14), [50], surfactant protein receptors A [51], the complement receptor CR3 [52], and CD43 [53 54].

It might be expected that binding and ingestion of *M.tb* to MΦ would trigger the induction of a number of genes to prepare the bacterium for the major changes in oxygen tension, nutrient availability and exposure to toxic molecules. Surprisingly, the attachment of *M.tb* to MΦ did not alter bacterial gene expression, as measured by microarray with a >1.5x fold change in expression compared to *M.tb* bacilli resuspended in MΦ culture media alone [55]. However, uptake of the bacteria over 2 hours resulted in transcriptional responses commencing within minutes and resulting in distinct changes in gene subsets over time [55]. These included members of the WhiB family of transcriptional regulators, members of the *devR* (also known as *dosR*) regulon involved in the response to hypoxia and ROI and >50% of the *phoPR* regulon, including genes involved in cell wall remodelling.

Several groups have defined the transcriptome of intracellular *M.tb* from 4h to 48h after MΦ infection, describing the changes in gene expression between broth grown and intracellular *M.tb*. Many additional studies have investigated specific parameters considered to be representative of the intracellular environment, such as hypoxia or low pH, and have been used to deconvolute the complex patterns of gene expression observed after macrophage uptake. Figure 2 illustrates the overlap of gene subsets between the intracellular transcriptional signatures derived from MΦ infection models and microarray experiments designed to mimic specific environmental stresses that *M.tb* may encounter during infection. Interestingly, there is limited overlap between all intracellular transcriptomes (A-D). However, common functionalities of regulated gene sets are repeatedly identified (E-S). These include genes indicative of fatty acid metabolism, cell wall metabolism and restructuring, iron metabolism, DNA damage repair, nutrient deprivation, transcriptional regulation and a response to hypoxia including the *devR* regulon [56 57 58 59 55 60]. These differences are taken to imply that the bacterium has moved to an environment low in carbohydrate yet relatively lipid rich, where it encounters a low oxygen tension, and is exposed to potentially toxic mechanisms requiring restructuring of the cell wall and the utilization of defence mechanisms. The specific events following uptake of *M.tb* by MΦ have been modelled and investigated in some detail.

RESIDENCE IN A PHAGOSOME AND THE ASSOCIATED LOW NUTRIENTS, INCREASED ACIDITY AND HYPOXIA.

The bacterial phagosome is a defining feature of *M.tb* pathogenesis as it differs from most particulate endosomes. There are numerous studies in which this compartment has been modelled in a cell free system. Following the phagocytosis of most particles, the phagosome will mature along the phagosomal-lysosomal pathway, sequentially acquiring markers from early endosomes (such as transferrin receptors, mannose receptors and Rab5), late endosomes (such as LAMPs, cathepsin D and Rab7) and lysosomes (such as LAMPs, and cathepsin D) via a “kiss

and run" phenomenon [61,62]. The phagosome also progressively acidifies down to pH5.5, or lower, following fusion with late endosomes and lysosomes [63,64]. However, pathogenic mycobacteria such as *M.tb* subvert this phagosome maturation, presumably to aid in their survival within the MΦ. Seminal work by Hart first demonstrated that mycobacterial phagosomes did not fuse with lysosomes [65]. This observation was extended to show that *M.tb* arrests maturation of the phagosome to reside in an early endosomal-like compartment that can interact with other endosomes, thereby obtaining nutrients for its survival. The *M.tb* phagosome contains the early endosome markers glycosphingolipids [66], transferrin [67] and Rab5 but excludes the late endosome marker Rab7 [68]. However, the late endosomal-lysosomal markers LAMP-1 and cathepsin D do appear to be found in the *M.tb* phagosome [69,70] (Fig 1).

The mycobacterial phagosome remains at pH 6.2-6.3 displaying a reduced acidification when compared to those containing control particles [71,72] . This has been attributed to a paucity of proton ATPase pumps on the *M.tb* phagosome [69,73]. Evidence has been presented to explain how mycobacteria bring about the reduced acidification and arrest of maturation of their phagosome. Galectin 3 [74], coronin1 (TACO) [75] and degraded cellubrevin [76] have all been found to accumulate on the *M.tb* phagosome but, as yet, no mechanism for their activity has been fully defined. The mycobacterial cell wall associated glycolipids, lipoarabinomannan [77,78] and cord factor [79] have been shown to inhibit phagosome maturation, though again, no mechanistic explanation has been determined. Inhibition of intracellular calcium by mycobacteria has also been shown to inhibit phagosome maturation [80] through the inhibition of sphingosine kinase [81]. In addition, transposon mutants of *M.tb* that fail to prevent the maturation of phagosome development have been identified and the genes involved were found to include putative transporters, lipid synthesis enzymes [82] and several mutations in the ESX-1 secretion pathway [83]. The importance of the proteins secreted by the ESX-1 pathway in down-regulating MΦ responses and in the virulence of *M.tb* has been recently reviewed [84]. That progression of endosomal maturation is deleterious to the survival of *M.tb* has been suggested by numerous studies [82,85,86,87], though it is difficult to differentiate whether the death of intracellular *M.tb* is the result of, or precedes the development of, phagosome maturation.

Exposure of *M.tb* to various models of the phagosome has resulted in a better understanding of the *M.tb* transcriptional response to this niche. Although the *M.tb* phagosome is held at a modified early endosome stage, thereby allowing access to some extracellular nutrients, there is still a level of nutrient deprivation the bacterium has to contend with, including low levels of iron. Growth of *M.tb* in a chemostat under nutrient depletion resulted in the bacteria entering a non-replicative state and an up-regulation of genes involved in β oxidation of fatty acids [88]. Starvation of *M.tb* led to a downregulation of 15% of the energy metabolism genes, 47% of the aerobic respiration genes, 59% of the genes involved in ribosomal protein synthesis and 16% of the lipid biosynthesis genes [89]. Interestingly, protein analysis identified significant upregulation of the *hspx* gene product, α-crystallin that is associated with a dormancy profile [89]. Iron starvation leads to upregulation of genes involved in iron acquisition (*mbtA, mbtB, mbtI*) [90] and the regulation of a variety of other proteins, including putative transporters, iron storage proteins, members of the PE/PPE family, transcriptional regulators, and enzymes involved in lipid metabolism [91]. Many of these transcriptional signatures are mirrored in the intracellular gene expression patterns of *M.tb* after MM infection [56,55,58,60,92].

The regulation of genes following pH reduction is dependent on the relative acidity that *M.tb* is exposed to. The mild acidification (pH 6.2-6.3) seen in the "normal" *M.tb* phagosome results in the regulation of 162 genes, whereas exposure to a pH 5.5, that may be seen in IFNγ-activated MMs, results in the differential regulation of 679 genes [55]. In another study in which *M.*tb was exposed to a pH of 5.5, 81 genes were differentially expressed with many of the highly induced genes showing homology with nonribosomal peptide synthetases/polyketide synthases [93]. Using a promoter trap system, it was shown that acid induced the expression of *lipF* (a putative lipase) and Rv0834c, though neither was induced in resting MΦ phagosomes [94]. Several members of the *phoP* regulon were induced by acid pH as was *whiB3* suggesting a role for these genes in regulation of the acid response [55]. A number of genes were only regulated by pH 5.5 and not pH6.5; these included repression of genes mediating protein synthesis, replication and energy metabolism along with induction of genes involved in stress responses, such as *sigH, sigB, groEL2* and *dnaJ* [55].

Hypoxia is considered to be one of the most important changes for *M.tb* to deal with when it enters the MΦ and also when it resides within the granuloma during chronic infection. This is reflected by the numerous studies investigating gene regulation in *M.tb* exposed to reduced oxygen tension [95,96,97,98,99,30]. The two-component response regulator *devR-devS* (*dosR-dosS; Rv3133c/Rv3132c*) was originally shown to be up regulated during hypoxia along with a regulon of around 50 genes including *hspX* (α-crystallin), *fdxA, narX, narK2, bfrB* and *ctpF* [95,96]. Later studies confirmed and extended these results using controlled oxygen depletion in a chemostat [97]. In addition to up-regulation of the *devR* regulon, six of the ten contiguous mycobactin synthesis genes (*mbtA- L*) were up-regulated. A comparison of aerobic cultures of *M.tb* with microaerophilic and anaerobic cultures [100] revealed similarities and differences between the two gene sets from the hypoxic *M.tb* [98]. Lycine dehydrogenase, nitrate reductase and α-crystallin were induced in both treatments, as were genes of the *devR* regulon and fatty acid metabolism genes including *fadD26*. Induction of the fumarate reductase operon (*frdA-D*) following hypoxia has been shown for *M.tb*, presumably as an adaptation to anaerobic respiration [30]. Interestingly, while *M.tb* grown under hypoxic conditions was more virulent in a guinea pig model [97], a *devR* deletion mutant had no decreased virulence in a murine model of TB [99], suggesting that the *devR* regulon may be redundant with regards bacterial virulence in mice. Additional analysis showed that induction of the *devR* regulon is transient and is replaced by an "enduring hypoxic response" of 230 genes induced at 4 and 7 days of hypoxia. A meta-analysis of the transcriptional network (origons) differentially expressed in response to hypoxia over time [101], identified the *devR* , *sigD* and *Rv0494* as early origons (response on or before day 6 of hypoxia). Intermediate (day 8-14) origons included *furB/zur, crp, sigH, kstR* and *sigE-mprA;* while *nadR, Rv1956* and *hrcA* were late origons (>20 days after hypoxia).

EXPOSURE TO POTENTIAL MYCOBACTERIACIDAL AGENTS AND PROCESSES

Invading microbes, including pathogens like *M.tb,* can be killed by MMs in a number of ways [102]. MΦ killing mechanisms that have been shown to be deleterious to *M.tb* include fatty acids [103], defensins [104] ATP-mediated killing [105], autophagy [106,107], glutathione and nitrosoglutathione [108], p47 GTPases [109] lysosomal ubiquitin derived peptides [110], the lysosomal enzyme beta-hexosaminidase [111] and the induction of endosomal maturation into phagolysosomes [112,113,114,82,115,86]. However, the major mechanisms that have repeatedly been described as having

antimycobacterial activity are the production of ROI and RNI [116 117 118 119] and the induction of apoptotic cell death of infected MMs [120 121 122 123].

ROI: MΦ produce ROI (superoxide anion, hydrogen peroxide, singlet oxygen) during the ingestion of many types of particles [124 116]. ROI are able to kill mycobacteria [125 126]. In addition, it has been shown that *phox* knockout mice, which are unable to produce ROI, are more susceptible to *M.tb* than their wild type littermates [127 128], demonstrating the involvement of ROI in killing *M.tb*. Moreover, a *katG* knockout strain of *M.tb* was markedly attenuated in wild-type C57Bl/6 mice and iNOS knockout mice (that can not make RNI), yet was indistinguishable from wild-type *M.tb* in its ability to replicate and persist in *phox* knockout mice [129]. As there is some redundancy in *phox* [116], it is most probable that ROI play a role in the killing of intracellular mycobacteria but that this is not an exclusive role.

RNI: RNI (nitric oxide, the nitrite ion, nitrous anhydride and peroxynitrite) are considered to be involved in the killing of mycobacteria and other microbes by MΦs, either independently or in conjunction with ROI [116 117 130 131]. RNI are produced by MΦs in response to multiple signals and, like ROI, are generally considered to be the result of MΦ activation by a priming (e.g IFNγ) and an activating signal (e.g.LPS) [117]. Studies using iNOS knockout mice [132 127 133] demonstrated that RNI are an important component of the antimycobacterial armature of the murine MΦ though, like ROI, there is some redundancy in iNOS [116 134]. Mice with a combined deficiency in Phox and iNOS die of spontaneous microbial infections, illustrating the need for at least one system to be present to fight infection [135]. Recent studies have shown that RNI do not gain access to the mycobacterial phagosome [136] and there is evidence that RNI do not mediate the killing of *M.tb* in MΦs via a direct action on the intracellular bacteria [137]. Thus, the mechanism of RNI toxicity to intracellular *M.tb* is more complex than was first thought. There is some controversy as to whether MΦ production of RNI is a killing mechanism that can be employed by human MΦs [138 139 140], however, evidence is accumulating that demonstrates that human phagocytes can produce RNI, albeit under tighter regulation than in the murine system [141 142 143 144 145 119]. Thus, whether RNI actually play a role in killing *M.tb* in human MΦs is still unresolved.

Apoptosis: Apoptosis is the process of programmed cell death involving a cascade of intracellular signals that results in the death of damaged, infected, or redundant cells without inducing an inflammatory response and the associated damage of surrounding cells. Ligation of apoptotic triggers, such as TNFα, with its receptor, TNFR, leads to the activation of caspases that are responsible for the regulated disassembly of the cell into apoptotic bodies [146]. More recently it has been shown that there are both caspase dependent [147] and independent [148] mechanisms of apoptosis within MΦ that can successfully control intracellular microbes. It has been shown that apoptosis is a mechanism that is employed by the host to eliminate MΦs infected with mycobacteria [149 150 151 152 123]. Moreover mycobacteria (and other pathogens) have been demonstrated to be able to inhibit the apoptotic pathways of MΦs, at least to some extent, resulting in survival of the intracellular bacteria [153 154 152 155]. Recent studies have identified mycobacterial genes (*nuoG* and *secA2*) that are involved in inhibiting apoptosis [156 157], confirming the importance of apoptosis in MΦ control of *M.tb*.

Changes to the transcriptome of *M.tb* exposed to multiple ROI and RNI donors have been investigated. Hydrogen peroxide was shown to induce the *devR* regulon, but at relatively low levels compared to other stress conditions, including nitrosoglutathione [158]. As would be expected, the catalase gene, *katG* is upregulated following H_2O_2 treatment [159]. Exposure of *M.tb* to RNI regulated a similar set of genes as did hypoxia, including *devR/devS, nrdZ, narK2, narX,*

pfkB, hspX, ctpF and a number of hypothetical proteins [160, 161]. An alternative approach, comparing transcriptomes of *M.tb* in iNOS knockout MΦs versus wild type MΦs gave similar results and identified an NO-induced response that represented a shift from aerobic to anaerobic respiration (induction of the *devR* regulon) and increased iron scavenging and utilisation of fatty acids as a carbon source. [56]. Unfortunately, no information is available for gene regulation of *M.tb* within apoptotic MΦ, although, as both ROI and RNI induce apoptosis, the studies comparing *M.tb* gene expression in the presence of these toxic molecules may reflect what would be found for bacteria within apoptotic MΦ.

THE INDUCTION OF ADAPTIVE IMMUNITY AND THE MOVE TO A DORMANCY PROFILE.

The early interactions of *M.tb* with MΦ are generally considered to take place in the absence of any adaptive immune response. Rather, the bacteria are exposed to elements of innate immunity, of which MΦ are an important component. Although innate immunity exerts a level of control on the replication of *M.tb* during the early acute phase of disease [123, 162], it is only when antigen specific adaptive immunity is initiated that bacterial replication dramatically slows down and *M.tb* enters into a chronic stage of disease. This is characterised by minimal increase, or even slow decline, in the bacterial burden and a move towards a physiological state that is often referred to as dormancy. That this control of bacterial replication is mediated by the immune system has been demonstrated by studies in knockout mice lacking CD4+ T cells, or with defects in the IFNγ activation pathway (IFNγ production, IFNγ receptor, IL-12), or with defects in TNFα or RNI production. These mice are significantly more susceptible to *M.tb* than intact littermates. Other components of the adaptive immune response such as CD8+ T cells are also important [163, 164] but do not appear to be as critical.[165, 166]. From these, and other studies, it is generally accepted that, while there are a number of contributory factors to adaptive immunity to *M.tb*, the most important elements are the production of CD4+ve TH_1 cells that produce IFNγ, CD8+ve cytolytic T lymphocytes (also IFNγ producers) and TNFα [167, 168, 169]. The most important way these critical elements mediate control of bacterial survival and replication is likely through the action of IFNγ and TNFα on the MΦ and the granuloma. Both IFNγ and TNFα can act alone or in synergy to activate infected MΦs, resulting in an increase in phagosomal maturation towards a phagolysosome [170], increased ROI and RNI production by the MΦ [116] and increased apoptosis [171, 121], ultimately resulting in a MΦ intracellular environment that is non-permissive for mycobacterial replication. To counter this response by the host, *M.tb* can down-regulate the induction of cytokines involved in the activation of MΦ. Studies using purified mycobacterial components or directed cell wall mutants of *M.tb* indicate that survival of intracellular *M.tb* is linked to the ability to down-regulate pro-inflammatory cytokines such as IL-12, IL-6 and TNFα [172-174] and inhibit the activation of MΦ by IFNγ [175]. However, even though *M.tb* can modulate the cytokine response of the host, it is evident from animal infection models that this is not sufficient to prevent the activation of MΦ to a state capable of controlling *M.tb* growth and survival *in vivo.*

In response to IFNγ-mediated activation of the MΦ, *M.tb* alters its physiology to enter into a dormancy phase characterised by a drastically reduced level of replication [176]or a balance between slow replication and killing [162], use of lipids as a carbon source through the glyoxalate shunt [177] and a shift towards microaerophilic respiration [178]. The IFNγ -mediated maturation of the phagosome will also lead to further nutrient deprivation and hypoxia. These signals are thought to lead to a dormancy regulon that enables *M.tb* to persist inside the MΦ and the

granuloma. Meta-analysis of microarray studies modelling dormancy [179], indicated several trends including up-regulation of 81% of the *devR* regulon, down-regulation of over 50% of the 30S and 50S ribosomal-protein genes and down regulation of ATP synthesis. This is indicative of a bacterium slowing down protein synthesis and shifting towards alternative electron acceptors such as nitrate or fumarate during respiration. *M.tb* is nominally an aerobe but is able to switch to a microaerophillic/anaerobic mode of respiration as indicated by the up-regulation of *fdxA, narX, narK2* and the *frd* operon. Other features of the dormancy regulon include the further induction of genes involved in the utilization of fatty acids as a carbon source and genes involved in mycolic acid modifications, indicating modification of the cell envelope [56]. As RNI are only produced by activated MΦ, it has been suggested that they may be an important trigger of dormancy in *M.tb* following the onset of adaptive immunity. RNI can inhibit respiration in bacteria and it is therefore not surprising that the RNI induced dormancy regulon closely resembled that induced by hypoxia and nutrient starvation as represented by the *devR* regulon [160 161 180].

CONCLUSIONS AND CAVEATS

The move of *M.tb* from extracellular to intracellular residence within the MΦ is a major change in environment, requiring major adjustments to enable the bacterium to survive and flourish. We have reviewed those environmental changes and the accompanying bacterial adaptations necessary for *M.tb* to succeed as a pathogen at the level of gene expression. While there is no complete agreement among the numerous studies as to the identity of genes regulated by this adaptation, nor for that matter as to the identity of the elements of the MΦ environment that induce this regulation or indeed the multiple microenvironments that may be encountered by infecting bacilli, there is a level of consensus. It is generally agreed that the move to an intramacrophage location will result in *M.tb* having to deal with changes in oxygen tension, nutrient depletion, increased acidity and exposure to immune defences such as ROI, RNI, apoptosis and phagosomal maturation. During the early, acute stage of infection, *M.tb* bacilli are likely exposed to the innate immune response which includes such elements as complement, NK cells, defensins, neutrophils and resident MΦs. Although these clearly have an effect, some bacteria seem able to adapt to or avoid these challenges and replicate relatively successfully. Following the development of adaptive immunity, the infection shifts into a chronic stage where the host's defences are more effective at controlling bacterial replication and survival. The bacteria are walled off in a granuloma and go into a dormant or latent phase that is maintained predominately by host IFNγ and TNFα.

M.tb responds to these changes by rapidly changing its transcriptome. Intracellular residence, hypoxia, nitrites and starvation all seem to induce genes characterized by the dormancy regulon that enable bacilli to undergo anaerobic respiration, utilize lipids as a carbon source and modify the cell envelope. Many studies have shown that the *devR* (*dosR*) regulon is heavily represented in all treatments that lead to a dormancy profile in *M.tb*, suggesting that these genes are very important in the continued survival of intracellular bacteria. However, recent data suggests that the *devR* regulon is not necessary for bacterial virulence and appears to be transiently expressed during early hypoxia (< 24 hours) and that an enduring hypoxic response of 230 genes contains key factors needed for *M.tb* persistence [99]. The *hspX* gene encoding the 16kD α-crystallin chaperonin is also commonly up-regulated in *M.tb* on entering MΦs and moving into dormancy. Indeed, its expression is often used as an indication of a dormant state. However, it has been shown that letting cultures stand for 30 minutes can result in 100 fold increases in *hspX* expression [158], which suggests that dormancy is not the only trigger leading to increased production of this chaperonin, It appears that *hspX*, along with many other genes differentially expressed intracellularly, are inextricably linked to a slowing of *M.tb* replication after multiple stresses. Many of the studies investigating gene expression by *M.tb* in MΦs have modelled the intracellular environment in axenic culture in order to determine the effect of a single variable over time, and to avoid the complications of isolating RNA from intracellular bacteria. This has often led to exposing the bacteria to a single trigger hypothesised to reflect the phagosomal

environment, such as acidity, hypoxia, H_2O_2 or nitrite. While this reductionist approach has produced valuable information, it has to be remembered that the intracellular *M.tb* bacilli are not exposed to each of these conditions in isolation and it is perhaps better to try and determine the bacteria's response to intracellular life by actually looking at bacteria within the MΦ. Perhaps more informative are studies aimed at identifying the functional significance of isolated elements of the host response by using MΦ with single gene deletants (for example, ROI or RNI) to yield single pathway inferences. Studies designed to investigate the multi-factorial nature of *M.tb* infection should provide new perspectives, but will have to negotiate increasingly complex networks of interaction.

The distinction between intracellular microenvironments permissive and non-permissive for *M.tb* growth has also been modelled by comparing the intracellular transcriptomes of different mycobacterial species and in different phagocytic cells, for example *M.tb* compared to BCG [55], and *M.tb* infection of MΦ compared to dendritic cells [60]. This raises an important element in studying the transcriptome of *M.tb* within the intracellular environment. The actual model utilised i.e. the mycobacterial species and phagocyte used will have significant impact on the results obtained. Many studies have been performed using *M. bovis* BCG as a model for *M.tb* because of the high degree of homology between the two bacterial genomes, coupled with the relative ease with which BCG can be used experimentally (it does not require containment level 3 laboratories). However, BCG lacks an important virulence region called RD1 [181], is attenuated in humans and does not result in the same pathogenesis as *M.tb.* While the genome sequence of *M.tb* and *M. bovis* are very similar, the transcriptomes of the 2 species show several differences [182] demonstrating the need to use *M.tb* in expression analysis of *M.tb* in MΦ. The other major source of variation in trying to understand the intracellular phenotype of *M.tb* is the choice of MΦ infection model itself. Commonly used models are the murine bone marrow-derived MΦ (BMM) [56 58 55], human monocyte-derived MΦ (MDM) [57 60] and the human MΦ-like cell line THP-1 [59]. Each of these models has strengths and weaknesses. For instance, when using MDM, significant variation between the MΦs from individual blood donors means that greater numbers of samples are required before variation is reduced enough to characterize specific patterns. The use of THP-1 cells overcomes this variation but introduces problems associated with it being a cell line that is not truly representative of a MΦ. Perhaps the BMM is the best model as it represents a reasonably uniform population of MΦ that are derived from *ex vivo* cells and, unlike human MΦ models, results obtained in BMM *in vitro* can be further tested in infection models *in vivo*. However, a long-standing issue with the use of murine MΦs is the strong inducible RNI response seen following immune activation. This has been identified as one of the major determinants resulting in the induction of the *M.tb* intracellular dormancy program. While it is becoming increasingly clear that human MΦs can make RNI [119], there are significant differences between human and mouse MΦ RNI production. Additionally, strain variation between mice may also affect the results using BMM.

Finally, it is necessary to ensure that comparisons between data sets of genes take into consideration the behaviour of *M.tb* in the MΦ model used. For instance, *M.tb* usually replicates efficiently in untreated "resting" murine BMM over 7 days *in vitro* and IFNγ treatment can slow this replication rate but does not result in bacterial elimination [183 109 123]. In one of the seminal studies on *M.tb* gene expression in MΦ [56], no replication was seen in resting murine BMM MΦ, and IFNγ stimulation resulted in bacterial elimination. While there are many possible explanations for this disparity, it is important to ensure that variations in the models used are considered when comparing *M.tb* intracellular RNA profiles. An additional source of variation, particularly applicable to the use of microarray platforms to study changes in gene expression, is the choice of comparator conditions. Typically, intracellular transcriptional profiles are compared to log phase axenic bacilli, or bacilli resuspended in tissue culture media alone. Transcriptional differences between these bacterial populations will necessarily affect the interpretation of intracellular RNA profiles, and make the meaningful comparison of intracellular datasets problematic. Figure 3 displays one such comparison of four publicly available microarray

datasets describing the global response of *M.tb* to macrophage infection. Significant technical variation between studies makes the direct comparison of transcriptional profiles unwise; however the similarities between models are clearly visible. Interestingly, it is also possible to identify host-specific patterns that are represented across several datasets. Further work will be required to ascertain the significance of such findings.

FUTURE PERSPECTIVES

An understanding of the transcriptome of *M.tb* in the MΦ is developing and has identified a number of physiological changes the bacterium must undergo to survive within its specialised phagosome. Most of this information comes from use of *in vitro* models of *M.tb* in MΦs or of axenic bacteria exposed to conditions considered to reflect the intracellular environment. There have been some important studies investigating gene expression of *M.tb* in whole animal models [184 36 185 186 187 39] and humans [188 38 189]. While *in vitro* studies are informative and an essential step in understanding the physiology of intracellular *M.tb*; the complexity of the environment in the host with the associated myriad of potential triggers of bacterial gene regulation mean that a full understanding of the intracellular transcriptomes of *M.tb* during its ongoing pathogenesis and continued survival in multiple microenvironments in the host will only be fully understood through *in vivo* investigations. Gene expression profiling of *M.tb* bacilli extracted from distinct stages of infection using human tissue and animal models will be required. Although the mouse, guinea pig and rabbit are useful models of tuberculosis, the primate model of tuberculosis better reflects disease in humans [190 191]. Although challenging, studying the transcriptome of *M.tb* during the development of tuberculosis in the primate may give a greater understanding of the disease process in humans.

There are still areas of the host/pathogen interaction in tuberculosis that will greatly benefit from *in vitro* studies. One area that has not been studied is the gene expression of *M.tb* exposed to apoptotic MΦs. Apoptosis is a major killing mechanism of MΦs that has been shown to be very important in the control of *M.tb* [149 123 192]. *M.*tb has evolved mechanisms to inhibit apoptosis (which is protective for the host); instead *M.tb* induces necrosis in infected MΦ, which is beneficial to bacterial survival [153 157 156 193 123]. However, although gene expression by MΦs undergoing apoptosis or necrosis following infection with *M.tb* has been investigated [194], no study of the bacterial transcriptome in apoptotic versus necrotic MΦs has been undertaken. As inhibition of apoptosis is a critical survival mechanism for *M.tb*, it will be of great interest to define the transcriptional basis of this process.

Another area that has not been extensively investigated at the level of bacterial gene expression is the comparison of virulent and avirulent *M.tb* in MΦs. Some work has been done comparing *M.tb* with the attenuated vaccine strain, *M.bovis* BCG [55] but there are significant differences between *M.tb* and BCG which makes comparing the two strains difficult. Recent work has compared the transcriptomes of a virulent strain of *M.tb* (H37Rv) with an avirulent strain (H37Ra) during growth in broth and identified a number of genes that are differentially regulated in the attenuated H37Ra that include members of the dormancy regulon and a number of genes connected to *phoP* expression [195 196 197]. Whether these differences are repeated when H37Rv and H37Ra are compared when inside MΦs awaits investigation, although one recent study has identified *frdA-D* among differentially regulated genes between intracellular H37Rv and H37Ra [30]. Transcriptional profiling of *M.tb* knockout mutants with specific *in vivo* phenotypes of compromised virulence [198] may become an important tool for defining the functional significance of single gene products and understanding the molecular determinants defining *M.tb* pathogenicity and virulence.

The intracellular transcriptomes of *M.tb* may help us to understand the physiological and metabolic states required by *M.tb* to successfully complete a cycle of human infection. They may also be utilised as a bioprobe to discover and define microenvironments encountered by

bacilli during infection. Additionally, they may facilitate the discovery of virulence mechanisms that enable *M.tb* bacilli to modulate the host immune response. The identification of genes induced intracellularly suggests that they are important for the survival of *M.tb* in the host environment, and would therefore be possible candidate targets for therapeutic discovery [179]. As novel therapeutics are critically needed to control tuberculosis, especially with XDR *M.tb* becoming so prevalent [2], any strategy that facilitates drug discovery should be actively pursued. Additionally, these targets could act as potential candidates in rational vaccine design either to replace or augment the current BCG vaccine.

EXECUTIVE SUMMARY

Overview of the infectious process

- *M.tb* binds to MΦ receptors and is ingested into a phagosome.
- *M.tb* inhibits the development of the phagosome, holding it at the early endosome stage.
- *M.tb* induces the production of cytokines that result in a TH_1 adaptive immune response.
- Through the activity of IFNγ and TNFα, infected MΦ become activated and are better able to control the replication of *M.tb* through the action of Reactive Nitrogen Intermediates , Reactive Oxygen Intermediates and apoptosis.
- *M.tb* then moves to a dormant state, often within a granuloma, and changes its physiology to combat the variation in oxygen tension, low carbohydrates and MΦ killing mechanisms.

Binding and uptake (movement from extracellular to intracellular)

- Non-opsonic uptake of *M.tb* is important in the serum free environment of the lung.
- *M.tb* moderates its non-opsonic uptake by MΦ.
- Transcriptional responses of *M.tb* begin within minutes of ingestion and include members of the *devRS* regulon and the *phoPR* regulon
- A comparison of numerous microarray experiments identified common functionalities in *M.tb* regulated gene sets indicating a move to an environment low in carbohydrate, rich in lipid, low in oxygen and with toxic molecules present.

Residence in a phagosome and the associated low nutrients, increased acidity and hypoxia.

- *M.tb* maintains the phagosome at a mildly acidic pH and with markers indicating arrest at the early endosome stage with access to extracellular nutrients.
- Nutrient deprivation, exposure to acidic pH and hypoxia have been used to model the phagosome.
- This resulted in *M.tb* regulation of genes involved in oxidation of fatty acids, aerobic respiration, energy metabolism and lipid biosynthesis.
- The *devRS* and *phoPR* regulons were upregulated.

Exposure to potential mycobacteriacidal agents and processes

- The major mycobacteriacidal mechanisms appear to be RNI, ROI and apoptosis.
- ROI induces the upregulation of *katG.*
- RNI induce the *devRS* regulon.

The induction of adaptive immunity and the move to a dormancy profile.

- Adaptive immunity is essential for the optimal control of *M.tb.*
- The most important components of the effective adaptive response are IFNγ producing CD4+ve TH_1 cells, CD8+ve cytolytic T cells and TNFα.
- IFNγ activates MΦ to better control the survival and replication of *M.tb.*
- This induces dormancy in *M.tb*, characterised by lower rates of replication, use of lipids as a carbon source and a shift to microaerophilic respiration.
- The dormancy regulon is dominated by the *devRS* regulon , genes involved in mycolic acid modifications and genes involved in the utilization of fatty acids.

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Figure Legends

Figure 1. **Interaction of *M.tb* with macrophages**.
M. tuberculosis (M. tb) expressing a variety of Pathogen Associated Molecular Patterns (PAMPs), is recognized by Pattern Recognition Receptors (PRR) on host macrophages leading to BINDING and then UPTAKE. During binding and uptake Reactive Oxygen Intermediates (ROI), Reactive Nitrogen Intermediates (RNI) and Cytokines are released by the macrophage. The INNATE immune response drives the ADAPTIVE response and, depending on the cytokine profile induced, will result in TH1 or TH2 and pro- or anti- inflammatory responses. The bacteria reside within an EARLY PHAGOSOME in which Rab5, coronin 1 and proCathepsin D (PCD) are found. The mycobacterial phagosome also has a relatively high pH and has access to the extracellular milieu as shown by the cycling of transferrin. Mycobacterial antigens are processed in some, as yet, poorly defined way leading to PRESENTATION. Under some circumstances, mycobacteria are killed (xxx) and are then found in LATE PHAGOSOMES which contain Lamp 1, Rab 7 and cathepsin D and also have a V-ATPase proton pump which acidifies the phagosome. The killing of intracellular mycobacteria can be mediated by RNI, ROI or through the INDUCTION OF APOPTOSIS which can be triggered by ROI, RNI and cytokines such as TNFα.

Figure 2. Dissecting the *M.tb* intracellular transcriptional profile.
535 genes identified as significantly differentially expressed in two or more studies describing the M.tb response to macrophage infection (A-D). Genes identified (by the study authors) to be significantly induced (marked in red) or repressed (marked in blue) from *in vitro* axenic models broadly representing low pH (E-H), hypoxia (I-M), nutrient starvation (N-P) and oxidative stress (Q-S) are mapped alongside the intracellular response. Conditions are detailed as columns, genes as rows. The asterisked column represents an additive model describing the sum induction or repression of each gene from the axenic conditions. This rudimentary model shows the extent to which current *in vitro* modelling helps to define the *M.tb* intracellular profile. For example, Box 1 highlights the hypoxic response as an integral element of the *M.tb* intracellular response. Box 2 marks a cluster of genes differentially regulated by multiple *in vitro* environments. Box 3 shows the repression of ATP synthase and NAD dehydrogenase I (*atp* and *nuo*) gene families intracellularly. All *M.tb* axenic conditions relative to log phase untreated controls unless otherwise stated. (A) Murine bone marrow-derived MΦ (BMDM) [56]. (B) Human monocyte-derived MΦ (MDM), [60]. (C) THP-1 human MΦ-like cells (THP-1), [92]. (D) Murine BMDM relative to media control, [55]. (E) Acid shock, [182]. (F) Low pH, [93] (G) pH 6.5, [55] (H) pH 5.5 [55]. (I) Hypoxia, [97] (J) Non-replicating persistence-1 [189] (K) Non-replicating persistence-2 [189] (L) Non-

1 replicating persistence or stationary phase [180] (M) Enduring hypoxic response [99] (N) Low glucose
2 [88] (O) Nutrient starvation [89] (P) Low copper [199] (Q) Reactive nitrogen intermediates [160] (R)
3 Oxidative stress [200] (S) SDS stress [201]
4
5 **Figure 3 The *M.tb* response to the intracellular environment.**
6 The transcriptional profile of 1121 genes (>2 fold differentially expressed in >2 conditions) were
7 extracted, combined and clustered from four publicly available microarray datasets.
8 Conditions/timepoints are displayed as columns, genes as rows. Red colouring indicates
9 induction, blue repression compared to aerobic log phase growth (unless otherwise stated).
10 MDM, human monocyte-derived MΦ infection model [60]. BMDM, naïve and IFNγ-activated
11 murine bone marrow-derived MΦs [56]. THP, THP-1 human MΦ -like cell line infection model [92].
12 BM, murine bone marrow-derived MΦ s infected with *M.tb* relative to bacilli resuspended in
13 tissue culture media [55]. Box A – genes differentially expressed after infection of human-derived
14 cells only (MDM/THP). Box B – temporal response to infection observed in murine-derived cells
15 only (BMDM/BM).
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Figure 1

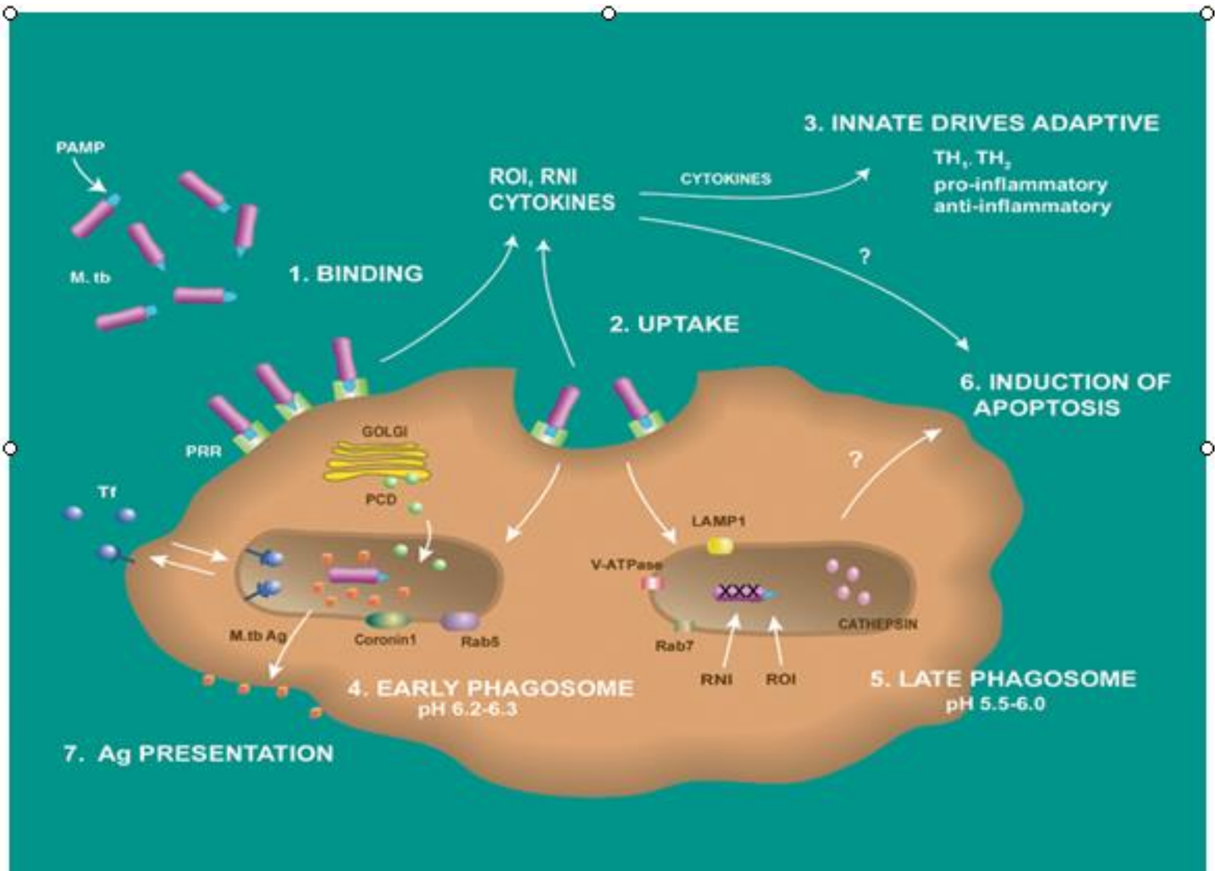

Figure 2

Intra Low pH Hypoxia Starv RO/NI *
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