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Non-coding RNA response of human monocyte-derived macrophages during mycobacterial infection

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To the Universe,
(This is just a small effort to know you better)

To my Parents and little Sister,
(To me, universe is nothing without you)

PS:
Numbers cannot be the only way to know the Universe.

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Abbreviations

%	Percent
°C	Degree centigrade
BCG	*Mycobacterium bovis* BCG
CTSS	Cathepsin S
g	Grams
h	Hour
HK	Heat killed
IFN-γ	Interferon gamma
lncRNAs	Long non-coding RNAs
M. Tuberculosis	*Mycobacterium tuberculosis*
min	Minutes
miRNAs	MicroRNAs
Mtb	*Mycobacterium tuberculosis*
ncRNA	Non-coding RNA
piRNAs	PIWI-interacting RNAs
RNA-seq	RNA sequencing
RNAi	RNA interference
rRNA	Ribosomal RNA
siRNA	Small interfering RNA
snoRNAs	Small nucleolar RNAs
TNF-α	Tumor necrosis factor alpha

1. Introduction

Modern humans (*Homo sapiens*) have been living on this earth for the past ~200,000 years. During this period of evolution, humans have encountered thousands of pathogenic organisms (e.g. bacteria, viruses, fungi, parasites etc.). Similar to humans, animals also confronted pathogen problems. Some pathogens destroy their mammalian host but for many of them, hosts have created invulnerability. When we look at the classification of microorganisms, we can see that the number of species is vast. As prokaryotes (microorganisms) evolved first, the immune system of mammals must have developed accordingly. However, during the evolution of mammals, microorganisms might also have co-evolved to survive within the host. On one hand, co-existence with microorganisms drove the development of the mammalian immune system and on the other hand, pathogens changed their strategies to overcome the immune response. There are also symbiotic bacteria that live as commensals within mammalian hosts, providing benefits for both. However, some microorganisms have mechanisms by which they trick the complex immune system, thus allowing them to survive and multiply. For example, there are a number of pathogenic species of mycobacteria that are found all over the world. Some of them are fast growing while others are slow growing. There are tremendous efforts to understand the pathogenesis of these species. Pathogenic bacteria cause changes inside host cells. From earlier studies, it was clear that infection not only affects host cells at the mRNA level but also at the non-coding RNA (ncRNA) level. Much is unknown about the role of ncRNAs (especially lncRNAs) in infection biology. This thesis will investigate the host response, at the ncRNA level, following mycobacterial infection. Following infection of human macrophages with *Mycobacterium bovis* BCG (BCG), expression of long ncRNAs (lncRNAs) and microRNAs (miRNAs) was studied and their physiological relevance was determined.

To provide a background for the present study, information on pathogenic mycobacteria and virulence factors, as well as two host defense mechanisms (phagocytosis and autophagy) in the context of mycobacterial infection, are described in brief. Also, two classes of ncRNAs (lncRNAs and miRNAs) and their functions in mycobacterial infection are described.

1.1 Mycobacteria

Mycobacteria are non-motile, rod-shaped, acid-fast bacteria with waxy outer coats (Figure 1). More than 70 species of mycobacteria can be found throughout the globe. Mycobacteria have evolved efficient strategies to overcome fundamental mechanisms of mammalian host immunity. The genus *Mycobacterium* comprises highly pathogenic species as well as opportunists, which are able to withstand the hostile phagosomal environment and cause infection. Mycobacteria are primarily environmental

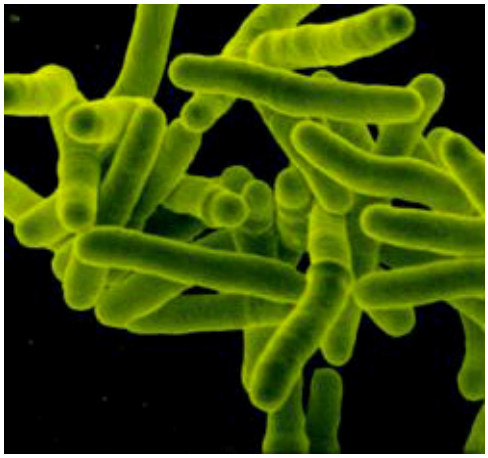


Figure 1: Scanning electron micrograph of *Mycobacterium tuberculosis*. (©:microbiologyspring2010.wikispaces.com)

organisms; however, several transitions occurred from environmental to pathogenic species approximately 2.5 million to 67,000 years ago, which includes transitions of *Mycobacterium avium* and the Mycobacterium tuberculosis complex (MTBC). In the evolution scale, animal-adapted *Mycobacterium bovis* ecotypes branched from a presumed human-adapted lineage of *Mycobacterium africanum* [1]. In addition to pathogenic species, the genus also consists of many non-pathogenic species e.g. *Mycobacterium smegmatis*, *Mycobacterium gilvum*, *Mycobacterium vanbaalenii* etc. [2]. There are several factors that have made mycobacteria successful pathogens.

1.2 Factors of mycobacterial pathogenicity

1.2.1 Cell components

Mycolic acids (MAs), 2-alkyl, 3-hydroxy long-chain fatty acids (FAs), are incorporated in the cell walls of *Mycobacterium tuberculosis (M. tuberculosis)*-related species and genera [3, 4]. Components of mycolic acid synthesis are responsible for mycobacterial virulence. *kasB* is one of two *M. tuberculosis* genes encoding distinct β-ketoacyl-acyl carrier protein synthases involved in mycolic acid synthesis. Deletion of the *kasB* gene resulted in a mutant strain that persisted in infected immunocompetent mice for up to 600 days without causing disease or mortality [5]. Inactivation of the *MmaA4* [methoxy mycolic acid synthase 4 (methyltransferase)] gene resulted in a profound alteration in cell wall permeability, and, in addition, the loss of oxygenated mycolic acids and bacteria showed an attenuated phenotype, suggesting that the oxygenated mycolic acids are important for infection [6]. Some earlier studies demonstrated that cyclopropyl modification of mycolates on trehalose dimycolates (TDM) not only modify innate immune recognition but also have a profound effect on the function of lipids as important virulence factors of mycobacteria [7]. It was also highlighted that the *mymA* operon has an important function in virulence, protecting the bacterium during unfavourable conditions and also its virulence factor is specifically required for growth of *M. tuberculosis* at late stages of disease [8].

In addition to mycolic acids, early secretory antigenic target protein 6 (ESAT-6), a protein that is profusely secreted by *M. tuberculosis,* is also an important virulence factor, inactivation of which leads to reduced virulence of *M. tuberculosis*. The ESAT-6 protein interacts with Beta-2-Microglobulin (β2M) that affects antigen presentation by macrophages [9]. In vivo, the culture filtrate protein 10 (CFP10) and ESAT6 form a 1:1 heterodimeric complex which functions as a regulator of macrophage cell death at different stages of tuberculosis infection [10]. It has been also demonstrated that ESAT6 protein induces apoptosis of macrophages by caspase activation [11]. In addition to these two components, there are several other factors, systematically described in an earlier review [12], which also contribute to mycobacterial pathogenicity.

1.2.2 Heterogeneity

A common survival strategy of microorganisms subjected to stress (depletion of nutrients, environmental fluctuations, lack of oxygen, treatment with antibiotic drugs) involves generation of phenotypic heterogeneity in the isogenic microbial population enabling a subset of the population to survive. Similar to other bacterial species, mycobacteria demonstrate heterogeneity within a single population [13]. The biochemical composition and physiology of microorganisms may vary, not only in the course of cell growth and division but also as a result of selective activation of specific metabolic processes. The mechanisms of so-called biostability in bacteria may be an example of such behaviour [14]. Persistence is where a subset of pathogens adapts to stressful conditions and can survive for years in a dormant state. Mycobacteria encounter a changed physical environment in the confined space of granulomas with a paucity of nutrients, oxygen and iron [15, 16]. *M. tuberculosis* has been found to persist for years in a dormant state characterised by the absence of active replication and metabolism [17]. Replicating bacteria, metabolically active bacteria, and dormant bacteria together may contribute to heterogeneity within mycobacterial populations. Recent studies, using microfluidics technology, have described how different approaches are being used to investigate mycobacterial heterogeneity [18-20].

1.2.3 Slow growth

Multiplication times of different mycobacterial species are different, e.g. *M. smegmatis* takes 3-4 hours and *M. tuberculosis* complex takes 14-15 hours [21]. It is still not entirely clear which factors limit the growth of *M. tuberculosis* complexes. Mycobacteria that can multiply within 5 hours and produce visible colonies in 7 days are considered to be fast-growing mycobacteria [22]. It is considered that the high-energy requirement for synthesis of mycolic acids in the lipid layer of the cell wall (which is approximately 30% to 40% of the total weight) contributes to limiting the growth rate of mycobacteria [23]. Compared to the cell wall of *Escherichia coli (E. coli)*, the mycobacterial cell wall is 100 to 1000 times less permeable for small charged molecules and therefore has been thought to restrict distribution permeability for nutrients, therefore limiting growth [24]. Porins play an important role in the growth of mycobacteria. MspA is a membrane porin produced by *M. smegmatis* and some related fast-growing mycobacteria, which allows hydrophilic nutrients to enter the bacterium [25]. Proof that porins are one of the factors that limit bacterial growth was demonstrated when the *mspA* gene from *M. smegmatis* was expressed in BCG. This resulted in a slightly accelerated growth rate in liquid culture [26] or on agar plates [27]. In addition, a number of ribosomal RNA genes (rRNA) in genomes, insertion elements, and reductive evolution in bacteria and metabolic benefits, may play a role in the reduced growth rate of mycobacteria [22].

1.2.4 Development of resistance

Mycobacteria may develop resistance against antibiotic treatments and survive in the host. *M. tuberculosis* has developed resistance to rifampicin and isoniazid, two key drugs in the treatment of the disease. Also, severe forms of drug resistance such as extensively drug-resistant (XDR) TB have been described [28]. Mutations in several genes, such as *katG*, *ahpC*, *inhA*, *kasA,* and *ndh*, have been linked with isoniazid resistance. The gene *rpoB*, which encodes the β-subunit of RNA polymerase, was mutated in the majority of *M. tuberculosis* clinical isolates that are responsible for producing resistance to rifampicin [29]. One earlier study showed that BDQ (Bedaquiline, an ATP synthase inhibitor, which is the first drug to be approved for treatment of multidrug-resistant tuberculosis) exposure triggers metabolic remodeling in mycobacteria, thereby enabling transient bacterial survival [20]. Other studies also reported mycobacterial resistance to commonly used drugs against human TB such as Pyrazinamide, Streptomycin, Ethambutol, Fluoroquinolones, Ethionamide, *p*-Amino salicylic acid and Macrolides [28].

In addition to the above factors, mycobacteria have also adapted to survive inside macrophages. One mechanism is to inhibit phagosomal maturation. Moreover, mycobacteria also affect the process of autophagy which enhances survival. Basic mechanisms of phagocytosis and autophagy during mycobacterial infection are discussed below.

1.3 Mycobacteria and phagocytosis

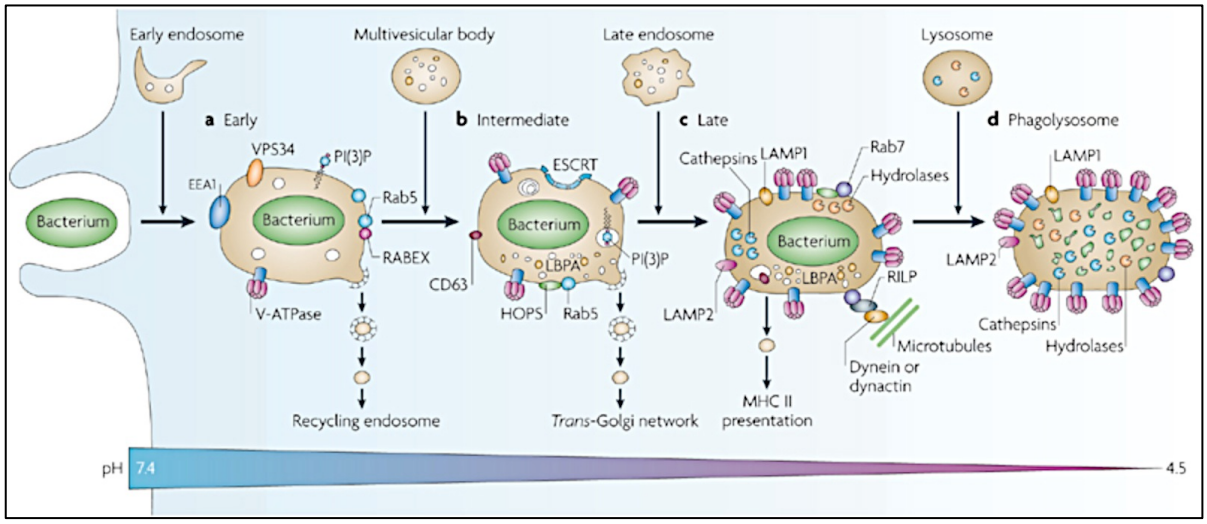


Figure 2: © Schematic representative diagram of phagocytosis of bacteria by macrophage obtained from [30]

Macrophages function as the first line of defense during bacterial invasion. They engulf bacteria by forming phagosomes around them. During the process of phagosomal maturation, multi-vesicular bodies such as endosomes fuse with, or pinch off from, phagosomes. Finally, phagosomes fuse with lysosomes, and engulfed bacteria are eliminated or destroyed. As shown in Figure 2, shortly after pathogen uptake, the phagosome undergoes a series of

transformations where there are fusions of sub-compartments of endocytic pathways as well as fission of small vesicles that are pinched out from phagosomes and transferred to the trans-Golgi network or recycled. As the phagosome matures, it also acquires or loses membrane proteins. There are different stages of maturation, which are early (a), intermediate (b) and late (c) phagosomes that culminate in the formation of phagolysosomes (d). The early phagosome is characterised by the presence of Rab5 and early endosomal autoantigen 1 (EEA1). During intermediate or late stages, Rab5 is replaced by Rab7, which can be detected on the phagosomal membrane. Finally, a fusion of late phagosomes and lysosomes [which is characterised by the presence of lysosome-associated membrane proteins (LAMPs)] triggers the release of several hydrolytic enzymes responsible for destroying the pathogen [31, 32].

As illustrated in Figure 2, with the progression of maturation events, the pH of the phagosome drops. However, mycobacteria such as *M. tuberculosis* and BCG inhibit phagosomal acidification to survive inside macrophages. In the case of *M. tuberculosis*, the bacterial protein tyrosine phosphatase (PtpA) is responsible for the exclusion of host vacuolar-H+-ATPase to inhibit phagosome acidification [33]. An attempt has been made to uncover the role of KefB in mycobacterial infection, as it inhibits phagosomal acidification, but its role was found to be unrelated to *M. tuberculosis* survival in the host [31]. During the course of phagosomal maturation, it has been shown that V-ATPases accumulate in the phagosomal membrane; therefore the activity of vacuolar type V-ATPases is principally related to the process of phagosomal acidification [34]. In the case of mycobacterial infection, exclusion of the vesicular proton-ATPase resulted in a lack of acidification in phagosomes [35]. However, delivery of acid (equivalents) to phagosomes is not necessarily accompanied by detectable levels of V-ATPases on the phagosomal membrane [36].

In a previous study, it was observed that the phagolysosome biogenesis block occurred between the maturation stages, which were controlled by the small GTP-binding protein Rab5 (early endocytosis) and Rab7 (late endosome) [32]. In addition, Rab5 was detected on mycobacterial phagosomes, while Rab7 was not expressed at times expected for its recruitment [32]. Conversion of Rab5 to Rab7 is essential for fusion of lysosomes and phagosomes [37]. However, a previous study observed that *M. tuberculosis* phagosomes were associated with lysosomal markers in the early stage of infection, suggesting that *M. tuberculosis* phagosomes fuse with lysosomes [11]. After studying the dynamics of Rab7 localisation on phagosomes, it was clear that Rab7 was present on the phagosome during earlier stages (30 min) of infection but disappeared at later stages (360 min). Also, the localisation of lysosomal associated membrane protein-2 (LAMP-2) on the phagosome was Rab7-independent, while that of the lysosomal protease cathepsin D was Rab7-dependent [38]. The phagosomes containing *M. tuberculosis* have been shown to acquire limited amounts of LAMP1 [39].

Phosphatidylinositol 3-phosphate (PI3P), which is associated with EEA1, is generated on organelle membranes by the action of the Rab5 effector, type III phosphatidylinositol 3-kinase (PI3K) hVPS34 [40]. Inhibition of phosphatidylinositol 3-phosphate (PI3P) by *M.*

tuberculosis products, lipid (liparabinomannan, LAM) [41] and an enzyme (a PI3P phosphatase, SapM)[42] plays a central role in mycobacterial phagolysosome biogenesis block.

1.4 Mycobacteria and Autophagy

Autophagy is a process of self-digestion where organelles or long-lived cellular proteins are encapsulated in a vesicle with a double membrane and delivered to lysosomes for degradation (to remove damaged organelles) or recycling (to provide amino acids during starvation). Depletion of total amino acids strongly induces autophagy in many types of cultured cells [43]. As shown in Figure 3, the process starts with the formation of a phagophore that expands into a double-membraned autophagosome [44]. The autophagosome may fuse with an endosome when the cell internalises and degrades material that originates outside of the cell. Then the autophagosome fuses with a lysosome, which supplies acid hydrolases. Hydrolytic enzymes degrade the content of the autophagosome. The resulting macromolecules are released and recycled in the cytosol [44]. Different (approximately 18) Atg proteins (Autophagy-related proteins) are involved in autophagosome formation and are called "AP-Atg proteins" [43, 45, 46]. The mammalian homolog of Atg8, LC3 protein has been identified on the autophagosomal inner membrane [47]. LC3 functions as a receptor for a selective substrate, p62/SQSTM1 [48]. During autophagy, p62/SQSTM1 binds to LC3 and is preferentially degraded in the process [48, 49]. It is known that when proteins are ubiquitinated, they bind to p62/SQSTM1, which has an ubiquitin-binding domain, and recruited to the autophagosome for degradation [48, 49]. At the molecular level, mTOR (mammalian target of rapamycin) is a key component that regulates the balance between growth and autophagy in response to cellular physiological conditions and environmental stress [43]. However, the regulation of autophagy is not always dependent on mTOR activity [50]. Proteins called small-molecule enhancers of the cytostatic effects of rapamycin (SMERs), are known to induce autophagy, and this process acts independently of mTOR [51]. As the autophagosome is trafficked to the lysosome where its contents are degraded via acidic lysosomal hydrolases [52], it is now recognised that autophagy plays a crucial role in pathogen capture and degradation [53, 54].

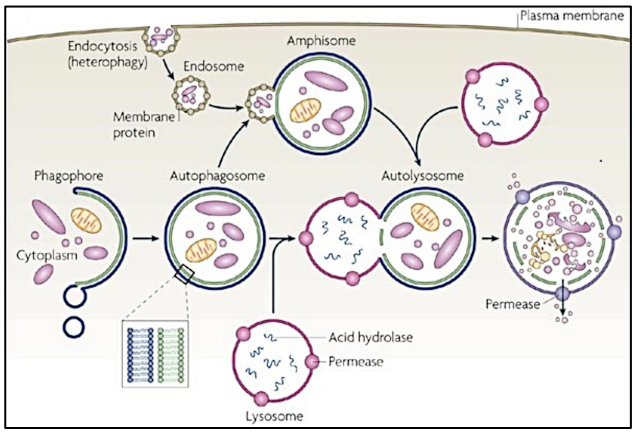


Figure 3: © Schematic diagram of autophagy mechanism [44]

A complex regulatory network manages autophagy with evidence of mTOR-dependent and independent pathways that are linked to tuberculosis (TB) defense [55]. Mycobacteria activate the cytosolic pathway, leading to ubiquitination of bacilli and delivery to autophagosomes. This process is dependent on the ubiquitin-autophagy receptors p62 and NDP52, and the

serine/threonine protein kinase TBK1 [56]. In a previous study, induction of autophagy in Mtb-infected macrophages by starvation or by treatment with the mTOR inhibitor rapamycin delivered bacilli to phagolysosomes and increased co-localisation of Mtb with LC3 and Beclin 1. In addition, after treatment with IFN-γ, autophagy was induced which reduced bacterial survival [57]. Induction of autophagy was observed in human monocyte-derived dendritic cells (DC) infected with H37Rv, H37Ra or BGG, and only H37Rv was able to inhibit autophagosome-lysosome fusion in DC. It was also observed that, after complementation with the Esx-1 region from *M. tuberculosis*, BCG was able to inhibit the induction of autophagy [58]. PI3P, which plays an important molecule in phagosomal maturation and mycobacterial phagolysosome biogenesis, also has a function in autophagy. In previous studies it was found that PI3P-dependent autophagy could bypass the mycobacterial phagolysosome biogenesis block [59, 60].

During mycobacterial infection, not only proteins and protein-coding RNAs are affected but also ncRNAs. An introduction to ncRNAs as well as their role in mycobacterial infection is discussed below.

1.5 Mycobacteria and ncRNAs

Different classes of ncRNAs are present in mammalian cells such as microRNAs (miRNAs), long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), PIWI-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs), etc. miRNAs are short, single-stranded ncRNA molecules approximately 22 nucleotides in length that play key roles in the regulation of gene expression by acting at the post-transcriptional level [61]. lncRNAs are non-protein coding transcripts longer than 200 nucleotides, which may influence post-transcriptional regulation by interfering with the miRNA pathways [62]. Circular RNAs (unlike linear RNAs) are round and can be produced by direct ligation of 5’ and 3’ ends of linear RNAs, or by “backsplicing” wherein a downstream 5’ splice site is joined to an upstream 3’ splice site [63]. Because of their structure, they are considered to be more resistant to RNA degradation. Studies have suggested that some circRNAs might regulate miRNA function at the transcriptional level [64]. piRNAs are small noncoding RNAs that act as guardians of the genome, protecting it from invasive transposable elements in the germline [65]. snoRNAs are a class of regulatory RNAs responsible for posttranscriptional maturation of ribosomal RNAs (rRNAs). snoRNAs direct site-specific modification of the pre-rRNA at positions of 2'-O-methylation and pseudouridine formation [66].

The focus of the present work was to define the role of lncRNAs and miRNAs in macrophages following mycobacterial infection.

1.5.1 Mycobacteria and lncRNA

Long non-coding RNAs (lncRNAs) are a large and diverse class of transcribed RNA molecules with a length of more than 200 nucleotides. With the advent of next generation

sequencing technology, many studies have identified thousands of lncRNAs [67, 68] in mammalian cells that regulate gene expression in a variety of immunological processes [69, 70].

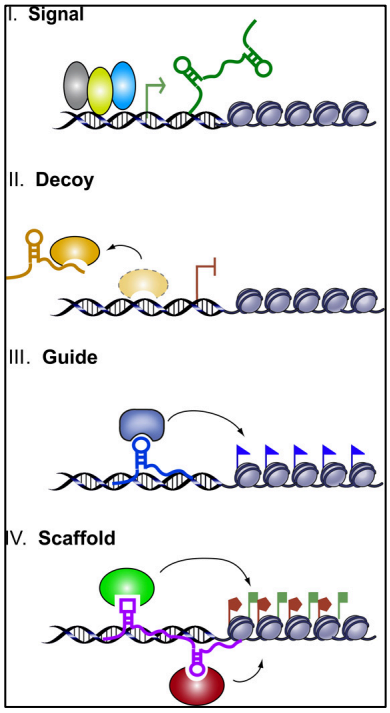


Figure 4: © Schematic diagram of the four archetypes of lncRNA mechanism [62]

Instead of taking part in protein coding, these RNAs function in regulatory mechanisms. Four different roles of lncRNAs have been proposed: Signals, Decoys, Guides, and Scaffolds. As shown in Figure 4, lncRNAs work in combination with transcription factors or signal to transcription factors resulting in regulation of genes (Signal). Sometimes lncRNAs remove transcription factors or other proteins that are involved in the transcription of a gene (Decoy). Moreover, lncRNAs recruit chromatin-modifying enzymes to target genes (Guide). Based on the unique structure of a lncRNA, it can also bind different proteins and form ribonucleoprotein complexes and function as histone modifiers of chromatin (Scaffold) [62]. lncRNAs function in different cellular processes from cell growth and differentiation to autophagy and cell death. There have been a few recent studies that focused on defining the role of lncRNAs in autophagy regulation. Recently, it has been found that a lncRNA, named autophagy-promoting factor (APF), regulates autophagic cell death by targeting miR-188-3p and ATG7. It was observed that miR-188-3p suppresses autophagy and myocardial infarction by targeting ATG7 [71]. In addition, it has been shown that decreased expression of the lncRNA MEG3 resulted in activation of autophagy in bladder cancer cells [72]. Moreover, overexpression of "highly up-regulated in liver cancer" (HULC), a lncRNA, was found to be involved in hepatocellular carcinoma development and progression, and it induced patterns of autophagy in a human gastric cancer cell line (SGC7901 cells) [73]. A lncRNA called NeST (nettoieSalmonella pas Theiler's [cleanup Salmonella not Theiler's]) was found to regulate epigenetic marking of IFN-γ-encoding chromatin, expression of IFN-γ, and susceptibility to a viral and bacterial pathogen [74]. However, the role of lncRNAs during infection is not known. An effort has been made to understand the function of lncRNAs during mycobacterial infection, as there is a need to understand molecular mechanisms of T-cell immune responses during tuberculosis (TB) infection. This study demonstrated that CD244 signalling in active human TB regulates repression of IFN-γ and TNF-α by lncRNA-CD244. In this regulation, lncRNA-CD244 modulates recruitment of EHZ2 to promoters of IFN-γ and TNF-α for potential trimethylation of H3K27 and repression of *infg* and *tnfa* gene expression [75]. As lncRNA regulations occur that are connected to related pathways, it is important to study them during infection, as lncRNAs could be a potential target for therapeutic intervention of disease.

A recent study, utilising dual RNA-seq, revealed *Salmonella enterica* serovar Typhimurium-specific alterations in multiple long noncoding RNAs. Out of ~44% affected lncRNAs, almost

half of them showed differences in expression between wild-type and ΔpinT infections. PinT was a PhoP-activated small RNA, which after bacterial internalisation temporally controls the expression of invasion-associated effectors as well as virulence genes required for intracellular survival. This study suggested that lncRNAs could act as sensitive markers for pathogen activities in the early phase of infection [76].

1.5.2 Mycobacteria and miRNA

Mature miRNAs are generally 22 nucleotides in length and are transcribed as a primary miRNA from DNA and processed by different proteins during maturation. As shown in Figure 5, they are transcribed by RNA polymerase II (Pol II). A protein called Drosha (a type of RNase III enzyme) cleaves the primary miRNA (pri-miRNA) and forms a ~70 nucleotide hairpin-shaped loop structure called pre-miRNA. This pre-miRNA is exported from the nucleus into the cytoplasm, where it is further cleaved by another RNase III enzyme, Dicer, yielding a miRNA–miRNA* duplex that is about 22 nucleotides in length. Out of two, only one strand is usually incorporated into the RNA-induced silencing complex (RISC), which recognises its target (mRNAs) with nucleotides 2–7 of miRNA (known as seed region). Association of a miRNA with its mRNA target results in degradation of the mRNA or translational inhibition [61].

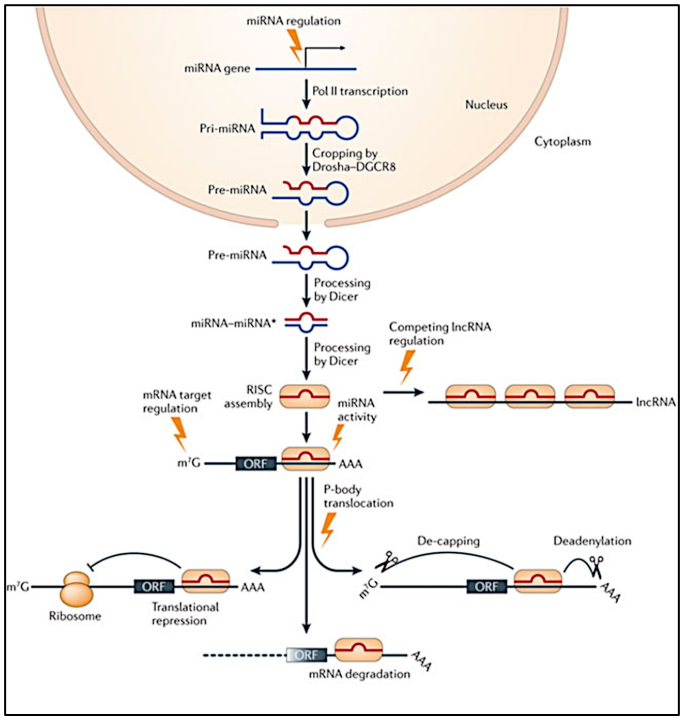


Figure 5: ©Schematic diagram of miRNA biogenesis [61]

Until now there have been no definitive miRNA signatures in mycobacterial infection, however, miR-155, miR-155* and miR-29a have been considered as biomarkers, because these miRNAs are highly expressed in patients with active TB [77, 78]. miRNA expression profiling of human macrophages upon infection with mycobacteria resulted in a signature of miRNA expression mostly overlapping between two mycobacteria (H37Rv and BCG) [79]. Human macrophages were infected with virulent *M. tuberculosis* and yielded increased hsa-miR-125b, which destabilised TNF-α mRNA and reduced hsa-miR-155 expression, thus reducing TNF-α production [80, 81]. miR-142-3p is another important miRNA which is regulated during mycobacterial infection. An interesting feature of this miRNA is that during the initial period of infection it is up-regulated in macrophages but down-regulated at a later stage of the

mycobacterial infection [82, 83]. Decreased expression of miR-142-3p corresponds to increased expression of its target gene IRAK-1, which is an inhibitor of TLR signalling [84]. In another study, miRNA and mRNA expression profiles of macrophages infected with *M. avium* subsp. *hominissuis* revealed two miRNAs, hsa-miR-29a, and let-7e, that target and inhibit the pro-apoptotic caspase 3 and caspase 7 networks [85]. miRNAs not only affect apoptotic pathways but also autophagy, as miRNA-125a inhibits autophagy activation and antimicrobial responses during mycobacterial infection [86]. Beside these studies, there are many more studies that have revealed new functions of miRNAs in mycobacterial infection. Gradually, there is a better understanding of the role of miRNAs in regulatory mechanisms of mycobacterial pathogenesis. However, there are still many areas where future research will improve our understanding of this complex interaction.

2. Aim of the study

Considering available knowledge on interaction between mycobacteria and macrophages, aims have been set to answer following questions to fill the gaps in knowledge. Primary aim of present study was to understand the role of ncRNAs in case of mycobacterial infection.

I. How long non-coding RNAs response in human macrophages infected with mycobacteria and what is their significance in infection?

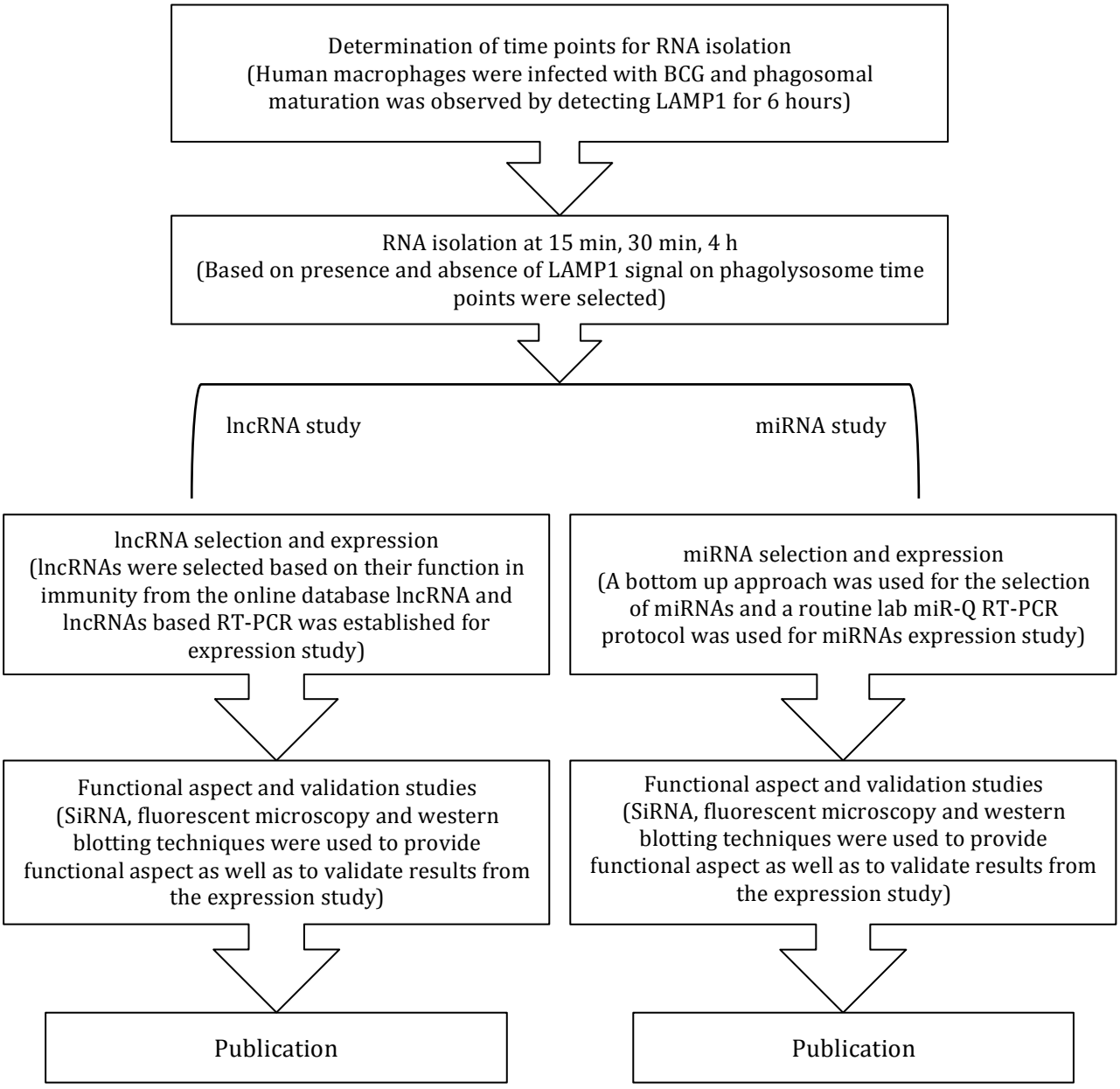
- Standardization of lncRNA based RT-qPCR
- Expression of immune related lncRNAs in mycobacterial infection during initial period of infection to macrophages
- Finding the functional aspect of lncRNAs in infection.

II. Are miRNAs involved in phagosomal maturation during mycobacterial infection?

- Bottom up approach to search miRNAs that are involved in phagocytosis related pathways.
- Finding targets of miRNAs and validation
- Biological relevance of the miRNA-mRNA interaction

3. Experimental design

Following flow chart was the design of experiment for current thesis details could be found in the published articles.



4. Subsuming the published work

Publication 1

Pawar K, Hanisch C, Palma Vera S E, Einspanier R, and Sharbati S. (2016). Down regulated lncRNA MEG3 eliminates mycobacteria in macrophages via autophagy. *Sci. Rep*. 6:19416. doi: 10.1038/srep19416

Down regulated lncRNA MEG3 eliminates mycobacteria in macrophages via autophagy

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Abstract

Small non-coding RNA play a major part in host response to bacterial agents. However, the role of long non-coding RNA (lncRNA) in this context remains unknown. LncRNA regulate gene expression by acting e.g. as transcriptional coactivators, RNA decoys or microRNA sponges. They control development, differentiation and cellular processes such as autophagy in disease conditions. Here, we provide an insight into the role of lncRNA in mycobacterial infections. Human macrophages were infected with *Mycobacterium bovis* BCG and lncRNA expression was studied early post infection. For this purpose, lncRNA with known immune related functions were preselected and a lncRNA specific RT-qPCR protocol was established. In addition to expression-based prediction of lncRNA function, we assessed strategies for thorough normalisation of lncRNA. Arrayed quantification showed infection-dependent repression of several lncRNA including MEG3. Pathway analysis linked MEG3 to mTOR and PI3K-AKT signalling pointing to regulation of autophagy. Accordingly, IFN-γ induced autophagy in infected macrophages resulted in sustained MEG3 down regulation and lack of IFN-γ allowed for counter regulation of MEG3 by viable *M. bovis* BCG. Knockdown of MEG3 in macrophages resulted in induction of autophagy and enhanced eradication of intracellular *M. bovis* BCG.

Keywords

Mycobacteria, *Mycobacterium bovis* BCG, lncRNA, microRNA, macrophage, regulation, gene expression, autophagy, MEG3, RT-qPCR, normalisation

Introduction

Mycobacterial species rank among the most potent bacterial pathogens threatening the health of both humans and animals. *M. tuberculosis* is the etiological agent of human tuberculosis and is the leading cause of death from a single infectious bacterial pathogen in humans. One-third of the world's population is estimated to have latent tuberculosis while multidrug resistance is occurring worldwide (WHO Fact sheet N°104). The vaccination based on using the attenuated strain *M. bovis* BCG (BCG) does not provide enough immunity [1] and treatment of latent as well as active tuberculosis implies prolonged medication using a combination of therapeutics. Tremendous efforts have been made to investigate the molecular details of host cell response to mycobacterial infections. However, there is still a need for understanding regulatory networks in infection that may lead to new approaches in translational medicine.

Cellular response to bacterial infections relies on well-defined networks of molecular interactions based on regulation of gene expression and protein function. Interestingly, recent RNAseq studies have shown that most of human genome is transcribed but very little of it has the ability to encode proteins [2]. Members of the non-coding genome include microRNA (miRNA) that are a major class of well characterised, conserved and endogenous small interfering RNA that regulate gene expression [3]. Several recent studies pointed out their involvement in mycobacterial infections [4,5].

Another class of regulating non-coding RNA is distinguished from the others primarily based on their size of larger than 200 nt. This class is referred to as lncRNA. Their numbers are estimated to match those of known protein coding genes but they are generally shorter, have fewer exons and possess low evolutionary conservation [6,7]. Furthermore, they have lower level of cellular concentration than protein coding transcripts but have higher degree of tissue specificity [8,9]. Global expression of lncRNA can be studied e.g. by whole-transcriptome sequencing. Different studies have used RNAseq to study lncRNA expression in different contexts [10-12]. Mentioned tissue specificity, low level of cellular concentrations and lack of understanding on mode of action in most cases raise the question whether a targeted approach based on e.g. RT-qPCR arrays shall be an alternate strategy to study lncRNA in a defined biological context.

LncRNA frequently localise in the nucleus functioning both in *cis* (at the site of their transcription) as well as in *trans* (at the sites on other chromosomes), which points to potential functions as interfaces with the epigenetic machinery, chromatin organisation and regulation of gene expression. They function e.g. as protein scaffolds, activators or inhibitors of transcription, antisense RNA or miRNA sponges, respectively [13,14]. The latter has been reported recently as a novel mode of action of lncRNA, where they act as a competing endogenous RNA (ceRNA). This suggests the existence of a network of lncRNA and mRNA, which crosstalk based on mutual miRNA response elements (MRE) [15]. In this context, susceptibility to ceRNA competition has been shown to differ depending on endogenous miRNA:target pool ratios [16].

Recent studies have demonstrated innate immune related expression of lncRNA following activation of monocytes, dendritic cells or macrophages [7]. Interestingly, a few studies have pointed out the role of lncRNA in regulating autophagy in cancer. For example, it has been shown that decreased expression of the lncRNA MEG3 results in activation of autophagy in bladder cancer cells [17]. However, the molecular details of mode of action are not understood. Rapamycin and other inhibitors of the Ser/Thr protein kinase 'mammalian target of rapamycin' (mTOR) are potent inducers of autophagy and inhibitors of phosphatidylinositol 3-kinases (PI3K) such as 3-methyladenine (3-MA) are well-known inhibitors of autophagy [18]. Autophagy is known to be an important antimicrobial process for eradication of *M. tuberculosis* in infected macrophages [19]. However, induction of autophagy during macrophage infection with *M. tuberculosis* or BCG depends on IFN-γ and is enhanced by the presence of 1,25-dihydroxyvitamin D3. This process depends on Beclin1 and Atg5 and therefore constitutes the canonical form [20]. Beclin1 functions as a scaffold for assembly of the PI3KC3 complex to initiate autophagosome formation [21]. Beclin1 was shown to have a role in the convergence between autophagy and apoptosis and to be a substrate of CASP3 [21,22]. Autophagy and apoptosis are two interconnected cellular processes that are both modulated by pathogenic mycobacteria. In this context, we have reported earlier that in *M. avium* infection of human macrophages inhibition of apoptosis is grounded in let-7e and miR-29a mediated regulation of CASP3 and 7 [5].

This study focuses mainly on two aspects, first the establishment of a RT-qPCR based approach to predict lncRNA function. Secondly, proof of principle by mechanistic studies. We hypothesise that the innate immune response of human macrophages to mycobacterial infections is controlled by lncRNA. Furthermore, we speculate that mycobacterial infection and IFN-γ stimulation of human macrophages leads to lncRNA-mediated induction of autophagy to combat intracellular bacteria. To address this, we preselected a list of lncRNA with known immune related functions and established a customised protocol for arrayed quantification of lncRNA expression based on RT-qPCR.

Results

A targeted approach for expression based prediction of lncRNA function

In this study, we aimed at designing a targeted approach based on selecting lncRNA, which are known to have immune related functions and have not been reported in context of mycobacterial infections. Therefore, an RT-qPCR based protocol for individual as well as arrayed quantification of lncRNA was established considering novel aspects for accurate normalisation of lncRNA expression. A list of 21 human lncRNA with immune related functions was compiled using the database lncRNAdb [23]. Initial testing resulted in selection of 17 lncRNA exhibiting harmonised annealing temperatures at 60°C, dynamic ranges of higher than 8 logs of magnitude and mean efficiency of 96.62% ± 4.42% (table 1).

Epithelial as well as immune cell lines provide widely recognised *in vitro* models for addressing different aspects of interactions between bacterial agents and host cells and help to reduce animal experiments. We applied a set of human monocytic (THP-1 and U937) as well as epithelial cell lines (A549.1, HeLa, HT-29 and HT-29/B6), which serve as common *in vitro* infection models and evaluated the performance of the presented approach. Furthermore, we aimed at developing a strategy for functional prediction and pathway annotation of selected lncRNA. Cell line specific expression was examined and lncRNA-clusters were deduced from determined expression patterns that were subjected to functional predictions. For this purpose, lncRNA expression in investigated cell lines (fold changes of a given sample vs. a common reference) was hierarchically clustered. Distinct groups of lncRNA such as H19, PTENP1 and GAS5 or MEG3 and NEAT1 were defined, which showed matched expression among cell lines and exhibited small distances in hierarchy (figure 1 a). Identified clusters were chosen for prediction and annotation of molecular pathways. We took advantage of the evolving concept that lncRNA, mRNA and pseudogenes talk to each other based on mutual MRE [15]. Consequently, CLIP-Seq based interactions of lncRNA and miRNA were obtained from starBase [24] to determine miRNA that target lncRNA of an identified cluster (figure 1 a and supplementary information). The resulting list of miRNA was subjected to target analysis and a network of mRNAs was generated that were inferred to cross talk to the lncRNA via mutual MRE. For this purpose, we employed Cytoscape [25] together with its app CyTargetLinker [26] and predicted miRNA-mRNA networks by considering regulatory interaction networks of target scan [27]. The resulting target networks (mRNA sharing MREs with lncRNA) of an

identified lncRNA cluster (figure 1 a and supplementary information) were intersected and subjected to ClueGo analysis [28] for determining significant KEGG [29] pathway enrichment (detailed information on the approach is provided in the supplementary information). For example, the cluster consisting of H19, PTENP1 and GAS5 was mainly assigned to gene ontologies 'specific DNA binding' and 'protein kinase activity' and genes were enriched in signalling pathways such as FoxO, HIF-1 or Estrogen (supplementary information). Our analysis pointed out a particular cluster with small distance among the members NEAT1 and MEG3. The introduced prediction strategy assigned this cluster inter alia to mTOR (13 genes; 22% genes/term; $P < 0.01$) and PI3K-AKT (30 genes; 9% gene/term; $P < 0.05$) signalling pathways, which are involved in regulation of autophagy (figure 1 b and supplementary information).

Table 1: Selected lncRNA, ncRNA and conventional reference genes for RT-qPCR arrays.

Targets (lncRNA & reference genes)	Accession No.	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon Size (bp)	Tm (°C)	Slope	Y-Intercept	R^2	Efficiency	Dyn. range
ANRIL (CDKN2B-AS1)	NR_003529.3	acccaggctggagtgtattg	tagtcccagctgctcaggtt	81	60	-3.384	30.149	0.999	97.457	8
AS UCHL1 (UCHL1-AS1)	NR_102709.1	gtcgtctgcccaaaactagc	aaggtggacaccagctcatc	124	60	-3.131	29.645	0.994	108.630	9
EGO (EGOT)	NR_004428.1	taatcagctcagggggtcac	atacccaattccctgccttc	133	60	-3.496	31.101	0.999	93.233	8
GAS5	NR_002578.2	agaaatgcaggcagacctgt	gcactctagcttgggtgagg	108	60	-3.394	30.804	1.000	97.082	8
H19	NR_002196.1	ttcaaagcctccacgactct	ctgagactcaaggccgtctc	101	60	-3.347	29.198	0.999	98.956	8
HOTAIRM1	GQ479958.1	gaagagcaaaagctgcgttc	cagacctctcgccagttcat	140	60	-3.511	29.864	0.998	92.669	8
RPS15AP25 (LETHE LIKE)	NG_011025.2	caggaacatccacaatgctaaa	cagtgtagccatgcttcatca	107	60	-3.612	28.697	0.999	89.161	8
MEG3	NR_003530.2	cagccaagcttcttgaaagg	ttccacggagtagagcgagt	103	60	-3.400	27.506	0.999	96.838	8
NEAT1	NR_028272.1	gtggctgttggagtcggtat	attcactccccacctctct	113	60	-3.427	29.274	0.998	95.806	9
NRON	NR_045006.1	ccccatttcacagaggaaga	ttgggccttctttacaccac	89	60	-3.448	30.652	0.999	95.010	8
NTT	U54776.1	ggtctccttaagggcaaagg	ccttgagaggccatttggta	86	60	-3.292	27.021	0.998	101.256	9
PRINS	NR_023388.1	ggaatgtggcctgtgttctt	agggacaaccacatcaaagc	116	60	-3.396	30.633	0.999	97.007	9
PTENP1	NR_023917.1	agttccctcagccgttacct	aggtttcctctggtcctggt	135	60	-3.334	29.109	0.999	99.503	8
RNCR3 (LINC00599)	NR_024281	agtcgttgggctatgtggac	ggatctctgcctgaaactgc	140	60	-3.490	26.643	0.999	93.430	8
TMEVPG1 (IFNG-AS1)	Ak124066	aaacgctggaggagaagtca	gttagcagttggtgggcttc	77	60	-3.311	27.708	0.998	100.466	8
TNCRNA (NEAT1)	AF080092	gacttggggatgatgcaaac	tcacaacagcatacccgaga	94	60	-3.397	28.417	1.000	96.944	8
WT1-AS	NR_023920	cctgagctaagcaccaggac	gtacaggagagcgcctatcg	85	60	-3.556	28.974	0.999	91.066	8
ZEB2NAT	HG494679	gaccgttattcctgcagagc	ccctccccacaaagataggt	73	60	-3.321	28.383	0.994	100.055	8
SNORD47	NR_002746.1	tgatgtaatgattctgccaaatg	acctcagaatcaaaatggaacg	77	60	-3.43	28.654	0.999	95.699	8
ACTB	NM_001101.3	ggacttcgagcaagagatgg	agcactgtgttggcgtacag	234	60	-3.295	31.373	1	101.1128	7
B2M	NM_004048.2	gtgctcgcgctactctctct	ggatggatgaaacccagaca	135	60	-3.577	32.358	1	90.369	7
GAPDH	NM_001256799.2	ccatcttccaggagcgagat	ctaagcagttggtggtgcag	249	60	-3.36	36.635	0.999	98.435	7
18S rRNA	NR_003286.2	atggccgttcttagttggtg	cgctgagccagtcagtgtag	217	60	-3.34	32.878	1	99.264	7

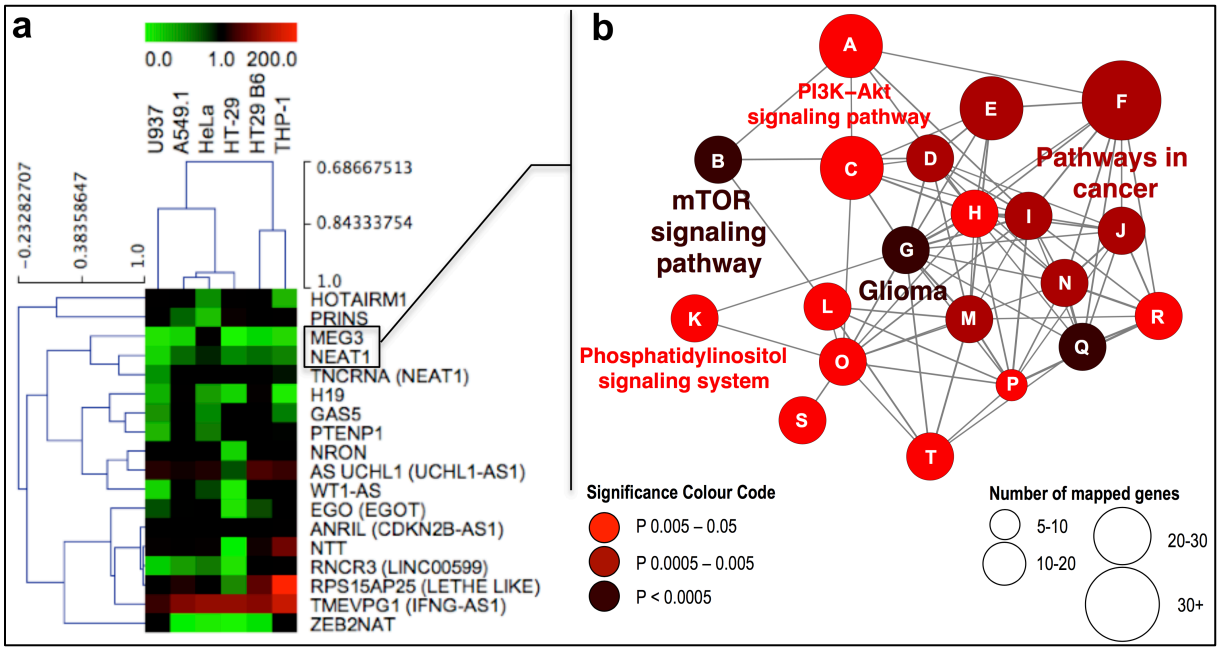

Figure 1: Hierarchical clustering of lncRNA expression and prediction of pathways. a) Expression of selected lncRNA was studied in six cell lines and mean fold changes of expression vs. a common reference were hierarchically clustered in a heat map (decreased expression is shown in green and increased in red according to the colour bar shown above). All experiments were performed at least in triplicates. B2M and ACTB were used as reference genes. b) *In silico* analysis assigned the cluster consisting of MEG3 and NEAT1 to mTOR and PI3K-AKT signalling pathways as indicated by the ClueGO network. Colours represent the P value of significant enrichment and size of circles describes number of mapped genes as indicated in the legend. Legend: A, PI3K-AKT signalling pathway; B, mTOR signalling pathway; C, Rap1 signalling pathway; D, HIF-1 signalling pathway; E, protoglycans in cancer; F, pathway in cancer; G, glioma; H, melanoma; I, prostate cancer; J, FoxO signalling pathway; K, Phosphatidylinositol signalling system; L, insulin signalling pathway; M, ErbB signalling pathway; N, pancreatic cancer; O, estrogen signalling pathway; P, endometrial cancer; Q, chronic myeloid leukaemia; R, colorectal cancer; S, adregenic signalling pathway; T, neutrophin signalling pathway.

Assessment of strategies for normalisation of lncRNA expression

lncRNA are known to exhibit lower cellular expression levels compared with protein coding genes. So we raised the question whether normalisation of lncRNA expression by means of conventional reference genes (mRNA) is advisable. To approach this, we tested the application of two sets of references: One set consisted of 3 stably expressed conventional reference genes and another set included stably expressed ncRNA. Except for the studied 17 lncRNA, we considered one small nuclear RNA (SNORD47) as well as 3 conventional reference mRNA (B2M, ACTB and GAPDH) together with 18S rRNA for evaluating normalisation strategies of lncRNA expression. Two successive rounds of geNorm analysis [30] were performed (considering the total number of 22 targets) to identify the most stable genes among both the conventional references and the entire list of ncRNA. At 30 min p.i., B2M, GAPDH and ACTB turned out to be stably expressed as represented by low geNorm M values (M_{B2M}=0.503, M_{ACTB}=0.557, M_{GAPDH}=0.521). Furthermore, geNorm analysis confirmed a group of three ncRNA including ZEB2NAT, SNORD47 and PTENP1 to be stably expressed ($M_{ZEB2NAT}$=0.487, $M_{SNORD47}$=0.565, M_{PTENP1}=0.581), which were chosen as ncRNA references. To evaluate differences in cellular abundance of studied RNA classes, raw Cq values of lncRNA were compared with identified ncRNA references as well as conventional references. As shown in figure 2 c, the mean raw Cq value of conventional reference genes was significantly lower compared with the selected ncRNA reference genes and remaining lncRNA (Mean Cq $_{conv.\ ref\ genes}$=11.2, Mean Cq $_{ncRNA\ ref\ genes}$=19.5, Mean Cq $_{lncRNAs}$=22.9, unpaired t test $P < 0.0001$). Consequently, the geometric mean of either reference ncRNA or conventional reference genes was used to normalise lncRNA expression. Obtained sets of data were plotted to determine the correlation. Strong linearity was observed between both sets of data as shown by the calculated values: slope=1.062 ± 0.016, R^2=0.9908 and Y-intercept=-0.02921 ± 0.01515 (figure 2 d). Although both sets of references were examined to be stable and suitable for normalisation of lncRNA expression, the distinct differences in cellular abundance (figure 2 c) call the use of conventional reference genes into question.

At 4 h p.i., geNorm analysis resulted in lower M values for the reference ncRNA ($M_{ZEB2NAT}$=0.536, $M_{WT1\text{-}AS}$=0.541, $M_{SNORD47}$=0.579) compared with the conventional references ($M_{18S\ rRNA}$=0.561, M_{GAPDH}=0.617, M_{B2M}=1.0). The raw Cq values were again significantly lower in the conventional reference genes compared with the other two groups (reference ncRNA and lncRNA) supporting the observations that were made above (figure 3 c: Mean Cq $_{conv.\ ref\ genes}$=11.1, Mean Cq $_{ncRNA\ ref\ genes}$=21.9, Mean Cq $_{lncRNAs}$=23.4, unpaired t test $P < 0.0001$). As shown in figure 3 d, the linear relationship between two data sets was not as pronounced as calculated above (slope=0.895 ± 0.078, R^2=0.7605 and Y-intercept=0.05127 ± 0.0769).

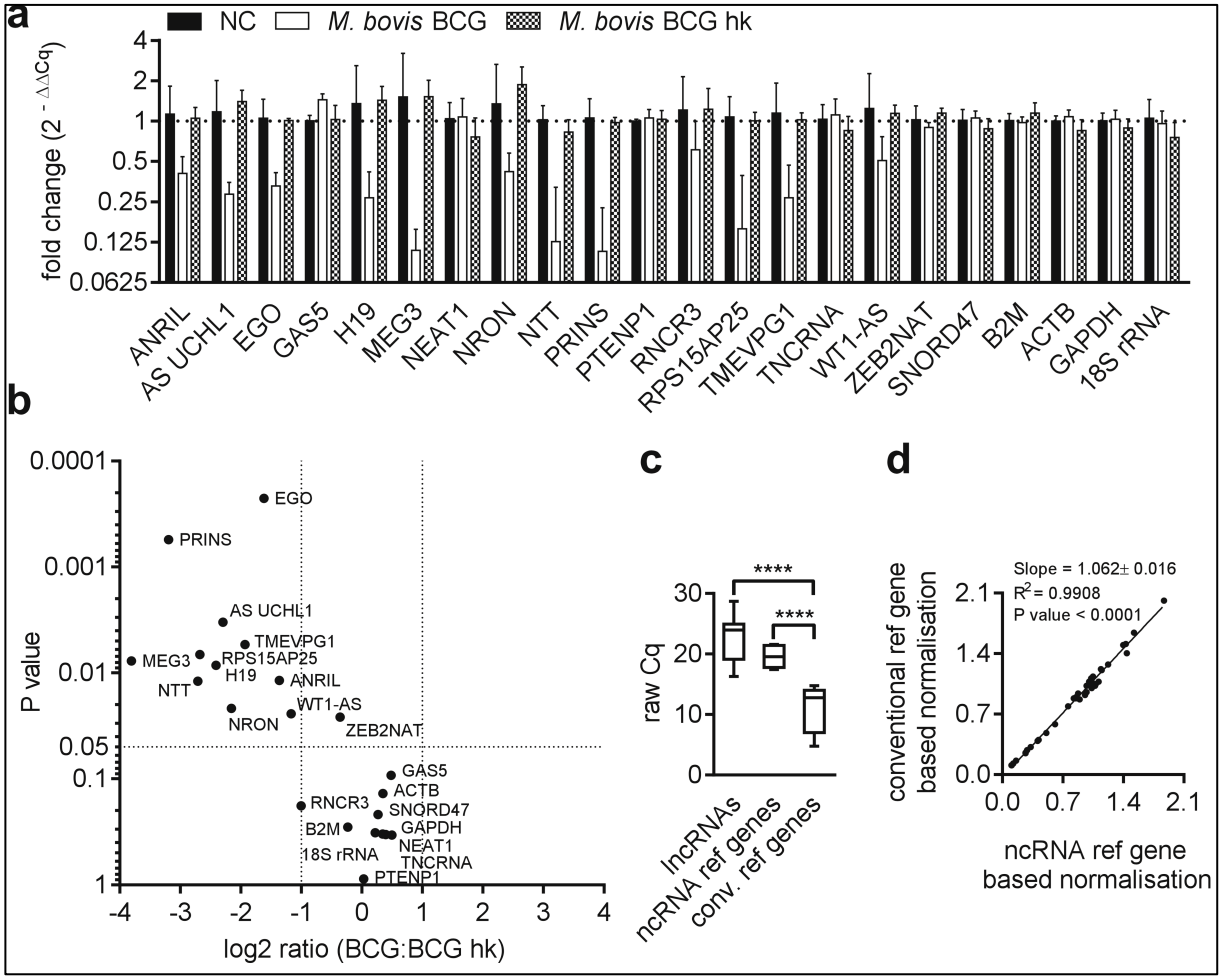

Figure 2: lncRNA expression and normalisation at 30 min p.i. of macrophages infected with viable and heat killed *M. bovis* BCG. a) In total, 11 lncRNA showed clearly decreased expression after infection with viable BCG when compared with BCG hk. Columns show mean fold change between samples and non infected controls (NC) of three biological replicates, each measured in triplicates, while bars indicate SD. ZEB2NAT, SNORD47 and PTENP1 were used as reference genes. b) The plot shows significantly (unpaired t test) dysregulated lncRNA in BCG infected macrophages compared with BCG heat killed (hk) treated cells. Dotted lines indicate statistically significant dysregulation ($P < 0.05$, log2 ratio < -1 or > 1). c) After identification of most stable genes by means of geNorm analysis two groups of reference genes and a remaining group were defined: ncRNA references (ZEB2NAT, SNORD47 and PTENP1), conventional references (B2M, GAPDH and ACTB) and lncRNAs (remaining 15 lncRNA). Box plots show raw Cq values of groups (****: $P < 0.0001$, unpaired t test). d) lncRNA expression was normalised with the geometric mean of either conventional references or ncRNA references and data were plotted to determine the coefficient of determination (R^2), slope and Y-intercept.

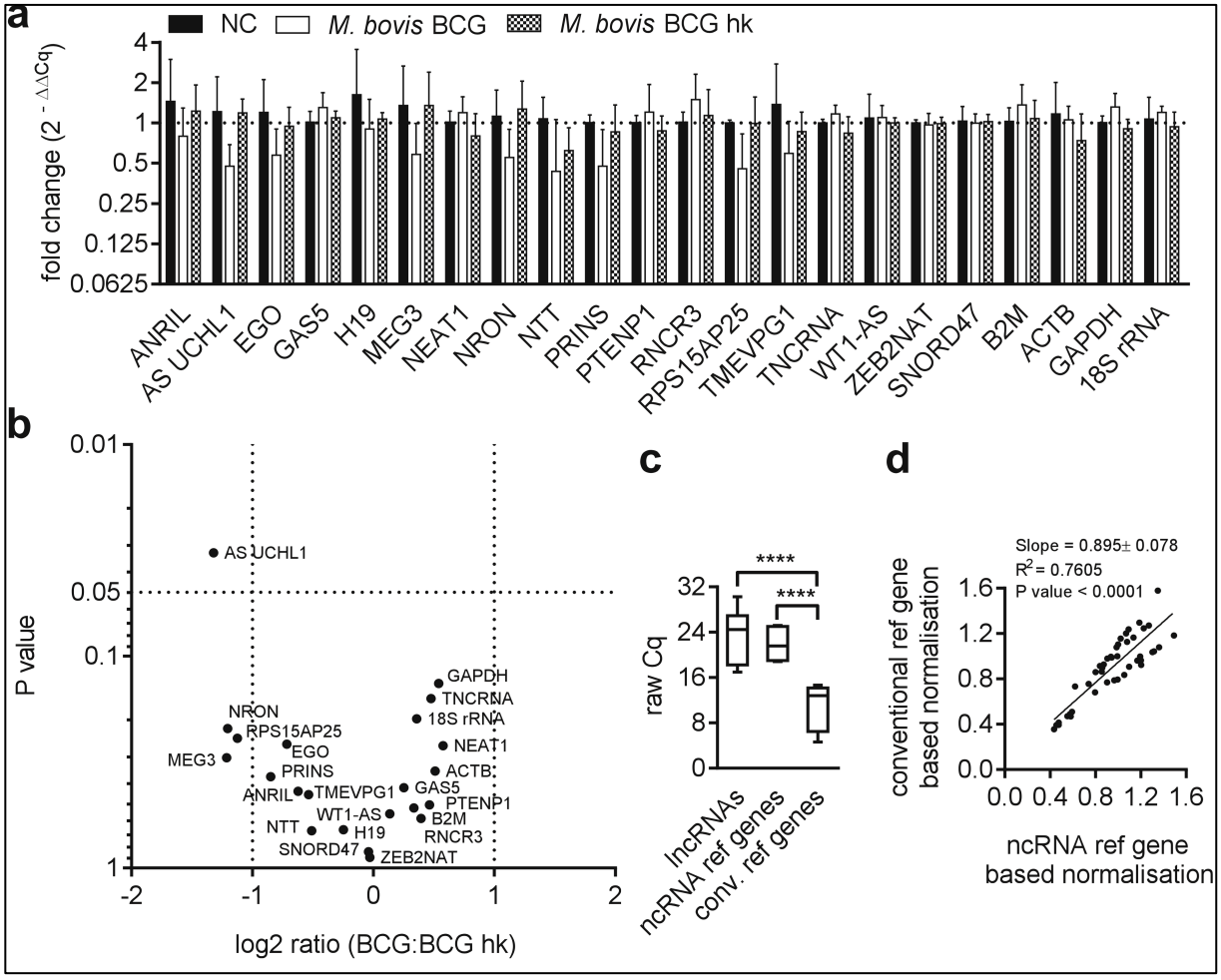

Figure 3: lncRNA expression and normalisation at 4 h p.i. of macrophages infected with viable and heat killed *M. bovis* BCG. a) A trend of decreased expression of several lncRNA was determined after infection with viable BCG at 4 h p.i. compared with BCG hk. Columns show mean fold change between samples and non infected controls (NC) of three biological replicates, each measured in triplicate, while bars indicate SD. ZEB2NAT, SNORD47 and WT1-AS were used as reference genes. b) The plot shows only one significantly (unpaired t test) dysregulated lncRNA in BCG infected macrophages compared with BCG heat killed (hk) treated cells. Dotted lines indicate statistically significant dysregulation ($P < 0.05$, log2 ratio < -1 or > 1). c) After identification of most stable genes by means of geNorm analysis two groups of reference genes and a remaining group were defined: ncRNA references (ZEB2NAT, SNORD47 and WT1-AS), conventional references (B2M, GAPDH and 18S rRNA) and lncRNAs (remaining 15 lncRNA). Box plots show raw Cq values of groups (****: $P < 0.0001$, unpaired t test). d) lncRNA expression was normalised with the geometric mean of either conventional references or ncRNA references and data were plotted to determine the coefficient of determination (R^2), slope and Y-intercept.

Induction of autophagy by IFN-γ in BCG infected macrophages accounts for sustained MEG3 down regulation

Autophagy is a cellular process for degradation and recycling of considerable cytoplasmic particles. Along these lines, recent studies have elucidated autophagy as a cell-autonomous defence mechanism for eradication of intracellular bacterial pathogens such as *Salmonella*, *Listeria*, *Shigella* and pathogenic *Mycobacterium* species [19]. It has been shown recently that MEG3 knock down in bladder cancer cells resulted in induction of autophagy, increased cell proliferation and inhibition of apoptosis [17]. For studying the effects of autophagy on MEG3 levels in the context of BCG infection, we inhibited or activated autophagy in macrophages by addition of 3-MA or IFN-γ, respectively. IFN-γ has been shown to be a potent inducer of autophagy in BCG infected macrophages [31]. Autophagy was examined by detecting the autophagosomal marker LC3A/B. As shown in figure 4 a and b, IFN-γ treatment along with BCG infection resulted in an increase of LC3A/B punctae and 3-MA compensated for it. The quantification of LC3A/B punctae proved IFN-γ as potent inducer of autophagy in macrophages both in infected and non infected samples (figure 4 b, unpaired t test $P < 0.05$). However, BCG without IFN-γ treatment was not sufficient to increase the number of punctae to the same extent. Interestingly, IFN-γ treatment resulted in efficient eradication of BCG as proven by significant decrease (unpaired t test, $P < 0.05$) of green fluorescence signals, while in other treatments sustained green fluorescence was detected over the course of experiment (figure 4 c). Induction of autophagy was proved by LC3A/B conversion and p62 degradation in infected and IFN-γ treated macrophages compared with the controls (figure 4 d). To determine effects of treatments on mTOR activation, p70-S6K phosphorylation was studied. Infection alone or along with 3-MA treatment caused mTOR activation shown by phosphorylation of p70-S6K (Thr389). However, treatment with IFN-γ along with infection distinctly decreased phosphorylation of Thr389 proving mTOR inactivation. Also phosphorylation of the residue Ser371 declined after treatment (figure 4 d). In the same set of experiments RNA was isolated to determine MEG3 and Beclin1 expression. Beclin1 is a key regulator of autophagy and has been described recently as an interface between apoptosis and autophagy [21]. Induction of autophagy by IFN-γ in infected macrophages was accompanied by sustained and significant (unpaired t test $P < 0.05$) MEG3 down regulation (over 24 h), while 3-MA administration partly restored MEG3 expression (figure 4 e). Combinations of both resulted also in partially restored MEG3 expression accompanied by inhibition of autophagy (figure 4 d). BCG infected and non treated macrophages exhibited clearly decreased MEG3 levels at 30 min p.i., however, MEG3 expression converged normal levels at 24 h. The treatments had no pronounced effects on Beclin1 expression (figure 4 f, unpaired t test $P > 0.05$). To rule out apoptotic effects on our observations, active CASP3 was examined by immunofluorescence. There was no obvious and general activation of CASP3 in any case of treatment (figure 4 g).

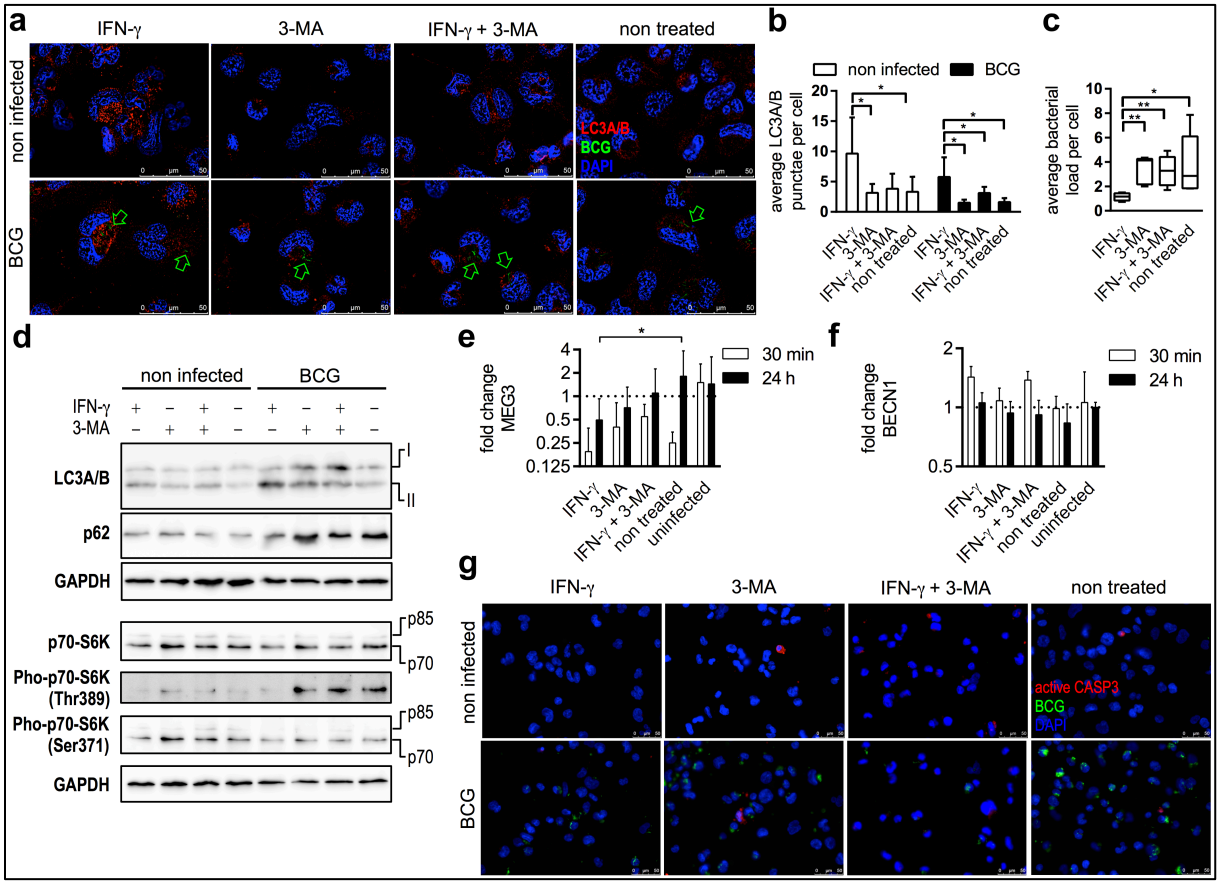

Figure 4: Induction of autophagy is associated with MEG3 down regulation without activation of Caspase 3. a) Autophagy in BCG infected cells and non infected controls (NC) was induced or inhibited with IFN-γ or 3-MA, respectively. Autophagy was tracked by detecting the marker LC3A/B (red) in macrophages. Green arrows indicate fluorescently labelled BCG (green, indicated by arrows). Nuclei were stained with DAPI (blue). Scale bars indicate 50 µm. b) LC3A/B punctae were quantified using ImageJ. IFN-γ treatment increased the average number of LC3A/B punctae in both infected cells and non infected controls (unpaired t test, $P < 0.05$). c) Number of intracellular BCG was significantly diminished in IFN-γ treated macrophages (unpaired t test, $P < 0.05$). d) Autophagy and mTOR activation was studied in infected and control cells by means of western blots. BCG infection along with IFN-γ induced autophagy was examined by LC3A/B conversion and p62 degradation. mTOR activation was reduced in same samples as shown by clearly decreased p70-S6K (Thr389) phosphorylation. GAPDH is shown last as respective loading reference. e and f) MEG3 and Beclin1 expressions were followed at 30 min and 24 h p.i. along with induction and inhibition of autophagy. IFN-γ treatment caused consistent and significant down regulation of MEG3 in infected cells compared with non treated controls (unpaired t test, $P < 0.05$). There was no significant change of Beclin1 expression. Columns show mean fold changes over non infected controls of at least three biological replicates normalised with SNORD47, each measured at least in triplicate. Bars indicate SD. g) Induction of apoptosis was tracked by detecting active CASP3 (red). BCG was fluorescently labelled (green) and nuclei counterstained with DAPI (blue). Scale bars indicate 50 µm.

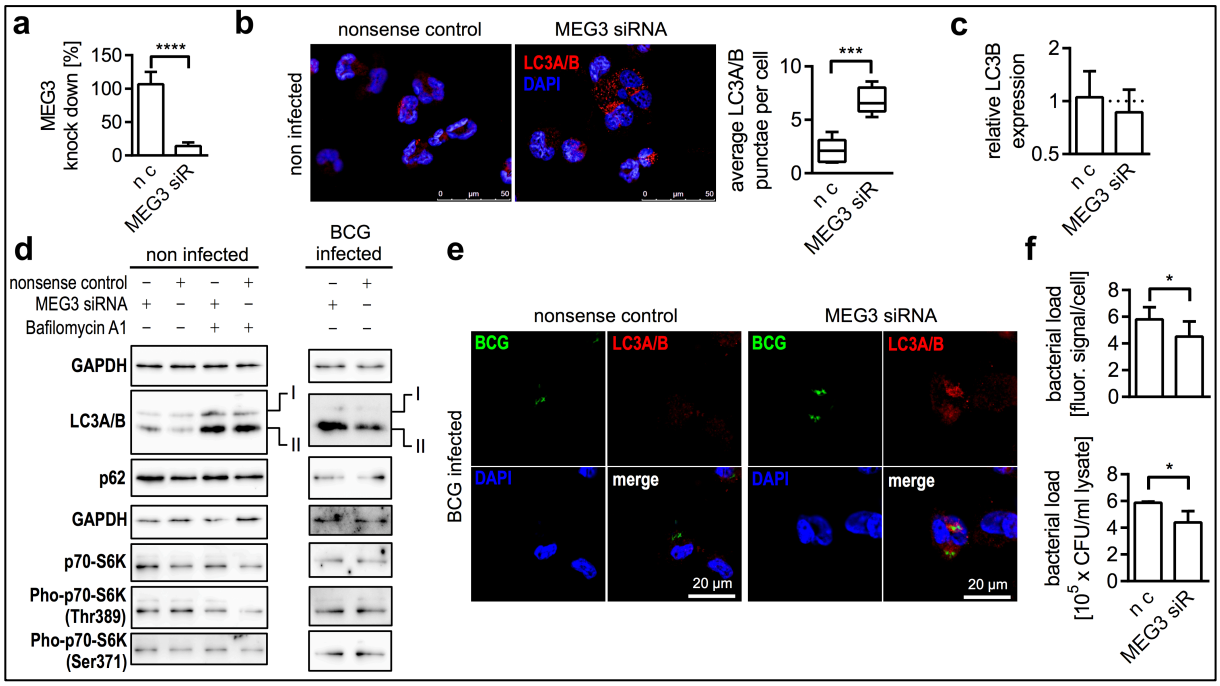

Figure 5: MEG3 knockdown induces autophagy. a) Knockdown in THP-1 using a specific siRNA resulted in significantly (unpaired t test, $P < 0.0001$) decreased cellular MEG3 levels compared with nonsense-transfected control.(n c). Experiments were repeated at least 3 times. b) MEG3 knockdown resulted in significantly (unpaired t test, $P < 0.001$) increased LC3A/B punctae compared with nonsense transfected controls (n c). Box plots show at least three biological replicates. c) MEG3 knockdown had no effect on LC3B transcriptional levels. d) MEG3 knockdown triggered autophagy as demonstrated by increased LC3A/B conversion in both infected and non infected macrophages. mTOR activation was determined by evaluating p70-S6K phosphorylation. GAPDH is shown first as respective loading reference. e) After knockdown LC3A/B punctae were co-localised with intracellular BCG d) MEG3 knockdown significantly decreased intracellular BCG numbers as determined by quantification of fluorescence signals as well as CFU (unpaired t test, $P < 0.05$).

MEG3 knockdown induces LC3A/B conversion in macrophages

To determine whether MEG3 is a regulator of autophagy in macrophages, we efficiently knocked down MEG3 (figure 5 a) and tested effects on autophagy by quantification of LC3A/B punctae per cell as well as by means of western blots examining its conversion. The application of siRNA potently decreased (unpaired t test, $P < 0.0001$) cellular levels of MEG3 in THP-1 derived macrophages and clearly increased LC3A/B punctae as represented by immunofluorescence detection (figure 5 b). Quantification resulted in significant increase of LC3A/B punctae after MEG3 knockdown compared with nonsense transfected controls (figure 5 b, unpaired t test $P < 0.001$). MEG3 knockdown had no obvious effects on LC3B mRNA expression (figure 5 c) and LC3A levels were under the detection limit.

In a guideline for autophagy detection, investigators were made aware of efficiency of transfections, because a western blot will detect LC3A/B in the entire cell population, including those that are not transfected [32]. The transfection resulted in more than 80% knockdown of MEG3 in THP-1 compared with nonsense transfected controls (figure 5 a). As shown in figure 5 d, MEG3 knockdown increased LC3A/B conversion compared with nonsense transfected controls as shown by enhanced intensity of LC3A/B-II in western blots. Addition of Bafilomycin A1 accumulated LC3A/B levels without clear differences between samples and controls. However, there was no obvious degradation of p62 after siRNA treatment in non infected cells. The levels of p62 slightly increased in MEG3 siRNA treated cells (figure 5 d). To determine whether MEG3 knockdown has effects on mTOR activation, we detected p70-S6K phosphorylation. MEG3 knockdown resulted in increased total cellular levels of p70-S6K in both Bafilomycin A1 treated and untreated cells compared with nonsense controls. P70-S6K (Thr389) phosphorylation was slightly decreased after MEG3 knockdown indicating mTOR inactivation (figure 5 d). Knockdown had no effect on phosphorylation of the residue Ser371 (figure 5 d). Moreover, MEG3 knockdown along with BCG infection resulted in clearly increased cellular LC3A/B-II levels compared with controls and p62 levels were slightly increased in MEG3 siRNA and infected controls (figure 5 d). RNAi resulted in accumulation of LC3A/B signals that were co-localised with BCG (figure 5 e), which went along with enhanced eradication of intracellular BCG as examined by two independent methods (detection of fluorescently labelled BCG as well as CFU). Both methods revealed significantly decreased bacterial loads after MEG3 knockdown in THP-1 derived macrophages compared with nonsense controls (figure 5 f, unpaired t test $P < 0.05$).

Discussions

Currently, about 9000 annotated human genes are known to produce more than 14000 lncRNA transcripts [33]. Although dysregulation of lncRNA has been reported recently in different aspects of innate immune response [7], the function of majority of these transcripts is yet to be determined. The mentioned tissue specific expression of lncRNA suggests the application of well-established cell lines as suitable models for studying molecular functions of lncRNA in a particular biological process. For this and other purposes, we developed a

targeted approach for RT-qPCR based expression analysis of lncRNA. The employed layout based on 96 well plates is extendable to include a total number of 26 lncRNA together with 5 reference RNAs each measured in triplicates. To our knowledge, this is the first non-commercial approach for arrayed RT-qPCR based quantification of lncRNA, which is fully scalable and automatable. We evaluated the application of both conventional and ncRNA reference genes for normalisation of lncRNA expression. A set of three ncRNA was considered for normalisation of lncRNA expression because they exhibited enhanced stability of expression compared with the conventional reference genes. More to the point, there were distinct and significant differences in cellular abundance of conventional references and ncRNA. Diverging cellular abundance of a reference gene and the gene of interest may bias the analysis based on differences in e.g. cellular turn over, RNA integrity or efficiencies of the enzymatic reaction as it has been discussed in the case of 18S rRNA [34]. We conclude that the application of a stability-tested set of ncRNA is indicated for thorough normalisation of lncRNA expression.

LncRNA constitute a heterogeneous class with low degree of evolutionary conservation and diverse modes of action [35]. This fact aggravates functional predictions and pathway involvement as it has been developed for miRNA and mRNA, respectively. However, in recent years the concept of a common RNA language has been developed, where shared MRE are suggested to serve as letters between different classes of RNA [15]. It has been shown that miRNA-ceRNA interaction depends on cellular ratios of both RNA classes, which aggravates a generalised model for miRNA sponges [16]. However, lncRNA have been reported to have several other modes of action. Our interpretation of the mentioned model considers lncRNA not just as miRNA decoys. LncRNA are known to function as protein scaffolds and are supposed to bind and control transcriptional coactivator or corepressor complexes [36]. Hence, we speculate that miRNA are able to indirectly influence cellular availability of mRNA by targeting e.g. lncRNA that are capable of stimulating or inhibiting transcription. The rationale of the study was to provide an expression-based approach that functionally assigns lncRNA clusters to molecular pathways and enables prediction of lncRNA function by considering lncRNA-miRNA-mRNA crosstalk. As we were analysing lncRNA clusters that were deduced from cell line specific expressions, we came across a particular cluster with small distance between NEAT1 and MEG3. Interestingly, *in silico* analysis linked them to mTOR and PI3K-AKT signalling pathways that are important for regulation of autophagy. For reducing the complexity of the study, we decided to focus only on mechanistic studies of MEG3. This does not necessarily rule out the fact that other investigated lncRNA may have different or cooperative functions. The data presented here provides a basis for our future research.

Infection of THP-1 derived macrophages with viable BCG caused remarkable dysregulation of several lncRNA. For example, NTT, PRINS, TMEVPG1 and MEG3 were significantly down regulated at 30 min p.i. compared with BCG hk stimulated cells but not at 4 h p.i.. We conclude that the pronounced down regulation at 30 min p.i. points to specific functions in responding to physiologically active BCG rather than effects being relevant to mechanisms of uptake. None of the above mentioned lncRNA has been reported in the context of

mycobacterial infection. However, recent studies have shown that herein investigated and dysregulated lncRNA are associated with IFN-γ signalling, NF-κB, apoptosis and autophagy [17,37,38]. NTT was 7-fold down regulated after infection. NTT is a single-copy gene residing in chromosome 6q23-q24, close to the IFN-γ receptor gene and its transcripts were found in activated, but not resting T cells [39]. NTT has been suggested to act as a regulatory RNA by activating PKR, which induces the degradation of I-κBβ with subsequent NF-κB activation [37]. PRINS showed 9-fold decreased expression in BCG infected macrophages. PRINS has been reported to regulate the antiapoptotic effects of the interferon-inducible gene IFI6 in psoriasis [40]. TMEVPG1 was 4-fold down regulated and is located in a cluster of cytokine genes including IFN-γ. TMEVPG1 transcription is dependent upon NF-κB while it contributes to IFN-γ expression as part of the Th1 differentiation program [41,42]. AS UCHL1 (antisense UCHL1) showed consistent down regulation at 30 min as well as at 4 h p.i.. UCHL1 will be a hot candidate for our future research, since it is the antisense product of the gene UCHL1 and its dysregulation has been related to defects in autophagy in diabetes mellitus [43].

BCG infection caused effective down regulation of MEG3, which is a tumour suppressor gene located in chromosome 14q32 that has been associated with carcinogenesis [44,45]. MEG3 is down regulated in case of human tumours including hepatocellular cancers, meningiomas, bladder cancer and gastric cancer [17,46-48]. The reason behind loss of MEG3 expression in tumours has been linked to hyper methylation of the promoter or differentially methylated regions upstream of the MEG3 gene [44,46]. There are no reports about involvement of MEG3 in any case of infection. However, decreased expression of MEG3 has been reported to activate autophagy in bladder cancer cells [17]. It is noteworthy to mention that several *M. tuberculosis* products, such as PI3P phosphatase SapM, glycosylated phosphatidylinositol-mimic LAM and phosphatidylinositol mannoside (PIM) have been reported to be able to suppress PI3K signalling and to counteract autophagy [49]. In line with this, BCG has been shown to oppose and inhibit induction of autophagy in macrophages while *M. smegmatis* was devoid of this characteristic [50]. It will be interesting to examine whether mycobacterial products are capable of directly manipulating cellular MEG3 levels. Induction of autophagy by rapamycin mediated inhibition of mTOR signalling has been shown to unblock phagosomal maturation in *M. tuberculosis* and BCG infection [31]. More recent studies have reported IFN-γ and vitamin D to be needed *in vivo* for Beclin 1 dependent autophagy and antimycobacterial activity of macrophages [19,20]. Beclin1 is a key regulator of autophagy and an interface between apoptosis and autophagy. CASP3 mediates the cleavage of Beclin1 and produced fragments are devoid of autophagy inducing capacities. However, the C-terminal cleavage product seems to be capable of triggering the release of proapoptotic factors [21]. Consequently, we tested for activation of executioner CASP3 to ensure apoptosis was not triggered. On one hand, IFN-γ induced autophagy in infected macrophages was associated with sustained down regulation of MEG3 over 24 h, while 3-MA partly compensated for the dysregulation. The absence of IFN-γ involved early decreased MEG3 expression, which was restored back to normal over 24 h pointing to a potential counter regulation by BCG. On the other hand, IFN-γ treatment along with BCG infection efficiently compromised p70-S6K (Thr389) phosphorylation indicating mTOR inactivation.

Specific knockdown of MEG3 had no effect on LC3B transcription. Therefore, increasing number of LC3A/B punctae per cell is unlikely to depend on MEG3 mediated expressional changes of LC3A/B. In line with this, increased LC3A/B conversion was observed after MEG3 knockdown in both infected and non infected macrophages. However, RNAi caused no obvious degradation of p62 in macrophages. In MEG3 knockdown cells the ratio of LC3A/B-II to LC3A/B-I increases. This can occur because of increased formation of autophagosomes, or decreased degradation of autophagosomal material in lysosomes. As shown in figure 5 d, the levels of p62, the autophagy substrate and adaptor for intracellular bacteria, seem to increase in MEG3 siRNA treated cells. This would be consistent with a block in lysosomal degradation and not an increase in autophagosome formation. Phosphorylation of p70-S6K (Thr389) was subtly different in non infected cells. It has been reported that p62 associates with Rags in lysosomes and is required both for the interaction of mTOR with Rag and for translocation of the mTORC1 complex to the lysosome, which is important for mTOR activation [51]. Inhibition of mTOR on the other hand induces autophagy [52]. Taken together, we conclude that MEG3 seems to block lysosomal degradation and has limited effects on mTOR activation. Our results show that IFN-γ mediated induction of autophagy in BCG infection happens in a MEG3 dependent manner. However, other lncRNA may have cooperative effects on autophagy and mTOR activation and will be a focus of our future research. Furthermore, repression of the lncRNA MEG3 results in enhanced eradication of intracellular mycobacteria. We speculate that in absence of IFN-γ, intracellular BCG are able to counter regulate early MEG3 dysregulation resulting in inhibition of autophagy. Presence of IFN-γ, on the other hand, allows for induction of autophagy by sustained MEG3 down regulation reinforcing the eradication of mycobacteria in infected macrophages.

Methods

Bacterial cultivation, cell culture and RNA isolation

M. bovis BCG (DSMZ No. DSM 43990) was grown as described previously [53]. For microscopy, BCG was fluorescently labelled with 5-(6)-carboxyfluorescein-succinylester (Sigma Aldrich) as described previously [54].

Human acute monocytic leukaemia cell line THP-1 (DSMZ No. ACC 16), human cervix carcinoma cell line HeLa (ATCC No. CCL-2), human monocytic cell line U937 (DSMZ No. ACC 5), human type II pneumocyte cell line A549.1 (ATCC No. CCL 185), human colorectal adenocarcinoma cells HT-29 (DSMZ No.: ACC 299) and its sub clone HT-29/B6 [55] were cultured as described previously [5,53,56,57] or following the protocols for nucleofection (Lonza). Total RNA from cell lines was isolated and quality controlled as described earlier [58].

Selection of lncRNA, oligonucleotides and RT-qPCR

A total of 17 known human lncRNA molecules were chosen from the lncRNAdb [23] by using search strings to extract entries with immune related functions. lncRNA sequences were

acquired from the same database. We also included a set of 5 non-coding as well as conventional reference mRNA for normalisation (table 1). All oligonucleotides were synthesised by Sigma Aldrich. A list of lncRNA including primer sequences and amplicon sizes is given in table1.

The cDNA was synthesised from individual samples using the Maxima First Strand cDNA synthesis kit (Thermo Scientific). Pooled cDNA was taken as template for testing the primers listed in table 1 via gradient RT-qPCR. Amplicons showing the expected size were isolated and sequenced to ensure specificity. Serial dilutions of cDNA pools spiked with specific amplicons (3 µM to 3 fM) were used to determine the dynamic range, efficiency, slope, Y-intersect and R^2 of lncRNA RT-qPCRs (table 1). Expression analysis was performed by means of SYBR Green detection chemistry using the SensiMix SYBR Hi-ROX Kit (Bioline GmbH) and the StepOnePlus Cycler (Life Technologies). All reactions were carried out using clear MicroAmp® Fast 96-Well Reaction Plates (Life Technologies) that were sealed with adhesive films. Real-time amplification was carried out via the first step at 95°C for 10 min, followed by 40 cycles with 15 s at 95°C, 20 s at 60°C and 30 s at 72°C. The fluorescence signal was acquired at 60°C. Quality control was performed by subsequent melting curve analysis by 95°C for 15 s, 60°C for 1 min, ramping from 60°C to 95°C at 5°C/min and acquiring the signal. RT-qPCR experiments and analysis of data were performed according to the MIQE guidelines [59].

Individual expression analysis of lncRNA was examined in 10 µl final reaction volume. Triplicate measurements were performed using a master mix containing 2x SensiMix SYBR Hi-ROX with 0.1 µM of each forward and reverse primer. After dispensing 9 µl of master mix in respective sample wells in a 96 well plate, 1 µl of a 1:5 dilution of respective RT-reaction was added. The amplification cycles and melting curves were carried out as mentioned above. Samples containing water instead of RT-reaction served as negative controls. Expression of lncRNA in each cell line was compared with a common reference composed of an *ana partes aequales* pool of all investigated RNA samples.

Arrayed RT-qPCR of lncRNA along with reference RNA was performed based on the same mentioned principle. A master mix was prepared in a 675 µl volume consisting of 2x SensiMix SYBR Hi-ROX and 20 µl of non diluted RT-reaction. After dispensing 9 µl of the master mix in wells of a 96 well plate, 1 µl of a 2 µM primer pool consisting of the respective lncRNA specific forward and reverse primer was added to corresponding wells of the array. The amplification cycles and melting curves were carried out as mentioned above. Arrayed quantification was performed in triplicate.

Normalisation of lncRNA expression was performed with a set of reference RNA using the geNorm algorithm [30]. A first round of geNorm analysis resulted in the selection of 10 most stable targets that were subjected to a second round of geNorm analysis to define the most stable 3. Relative quantification (ΔΔCq) was determined after calculation of the difference

between $\Delta Cq_{\text{ sample}}$ and $\Delta Cq_{\text{ calibrator}}$. Fold differences were determined and corrected for efficiency of reactions by calculating $\left(1 + \frac{efficiency\,[\%]}{100}\right)^{-\Delta\Delta Cq}$.

Infection experiments

Monocytic THP-1 cells were seeded without gentamycin in 6 well plates at a density of 1 x 10^6 cells per well. Cells were differentiated into macrophages by adding 10 nM phorbol-12-myristate-13-acetate (PMA, Sigma Aldich). PMA was removed after 24 h of incubation by washing the cells with warm PBS three times. Cells were incubated for another 24 h in RPMI 1640 supplemented with FBS Superior (Biochrom). Afterwards, macrophages were infected with viable as well as hk BCG at a multiplicity of infection (MOI) of 10. Synchronised infections [60] were performed by cooling down to 4°C for 10 min followed by addition of mycobacteria and another incubation at 4°C for 10 min. Afterwards, cells were immediately transferred to the incubator and warmed up to 37°C and 5% CO_2. Non infected cells were treated equally and served as a control. Samples were taken at 30 min and 4 h for RT-qPCR. For immunostaining, cells were infected as described earlier [53] and samples were taken at 24 h. All experiments were performed at least in triplicate.

Induction and inhibition of autophagy

3-MA (R & D Systems) is an inhibitor of PI3K. 3-MA inhibits autophagy by blocking autophagosome formation via the inhibition of PI3KC3. 3-MA was resuspended in water because organic solvents such as DMSO display cytotoxicity. Due to its instability in aqueous solutions, 50 mM stocks were prepared freshly; cells were treated with 5 mM 3-MA to inhibit autophagy. Same final water concentrations were used for negative controls. 200 U/ml IFN-γ (Sigma Aldrich) was also dissolved in water and used as an inducer of autophagy in mycobacterial infection experiments [61]. The inhibitor and inducer of autophagy or combinations thereof were added to the cells 24 h ahead of infection (as well as during infection) and samples for RT-qPCR or immunostaining were taken at indicated time points. All experiments were performed at least in triplicate. Expression level of Beclin1 mRNA was observed by means of RT-qPCR (5'-caggagagacccaggaggaa-3' and 5'-gctgttggcactttctgtgg-3').

Autophagy was followed in infected cells by means of immunostaining along with fluorescently labelled BCG. For this purpose, THP-1 derived macrophages were fixated with 4% Roti-Histofix (Carl Roth) and immunostaining was performed as described previously [62] using a 1:100 dilution of the antibody LC3A/B (D3U4C) XP® Rabbit mAb (Cell Signaling Technologies) together with the secondary antibody Goat-anti-Rabbit, DyLight 594, #35560 (Thermo Scientific) using a 1:200 dilution. Caspase 3 activation was studied as described previously [62]. Microscopic photographs were taken with a fluorescence inverted microscope DMI6000 B (Leica). DAPI staining (Sigma-Aldrich, Phalloidin Atto 488) was used to stain

nuclei. 3D Deconvolution blind method was used with total iterations of 10, refractive index 1.52 and remove background checked. Experiments were repeated three times.

MEG3 knockdown and detection of autophagy

For knockdown of MEG3, siRNA were custom designed and synthesised by Sigma Aldrich (5'-gacuuaaaccaaugcccua-3'). 10^6 THP-1 cells were transfected using the Nucleofector Technology (Lonza) together with 100 pmol siRNA and Kit V, respectively. Experiments were repeated at least three times. RNA samples were taken after 24h of incubation. Along with MEG3 quantification, mRNA expression of LC3B (5'-ccgcaccttcgaacaaagag-3' and 5'-ttgagctgtaagcgccttct-3') was also measured in the samples.

For infection experiments, 24 h post nucleofection THP-1 cells were incubated for 1 h with BCG at MOI of 10. Afterwards, non-phagocytised BCG were washed and cells were further incubated for 24 h. THP-1 were lysed in 500 µl ultra pure water and 100 µl of 10^{-2} diluted lysates were plated in triplicate on middlebrook agar plate for bacterial counting as described earlier [63].

For detection of autophagy after MEG3 knockdown, THP-1 cells were washed with PBS and lysed in RIPA buffer supplemented with protease inhibitor cocktail (Cell Signaling Technology #5871S). If needed, 100nM Bafilomycin A1 (TOCRIS bioscience) was added 24 h after transfection and samples were taken after 3 hours. Protein concentrations were measured using a protein assay kit (2D quant, GE Healthcare). Cell lysates (10–15 µg protein/lane) were separated by SDS-PAGE (13.5% gels) and transferred to 0.45 µm polyvinylidene difluoride membranes (PVDF) (GE Healthcare) as described previously [64]. The membrane was blocked by blocking buffer (5% skim milk powder in TBST) for 60 min at room temperature and then incubated with rabbit anti-LC3A/B (1:1000, Cell Signaling Technology, 12741S) or rabbit anti-p62 (1:1000, Cell Signaling Technology, 5114S) overnight. mTOR activation was studied using the antibodies pan-P70-S6K (1:1000, Cell Signaling Technology #49D7), pho-P70-S6K (1:1000, Ser371, Cell Signaling Technology #9208), pho-P70-S6K (1:1000, Thr389, CST #108D2). After proper washing, membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2000, Cell Signaling Technology), and chemiluminescence was detected using western blotting reagents (Cell Signaling Technology). After detection of LC3A/B or p62, membrane was stripped and again processed for GAPDH detection by using primary mouse mAb anti-GAPDH (1:10000, Abcam, ab8245) antibody and sheep anti-mouse secondary antibody (1:20000, GE Healthcare). Experiments were repeated at least three times.

For counting LC3 punctae, at least 3 randomly selected microscopic photographs of each biological replicate were analysed using ImageJ 1.48v [65]. Threshold value was adjusted carefully for strong autophagy signal and a particle size of 10-60 pixel^2 for 1392 x 1040 resolution photograph was taken into consideration. Data is represented as average LC3 punctae per cell. An average of 12 cells per section were counted. For quantification of

cellular bacterial loads after MEG3 knockdown, THP-1 cells were transfected as described above. After 24 h of incubation, they were infected with fluorescently labelled BCG at MOI of 10 as described above. After fixation, microphotographs were taken and the number of BCG per cell was counted manually. Triplicate experiments were performed. To prove the fluorescence based generation of bacterial counts, infected macrophages (three biological replicates) were lysed and CFU/ml were determined as described earlier [63].

Statistical and *in silico* analysis

Unpaired t tests were conducted applying GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Asterisks in figures summarise P values (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$).

Heatmap and hierarchical clustering analysis were performed using the MultiExperiment Viewer (MeV) from the TM4 software package [66]. For this purpose, the fold differences of means (triplicate RT-qPCR measurements) were calculated and were subjected to MeV analysis. *In silico* analysis of lncRNA networks was performed by retrieving CLIP-Seq data based prediction of lncRNA-miRNA interactions using starBase v2.0 [24]. Networks were extended by target analysis of miRNA lists using the CyTargetLinker [26] together with Cytoscape [25] and ClueGO analysis [28] annotated functionally grouped gene ontologies and pathways. Following settings were considered for ClueGo analysis: significant KEGG pathways [29] enrichment ($P < 0.05$), GO Term/Pathway Selection (3/4%), Kappa Score ≥ 0.3 and GO Term grouping.

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Author contributions

SS conceived the study and experiments. KP and CH performed experiments. SS, KP and SEPV analysed the data. SS and RE contributed reagents, materials and analysis tools. SS and KP wrote and edited the manuscript.

Competing financial interests

The authors declare no competing financial interests.

References

1 Orme, I. M. Vaccine development for tuberculosis: current progress. *Drugs* 73, 1015-1024, doi:10.1007/s40265-013-0081-8 (2013).

2 Birney, E. *et al.* Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature* 447, 799-816, doi:10.1038/nature05874 (2007).

3 Huntzinger, E. & Izaurralde, E. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet* 12, 99-110 (2011).

4 Rajaram, M. V. *et al.* Mycobacterium tuberculosis lipomannan blocks TNF biosynthesis by regulating macrophage MAPK-activated protein kinase 2 (MK2) and microRNA miR-125b. *Proc Natl Acad Sci U S A* 108, 17408-17413 (2011).

5 Sharbati, J. *et al.* Integrated MicroRNA-mRNA-Analysis of Human Monocyte Derived Macrophages upon Mycobacterium avium subsp. hominissuis Infection. *PloS one* 6, e20258 (2011).

6 Bertone, P. *et al.* Global identification of human transcribed sequences with genome tiling arrays. *Science* 306, 2242-2246, doi:10.1126/science.1103388 (2004).

7 Heward, J. A. & Lindsay, M. A. Long non-coding RNAs in the regulation of the immune response. *Trends in immunology* 35, 408-419, doi:10.1016/j.it.2014.07.005 (2014).

8 Marques, A. C. & Ponting, C. P. Catalogues of mammalian long noncoding RNAs: modest conservation and incompleteness. *Genome Biol* 10, R124, doi:10.1186/gb-2009-10-11-r124 (2009).

9 Pang, K. C., Frith, M. C. & Mattick, J. S. Rapid evolution of noncoding RNAs: lack of conservation does not mean lack of function. *Trends in Genetics* 22, 1-5, doi:DOI 10.1016/j.tig.2005.10.003 (2006).

10 Billerey, C. *et al.* Identification of large intergenic non-coding RNAs in bovine muscle using next-generation transcriptomic sequencing. *BMC genomics* 15, doi:Doi 10.1186/1471-2164-15-499 (2014).

11 Carpenter, S. *et al.* A Long Noncoding RNA Mediates Both Activation and Repression of Immune Response Genes. *Science* 341, 789-792, doi:DOI 10.1126/science.1240925 (2013).

12 Peng, X. *et al.* Unique signatures of long noncoding RNA expression in response to virus infection and altered innate immune signaling. *mBio* 1, doi:10.1128/mBio.00206-10 (2010).

13 Cech, T. R. & Steitz, J. A. The noncoding RNA revolution-trashing old rules to forge new ones. *Cell* 157, 77-94, doi:10.1016/j.cell.2014.03.008 (2014).

14 Rinn, J. L. lncRNAs: linking RNA to chromatin. *Cold Spring Harbor perspectives in biology* 6, doi:10.1101/cshperspect.a018614 (2014).

15 Salmena, L., Poliseno, L., Tay, Y., Kats, L. & Pandolfi, P. P. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? *Cell* 146, 353-358, doi:10.1016/j.cell.2011.07.014 (2011).

16 Bosson, A. D., Zamudio, J. R. & Sharp, P. A. Endogenous miRNA and target concentrations determine susceptibility to potential ceRNA competition. *Molecular cell* 56, 347-359, doi:10.1016/j.molcel.2014.09.018 (2014).

17 Ying, L. *et al.* Downregulated MEG3 activates autophagy and increases cell proliferation in bladder cancer. *Molecular bioSystems* 9, 407-411, doi:10.1039/c2mb25386k (2013).
18 Mizushima, N., Yoshimori, T. & Ohsumi, Y. The role of Atg proteins in autophagosome formation. *Annual review of cell and developmental biology* 27, 107-132, doi:10.1146/annurev-cellbio-092910-154005 (2011).
19 Bradfute, S. B. *et al.* Autophagy as an immune effector against tuberculosis. *Current opinion in microbiology* 16, 355-365, doi:10.1016/j.mib.2013.05.003 (2013).
20 Fabri, M. *et al.* Vitamin D is required for IFN-gamma-mediated antimicrobial activity of human macrophages. *Science translational medicine* 3, 104ra102, doi:10.1126/scitranslmed.3003045 (2011).
21 Wirawan, E. *et al.* Caspase-mediated cleavage of Beclin-1 inactivates Beclin-1-induced autophagy and enhances apoptosis by promoting the release of proapoptotic factors from mitochondria. *Cell death & disease* 1, e18, doi:10.1038/cddis.2009.16 (2010).
22 Zhu, Y. *et al.* Beclin 1 cleavage by caspase-3 inactivates autophagy and promotes apoptosis. *Protein & cell* 1, 468-477, doi:10.1007/s13238-010-0048-4 (2010).
23 Amaral, P. P., Clark, M. B., Gascoigne, D. K., Dinger, M. E. & Mattick, J. S. lncRNAdb: a reference database for long noncoding RNAs. *Nucleic Acids Res* 39, D146-151, doi:10.1093/nar/gkq1138 (2011).
24 Li, J. H., Liu, S., Zhou, H., Qu, L. H. & Yang, J. H. starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. *Nucleic Acids Res* 42, D92-97, doi:10.1093/nar/gkt1248 (2014).
25 Cline, M. S. *et al.* Integration of biological networks and gene expression data using Cytoscape. *Nature protocols* 2, 2366-2382 (2007).
26 Kutmon, M., Kelder, T., Mandaviya, P., Evelo, C. T. & Coort, S. L. CyTargetLinker: a cytoscape app to integrate regulatory interactions in network analysis. *PloS one* 8, e82160, doi:10.1371/journal.pone.0082160 (2013).
27 Lewis, B. P., Burge, C. B. & Bartel, D. P. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120, 15-20 (2005).
28 Bindea, G. *et al.* ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics (Oxford, England)* 25, 1091-1093 (2009).
29 Kanehisa, M., Goto, S., Furumichi, M., Tanabe, M. & Hirakawa, M. KEGG for representation and analysis of molecular networks involving diseases and drugs. *Nucleic Acids Res* 38, D355-360 (2010).
30 Sharbati, S., Sharbati, J., Hoeke, L., Bohmer, M. & Einspanier, R. Quantification and accurate normalisation of small RNAs through new custom RT-qPCR arrays demonstrates Salmonella-induced microRNAs in human monocytes. *BMC genomics* 13, 23 (2012).
31 Gilchrist, M. *et al.* Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. *Nature* 441, 173-178, doi:10.1038/nature04768 (2006).

32 Seto, S., Tsujimura, K. & Koide, Y. Rab GTPases regulating phagosome maturation are differentially recruited to mycobacterial phagosomes. *Traffic* 12, 407-420, doi:10.1111/j.1600-0854.2011.01165.x (2011).

33 Beatty, W. L. & Russell, D. G. Identification of mycobacterial surface proteins released into subcellular compartments of infected macrophages. *Infect Immun* 68, 6997-7002 (2000).

34 Vandesompele, J. *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3, RESEARCH0034 (2002).

35 Gutierrez, M. G. *et al.* Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell* 119, 753-766, doi:10.1016/j.cell.2004.11.038 (2004).

36 Klionsky, D. J. *et al.* Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* 8, 445-544 (2012).

37 Derrien, T. *et al.* The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. *Genome research* 22, 1775-1789, doi:10.1101/gr.132159.111 (2012).

38 Kozera, B. & Rapacz, M. Reference genes in real-time PCR. *J Appl Genet* 54, 391-406, doi:10.1007/s13353-013-0173-x (2013).

39 Fitzgerald, K. A. & Caffrey, D. R. Long noncoding RNAs in innate and adaptive immunity. *Current opinion in immunology* 26, 140-146, doi:10.1016/j.coi.2013.12.001 (2014).

40 Kurokawa, R., Rosenfeld, M. G. & Glass, C. K. Transcriptional regulation through noncoding RNAs and epigenetic modifications. *RNA biology* 6, 233-236 (2009).

41 Delgado Andre, N. & De Lucca, F. L. Non-coding transcript in T cells (NTT): antisense transcript activates PKR and NF-kappaB in human lymphocytes. *Blood cells, molecules & diseases* 40, 227-232, doi:10.1016/j.bcmd.2007.08.005 (2008).

42 Szegedi, K. *et al.* Expression and Functional Studies on the Noncoding RNA, PRINS. *International journal of molecular sciences* 14, 205-225, doi:10.3390/ijms14010205 (2012).

43 Liu, A. Y., Torchia, B. S., Migeon, B. R. & Siliciano, R. F. The human NTT gene: identification of a novel 17-kb noncoding nuclear RNA expressed in activated CD4+ T cells. *Genomics* 39, 171-184, doi:10.1006/geno.1996.4463 (1997).

44 Szegedi, K. *et al.* The anti-apoptotic protein G1P3 is overexpressed in psoriasis and regulated by the non-coding RNA, PRINS. *Experimental dermatology* 19, 269-278, doi:10.1111/j.1600-0625.2010.01066.x (2010).

45 Collier, S. P., Collins, P. L., Williams, C. L., Boothby, M. R. & Aune, T. M. Cutting edge: influence of Tmevpg1, a long intergenic noncoding RNA, on the expression of Ifng by Th1 cells. *J Immunol* 189, 2084-2088, doi:10.4049/jimmunol.1200774 (2012).

46 Collier, S. P., Henderson, M. A., Tossberg, J. T. & Aune, T. M. Regulation of the Th1 Genomic Locus from Ifng through Tmevpg1 by T-bet. *J Immunol* 193, 3959-3965, doi:10.4049/jimmunol.1401099 (2014).

47 Costes, S., Gurlo, T., Rivera, J. F. & Butler, P. C. UCHL1 deficiency exacerbates human islet amyloid polypeptide toxicity in beta-cells: evidence of interplay between the ubiquitin/proteasome system and autophagy. *Autophagy* 10, 1004-1014, doi:10.4161/auto.28478 (2014).
48 Benetatos, L., Vartholomatos, G. & Hatzimichael, E. MEG3 imprinted gene contribution in tumorigenesis. *Int J Cancer* 129, 773-779, doi:10.1002/ijc.26052 (2011).
49 Zhou, Y., Zhang, X. & Klibanski, A. MEG3 noncoding RNA: a tumor suppressor. *Journal of molecular endocrinology* 48, R45-53, doi:10.1530/JME-12-0008 (2012).
50 Anwar, S. L. *et al.* Loss of Imprinting and Allelic Switching at the DLK1-MEG3 Locus in Human Hepatocellular Carcinoma. *PloS one* 7, doi:DOI 10.1371/journal.pone.0049462 (2012).
51 Balik, V. *et al.* MEG3: a novel long noncoding potentially tumour-suppressing RNA in meningiomas. *Journal of neuro-oncology* 112, 1-8, doi:10.1007/s11060-012-1038-6 (2013).
52 Sun, M. *et al.* Downregulated long noncoding RNA MEG3 is associated with poor prognosis and promotes cell proliferation in gastric cancer. *Tumor Biol* 35, 1065-1073, doi:DOI 10.1007/s13277-013-1142-z (2014).
53 Deretic, V. Autophagy, an immunologic magic bullet: Mycobacterium tuberculosis phagosome maturation block and how to bypass it. *Future microbiology* 3, 517-524, doi:10.2217/17460913.3.5.517 (2008).
54 Zullo, A. J. & Lee, S. Mycobacterial induction of autophagy varies by species and occurs independently of mammalian target of rapamycin inhibition. *J Biol Chem* 287, 12668-12678, doi:10.1074/jbc.M111.320135 (2012).
55 Duran, A. *et al.* p62 is a key regulator of nutrient sensing in the mTORC1 pathway. *Molecular cell* 44, 134-146, doi:10.1016/j.molcel.2011.06.038 (2011).
56 Jung, C. H., Ro, S. H., Cao, J., Otto, N. M. & Kim, D. H. mTOR regulation of autophagy. *FEBS letters* 584, 1287-1295, doi:10.1016/j.febslet.2010.01.017 (2010).
57 Sharbati-Tehrani, S., Meister, B., Appel, B. & Lewin, A. The porin MspA from Mycobacterium smegmatis improves growth of Mycobacterium bovis BCG. *Int J Med Microbiol* 294, 235-245 (2004).
58 Agerer, F., Waeckerle, S. & Hauck, C. R. Microscopic quantification of bacterial invasion by a novel antibody-independent staining method. *Journal of microbiological methods* 59, 23-32, doi:10.1016/j.mimet.2004.05.008 (2004).
59 Kreusel, K. M., Fromm, M., Schulzke, J. D. & Hegel, U. Cl- secretion in epithelial monolayers of mucus-forming human colon cells (HT-29/B6). *The American journal of physiology* 261, C574-582 (1991).
60 Bohmer, M., Sharbati, J., Zur Bruegge, J., Einspanier, R. & Sharbati, S. Structural Analysis of microRNA-Target Interaction by Sequential Seed Mutagenesis and Stem-Loop 3' RACE. *PloS one* 8, e81427, doi:10.1371/journal.pone.0081427 (2013).
61 Hoeke, L. *et al.* Intestinal Salmonella typhimurium Infection Leads to miR-29a Induced Caveolin 2 Regulation. *PloS one* 8, e67300, doi:10.1371/journal.pone.0067300 (2013).

62 Sharbati-Tehrani, S., Kutz-Lohroff, B., Bergbauer, R., Scholven, J. & Einspanier, R. miR-Q: a novel quantitative RT-PCR approach for the expression profiling of small RNA molecules such as miRNAs in a complex sample. *BMC molecular biology* 9, 34 (2008).
63 Bustin, S. A. *et al.* The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical chemistry* 55, 611-622 (2009).
64 Stewart, G. R., Patel, J., Robertson, B. D., Rae, A. & Young, D. B. Mycobacterial mutants with defective control of phagosomal acidification. *PLoS pathogens* 1, 269-278, doi:10.1371/journal.ppat.0010033 (2005).
65 Dutta, R. K., Kathania, M., Raje, M. & Majumdar, S. IL-6 inhibits IFN-gamma induced autophagy in Mycobacterium tuberculosis H37Rv infected macrophages. *The international journal of biochemistry & cell biology* 44, 942-954, doi:10.1016/j.biocel.2012.02.021 (2012).
66 Bruegge, J. Z. *et al.* Arcobacter butzleri induces a pro-inflammatory response in THP-1 derived macrophages and has limited ability for intracellular survival. *Int J Med Microbiol*, doi:10.1016/j.ijmm.2014.08.017 (2014).
67 Sharbati-Tehrani, S. *et al.* Porins limit the intracellular persistence of Mycobacterium smegmatis. *Microbiology* 151, 2403-2410 (2005).
68 Scholven, J. *et al.* Intestinal expression of TFF and related genes during postnatal development in a piglet probiotic trial. *Cell Physiol Biochem* 23, 143-156 (2009).
69 Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9, 671-675 (2012).
70 Saeed, A. I. *et al.* TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* 34, 374-378 (2003).

Publication 2

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Mycobacterium bovis BCG interferes with miR-3619-5p control of Cathepsin S in the process of autophagy

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Abstract

Main survival mechanism of pathogenic mycobacteria is to escape inimical phagolysosomal environment inside the macrophages. Many efforts have been made to unravel the molecular mechanisms behind this process. However, little is known about the involvement of microRNAs (miRNAs) in the regulation of phagolysosomal biosynthesis and maturation. Based on a bottom up approach, we searched for miRNAs that were involved in phagolysosomal processing events in the course of mycobacterial infection of macrophages. After infecting THP-1 derived macrophages with viable and heat killed *Mycobacterium bovis* BCG (BCG), early time points were identified after co-localization studies of the phagosomal marker protein LAMP1 and BCG. Differences in LAMP1 localization on the phagosomes of both groups were observed at 30 min and 4 h. After *in silico* based pre-selection of miRNAs, expression analysis at the identified time points revealed down-regulation of three miRNAs: miR-3619-5p, miR-637 and miR-324-3p. Consequently, most likely targets were predicted that were supposed to be mutually regulated by these three studied miRNAs. The lysosomal cysteine protease Cathepsin S (CTSS) and Rab11 family-interacting protein 4 (RAB11FIP4) were up-regulated and were considered to be connected to lysosomal trafficking and autophagy. Interaction studies verified the regulation of CTSS by miR-3619-5p. Down-regulation of CTSS by ectopic miR-3619-5p as well as its specific knockdown by siRNA affected the process of autophagy in THP-1 derived macrophages.

Keywords

microRNA, *Mycobacterium bovis* BCG, phagocytosis, Cathepsin S, miR-3619-5p, autophagy

Introduction

Mycobacteria consist of both highly pathogenic species e.g. *Mycobacterium tuberculosis* and opportunist species such as *M. avium*. Once entered into the host cell, most of the bacteria are trafficked into a series of increasingly acidified membrane-bound structures that lead to eradication of bacteria, by a process called phagosomal maturation in macrophages. However, pathogenic mycobacteria have the ability to survive in this harsh environment. Phagosomal maturation is divided into three different stages, which are early, intermediate and late that culminate with the formation of phagolysosomes (Flannagan et al., 2009). The phagosome acquires the proteins Rab5 and EEA on its membrane during early maturation stages, whereas LAMP1 and Rab7 are present on late maturation stages (Gorvel et al., 1991;Vieira et al., 2003;Flannagan et al., 2009). To understand mycobacterial survival strategies, a number of different proteins (e.g. Rab5, Rab7, EEA1, hVPS34 etc.) that are involved in phagosomal maturation have been studied on the surface of mycobacteria containing phagosomes (Gorvel et al., 1991;Christoforidis et al., 1999;Bucci et al., 2000;Fratti et al., 2001;Vieira et al., 2003). Studies have focused on how mycobacteria change phagosomal maturation to avoid its eradication (Vergne et al., 2004). Mycobacteria manipulate acidic environment of phagosomes by affecting several mechanisms. It has been shown that phagosomes

containing *M. tuberculosis* and *M. bovis* BCG (BCG) interact normally with early endosomes but fail to fuse with late endosomes and lysosomes (Sun et al., 2007). Moreover, it has been documented that mycobacteria interact with endosomal-lysosomal pathways (Clemens and Horwitz, 1995) but prevent the acidification of phagosomes to survive and multiply inside the host cells (Sturgill-Koszycki et al., 1994;Xu et al., 1994). Relative to phagosomes containing latex beads, BCG phagosomes have been shown to be depleted in LAMP2, NPC1, flotillin-1, vATPase, and syntaxin 3 (Lee et al., 2010). Beside these differences, novel proteins have been detected on BCG phagosomes but not on latex bead containing ones, which include CD44, ICAM-1, FAM3C, RALA/RALB, STIP1, EPB41L3, SEPT7, IQGAP1, Rab-6A, erlin-2, and Tpd52l2 (Lee et al., 2010). However, there are still gaps in understanding how mycobacteria interfere with endosomal trafficking and its regulation.

Apart from several other diseases, involvement of miRNAs in the immune response to pathogens provided allusion of their role in mycobacterial pathogenesis. When human macrophages were infected with virulent *M. tuberculosis,* high miR-125b and low miR-155 expression was observed compared to infection with the non-virulent *M. smegmatis* (Tili et al., 2007;Rajaram et al., 2011). BCG suppressed IFN-γ production by down-regulating miR-29 expression in IFN-γ-producing natural killer cells (Ma et al., 2011). An integrated miRNA-mRNA analysis of infected human macrophages provided insights into the cumulative impact of miRNA regulation during *M. avium* infection of human macrophages (Sharbati et al., 2011). BCG triggers TLR2-dependent miR-155 expression in macrophages, which involves signaling cross talk among PI3K, PKCδ and MAPKs, and recruitment of NF-κB and c-ETS to miR-155 promoter. This BCG-driven miR-155 expression acts as a crucial regulator of cell fate decisions of infected macrophages (Ghorpade et al., 2012). Intracellular survival of *M. tuberculosis* was decreased when mouse macrophages were transfected with miR-155 (Kumar et al., 2012). The reason might be because miR-155 promotes autophagy to eliminate intracellular mycobacteria by targeting RHEB, which is a negative regulator of autophagy (Wang et al., 2013).

Autophagy is a process where cells remove or recycle damaged and unwanted cytosolic components to provide amino acids during starvation. It also helps disposing microorganisms. An important marker for autophagy is LC3. After lipidation of the cytosolic form (LC3-I), LC3-II is recruited to autophagic membranes (Klionsky et al., 2007). In case of BCG infection, induction of autophagy by IFN-γ leads to engulfment of bacteria in autophagosomes and reduced viability in macrophages (Gutierrez et al., 2004). Autophagy, phagocytosis and endocytosis are interconnected cellular processes as phagosomal membranes have been shown to recruit LC3 in a TLR-dependent fashion (Sanjuan et al., 2007). TLR-induced association of autophagic components promotes phagosome–lysosome fusion. So, TLR signaling enhances phagosomal maturation and thereby pathogen defense coinciding with autophagy related pathways (Sanjuan et al., 2007). On a different note, few recent studies have suggested that inhibition of the lysosomal cysteine protease Cathepsin S (CTSS) affects autophagy in different cells (Chen et al., 2012;Huang et al., 2013;Zhang et al., 2014;Huang et al., 2015) as observed by enhanced LC3 conversion.

MiRNAs are considered to fine tune the regulation of gene expression and are involved in various processes in mycobacterial infection. Finding new miRNAs in mycobacterial infection gave new dimensions to study its pathogenesis, however few have focused on regulation of phagolysosomal processing and autophagy by miRNAs. The present study mainly focused on an approach to find miRNAs in phagolysosomal maturation during infection of THP-1 derived macrophages with viable and heat killed (HK) BCG and provided functional aspects to the regulated RNAs. Beside *in silico* predictions of relevant miRNAs, time points of investigation were identified, based upon presence or absence of LAMP1 as a marker for phogolysosome formation on HK and viable BCG phagosomes. After *in silico* prediction of binding partners, infection dependent dysregulation of predicted miRNAs as well as targets was examined and validated experimentally. In the end, functional relevance of a regulated miRNA was associated with the process of autophagy.

Materials and Methods

Bacterial cultivation, cell culture and infection experiments

Mycobacterium bovis BCG (DSMZ No. DSM 43990) was grown as previously described (Sharbati-Tehrani et al., 2004). For microscopy, BCG was fluorescently labeled with 5-(6)-carboxyfluorescein-succinylester (Sigma Aldrich) following an earlier described protocol (Agerer et al., 2004). The human acute monocytic leukemia cell line THP-1 (DSMZ ACC 16) was cultured in suspension using RPMI 1640 (Biochrom AG) supplemented with 10% fetal bovine serum superior (Biochrom AG) and gentamycin (zur Bruegge et al., 2014). Infection experiments were performed as described previously (Pawar et al., 2016).

LAMP1 detection and determining time points for RNA isolation

For selection of time points for RNA isolation, LAMP1 signal was observed during mycobacterial infection. In brief, differences in LAMP1 signal were observed on phagosome containing viable and HK mycobacteria and time points for RNA isolation were selected based on presence or absence of LAMP1 signal. To coordinate phagocytosis, cell and mycobacterial interaction was synchronized by incubating THP-1 derived macrophages at 4°C for 10 min without mycobacteria then at 4°C with mycobacteria for 10 min (Stewart et al., 2005). Afterwards, cells were incubated at 37°C in a CO_2 incubator and presence or absence of LAMP1 signal on phagosomal membranes was observed every 15 min after washing and fixing the cells in 4% Roti-Histofix (Carl Roth). Immunostaining was performed as described previously (Sun et al., 2007;Sharbati et al., 2015) using a 1:300 dilution of the antibody LAMP1 [1D4B] (ab25245) Rat mAb (Abcam) together with the secondary antibody Goat pAb to Rat IgG, Alexa Fluor 594 (ab150160) (Abcam) using a 1:300 dilution. Microscopic photographs were taken with an inverted fluorescence microscope DMI6000 B (Leica). Nuclei were counterstained using DAPI (Sigma-Aldrich). Consequently, 15 min, 30 min and 4 h were chosen for RNA isolation. Total RNA was isolated using the miRVana Isolation Kit (Life Technologies). The quality and quantity was controlled as described

previously (Sharbati et al., 2010).

Approach for selecting miRNAs and protein-coding genes

(1) The search for protein-coding genes involved in the pathways: Three pathways [Phagosome (ko04145), Lysosome (ko04142), and Tuberculosis (hsa05152)] from KEGG pathway database (Kanehisa and Goto, 2000;Kanehisa et al., 2014) were searched for protein-coding genes based on their direct involvement in the phagosomal maturation process and were backed up by literature (Supplementary Table 1). (2) miRNAs targeting the selected genes were chosen using miRmap (Vejnar and Zdobnov, 2012) possessing a score of 90 and above (Data Sheet 1). (3) Enriched miRNAs were selected with the help of the Galaxyproject (Blankenberg et al., 2010). Top 10 mostly enriched miRNAs were chosen for the study (Supplementary material 1, Table 2). (4) After finding regulated miRNAs, corresponding targets were again searched in miRmap using a stringent score of 99 and above (Data Sheet 2). (5) List of identified targets was loaded on the database for annotation, visualization and integrated discovery (DAVID) (Dennis et al., 2003) and genes were selected based on their involvement in phagosomal maturation related pathways [Lysosome (ko04142), Phagosome (ko04145), Endocytosis (ko04144), Antigen presentation (hsa04612)] from KEGG pathway classification in annotation summary result. Genes were selected for the expression study based on the criteria that at least two mutual miRNAs target one protein-coding gene with a miRmap score of 80 and above (Table 1).

Quantification of mRNA and miRNA by RT-qPCR

Quantification of mRNA as well as miRNA expression by means of RT-qPCR was performed as described earlier by triplicate measurement of individual samples (Sharbati-Tehrani et al., 2008;Sharbati et al., 2010;Sharbati et al., 2011). All experiments were performed according to MIQE guidelines (Bustin et al., 2009) and based on the ΔΔCq method (Livak and Schmittgen, 2001) following the protocols described previously (Sharbati et al., 2012;Pawar et al., 2016). For normalization of expression data, the stability of reference RNAs was tested using the geNorm algorithm (Vandesompele et al., 2002) and the geometric means of reference RNAs were calculated for normalization. For normalization of mRNA expression, GAPDH, B2M as well as ACTB were used, which all possessed stable expression. For normalization of miRNA expression, SNORD44 and SNORD52 were used as reference RNAs. The entire set of oligonucleotides used in this study is provided in the Supplementary Table 2. All oligonucleotides were synthesized by Sigma Aldrich.

RNAi Experiments, detection of autophagy and western blots

THP-1 cells ($1x10^6$) were transfected using the Nucleofector Technology (Lonza) together with 50 pmol miRNA mimic miR-3619-5p (5'-ucagcaggcaggcuggugcagc-3'), miR-637 (5'-acuggggcuuucgggcucugcgu-3') or 100 pmol of On-Targetplus human CTSS siRNA (SO-2494920G, Dharmacon) using Kit V (Lonza). After transfection, THP-1 cells were

differentiated into macrophages using 10 ng/ml phorbol-12-myristate-13-acetate (PMA, Sigma Aldich) and incubated for 24 h and RNA was isolated to observe expression level of RAB11FIP4 and CTSS. Knockdown of CTSS was evaluated by means of western blot experiments using a 1:500 dilution of rabbit anti-CTSS primary antibody (ab134157, Abcam) together with a 1:10000 dilution of the horseradish peroxidase-conjugated anti-rabbit secondary antibody (Cell Signaling Technology). Experiments were performed three times for statistical analysis. For detection of autophagy after transfection with miR-3619-5p mimics and CTSS siRNA, LC3A/B and p62 proteins were detected by means of western blotting as described earlier (Pawar et al., 2016). For counting LC3 puncta, at least six randomly selected microscopic photographs of each biological replicate were analyzed using ImageJ 1.48v (Schneider et al., 2012). Fluorescent staining for microscopy and method of analysis for counting LC3 puncta was done as described earlier (Pawar et al., 2016). To validate the autophagy signal produced in transfected experiment, another approach for detection of LC3 signal was used. THP-1 cells were co-transfected with miR-3619-5p and the reporter plasmid pEX-GFP-hLC3-WT (Addgene). Autophagy signal (number of LC3-GFP puncta) produced in above cells was compared with that of THP-1 cells co-transfected with nonsense control and pEX-GFP-hLC3-WT plasmid. Minimum of 15 autophagic cells were counted per biological replicate (n=3) in each group.

Reporter gene assay

Reporter gene assays were performed as described previously (Sharbati et al., 2011). HeLa cells were transfected using the Nucleofector Technology (Lonza). Nucleofection was performed with $5x10^5$ cells per transfection using 2 µg reporter plasmid (pTK-Gluc derivatives, NEB GmbH), 200 ng normalization plasmid (pTK-Cluc, NEB GmbH) and 100 pmol miRNA mimic according to the manufacturer's instructions. In brief, three miR-3619-5p target sites of human CTSS were identified (position 38, 1656 and 2477) and used as a single unit oligonucleotide – NotIhCTSS-ts-sense and XbaIhCTSS-ts-antisense (Supplementary Table 2) – that were hybridized and cloned in pTK-Gluc as described earlier (Hoeke et al., 2013). Endotoxin-free reporter plasmids (pTKGhCTSS) were produced for transfection.

Statistical analysis

Unpaired t tests were conducted to test significant differences between two treatments. Asterisks in figures summarize P values (*: $P < 0.05$). Statistical tests were conducted applying GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

Results

LAMP1 was absent on phagosomes containing viable BCG

Synchronized infection experiments were performed and LAMP1 signal (as a late phagosomal marker) was studied on viable and heat killed (HK) BCG containing phagosomes for up to 4 h post infection. There was no LAMP1 signal observed in initial period of time (at 15 min) in both types of infection (Figure 1). However, the difference in signals was observed after 30 min and onward. At 30 min and 4 h, LAMP1 signal was visible in HK BCG infection co-localizing with BCG and no equivalent signal was seen in viable BCG infection at 30 min and 4 h. Therefore, 30 min and 4 h were chosen representing the time points, where BCG actively manipulate endosomal trafficking. The time point 15 min served as an additional early control.

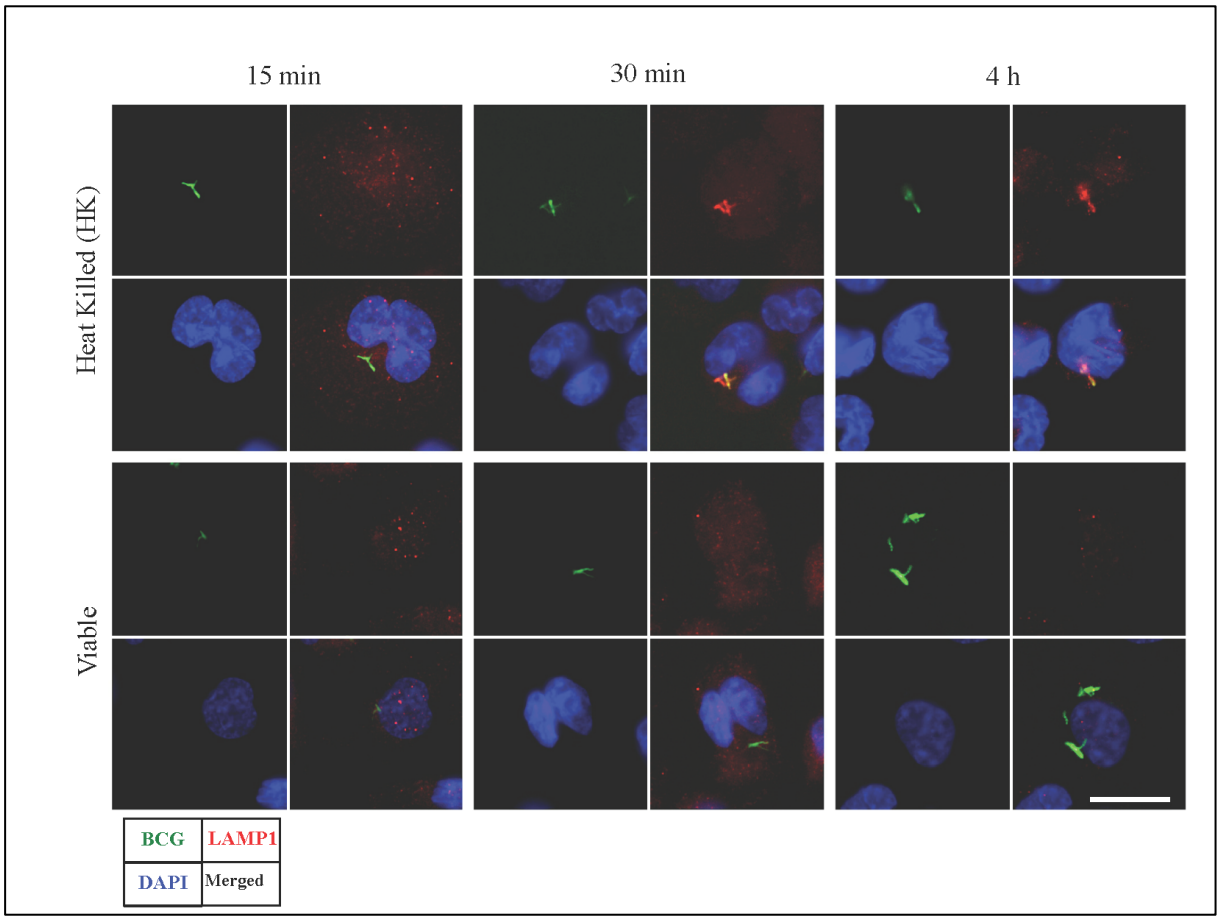

Figure 1: Detection of LAMP1 signal in macrophages infected with viable and heat killed (HK) *Mycobacterium bovis* BCG. Bacteria were stained with FITC (green fluorescent). At 15 min, no visible LAMP1 signal was observed that are co-localized with BCG in both viable and HK bacterial infection. At 30 min and 4 h, LAMP1 signal was visible in HK samples co-localizing with HK BCG, however no equivalent signal was seen in viable bacterial infection at 30 min and 4 h. Bar measures 25 micron.

Selection of interacting miRNAs and mRNAs by means of a bottom up approach

A total of 15 different proteins were selected based on their direct involvement in the phagosomal maturation process as well as on literature search. Selected protein-coding genes are shown in Supplementary Table 1. After searching miRNAs targeting each 15 different genes, lists of identified miRNAs were merged (resulted in a total of 1840 miRNAs) (Data Sheet 1). Out of 1840 miRNAs, few miRNAs were targeting more than one protein-coding gene. For example, miR-3619-5p, miR-4739 and miR-637 were predicted to target 6 different genes, whereas miR-324-3p, miR-4446-5p, miR-4690-5p, miR-4709-3p, miR-5006-5p, miR-654-5p and miR-761 were supposed to target 5 different genes. Remaining miRNAs targeted 4 or less different genes. Most enriched miRNAs (having 6 and 5 targets) were considered to have relevant functions for the study. So, top 10 most enriched miRNAs were chosen for infection based expression studies by means of RT-qPCR (Supplementary Table 2). Out of 10, three miRNAs (miR-3619-5p, miR-637 and miR-324-3p) were down-regulated compared to non-infected controls (Figure 2 A). Other 4 miRNAs (miR-4446-5p. miR-654-5p, miR-4709-3p, and miR-761) out of 10 were not regulated (Supplementary Figure 1 A) and 3 miRNAs (miR-5006-5p, miR-4690-5p and miR-637) were below the detection limit. miRNAs that were dysregulated, were used for further *in silico* analysis. Again highly potential targets of three dysregulated miRNAs (miR-3619-5p, miR-637 and miR-324-3p) were searched using miRmap with a score of 99 and above (Data Sheet 2). The predicted gene list was searched for protein-coding genes that were supposed to be mutually targeted by at least two miRNAs where the subsequent interactions had a miRmap score of 80 and above (Table 1). This resulted in identification of protein-coding genes (RAB11FIP4, PIP4K2B, RAB11FIP1, ZFYVE20, TRAF6 and CTSS) and their expressions were studied in same RNA samples that were used for miRNA expression studies helping to determine anti-correlated expression of supposed targets. Expression of other mutually targeted protein-coding genes (EHD3, IQSEC3, ARFGAP2 and GGA) was below detection limit.

miR-3619-5p, miR-637 and miR-324-3p and the proposed targets RAB11FIP4 and CTSS exhibited anti-correlated expressions

MiRNAs bind to respective mRNAs causing inter alia their degradation. In our experiments, down-regulation of 3 miRNAs miR-3619-5p, miR-637 and miR-324-3p was observed, and 2 mRNAs RAB11FIP4 and CTSS were up-regulated or at least showed a trend in up-regulation. Expression of miRNAs as well as mRNAs from same RNA samples is given in Figure 2 A and 2 B, respectively. Based on miRmap score analysis, all three miRNAs were supposed to target RAB11FIP4, however CTSS was predicted to be targeted by miR-3619-5p and miR-637, respectively. Therefore, we concentrated on studying the mutual targets RAB11FIP4 and CTSS of miR-3619-5p and miR-637. Consequently, transfection experiments with miR-3619-5p and miR-637 mimics were performed and cellular levels of both identified targets were examined. As shown in figure 2 C, only transfection of THP-1 with miR-3619-5p mimics resulted in significantly reduced levels of cellular CTSS and there was no effect of miR-3619-

5p on RAB11FIP4 and miR-637 on both CTSS and RAB11FIP4, respectively (Supplementary Figure 2).

Table 1: miRmap score of miRNAs targeting the genes

Name of gene	miR-3619-5p (miRmap score)	miR-637 (miRmap score)	miR-324-3p (miRmap score)
GIT1	15.19	13.98	99 and above
IQSEC3	**96.56**	**95.37**	**99 and above**
RAB11FIP1	**99.35**	**no target**	**99 and above**
RAB11FIP3	47.27	4.20	99 and above
RAB11FIP4	**99.82**	**99.97**	**99 and above**
ARRB1	57.01	27.47	99 and above
PIP5K1C	47.84	0.25	99 and above
PIP4K2B	**98.34**	**99.38**	**99 and above**
ZFYVE20	**90.99**	**No target**	**99 and above**
EHD3	**3.52**	**99 and above**	**96.15**
RAB11FIP4	**99.82**	**99 and above**	**99.80**
TRAF6	**82.54**	**99 and above**	**No target**
GGA1	40.33	99 and above	46.91
PIP4K2B	**98.34**	**99 and above**	**99.64**
ARFGAP2	**99 and above**	**89.29**	**No target**
RAB11FIP1	**99 and above**	**no target**	**99.64**
RAB11FIP4	**99 and above**	**99.97**	**99.80**
AP3B1	99 and above	no target	No target
CTSS	**99 and above**	**98.32**	**No target**
GGA3	**99 and above**	**95.94**	**No target**
PSD3	99 and above	no target	69.50

Bold fonts indicate "Protein-coding genes" that have at least two mutual miRNA targets with a miRmap score of 80 and above.

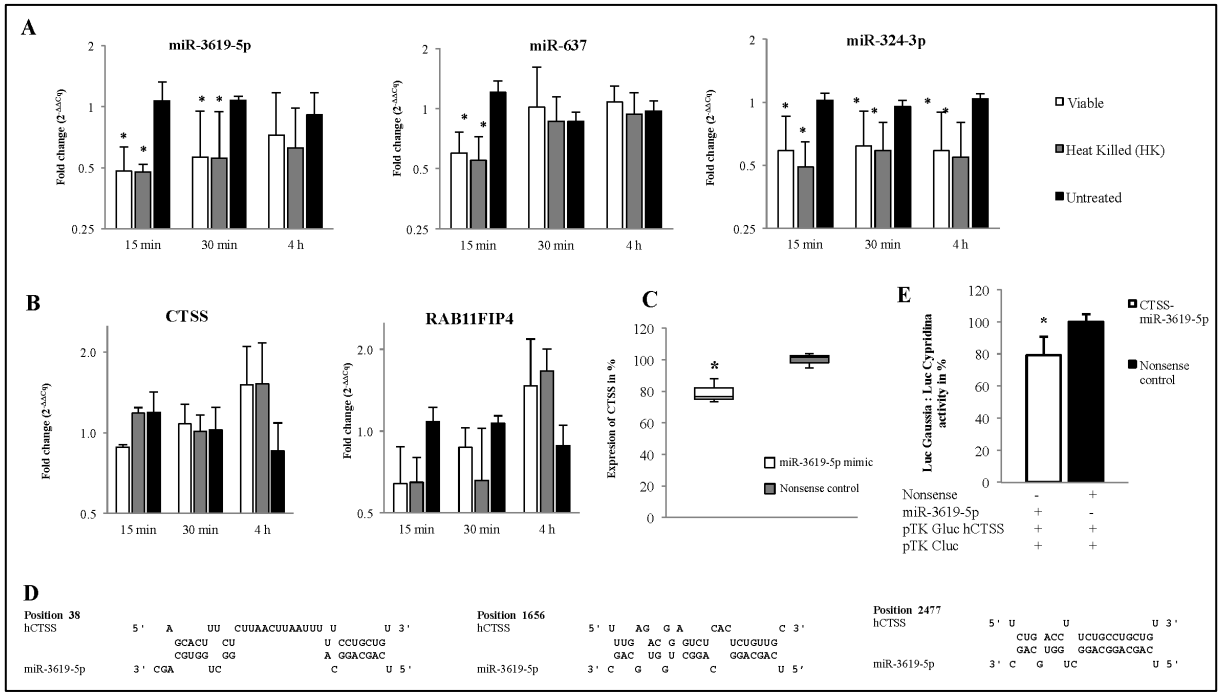

Figure 2: Expression of miRNAs and their predicted targets at 15 min, 30 min and 4 h in viable and heat killed (HK) mycobacterial infection to macrophages. (A) The miRNAs, miR-3619-5p and miR-324-3p were down-regulated in all time points whereas miR-637 was down-regulated only at initial period of time (15 min) in both viable and HK bacterial infections. (B) The predicted targets, CTSS and RAB11FIP4 were up-regulated in later period of time points (4 h) in both viable and HK bacterial infections. (C) Expression of CTSS in THP-1 cells was down-regulated after transfection with miR-3619-5p mimics. (D) Three different sites were found after screening the 3′ UTR region of CTSS for potential miR-3619-5p binding sites using RNAhybrid. (E) The interaction between miR-3619-5p and human CTSS was verified by means of reporter gene assays using HeLa cells. Identified target sites between miR-3619-5p and CTSS were analyzed using RNAhybrid. Relative luciferase activity (Luc Gaussia: Luc Cypridina) was determined respective to nonsense miRNA mimics.

The columns show means of three biological replicates each measured in triplicates while error bars show the standard deviation. Asterisks indicate statistical significance between samples (*: $P < 0.05$).

miR-3619-5p targets CTSS

Three different binding sites were found (Figure 2 D) after screening the 3′ UTR of CTSS for potential miR-3619-5p interaction using RNAhybrid (Kruger and Rehmsmeier, 2006). Calculated minimal free energies for interactions with miR-3619-5p were -31.2 kcal/mol, -22.1 kcal/mol and -17.1 kcal/mol, respectively (Figure 2 D). Reporter gene assays with identified binding sites in CTSS were performed (Supplementary Table 2). After co-transfection of miR-3619-5p mimic or nonsense controls together with the reporter plasmid, a significant reduction in luciferase activity ($Luc_{Gaussia}$: $Luc_{Cypridina}$) was measured for the interaction ($P < 0.05$, paired t test) compared to nonsense controls (Figure 2 E). This down-regulation (20%) of CTSS was comparable to the reduction of CTSS transcript levels in transfected THP-1 cells by miR-3619-5p mimic. This experiment proved the interaction between CTSS binding sites and miR-3619-5p to be specific.

miR-3619-5p as well as siRNA-mediated knockdowns of CTSS affect the process of autophagy

Ectopic expression of miR-3619-5p in THP-1 derived macrophages reduced the level of CTSS transcripts (Figure 2 C). As mentioned above, CTSS has been connected to autophagy. To test for functional effects of miR-3619-5p mediated CTSS regulation in macrophages; we examined the effect of miR-3619-5p transfection on autophagy. For this purpose, LC3A/B conversion was determined by means of western blots showing clearly increased LC3A/B-II levels after miR-3619-5p transfection compared with nonsense treated controls (Figure 3 A). To test if the observed effect is grounded in decreased cellular levels of CTSS, we used specific siRNA, which again enhanced conversion of LC3A/B (Figure 3 A). Western blots proved clearly decreased cellular levels of CTSS after both miR-3619-5p and siRNA transfection (Figure 3 A). The degradation of p62 is regarded to be a marker for progression of autophagy. Therefore, we examined cellular p62 levels after RNAi. Interestingly, there was no degradation of p62 after miR-3619-5p transfection and p62 even increased after specific knockdown of CTSS (Figure 3 A) indicating block of lysosomal processing after CTSS knockdown.

As shown in figure 3 B, the process of autophagy was also examined by immunofluorescence quantification of LC3A/B puncta. Significantly increased LC3 puncta were observed in mimic transfected cells compared to nonsense-transfected cells (Figure 3 B and C) as determined by ImageJ. Accumulation of LC3 puncta determined by immunofluorescence after transfection with miR3619-5p was confirmed by co-transfecting THP-1 derived macrophages with the GFP-LC3 reporter plasmid pEX-GFP-hLC3-WT (Tanida et al., 2008). Co-transfection of cells with miR-3619-5p and pEX-GFP-hLC3-WT produced increased number of GFP-LC3 puncta compared to co-transfected cells with nonsense controls and pEX-GFP-hLC3-WT (Figure 3 D). Significant difference in number of autophagy LC3 puncta was observed between two groups determined by ImageJ (Figure 3 E).

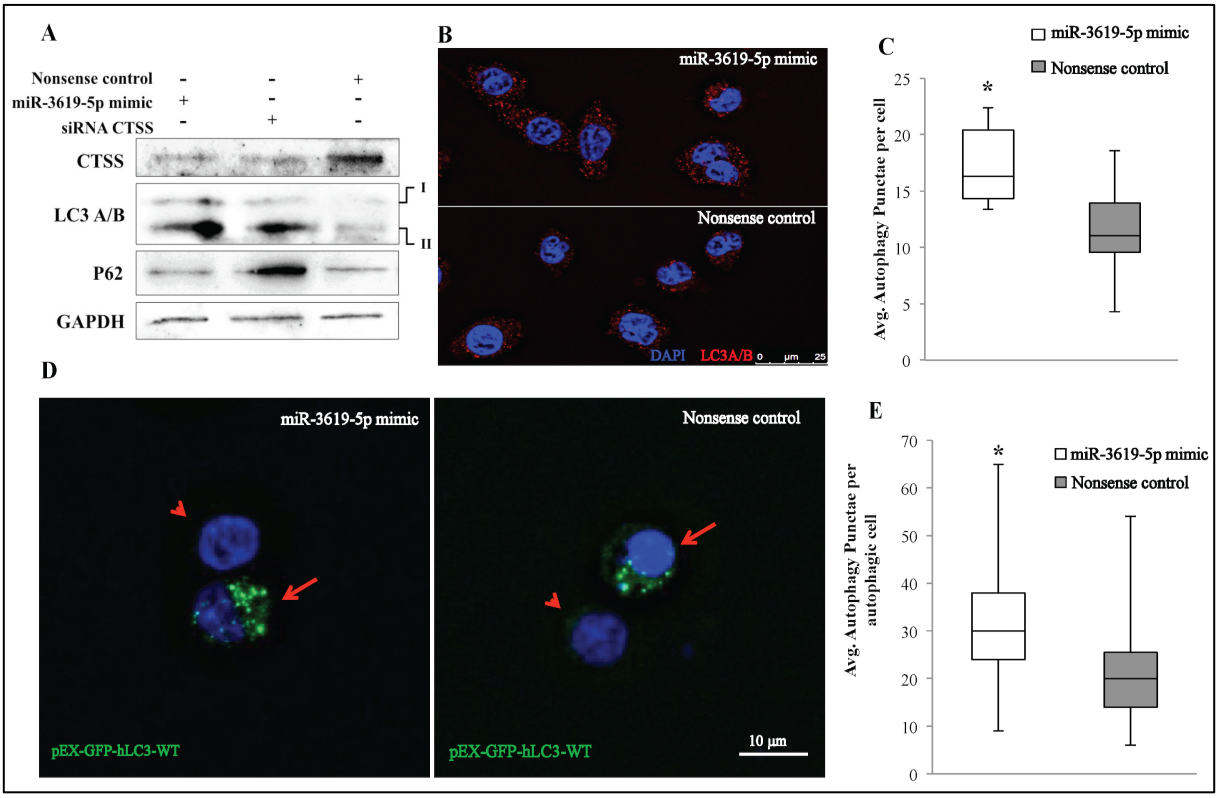

Figure 3: Detection of autophagy in THP-1 cells transfected with miR-3619-5p mimics. (A) Expression of CTSS in THP-1 cells was down-regulated after transfection with miR-3619-5p mimics as well as siRNA, which affected the process of autophagy observed by LC3A/B conversion on western blots. (B) LC3 autophagy puncta were observed under the microscope, (C) and were significantly increased in miR-3619-5p mimics transfected cells compared to nonsense controls as calculated by ImageJ. (D) LC3-GFP autophagy puncta were also observed with pEX-GFP-hLC3-WT plasmids. Co-transfecting cells with miR-3619-5p with pEX-GFP-hLC3-WT plasmid produced more autophagy GFP-LC3 puncta than that of co-transfected cells with nonsense control and pEX-GFP-hLC3-WT plasmid as observed under fluorescent microscope. Arrow indicates co-transfected cells and arrowhead indicates non-transfected population. (E) Significant differences in number of autophagic GFP-LC3 puncta was observed between two groups counted by ImageJ.

The columns show means of three biological replicates each measured in triplicates while error bars show the standard deviation. Asterisks indicate statistical significance between samples (*: $P < 0.05$).

Discussion

Phagosomal maturation goes along with changes in the protein composition of phagosomal membranes. Lysosomal fusion with phagosomes is an important stage in maturation, which is required for degradation or digestion of foreign particles as well as apoptotic bodies. Gaining LAMP1 protein on phagosomal membrane indicates phagosomal fusion with lysosomes. However, progression of phagosomal maturation fails when pathogenic mycobacterial species are phagocytized. In present investigation, based on knowledge of phagosomal maturation in mycobacterial infection, three different time points were selected, where absence and presence of LAMP1 proteins on phagosomes containing viable and HK BCG was observed. In this study, LAMP1 signal was noticeably present at 30 min, which co-localized with HK BCG. However, same signal was absent in case of viable BCG at 30 min and onwards. This indicates that phagosomes containing viable BCG failed to fuse with lysosomes compared to phagosomes containing HK BCG. As observed previously, wild-type BCG were associated with LAMP1-negative vesicles while BCG with inactivated protein serine/threonine kinase G (ΔpknG) co-localized predominantly with the lysosomal marker LAMP1 (Walburger et al., 2004). It was concluded that the mycobacterial protein serine/threonine kinase G (PknG) in BCG carries out the biological function in blocking lysosomal delivery of mycobacteria (Houben et al., 2009). In present case, heat killing of BCG might have inactivated PknG in BCG leading to fusion of phagosome containing HK BCG with lysosome. However, this finding was not in agreement with some other studies that showed BCG was associated with LAMP1 positive phagosomal compartments (Billeskov et al., 2010;Lee et al., 2010). Different bacterial strains or cell lines might be the cause for these differences. Primary aim of studying phagosomal LAMP1 was to select time points for RNA isolation during mycobacterial infection, which was used in a bottom up approach to select miRNAs that might be involved in the process of phagolysosomal trafficking. It was important to determine the exact time for RNA isolation to increase the chance for observing distinct differences in miRNA activity in infections with viable and HK BCG. As there was a difference in LAMP1 signal between phagosomes containing HK and viable BCG, we hypothesized that these differences might be regulated by miRNAs that control phogosomal processing. The direction of present bottom up approach was from protein-coding genes towards finding miRNAs, which is an opposite method to classical studies, where identification of given miRNAs involves association of targets (Sharbati et al., 2011;Hoeke et al., 2013). This approach is versatile and can also be used for other pathways, however the possibilities of finding regulatory miRNAs can be enhanced by pre-defining experimental conditions as it was shown by selection of time points for RNA isolation. The approach is very flexible at each step, e.g. selection criteria for protein-coding genes and selection of miRmap scores.

Next step in our approach was to search for miRNAs that target selected genes. In the present study, after selecting 10 top candidate miRNAs, three of them (miR-3619-5p, miR-637 and miR-324-3p) turned out to be regulated upon infection. The approach has narrowed down the study of vast number of miRNAs, leading to straightforward identification of regulated miRNAs. However, the method has failed to distinguish between regulating miRNAs after

phagocytosis of viable and HK BCG. The reason could be due to narrowed selection of proteins. Selection of an increased number of protein-coding genes in the beginning as well as inclusion of other closely related pathways to phagocytosis might have helped finding differences in regulated miRNAs between viable and HK BCG infection. Nevertheless, the presented approach helped to detect regulated miRNAs, which potentially target protein-coding genes involved in phagolysosomal trafficking and processing. On a different note, PMA is a known PKC activator, and PKC has been reported to interfere with autophagic signaling during mycobacterial infection of macrophages (Kumar et al., 2015). However, in our experiments PMA was only taken for differentiation and was removed 24 h before infection/treatment of THP-1 derived macrophages and therefore is not thought to influence the experiments.

Generally, down-regulation of miRNAs corresponds to up-regulation of respective targets. After *in silico* and RT-qPCR based analysis, increased and anti-correlated expression of two predicted targets CTSS and RAB11FIP4 was observed in same RNA samples. Both protein-coding genes (CTSS and RAB11FIP4) were predicted to be mutually targeted by miR-3619-5p and miR-637 with miRmap scores more than 90. Based on KEGG pathway database, these two proteins have functional involvement in lysosome and endocytosis pathways, respectively (Kanehisa and Goto, 2000). In present study, increased CTSS expression at 4 h post infection was observed and miR-3619-5p was down-regulated already at 15 min. Lagged regulation of the target CTSS is a characteristic of miRNA regulation. The lysosomal cysteine proteinase CTSS acts as a late event after fusion of autophagosomes and lysosomes. Therefore, regulation of CTSS at 4 h post infection is consistent with the co-localization of LAMP1 signals as a late marker of phagolysosomal processing. In an earlier study, CTSS was up-regulated when mica fine particles were phagocytized by murine macrophage cell line, RAW 264.7 (Jung et al., 2015). Interestingly, over-expression of CTSS was found to be responsible for the regulation of autophagy and apoptosis (Chen et al., 2012). After transfection of THP-1 derived macrophages with miR-3619-5p and miR-637 mimics, down-regulation of CTSS but not that of RAB11FIP4 was observed only in miR-3619-5p mimics transfected cells. Down-regulation of CTSS by miR-3619-5p mimic was around 20%. In reporter gene assays, also 20% decreased luciferase activity was observed in miR-3619-5p transfected cells corresponding well to the moderately decreased cellular levels of CTSS transcripts. In an earlier study, after transfecting human cancer cell lines HONE 1 with CTSS siRNA, LC3B-II protein was accumulated as determined by western blots (Chen et al., 2012). Other recent studies also have suggested that inhibition of CTSS induces autophagy in different cells (Chen et al., 2012;Zhang et al., 2014;Huang et al., 2015). Based on KEGG pathway database, CTSS is involved in 4 different pathways (lysosome, phagosome, antigen processing and presentation and tuberculosis). It is known that CTSS is involved in antigen processing and presentation (Riese et al., 1998;Driessen et al., 1999;Saegusa et al., 2002;Beers et al., 2005). So, in both infection groups (viable and HK), up-regulation of CTSS might be linked to degradation of phagocytized particles and antigen presentation. In another study, down-regulation of CTSS along with LAMP1 and Cathepsin D (CTSD) caused reduction in autophagosome-lysosome fusion (Burgos-Portugal et al., 2014). CTSS deficient mouse hearts

were associated with abnormal accumulation of autophagosomes. As CTSS in lysosomes was essential for mitophagy processing in macrophages, its deficiency increased the damage to mitochondria and elevated ROS levels (Pan et al., 2012). In our experiments, miR-3619-5p as well as siRNA mediated CTSS knockdown resulted in increased number of LC3 puncta. Western blots showed increased cellular LC3-II levels but there was no reduction of p62 levels. p62 was even accumulated after specific knockdown of CTSS pointing to a block of lysosomal processing. This points out that the increased number of LC3 puncta as well as conversion might rather be grounded in blockage of lysosomal trafficking after CTSS down-regulation than induction of autophagy. Decreased levels of the lysosomal cysteine proteinase CTSS is likely to affect degradation of autophagosomal contents. In a recent publication it has been shown that Atg5 but not other authophagy genes plays a key role in the host response to mycobacteria (Behar and Baehrecke, 2015;Kimmey et al., 2015). On a different note, lysosome activation induced by mTOR inhibition has been shown to depend on Atg5/7, suggesting that lysosomal activation depends upon autophagosome formation. The authors have shown that activity of the lysosomal proteinases cathepsin B and L depends upon Atg5/7 mediated autophagosome-lysosome fusion, meaning that cathepsins act downstream of Atg5 (Zhou et al., 2013). Our study suggests that miR-3619-5p mediated down-regulation of CTSS impairs degradation of autophagic substrates thus blocking autophagosome-lysosome processing. This results rather in accumulation of autophagic bodies than induction of autophagy, which is consistent with recent observations of Kimmey et al. since Atg5 is also needed for lysosomal cathepsin activation.

In conclusion, the presented bottom up approach was successful in finding regulated miRNAs and targets, which have functions in phagocytosis related pathways. Besides its potential role in other pathways, down-regulation of miR-3619-5p and consequently accumulated CTSS seems to be a new regulatory axis relevant for the innate immune response to pathogenic mycobacteria.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Authors and contributors

KP: Performed experiments, analyzed data and wrote manuscript
JS: Performed microscopy
RE: Contributed reagents, materials, analysis tools and writing.
SS: Conceived the study, designed experiments, analyzed data, wrote manuscript and contributed reagents, materials and analysis tools.

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References

Agerer, F., Waeckerle, S., and Hauck, C.R. (2004). Microscopic quantification of bacterial invasion by a novel antibody-independent staining method. *J Microbiol Methods* 59**,** 23-32. doi: 10.1016/j.mimet.2004.05.008.

Beers, C., Burich, A., Kleijmeer, M.J., Griffith, J.M., Wong, P., and Rudensky, A.Y. (2005). Cathepsin S controls MHC class II-mediated antigen presentation by epithelial cells in vivo. *J Immunol* 174**,** 1205-1212.

Behar, S.M., and Baehrecke, E.H. (2015). Tuberculosis: Autophagy is not the answer. *Nature* 528**,** 482-483. doi: 10.1038/nature16324.

Billeskov, R., Grandal, M.V., Poulsen, C., Christensen, J.P., Winther, N., Vingsbo-Lundberg, C., Hoang, T.T., van Deurs, B., Song, Y.H., Aagaard, C., Andersen, P., and Dietrich, J. (2010). Difference in TB10.4 T-cell epitope recognition following immunization with recombinant TB10.4, BCG or infection with Mycobacterium tuberculosis. *Eur J Immunol* 40**,** 1342-1354. doi: 10.1002/eji.200939830.

Blankenberg, D., Von Kuster, G., Coraor, N., Ananda, G., Lazarus, R., Mangan, M., Nekrutenko, A., and Taylor, J. (2010). Galaxy: a web-based genome analysis tool for experimentalists. *Curr Protoc Mol Biol* Chapter 19**,** Unit 19 10 11-21. doi: 10.1002/0471142727.mb1910s89.

Bucci, C., Thomsen, P., Nicoziani, P., McCarthy, J., and van Deurs, B. (2000). Rab7: a key to lysosome biogenesis. *Mol Biol Cell* 11**,** 467-480.

Burgos-Portugal, J.A., Mitchell, H.M., Castano-Rodriguez, N., and Kaakoush, N.O. (2014). The role of autophagy in the intracellular survival of Campylobacter concisus. *FEBS Open Bio* 4**,** 301-309. doi: 10.1016/j.fob.2014.03.008.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., and Wittwer, C.T. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 55**,** 611-622.

Chen, K.L., Chang, W.S., Cheung, C.H., Lin, C.C., Huang, C.C., Yang, Y.N., Kuo, C.P., Kuo, C.C., Chang, Y.H., Liu, K.J., Wu, C.M., and Chang, J.Y. (2012). Targeting cathepsin S induces tumor cell autophagy via the EGFR-ERK signaling pathway. *Cancer Lett* 317**,** 89-98. doi: 10.1016/j.canlet.2011.11.015.

Christoforidis, S., Miaczynska, M., Ashman, K., Wilm, M., Zhao, L., Yip, S.C., Waterfield, M.D., Backer, J.M., and Zerial, M. (1999). Phosphatidylinositol-3-OH kinases are Rab5 effectors. *Nat Cell Biol* 1**,** 249-252. doi: 10.1038/12075.

Clemens, D.L., and Horwitz, M.A. (1995). Characterization of the Mycobacterium tuberculosis phagosome and evidence that phagosomal maturation is inhibited. *J Exp Med* 181**,** 257-270.

Dennis, G., Jr., Sherman, B.T., Hosack, D.A., Yang, J., Gao, W., Lane, H.C., and Lempicki, R.A. (2003). DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 4**,** P3.

Driessen, C., Bryant, R.A., Lennon-Dumenil, A.M., Villadangos, J.A., Bryant, P.W., Shi, G.P., Chapman, H.A., and Ploegh, H.L. (1999). Cathepsin S controls the trafficking and maturation of MHC class II molecules in dendritic cells. *J Cell Biol* 147**,** 775-790.

Flannagan, R.S., Cosio, G., and Grinstein, S. (2009). Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nat Rev Microbiol* 7**,** 355-366. doi: 10.1038/nrmicro2128.

Fratti, R.A., Backer, J.M., Gruenberg, J., Corvera, S., and Deretic, V. (2001). Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. *J Cell Biol* 154**,** 631-644. doi: 10.1083/jcb.200106049.

Ghorpade, D.S., Leyland, R., Kurowska-Stolarska, M., Patil, S.A., and Balaji, K.N. (2012). MicroRNA-155 is required for Mycobacterium bovis BCG-mediated apoptosis of macrophages. *Mol Cell Biol* 32**,** 2239-2253. doi: 10.1128/MCB.06597-11.

Gorvel, J.P., Chavrier, P., Zerial, M., and Gruenberg, J. (1991). rab5 controls early endosome fusion in vitro. *Cell* 64**,** 915-925.

Gutierrez, M.G., Master, S.S., Singh, S.B., Taylor, G.A., Colombo, M.I., and Deretic, V. (2004). Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell* 119**,** 753-766. doi: Doi 10.1016/J.Cell.2004.11.038.

Hoeke, L., Sharbati, J., Pawar, K., Keller, A., Einspanier, R., and Sharbati, S. (2013). Intestinal Salmonella typhimurium infection leads to miR-29a induced caveolin 2 regulation. *PLoS One* 8**,** e67300. doi: 10.1371/journal.pone.0067300.

Houben, E.N., Walburger, A., Ferrari, G., Nguyen, L., Thompson, C.J., Miess, C., Vogel, G., Mueller, B., and Pieters, J. (2009). Differential expression of a virulence factor in pathogenic and non-pathogenic mycobacteria. *Mol Microbiol* 72**,** 41-52. doi: 10.1111/j.1365-2958.2009.06612.x.

Huang, C.C., Chen, K.L., Cheung, C.H., and Chang, J.Y. (2013). Autophagy induced by cathepsin S inhibition induces early ROS production, oxidative DNA damage, and cell death via xanthine oxidase. *Free Radic Biol Med* 65**,** 1473-1486. doi: 10.1016/j.freeradbiomed.2013.07.020.

Huang, C.C., Lee, C.C., Lin, H.H., Chen, M.C., Lin, C.C., and Chang, J.Y. (2015). Autophagy-Regulated ROS from Xanthine Oxidase Acts as an Early Effector for Triggering Late Mitochondria-Dependent Apoptosis in Cathepsin S-Targeted Tumor Cells. *PLoS One* 10**,** e0128045. doi: 10.1371/journal.pone.0128045.

Jung, M., Shin, M.K., Jung, Y.K., and Yoo, H.S. (2015). Modulation of macrophage activities in proliferation, lysosome, and phagosome by the nonspecific immunostimulator, mica. *PLoS One* 10**,** e0117838. doi: 10.1371/journal.pone.0117838.

Kanehisa, M., and Goto, S. (2000). KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 28**,** 27-30.

Kanehisa, M., Goto, S., Sato, Y., Kawashima, M., Furumichi, M., and Tanabe, M. (2014). Data, information, knowledge and principle: back to metabolism in KEGG. *Nucleic Acids Res* 42**,** D199-205. doi: 10.1093/nar/gkt1076.

Kimmey, J.M., Huynh, J.P., Weiss, L.A., Park, S., Kambal, A., Debnath, J., Virgin, H.W., and Stallings, C.L. (2015). Unique role for ATG5 in neutrophil-mediated immunopathology during M. tuberculosis infection. *Nature* 528**,** 565-569. doi: 10.1038/nature16451.

Klionsky, D.J., Cuervo, A.M., and Seglen, P.O. (2007). Methods for monitoring autophagy from yeast to human. *Autophagy* 3**,** 181-206.

Kruger, J., and Rehmsmeier, M. (2006). RNAhybrid: microRNA target prediction easy, fast and flexible. *Nucleic Acids Res* 34**,** W451-454.

Kumar, R., Halder, P., Sahu, S.K., Kumar, M., Kumari, M., Jana, K., Ghosh, Z., Sharma, P., Kundu, M., and Basu, J. (2012). Identification of a novel role of ESAT-6-dependent miR-155 induction during infection of macrophages with Mycobacterium tuberculosis. *Cell Microbiol* 14**,** 1620-1631. doi: 10.1111/j.1462-5822.2012.01827.x.

Kumar, R., Sahu, S.K., Kumar, M., Jana, K., Gupta, P., Gupta, U.D., Kundu, M., and Basu, J. (2015). MicroRNA 17-5p regulates autophagy in Mycobacterium tuberculosis-infected macrophages by targeting Mcl-1 and STAT3. *Cell Microbiol*. doi: 10.1111/cmi.12540.

Lee, B.Y., Jethwaney, D., Schilling, B., Clemens, D.L., Gibson, B.W., and Horwitz, M.A. (2010). The Mycobacterium bovis bacille Calmette-Guerin phagosome proteome. *Mol Cell Proteomics* 9**,** 32-53. doi: 10.1074/mcp.M900396-MCP200.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25**,** 402-408. doi: 10.1006/meth.2001.1262.

Ma, F., Xu, S., Liu, X., Zhang, Q., Xu, X., Liu, M., Hua, M., Li, N., Yao, H., and Cao, X. (2011). The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon-gamma. *Nat Immunol* 12**,** 861-869. doi: 10.1038/ni.2073.

Pan, L., Li, Y., Jia, L., Qin, Y., Qi, G., Cheng, J., Qi, Y., Li, H., and Du, J. (2012). Cathepsin S deficiency results in abnormal accumulation of autophagosomes in macrophages and enhances Ang II-induced cardiac inflammation. *PLoS One* 7**,** e35315. doi: 10.1371/journal.pone.0035315.

Pawar, K., Hanisch, C., Palma Vera, S.E., Einspanier, R., and Sharbati, S. (2016). Down regulated lncRNA MEG3 eliminates mycobacteria in macrophages via autophagy. *Sci Rep* 6**,** 19416. doi: 10.1038/srep19416.

Rajaram, M.V., Ni, B., Morris, J.D., Brooks, M.N., Carlson, T.K., Bakthavachalu, B., Schoenberg, D.R., Torrelles, J.B., and Schlesinger, L.S. (2011). Mycobacterium tuberculosis lipomannan blocks TNF biosynthesis by regulating macrophage MAPK-activated protein kinase 2 (MK2) and microRNA miR-125b. *Proc Natl Acad Sci U S A* 108**,** 17408-17413. doi: 10.1073/pnas.1112660108.

Riese, R.J., Mitchell, R.N., Villadangos, J.A., Shi, G.P., Palmer, J.T., Karp, E.R., De Sanctis, G.T., Ploegh, H.L., and Chapman, H.A. (1998). Cathepsin S activity regulates antigen presentation and immunity. *J Clin Invest* 101**,** 2351-2363. doi: 10.1172/JCI1158.

Saegusa, K., Ishimaru, N., Yanagi, K., Arakaki, R., Ogawa, K., Saito, I., Katunuma, N., and Hayashi, Y. (2002). Cathepsin S inhibitor prevents autoantigen presentation and autoimmunity. *J Clin Invest* 110**,** 361-369. doi: 10.1172/JCI14682.

Sanjuan, M.A., Dillon, C.P., Tait, S.W., Moshiach, S., Dorsey, F., Connell, S., Komatsu, M., Tanaka, K., Cleveland, J.L., Withoff, S., and Green, D.R. (2007). Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature* 450**,** 1253-1257. doi: 10.1038/nature06421.

Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9**,** 671-675.

Sharbati, J., Hanisch, C., Pieper, R., Einspanier, R., and Sharbati, S. (2015). Small molecule and RNAi induced phenotype transition of expanded and primary colonic epithelial cells. *Sci Rep* 5**,** 12681. doi: 10.1038/srep12681.

Sharbati, J., Lewin, A., Kutz-Lohroff, B., Kamal, E., Einspanier, R., and Sharbati, S. (2011). Integrated microRNA-mRNA-analysis of human monocyte derived macrophages upon Mycobacterium avium subsp. hominissuis infection. *PLoS One* 6**,** e20258. doi: 10.1371/journal.pone.0020258.

Sharbati, S., Friedlander, M.R., Sharbati, J., Hoeke, L., Chen, W., Keller, A., Stahler, P.F., Rajewsky, N., and Einspanier, R. (2010). Deciphering the porcine intestinal microRNA transcriptome. *BMC Genomics* 11**,** 275. doi: 10.1186/1471-2164-11-275.

Sharbati, S., Sharbati, J., Hoeke, L., Bohmer, M., and Einspanier, R. (2012). Quantification and accurate normalisation of small RNAs through new custom RT-qPCR arrays demonstrates Salmonella-induced microRNAs in human monocytes. *BMC Genomics* 13**,** 23.

Sharbati-Tehrani, S., Kutz-Lohroff, B., Bergbauer, R., Scholven, J., and Einspanier, R. (2008). miR-Q: a novel quantitative RT-PCR approach for the expression profiling of small RNA molecules such as miRNAs in a complex sample. *BMC Mol Biol* 9**,** 34. doi: 10.1186/1471-2199-9-34.

Sharbati-Tehrani, S., Meister, B., Appel, B., and Lewin, A. (2004). The porin MspA from Mycobacterium smegmatis improves growth of Mycobacterium bovis BCG. *Int J Med Microbiol* 294**,** 235-245. doi: 10.1016/j.ijmm.2004.02.001.

Stewart, G.R., Patel, J., Robertson, B.D., Rae, A., and Young, D.B. (2005). Mycobacterial mutants with defective control of phagosomal acidification. *PLoS Pathog* 1**,** 269-278. doi: 10.1371/journal.ppat.0010033.

Sturgill-Koszycki, S., Schlesinger, P.H., Chakraborty, P., Haddix, P.L., Collins, H.L., Fok, A.K., Allen, R.D., Gluck, S.L., Heuser, J., and Russell, D.G. (1994). Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. *Science* 263**,** 678-681.

Sun, J., Deghmane, A.E., Soualhine, H., Hong, T., Bucci, C., Solodkin, A., and Hmama, Z. (2007). Mycobacterium bovis BCG disrupts the interaction of Rab7 with RILP

contributing to inhibition of phagosome maturation. *J Leukoc Biol* 82**,** 1437-1445. doi: 10.1189/jlb.10.1189.

Tanida, I., Yamaji, T., Ueno, T., Ishiura, S., Kominami, E., and Hanada, K. (2008). Consideration about negative controls for LC3 and expression vectors for four colored fluorescent protein-LC3 negative controls. *Autophagy* 4**,** 131-134.

Tili, E., Michaille, J.J., Cimino, A., Costinean, S., Dumitru, C.D., Adair, B., Fabbri, M., Alder, H., Liu, C.G., Calin, G.A., and Croce, C.M. (2007). Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. *J Immunol* 179**,** 5082-5089.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3**,** RESEARCH0034.

Vejnar, C.E., and Zdobnov, E.M. (2012). MiRmap: comprehensive prediction of microRNA target repression strength. *Nucleic Acids Res* 40**,** 11673-11683. doi: 10.1093/nar/gks901.

Vergne, I., Chua, J., Singh, S.B., and Deretic, V. (2004). Cell biology of mycobacterium tuberculosis phagosome. *Annu Rev Cell Dev Biol* 20**,** 367-394. doi: 10.1146/annurev.cellbio.20.010403.114015.

Vieira, O.V., Bucci, C., Harrison, R.E., Trimble, W.S., Lanzetti, L., Gruenberg, J., Schreiber, A.D., Stahl, P.D., and Grinstein, S. (2003). Modulation of Rab5 and Rab7 recruitment to phagosomes by phosphatidylinositol 3-kinase. *Mol Cell Biol* 23**,** 2501-2514.

Walburger, A., Koul, A., Ferrari, G., Nguyen, L., Prescianotto-Baschong, C., Huygen, K., Klebl, B., Thompson, C., Bacher, G., and Pieters, J. (2004). Protein kinase G from pathogenic mycobacteria promotes survival within macrophages. *Science* 304**,** 1800-1804. doi: 10.1126/science.1099384.

Wang, J., Yang, K., Zhou, L., Minhaowu, Wu, Y., Zhu, M., Lai, X., Chen, T., Feng, L., Li, M., Huang, C., Zhong, Q., and Huang, X. (2013). MicroRNA-155 promotes autophagy to eliminate intracellular mycobacteria by targeting Rheb. *PLoS Pathog* 9**,** e1003697. doi: 10.1371/journal.ppat.1003697.

Xu, S., Cooper, A., Sturgill-Koszycki, S., van Heyningen, T., Chatterjee, D., Orme, I., Allen, P., and Russell, D.G. (1994). Intracellular trafficking in Mycobacterium tuberculosis and Mycobacterium avium-infected macrophages. *J Immunol* 153**,** 2568-2578.

Zhang, L., Wang, H., Xu, J., Zhu, J., and Ding, K. (2014). Inhibition of cathepsin S induces autophagy and apoptosis in human glioblastoma cell lines through ROS-mediated PI3K/AKT/mTOR/p70S6K and JNK signaling pathways. *Toxicol Lett* 228**,** 248-259. doi: 10.1016/j.toxlet.2014.05.015.

Zhou, J., Tan, S.H., Nicolas, V., Bauvy, C., Yang, N.D., Zhang, J., Xue, Y., Codogno, P., and Shen, H.M. (2013). Activation of lysosomal function in the course of autophagy via mTORC1 suppression and autophagosome-lysosome fusion. *Cell Res* 23**,** 508-523. doi: 10.1038/cr.2013.11.

zur Bruegge, J., Hanisch, C., Einspanier, R., Alter, T., Golz, G., and Sharbati, S. (2014). Arcobacter butzleri induces a pro-inflammatory response in THP-1 derived macrophages and has limited ability for intracellular survival. *Int J Med Microbiol* 304**,** 1209-1217. doi: 10.1016/j.ijmm.2014.08.017.

5. Discussion

In this thesis, we have presented data on the expression and function of two different classes of ncRNAs: lncRNAs and miRNAs, in mycobacterial infection. In the first section, expression patterns in different cell lines along with in silico analysis of lncRNAs provided us with a basis for discovering functions of lncRNAs after performing lncRNA-based qPCR in infected macrophages, which was validated with further experiments. We observed different expressions of preselected lncRNAs at different time points (30 min and 4 h) following BCG infection of macrophages. In the second section, we used a bottom-up approach to sort miRNAs in phagosomal maturation. After in silico analysis and validation, three miRNAs along with two mutual mRNA targets were identified. We speculate that these approaches will have a broad spectrum of use in discovering the role of ncRNAs in other studies.

Based on current findings, for the first time, we have demonstrated the role of lncRNAs in BCG infection. Distinct regulatory functions of MEG3 and miRNA-3619-5p in autophagy have been revealed during BCG infection.

5.1 Inhibition of phagosomal maturation by viable BCG provided timepoints for RNA isolation

Mycobacteria are known to affect phagolysosomal fusion; therefore, we studied phagosomal maturation in macrophages infected with viable and heat-killed (HK) BCG. Acquiring LAMP1 protein on phagosomal membrane indicates phagosomal fusion with lysosomes, which is the final stage of phagosomal maturation. However, it is known that viable BCG inhibits phagosomal maturation following engulfment by macrophages [87]. In the present study, we observed an absence of LAMP1 signal on viable BCG phagosomal membrane at 30 min and 4 h compared with HK BCG phagosomes. An earlier study revealed that BCG lacks region of difference 1 (RD1) locus, which is present in *M. bovis* and virulent *M. tuberculosis* strains [88], and is known to contain the ESX-1 type VII secretion system, responsible for the secretion of ESAT-6/CFP-10 [89]. ESAT-6 facilitates the escape of virulent bacteria from macrophage phagolysosomal degradation. Although *M. bovis* BCG lacks the RD1 locus, yet it can survive inside macrophage phagosomes and affect phagosomal maturation. A few proteins such asα-crystallin (HspX), GroEL-1 and GroEL-2 were produced by BCG only following entry to macrophages, but not during normal growth in media [90]. Production of these proteins could be responsible for the survival of BCG inside phagosomes and escape from phagolysosome fusion. Moreover, wild-type BCG was associated with LAMP1-negative phagosomes while BCG with inactivated protein serine/threonine kinase G (ΔpknG) co-localised predominantly with LAMP1 [91]. In our study, we did not see a difference in LAMP1 signal at 15 min but a difference was observed at 30 min and 4 h. We assumed that this difference in LAMP1 signal could not be the only factor controlling the mycobacterial protein serine/threonine kinase G (PknG) in BCG [92], but also by ncRNAs which block lysosomal delivery of mycobacteria. Therefore, we investigated the expression of ncRNAs by

choosing time points of 15 min, 30 min and 4 h for RNAs isolation in different infections (Viable BCG versus HK BCG infections) of macrophages.

On the other hand, BCG is recognised by macrophages via toll-like receptors (TLR) [93] that produce a series of changes in downstream pathways, which also gave us a rationale for choosing early time points in the present infection study. Pattern recognition receptors (PRRs) such as TLRs (TLR2 and 4) are essential for immune defense against BCG infection. TLR2 and 4 are expressed by macrophages and play an important role in the first line of defense against BCG infections. It has been shown that TLR2 and TLR4 serve distinct roles in the host immune response against BCG [94], which affects downstream signalling pathways, indicating that the initial period of activation is an important event [95].

Therefore, we used an initial period of time for RNA isolation for expression studies to determine whether there are any ncRNAs involved in carrying out changes in phagosomal maturation or related pathways that occurred in the initial period of time.

5.2 Expression of ncRNAs

After performing lncRNA based RT-qPCR we observed differential lncRNA expression in BCG-infected macrophages at 30 min and 4 h. At 30 min, most of the lncRNAs were downregulated in viable BCG; however, expression of all lncRNAs in HK BCG was not regulated. Interestingly, at this early period of infection (30 min), viable BCGs but not HK BCGs had the ability to downregulate immune function-related lncRNAs in macrophages. However, the expression of the same lncRNAs in viable BCG was restored to normal levels (no regulation) after 4 h of infection. This signifies the importance of selection of timepoints for RNA isolation because ncRNAs could be regulated differently at different times of infection. In addition, after looking at miRNA data from matching isolated RNA samples, it was clear that there was a difference in expression of miRNAs at different timepoints. miR-3619-5p was downregulated at 15 min and 30 min but not at 4 h in both BCG infection groups compared to the non-infected group. Expression of miR-637 was downregulated only at 15 min but not in the rest of the isolated RNA samples. These observations indicate that there may be different ncRNAs appointed to carry out functions at different stages of infection, which can be investigated using further functional studies for each specific ncRNA. In the present study, we observed phagosomal maturation differences at different timepoints (no LAMP1 signal on phagosomes at 15 min but detectable signals at 30 min and 4 h) as well as in different treatments (no LAMP1 signal on phagosomes containing viable BCG but not on phagosomes containing HK BCG), which may be linked to differential expression of observed ncRNAs. In a previous study, expression of miR-142-3p was negatively correlated to expression of its target gene IRAK-1, which is an inhibitor of TLR signalling [84]. Interestingly, miR-142-3p was upregulated at an initial period of time in macrophages but downregulated at a later stage of the mycobacterial infection [82, 83]. This highlights the importance of selecting specific time points for RNA isolation in infection experiments to maximise the chance of finding differentially regulated RNAs.

The present study has demonstrated, for the first time, regulation of lncRNAs in BCG infection. Of preselected lncRNAs (n=17), in total, 11 lncRNA (ANRIL, AS UCHL1, EGO, H19, MEG3, NRON, NTT, PRINS, RPS15AP25, TMEVPG1, WT1-AS) showed decreased expression after infection with viable BCG compared to that of with HK BCG. This suggests that the downregulation of lncRNAs was because of BCG pathogenesis but not any other physiological processes such as phagocytosis. Previous studies revealed that a reduced level of ANRIL has been associated with reduced cell proliferation, suggesting a pro-cancerogenic role [96]. On the other hand, over-expression of ANRIL was linked to uncontrolled cell proliferation (cancer) such as leukemia [97] or prostate cancer [98]. AS UCHL1 levels were strongly downregulated in neurochemical models of Parkinson's disease in vitro and in vivo [99]. Also, dysregulation of UCHL1 has been related to defects in autophagy in diabetes mellitus [100]. lncRNA EGO stimulates differentiation and mature cell function of hematopoietic progenitor cells (CD34+) by regulating eosinophil granule protein expression at the transcriptional level [101]. Knockdown of EGO by siRNAs in CD34+ progenitors impairs the expression of genes that are critical for eosinophil development such as major basic protein and eosinophil-derived neurotoxin [101]. lncRNA H19 has an important role in embryogenesis and foetal development [102] and suppression of H19 expression in Parthenogenetic embryonic stem cells (P-ESCs) allowed them to alleviate their limitations and differentiate at a similar rate to biparental embryonic stem cells (B-ESCs) [103]. Differential expression of MEG3 was associated with different cancer tissues such as bladder cancer [72], hepatocellular cancer [104] and gastric cancer [105]. NRON [106] and NTT [107] were the earliest lncRNA genes identified in immune cells (expressed in T-cells). NRON regulates the Ca2+-activated transcription factor NFAT and modulates the expression of IL-2 in activated T-cells [106]. NTT is primarily expressed in activated human CD4+ T cells; however, its function is still unclear. There may be a potential functional link between NTT and the interferon-γ receptor (IFN-γR) gene, as these genes share the same genomic locus (6q23-q24) [107]. Expression of lncRNA in infection has also been previously described, for example, lncRNA PRINS was upregulated upon infection with herpes simplex virus [108] and treatment with bacterial cell wall extracts [109]. However, regulation of lncRNA RPS15AP25 in infection has not yet been described. Regions of TMEVPG1 are positioned near the IFN-γ gene (IFNG) and it contributes to IFN-γ expression as part of the Th1 differentiation programme [110]. It has been demonstrated that WT1-AS transcripts upregulate WT1 protein levels, which co-localise with WT1 protein and RNA in kidney development [111]. An lncRNA-driven epigenetic program of T-cell immunity against TB infection has previously been shown [75]. As lncRNAs have different functions, studying individual dysregulated lncRNAs may reveal unidentified host responses to mycobacterial infection that may help our understanding of its pathogenesis.

The present study also identified three differentially regulated miRNAs: miR-3619-5p, miR-637 and miR-324-3p, in macrophages infected with mycobacteria compared with non-infected macrophages. The downregulation of all three miRNAs in macrophages infected with BCGs was not only caused by viable but also HK BCG. To rule out whether this effect is caused only by BCG and not by any other physiological processes such as phagocytosis,

further experiments are necessary such as infecting macrophages with other bacteria or with latex beads. In a previous study, the level of miR-3619-5p was observed to be significantly less in Non-Small Cell Lung Cancer (NSCLC) cells compared to non-tumour lung tissue (NLT) [112]. Also, expression of miR-3619-5p was negatively correlated with β-catenin levels, measured by western blotting. These results demonstrated the role of miR-3619-5p in suppression of β-catenin-mediated cancer growth and invasion in NSCLC cells, highlighting miR-3619-5p as a cancer suppressor in NSCLC. In our study, three miRNAs including miR-3619-5p were sorted based on a given bottom up approach, where in silico analysis was performed followed by experimental validation. Earlier, a novel integrative genomic tool called GRANITE (Genetic Regulatory Analysis of Networks Investigational Tool Environment) was used for expression profiling of both miRNA and mRNA genes in vehicle versus chronic lithium treatment in patient-derived lymphoblastoid cells (LCLs) (derived from either Li treatment responders or non-responders) [113]. miR-3619-5p, among a few other miRNAs, was highly expressed by both responders and non-responders, and it had the potential to control many lithium-regulated mRNA transcripts. miRNA miR-637 was shown to have a role in adipogenesis and osteogenesis, whereas miR-637 promoted formation of adipocytes and suppressed osteoblasts in human mesenchymal stem cells (hMSCs) derived from bone marrow [114]. In another study, expression level of miR-637 was significantly lower in clinical glioma tissues compared with normal brain tissues; however, ectopic expression of miR-637 suppressed glioma cell growth, migration and invasion in vitro as well as in vivo [115]. miRNA miR-324-3p can target 3'UTR of 'Mothers against decapentaplegic homolog 7' (SMAD7) and modulate growth and apoptosis of nasopharyngeal carcinoma cells [116]. Also, miR-324-3p was the most upregulated in micro-dissected glomeruli [117]. In our study, a target of miR-3619-5p was identified and experimentally validated. Based on further investigations, CTSS, a target of miR-3619-5p, had a role in autophagy, which is described later in the discussion.

In addition to the above three miRNAs, previous studies also identified other dysregulated miRNAs in BCG infection. Generally, during bacterial infection, some miRNAs, including miR-146, miR-155, miR-125, let-7 and miR-21, that contribute to anti-inflammatory immune responses, were commonly affected [118]. In BCG infection, miRNA miR-155 was upregulated, which was required for BCG-mediated apoptosis of macrophages [119]. Also, miR-155 enhanced the ROS-mediated mycobactericidal mechanism of macrophages by targeting src homology 2 (SH2) containing inositol 5-phosphatase1 (SHIP1) [120]. Studies investigating TLR signalling in BCG infection showed that miR-124 negatively regulates TLR signalling in alveolar macrophages in response to BCG infection by targeting multiple components of the pathway, including Tlr6, Myd88, Traf6 mRNAs. Moreover, miR-31 and miR-150 were also found to regulate TLR2 responses in BCG infected cells.

In our bottom up approach, in silico analysis predicted that miR-3619-5p and miR-637 mutually targeted CTSS and RAB11FIP4. Experimental validation proved that CTSS was a target for miR-3619-5p. CTSS is a lysosomal and endosomal cysteine protease expressed in antigen presenting cells (APCs) including macrophages, B cells and dendritic cells [121].

CTSS is the main regulator for MHC class II in APCs. It controls the maturation of MHC class II molecules by generating the class II-associated invariant chain peptide (CLIP) fragment through invariant chain (II) proteolysis. This is required for loading of antigenic peptide to MHC class II molecule where antigen is presented to T cells [122]. Upregulation of CTSS in our study may indicate that macrophages are preparing for presentation of BCG proteins through MHC class II molecule. From these studies, we concluded that miR-3619-5p targets CTSS, which is involved in the phagosomal maturation process (preparation for antigen presentation). In the present investigation, we explored the role of CTSS by down-regulating it via ectopic expression of miR-3619-5p as well as by using siRNA against CTSS, which was responsible for induction of autophagy in macrophages. Previous studies showed that inhibition of CTSS induced autophagy and mitochondrial apoptosis in human glioblastoma cells [123]. In addition, CTSS-mediated autophagy was responsible for early ROS production, oxidative DNA damage, and cell death via xanthine oxidase [123]. This has highlighted a new role for CTSS as an autophagy modulator in BCG infection. Similar to miRNA studies, to find the role of regulated lncRNAs in BCG infected macrophages, in silico analysis was performed from the data obtained from expression patterns of selected lncRNAs in different cell lines. This provided hierarchical clusters of different lncRNAs where MEG3 and NEAT1 were in one cluster. MEG3 was highly downregulated in viable BCG infection and in silico analysis predicted its role in autophagy. Based on the results obtained, both ncRNAs (MEG3 and miR-3619-5p) had roles in autophagy.

5.3 ncRNAs, BCG infection and autophagy

Following treatment with IFN-γ, autophagy was induced in THP-1 cells infected with viable BCG. A previous study demonstrated that BCG was responsible for reduced autophagy responses in macrophages compared to *M. smegmatis* suggesting that BCG minimised host cell autophagy by using specific mechanisms [124]. The enhanced intracellular survival (eis) gene of *M. tuberculosis* was responsible for suppression of host innate immune defenses by modulating autophagy [125]. Also, other products of *M. tuberculosis*, such as PI3P phosphatase SapM, glycosylated phosphatidylinositol-mimic LAM and phosphatidylinositol mannoside (PIM) have been reported to suppress PI3K signalling to reduce autophagy [126]. However, induction of autophagy can be achieved following addition of IFN-γ in infected macrophages, which affected *M. tuberculosis* and BCG survival inside macrophages [59]. Our study also reported that, after addition of IFN-γ, autophagy was induced via inactivation of mTOR, and this enhanced BCG clearance from macrophages. Interestingly, along with induction of autophagy, addition of IFN-γ also maintained downregulation of MEG3 even after 24 h in BCG infected macrophages. In initial experiments, expression of MEG3 was significantly downregulated at 30 min but not at 4 h in BCG infected macrophages. From these observations, we concluded that sustained downregulation of MEG3 was likely responsible for induction of autophagy. An earlier study reported that downregulated MEG3 activated autophagy and increased cell proliferation in bladder cancer [72]. In the present study, downregulation of MEG3 via siRNA was responsible for conversion of LC3-I to LC3-II, which is an indication of autophagy induction. Co-localisation of LC3 with BCG was

observed in infected THP-1 cells that were specifically knocked down for MEG3 that enhanced the eradication of BCG from macrophages. This is the first time that eradication of BCG after down-regulation of MEG3 in macrophages has been reported. However, knock down of MEG3 caused no obvious degradation of p62 in BCG macrophages; however, in non-BCG infected cells, p62 levels were increased. This could be due to a block in lysosomal degradation but not an increase in autophagosome formation. Also, MEG3 knockdown had a limited effect on mTOR inactivation. We speculate that MEG3 has a role in autophagy induction; however, other lncRNAs may also function in the process of eliminating BCG via IFN-γ. This can be further investigated by studying remaining lncRNAs in regard to IFN-γ mediated autophagy.

It has been proposed that not only IFN-γ [127] but also miRNAs [128] induced autophagy in infected cells that helped to eliminate mycobacteria [59]. Our study suggests that miR-3619-5p mediated downregulation of CTSS impairs degradation of autophagic substrates thus blocking autophagosome-lysosome processing. We observed conversion of LC3-I to LC3-II in macrophages where CTSS was downregulated via ectopic expression of miR-3619-5p. However, there was no obvious degradation of p62 protein. A previous study reported that miRNA miR-155 attaches to the 3'-untranslated region of a Ras homologue enriched in brain (Rheb), a negative regulator of autophagy, which promotes the process of autophagy that helps to eliminate *M. tuberculosis* from macrophages [128]. miRNAs miR-30a, miR-30d, miR-181a and miR-374a target the Autophagy protein 5 (Atg5) gene which is one of the important proteins in autophagy [129-131]. It has been shown that Atg5, but not other autophagy genes, play a key role in the host response to mycobacteria [132]. Our results suggest that high levels of expression of miR-3619-5p in macrophages resulted in accumulation of autophagic bodies rather than induction of autophagy, which is consistent with recent observations since Atg5 is also needed for lysosomal cathepsin activation.

5.4 Conclusion.

In conclusion, the present study has demonstrated, for the first time, the role of lncRNA MEG3 in BCG infection. Besides MEG3, we have also shown differential time-based expression of other lncRNAs, which were selected based on having functions related to immunity. With further investigation, each lncRNA may reveal unique functions in BCG infection as we have shown a role for MEG3 in autophagy. In addition, we have identified miRNA miR-3619-5p, which was involved in the phagosomal maturation process in mycobacterial infection. This demonstrates that miRNAs could be a regulatory element in phagosomal maturation, which is a crucial process for degradation of engulfed bacteria. Also, miR-3619-5p interferes with autophagy by targeting CTSS in BCG infected macrophages. With the advance of "miRNA therapeutics", it may be possible to control phagosomal maturation by targeting specific miRNAs such as miR-3619-5p. In addition, identifying regulated lncRNAs in mycobacterial diseases may be useful in "RaNA Therapeutics" that uses lncRNAs to develop treatments for diseases that have often been challenging to target.

6. Summary

Non-coding RNA response of human monocyte-derived macrophages during mycobacterial infection

Non-coding RNAs are a class of RNAs that generally do not code for any protein. Different types of ncRNAs are present in mammalian cells including miRNA and lncRNAs. miRNAs are small ncRNA containing about 22 nucleotides. They regulate post-transcriptional gene expression by a mechanism that is known as "RNA interference". lncRNAs are relatively large RNAs with a length of more than 200 nucleotides and have various functions such as carrying out both gene inhibition and gene activation through diverse mechanisms. miRNAs have been shown to have a role in mycobacterial pathogenesis; however, little is known about lncRNAs in mycobacterial infection. Mycobacteria are intracellular pathogens that are hard to eradicate because of their ability to inhibit phagolysosome formation. Approximately one-third of the global population is affected by mycobacterial infections. Nothing was known about involvement of lncRNAs and miRNAs in phagosomal maturation upon mycobacterial infection. In the present study, following infection of monocyte derived macrophages with BCG, expression of lncRNAs as well as miRNAs were studied at different time points of incubation. Time points for RNA isolation were selected based on two aspects: Co-localisation studies of the phagosomal marker protein LAMP1 with BCG, and cascades of TLR4 signalling, which occurs in the initial period of mycobacterial infection. Differences in LAMP1 localisation on the phagosomes of both groups were observed at 30 min and 4 h.

In the first part of the work, after establishing a lncRNA-based RT-qPCR protocol, several preselected lncRNAs that had functions related to immunity were observed to be downregulated at 30 min in BCG infected macrophages compared to non-infected cells as well as heat-killed controls. Among all of them, MEG3 was a highly downregulated lncRNA. In silico analysis predicted that MEG3 had functions in mTOR and PI3K-AKT signalling pathways suggesting its role in autophagy. Induction of autophagy with IFN-γ in infected macrophages resulted in sustained MEG3 downregulation and lack of IFN-γ allowed for counter-regulation of MEG3 by viable BCG. Knockdown of MEG3 in macrophages resulted in induction of autophagy and enhanced eradication of intracellular BCG.

In the second part of the work, a bottom up approach was used to identify miRNAs and respective targets that are involved in phagosomal maturation processes in mycobacterial infection. In silico analysis sorted most enriched miRNAs, and expression studies identified downregulated miRNAs (miR-3619-5p, miR-637 and miR-324-3p) at different time points in BCG infected cells (irrespective of viable or HK BCG) compared to non-infected cells. After identifying their targets, expression studies showed upregulation of lysosomal cysteine protease Cathepsin S (CTSS) and Rab11 family-interacting protein 4 (RAB11FIP4). Reporter gene assays verified regulation of CTSS by miR-3619-5p. Finally, downregulated miR-3619-5p affected autophagy in macrophages.

In conclusion, the present study has identified a role of lncRNA MEG3 in mycobacterial infection, as well as that of miRNA miR-3619-5p along with its target CTSS. Both ncRNAs had functions in autophagy-related pathways during mycobacterial infection.

6. Zusammenfassung

Die Regulation nicht-kodierender RNAs nach mykobakterieller Infektion in Humanen Makrophagen

Nicht-kodierende RNAs gehören zu einer Gruppe RNAs, die nicht für Proteine kodieren. In Säugerzellen befinden sich verschiedene Arten nicht-kodierender RNAs, wie z.B. miRNAs und lncRNAs. MiRNAs sind kleine nicht-kodierende RNAs, ihre Länge umfasst ca. 22 Nukleotide. Sie regulieren post-transkriptionell die Genexpression über einen Mechanismus, der als "RNA-Interferenz" bezeichnet wird. LncRNAs sind lange nicht-kodierende RNAs. Sie bestehen aus mehr als 200 Nukleotiden und haben verschiedene Funktionen wie z.B. Hemmung aber auch Aktivierung von Genen. Verschiedene Studien haben gezeigt, dass miRNAs in der Pathogenese mykobakterieller Infektionen eine wichtige Rolle spielen, wohingegen über die Beteiligung von lncRNAs sehr wenig bekannt ist.

Mykobakterien sind intrazelluläre Pathogene. Durch ihre Fähigkeit die Phagosomenreifung zu unterbinden, können sie in Makrophagen überleben. Ungefähr ein Drittel der Weltbevölkerung ist infiziert. Die Beteiligung von lncRNAs und miRNAs am Vorgang der Phagosomenreifung wurde bisher nicht untersucht.

In der vorliegenden Studie wurden Makrophagen mit BCG infiziert und die Expression von lncRNAs und miRNAs zu verschiedenen Zeitpunkten analysiert. Die Zeitpunkte für die RNA-Isolation wurden basierend auf Co-Lokalisationsexperimenten von LAMP1 (einem phagosomalen Marker) und BCG ausgewählt. Unterschiede der LAMP1 Lokalisation auf Phagosomen beider Gruppen wurden bei 30 min und 4 h festgestellt.

Im ersten Teil der Arbeit wurden lncRNAs untersucht, von denen bekannt ist, dass sie in der Immunantwort von Makrophagen eine Rolle spielen. Mittels RT-qPCR konnte nachgewiesen werden, dass einige dieser lncRNAs in BCG-infizierten Makrophagen 30 Minuten nach der Infektion herabreguliert waren (im Vergleich zu nicht-infizierten Zellen sowie hitzeinaktivierten BCG-infizierten Zellen). Von allen getesteten lncRNAs war MEG3 am stärksten herabreguliert. Eine *in silico* Analyse ergab, dass MEG3 in der mTOR und PI3K-AKT Signalkaskade involviert ist und daher eine Rolle bei der Autophagy spielen könnte. Eine Induktion von Autophagie durch IFN-γ in infizierten Makrophagen resultierte in anhaltender MEG3 Herabregulation. Ein Knockdown von MEG3 in Makrophagen resultierte in der Induktion von Autophagie und verstärkter Eliminierung intrazellulärer BCG.

Im zweiten Teil der Arbeit wurde ein „bottom up"-Vorgehen gewählt, um miRNAs und Zielgene zu identifizieren, die im Prozess der Phagosomenreifung eine Rolle spielen. Eine zunächst durchgeführte *in silico* Analyse identifizierte die am stärksten involvierten miRNAs. Anschließende Expressionsstudien ergaben, dass die miRNAs miR-3619-5p, miR-637 und miR-324-3p in BCG infizierten Zellen im Vergleich zu nicht-infizierten Zellen herabreguliert waren. Eine Analyse der Expression potentieller Zielgene ergab, dass CTSS (lysosomal cysteine protease S) und RAB11FIP4 (Rab11 family-interacting protein 4) heraufreguliert

waren. Eine Regulation von CTSS durch miR-3619-5p konnte mittels Reportergenassays verifiziert werden.

Die vorliegende Studie konnte zeigen, dass Autophagie-assoziierte Signalwege in Mykobakterien-infizierten Makrophagen durch die lange nicht-kodierende RNA MEG3 und die miRNA miR-3619-5p beeinflusst werden.

7. Outlook

To understand how RNA networks regulate autophagy process in mycobacterial infection, there is need of finding all other RNAs which influence autophagy. Therefore, based on current findings next step could be in the direction of investigating those remaining RNAs.

As discussed earlier, finding expression of miRNA-29 as well as miR-181a along with their influence on MEG3 expression in BCG infection could be an important study to address.

Except for AS UCHL1, expressions of all other 10 lncRNAs were counter-regulated at 4 h time points. Expression study of AS UCHL1 in further time point (e.g. at 24 h or 48 h) in mycobacterial infection could be addressed to know its significance. If the down-regulation of AS UCHL1 remains constant though out further time points then this is an important candidate to look into details with respect to mycobacterial infection.

Among 11 down-regulated lncRNAs, the current thesis has focused only on the expression of MEG3 and its role in autophagy. Remaining 10 lncRNAs could be studied for their response towards autophagy induction.

Further, finding a correlation between expression of lncRNAs and phagocytosis process may be an interesting area to focus.

To find specific miRNAs in phagosomal maturation, the bottom-up approach may be modified (e.g. considering more number of proteins for selection) and further expression studies for newly sorted miRNAs and their targets can be planned.

8. References

1. Galagan, J.E., *DISEASE MECHANISMS Genomic insights into tuberculosis.* Nature Reviews Genetics, 2014. **15**(5): p. 307-320.
2. Prasanna, A.N. and S. Mehra, *Comparative phylogenomics of pathogenic and non-pathogenic mycobacterium.* PLoS One, 2013. **8**(8): p. e71248.
3. Marrakchi, H., M.A. Laneelle, and M. Daffe, *Mycolic acids: structures, biosynthesis, and beyond.* Chem Biol, 2014. **21**(1): p. 67-85.
4. Daffe, M. and P. Draper, *The envelope layers of mycobacteria with reference to their pathogenicity.* Adv Microb Physiol, 1998. **39**: p. 131-203.
5. Bhatt, A., et al., *Deletion of kasB in Mycobacterium tuberculosis causes loss of acid-fastness and subclinical latent tuberculosis in immunocompetent mice.* Proc Natl Acad Sci U S A, 2007. **104**(12): p. 5157-62.
6. Dubnau, E., et al., *Oxygenated mycolic acids are necessary for virulence of Mycobacterium tuberculosis in mice.* Mol Microbiol, 2000. **36**(3): p. 630-7.
7. Rao, V., et al., *Mycobacterium tuberculosis controls host innate immune activation through cyclopropane modification of a glycolipid effector molecule.* J Exp Med, 2005. **201**(4): p. 535-43.
8. Singh, A., et al., *Requirement of the mymA operon for appropriate cell wall ultrastructure and persistence of Mycobacterium tuberculosis in the spleens of guinea pigs.* J Bacteriol, 2005. **187**(12): p. 4173-86.
9. Sreejit, G., et al., *The ESAT-6 protein of Mycobacterium tuberculosis interacts with beta-2-microglobulin (beta2M) affecting antigen presentation function of macrophage.* PLoS Pathog, 2014. **10**(10): p. e1004446.
10. Guo, S., et al., *The CFP10/ESAT6 complex of Mycobacterium tuberculosis may function as a regulator of macrophage cell death at different stages of tuberculosis infection.* Medical Hypotheses, 2012. **78**(3): p. 389-392.
11. Derrick, S.C. and S.L. Morris, *The ESAT6 protein of Mycobacterium tuberculosis induces apoptosis of macrophages by activating caspase expression.* Cell Microbiol, 2007. **9**(6): p. 1547-55.
12. Forrellad, M.A., et al., *Virulence factors of the Mycobacterium tuberculosis complex.* Virulence, 2013. **4**(1): p. 3-66.
13. Ghosh, S., et al., *Phenotypic heterogeneity in mycobacterial stringent response.* BMC Syst Biol, 2011. **5**: p. 18.
14. Magdanova, L.A. and N.V. Golyasnaya, *Heterogeneity as an Adaptive Trait of Microbial Populations.* Microbiology, 2013. **82**(1): p. 1-10.
15. Bishai, W., *Lipid lunch for persistent pathogen.* Nature, 2000. **406**(6797): p. 683-5.
16. De Voss, J.J., et al., *Iron acquisition and metabolism by mycobacteria.* J Bacteriol, 1999. **181**(15): p. 4443-51.
17. Nyka, W., *Studies on the effect of starvation on mycobacteria.* Infect Immun, 1974. **9**(5): p. 843-50.
18. Golchin, S.A., et al., *A microfluidic system for long-term time-lapse microscopy studies of mycobacteria.* Tuberculosis (Edinb), 2012. **92**(6): p. 489-96.
19. Parish, T. and D.M. Roberts, *Mycobacteria protocols*. Third edition. ed. Methods in molecular biology,. 2015, New York: Humana Press. xii, 416 pages.
20. Koul, A., et al., *Delayed bactericidal response of Mycobacterium tuberculosis to bedaquiline involves remodelling of bacterial metabolism.* Nat Commun, 2014. **5**: p. 3369.
21. Murray, P.R., K.S. Rosenthal, and M.A. Pfaller, *Medical microbiology*. 7th Edition ed. 2013, Philadelphia: Elsevier/Saunders. x, 874 pages.

22. Lewin, A. and S. Sharbati-Tehrani, *[Slow growth rate of mycobacteria. Possible reasons and significance for their pathogenicity].* Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz, 2005. **48**(12): p. 1390-9.
23. Rastogi, N., E. Legrand, and C. Sola, *The mycobacteria: an introduction to nomenclature and pathogenesis.* Revue Scientifique Et Technique-Office International Des Epizooties, 2001. **20**(1): p. 21-54.
24. Jarlier, V. and H. Nikaido, *Permeability barrier to hydrophilic solutes in Mycobacterium chelonei.* J Bacteriol, 1990. **172**(3): p. 1418-23.
25. Niederweis, M., *Mycobacterial porins--new channel proteins in unique outer membranes.* Mol Microbiol, 2003. **49**(5): p. 1167-77.
26. Mailaender, C., et al., *The MspA porin promotes growth and increases antibiotic susceptibility of both Mycobacterium bovis BCG and Mycobacterium tuberculosis.* Microbiology, 2004. **150**(Pt 4): p. 853-64.
27. Sharbati-Tehrani, S., et al., *The porin MspA from Mycobacterium smegmatis improves growth of Mycobacterium bovis BCG.* Int J Med Microbiol, 2004. **294**(4): p. 235-45.
28. Almeida Da Silva, P.E. and J.C. Palomino, *Molecular basis and mechanisms of drug resistance in Mycobacterium tuberculosis: classical and new drugs.* J Antimicrob Chemother, 2011. **66**(7): p. 1417-30.
29. Telenti, A., et al., *Direct, automated detection of rifampin-resistant Mycobacterium tuberculosis by polymerase chain reaction and single-strand conformation polymorphism analysis.* Antimicrob Agents Chemother, 1993. **37**(10): p. 2054-8.
30. Flannagan, R.S., G. Cosio, and S. Grinstein, *Antimicrobial mechanisms of phagocytes and bacterial evasion strategies.* Nat Rev Microbiol, 2009. **7**(5): p. 355-66.
31. Khare, G., et al., *KefB inhibits phagosomal acidification but its role is unrelated to M. tuberculosis survival in host.* Scientific Reports, 2013. **3**.
32. Via, L.E., et al., *Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7.* Journal of Biological Chemistry, 1997. **272**(20): p. 13326-13331.
33. Wong, D., et al., *Mycobacterium tuberculosis protein tyrosine phosphatase (PtpA) excludes host vacuolar-H+-ATPase to inhibit phagosome acidification.* Proc Natl Acad Sci U S A, 2011. **108**(48): p. 19371-6.
34. Pitt, A., et al., *Alterations in the protein composition of maturing phagosomes.* J Clin Invest, 1992. **90**(5): p. 1978-83.
35. Sturgill-Koszycki, S., et al., *Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase.* Science, 1994. **263**(5147): p. 678-81.
36. Hackam, D.J., et al., *Regulation of phagosomal acidification. Differential targeting of Na+/H+ exchangers, Na+/K+-ATPases, and vacuolar-type H+-atpases.* J Biol Chem, 1997. **272**(47): p. 29810-20.
37. Fairn, G.D. and S. Grinstein, *How nascent phagosomes mature to become phagolysosomes.* Trends in Immunology, 2012. **33**(8): p. 397-405.
38. Seto, S., et al., *Dissection of Rab7 localization on Mycobacterium tuberculosis phagosome.* Biochem Biophys Res Commun, 2009. **387**(2): p. 272-7.
39. Clemens, D.L. and M.A. Horwitz, *Characterization of the Mycobacterium tuberculosis phagosome and evidence that phagosomal maturation is inhibited.* J Exp Med, 1995. **181**(1): p. 257-70.
40. Christoforidis, S., et al., *Phosphatidylinositol-3-OH kinases are Rab5 effectors.* Nat Cell Biol, 1999. **1**(4): p. 249-52.

41. Kang, P.B., et al., *The human macrophage mannose receptor directs Mycobacterium tuberculosis lipoarabinomannan-mediated phagosome biogenesis.* J Exp Med, 2005. **202**(7): p. 987-99.
42. Vergne, I., et al., *Mechanism of phagolysosome biogenesis block by viable Mycobacterium tuberculosis.* Proc Natl Acad Sci U S A, 2005. **102**(11): p. 4033-8.
43. Mizushima, N., *Autophagy: process and function.* Genes Dev, 2007. **21**(22): p. 2861-73.
44. Klionsky, D.J., *Autophagy: from phenomenology to molecular understanding in less than a decade.* Nature Reviews Molecular Cell Biology, 2007. **8**(11): p. 931-937.
45. Kabeya, Y., et al., *Cis1/Atg31 is required for autophagosome formation in Saccharomyces cereviside.* Biochemical and Biophysical Research Communications, 2007. **356**(2): p. 405-410.
46. Suzuki, K. and Y. Ohsumi, *Molecular machinery of autophagosome formation in yeast, Saccharomyces cerevisiae.* FEBS Lett, 2007. **581**(11): p. 2156-61.
47. Kabeya, Y., et al., *LC3, a mammalian homolog of yeast Apg8p, is localized in autophagosome membranes after processing (vol 19, pg 5720, 2000).* Embo Journal, 2003. **22**(17): p. 4577-4577.
48. Bjorkoy, G., et al., *p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death.* J Cell Biol, 2005. **171**(4): p. 603-14.
49. Pankiv, S., et al., *p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy.* J Biol Chem, 2007. **282**(33): p. 24131-45.
50. Mordier, S., et al., *Leucine limitation induces autophagy and activation of lysosome-dependent proteolysis in C2C12 myotubes through a mammalian target of rapamycin-independent signaling pathway.* J Biol Chem, 2000. **275**(38): p. 29900-6.
51. Sarkar, S., et al., *Small molecules enhance autophagy and reduce toxicity in Huntington's disease models.* Nat Chem Biol, 2007. **3**(6): p. 331-8.
52. He, C. and D.J. Klionsky, *Regulation mechanisms and signaling pathways of autophagy.* Annu Rev Genet, 2009. **43**: p. 67-93.
53. Pareja, M.E. and M.I. Colombo, *Autophagic clearance of bacterial pathogens: molecular recognition of intracellular microorganisms.* Front Cell Infect Microbiol, 2013. **3**: p. 54.
54. Huang, J. and J.H. Brumell, *Bacteria-autophagy interplay: a battle for survival.* Nat Rev Microbiol, 2014. **12**(2): p. 101-14.
55. Moraco, A.H. and H. Kornfeld, *Cell death and autophagy in tuberculosis.* Semin Immunol, 2014. **26**(6): p. 497-511.
56. Watson, R.O., P.S. Manzanillo, and J.S. Cox, *Extracellular M. tuberculosis DNA targets bacteria for autophagy by activating the host DNA-sensing pathway.* Cell, 2012. **150**(4): p. 803-15.
57. Gutierrez, M.G., et al., *Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages.* Cell, 2004. **119**(6): p. 753-66.
58. Romagnoli, A., et al., *ESX-1 dependent impairment of autophagic flux by Mycobacterium tuberculosis in human dendritic cells.* Autophagy, 2012. **8**(9): p. 1357-70.
59. Gutierrez, M.G., et al., *Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages.* Cell, 2004. **119**(6): p. 753-766.

60. Deretic, V., *Autophagy in innate and adaptive immunity.* Trends Immunol, 2005. **26**(10): p. 523-8.
61. van Rooij, E. and E.N. Olson, *MicroRNA therapeutics for cardiovascular disease: opportunities and obstacles.* Nat Rev Drug Discov, 2012. **11**(11): p. 860-72.
62. Wang, K.C. and H.Y. Chang, *Molecular mechanisms of long noncoding RNAs.* Mol Cell, 2011. **43**(6): p. 904-14.
63. Lasda, E. and R. Parker, *Circular RNAs: diversity of form and function.* RNA, 2014. **20**(12): p. 1829-42.
64. Jeck, W.R. and N.E. Sharpless, *Detecting and characterizing circular RNAs.* Nat Biotechnol, 2014. **32**(5): p. 453-61.
65. Ishizu, H., H. Siomi, and M.C. Siomi, *Biology of PIWI-interacting RNAs: new insights into biogenesis and function inside and outside of germlines.* Genes & Development, 2012. **26**(21): p. 2361-2373.
66. Stepanov, G.A., et al., *Regulatory role of small nucleolar RNAs in human diseases.* Biomed Res Int, 2015. **2015**: p. 206849.
67. Mattick, J.S. and M.J. Gagen, *The evolution of controlled multitasked gene networks: the role of introns and other noncoding RNAs in the development of complex organisms.* Mol Biol Evol, 2001. **18**(9): p. 1611-30.
68. Guo, X., et al., *Advances in long noncoding RNAs: identification, structure prediction and function annotation.* Brief Funct Genomics, 2015.
69. Turner, M., A. Galloway, and E. Vigorito, *Noncoding RNA and its associated proteins as regulatory elements of the immune system.* Nature Immunology, 2014. **15**(6): p. 484-491.
70. Wang, P., et al., *The STAT3-binding long noncoding RNA lnc-DC controls human dendritic cell differentiation.* Science, 2014. **344**(6181): p. 310-3.
71. Wang, K., et al., *APF lncRNA regulates autophagy and myocardial infarction by targeting miR-188-3p.* Nat Commun, 2015. **6**: p. 6779.
72. Ying, L., et al., *Downregulated MEG3 activates autophagy and increases cell proliferation in bladder cancer.* Mol Biosyst, 2013. **9**(3): p. 407-11.
73. Zhao, Y., et al., *Role of long non-coding RNA HULC in cell proliferation, apoptosis and tumor metastasis of gastric cancer: a clinical and in vitro investigation.* Oncol Rep, 2014. **31**(1): p. 358-64.
74. Gomez, J.A., et al., *The NeST long ncRNA controls microbial susceptibility and epigenetic activation of the interferon-gamma locus.* Cell, 2013. **152**(4): p. 743-54.
75. Wang, Y., et al., *Long noncoding RNA derived from CD244 signaling epigenetically controls CD8+ T-cell immune responses in tuberculosis infection.* Proc Natl Acad Sci U S A, 2015. **112**(29): p. E3883-92.
76. Westermann, A.J., et al., *Dual RNA-seq unveils noncoding RNA functions in host-pathogen interactions.* Nature, 2016. **529**(7587): p. 496-501.
77. Wu, J., et al., *Analysis of microRNA expression profiling identifies miR-155 and miR-155* as potential diagnostic markers for active tuberculosis: a preliminary study.* Hum Immunol, 2012. **73**(1): p. 31-7.
78. Fu, Y., et al., *Circulating microRNAs in patients with active pulmonary tuberculosis.* J Clin Microbiol, 2011. **49**(12): p. 4246-51.
79. Furci, L., et al., *Alteration of human macrophages microRNA expression profile upon infection with Mycobacterium tuberculosis.* Int J Mycobacteriol, 2013. **2**(3): p. 128-34.
80. Tili, E., et al., *Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock.* J Immunol, 2007. **179**(8): p. 5082-9.

81. Bala, S., et al., *Up-regulation of microRNA-155 in macrophages contributes to increased tumor necrosis factor {alpha} (TNF{alpha}) production via increased mRNA half-life in alcoholic liver disease.* J Biol Chem, 2011. **286**(2): p. 1436-44.
82. Bettencourt, P., et al., *Actin-binding protein regulation by microRNAs as a novel microbial strategy to modulate phagocytosis by host cells: the case of N-Wasp and miR-142-3p.* Frontiers in Cellular and Infection Microbiology, 2013. **3**.
83. Martinez, A.N., S. Mehra, and D. Kaushal, *Role of interleukin 6 in innate immunity to Mycobacterium tuberculosis infection.* J Infect Dis, 2013. **207**(8): p. 1253-61.
84. Xu, G., et al., *microR-142-3p down-regulates IRAK-1 in response to Mycobacterium bovis BCG infection in macrophages.* Tuberculosis (Edinb), 2013. **93**(6): p. 606-11.
85. Sharbati, J., et al., *Integrated MicroRNA-mRNA-Analysis of Human Monocyte Derived Macrophages upon Mycobacterium avium subsp hominissuis Infection.* Plos One, 2011. **6**(5).
86. Kim, J.K., et al., *MicroRNA-125a Inhibits Autophagy Activation and Antimicrobial Responses during Mycobacterial Infection.* J Immunol, 2015. **194**(11): p. 5355-65.
87. Sun, J., et al., *Mycobacterium bovis BCG disrupts the interaction of Rab7 with RILP contributing to inhibition of phagosome maturation.* J Leukoc Biol, 2007. **82**(6): p. 1437-45.
88. Pym, A.S., et al., *Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines Mycobacterium bovis BCG and Mycobacterium microti.* Mol Microbiol, 2002. **46**(3): p. 709-17.
89. Hunt, D.M., et al., *Long-range transcriptional control of an operon necessary for virulence-critical ESX-1 secretion in Mycobacterium tuberculosis.* J Bacteriol, 2012. **194**(9): p. 2307-20.
90. Monahan, I.M., et al., *Differential expression of mycobacterial proteins following phagocytosis by macrophages.* Microbiology, 2001. **147**(Pt 2): p. 459-71.
91. Walburger, A., et al., *Protein kinase G from pathogenic mycobacteria promotes survival within macrophages.* Science, 2004. **304**(5678): p. 1800-1804.
92. Houben, E.N., et al., *Differential expression of a virulence factor in pathogenic and non-pathogenic mycobacteria.* Mol Microbiol, 2009. **72**(1): p. 41-52.
93. Heldwein, K.A. and M.J. Fenton, *The role of Toll-like receptors in immunity against mycobacterial infection.* Microbes Infect, 2002. **4**(9): p. 937-44.
94. Heldwein, K.A., et al., *TLR2 and TLR4 serve distinct roles in the host immune response against Mycobacterium bovis BCG.* J Leukoc Biol, 2003. **74**(2): p. 277-86.
95. Gilchrist, M., et al., *Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4 (vol 441, pg 173, 2006).* Nature, 2008. **451**(7181): p. 1022-1022.
96. Congrains, A., et al., *ANRIL: molecular mechanisms and implications in human health.* Int J Mol Sci, 2013. **14**(1): p. 1278-92.
97. Yu, W., et al., *Epigenetic silencing of tumour suppressor gene p15 by its antisense RNA.* Nature, 2008. **451**(7175): p. 202-6.
98. Yap, K.L., et al., *Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a.* Mol Cell, 2010. **38**(5): p. 662-74.
99. Carrieri, C., et al., *Expression analysis of the long non-coding RNA antisense to Uchl1 (AS Uchl1) during dopaminergic cells' differentiation in vitro and in neurochemical models of Parkinson's disease.* Front Cell Neurosci, 2015. **9**: p. 114.
100. Costes, S., et al., *UCHL1 deficiency exacerbates human islet amyloid polypeptide toxicity in beta-cells: evidence of interplay between the ubiquitin/proteasome system and autophagy.* Autophagy, 2014. **10**(6): p. 1004-14.

101. Wagner, L.A., et al., *EGO, a novel, noncoding RNA gene, regulates eosinophil granule protein transcript expression.* Blood, 2007. **109**(12): p. 5191-8.
102. Monnier, P., et al., *H19 lncRNA controls gene expression of the Imprinted Gene Network by recruiting MBD1.* Proc Natl Acad Sci U S A, 2013. **110**(51): p. 20693-8.
103. Ragina, N.P., et al., *Downregulation of H19 improves the differentiation potential of mouse parthenogenetic embryonic stem cells.* Stem Cells Dev, 2012. **21**(7): p. 1134-44.
104. Braconi, C., et al., *microRNA-29 can regulate expression of the long non-coding RNA gene MEG3 in hepatocellular cancer.* Oncogene, 2011. **30**(47): p. 4750-6.
105. Peng, W., et al., *Long non-coding RNA MEG3 functions as a competing endogenous RNA to regulate gastric cancer progression.* J Exp Clin Cancer Res, 2015. **34**: p. 79.
106. Willingham, A.T., et al., *A strategy for probing the function of noncoding RNAs finds a repressor of NFAT.* Science, 2005. **309**(5740): p. 1570-3.
107. Liu, A.Y., et al., *The human NTT gene: identification of a novel 17-kb noncoding nuclear RNA expressed in activated CD4+ T cells.* Genomics, 1997. **39**(2): p. 171-84.
108. Sonkoly, E., et al., *Identification and characterization of a novel, psoriasis susceptibility-related noncoding RNA gene, PRINS.* J Biol Chem, 2005. **280**(25): p. 24159-67.
109. Bari, L., et al., *Comparison of stress-induced PRINS gene expression in normal human keratinocytes and HaCaT cells.* Arch Dermatol Res, 2011. **303**(10): p. 745-52.
110. Collier, S.P., et al., *Cutting edge: influence of Tmevpg1, a long intergenic noncoding RNA, on the expression of Ifng by Th1 cells.* J Immunol, 2012. **189**(5): p. 2084-8.
111. Dallosso, A.R., et al., *Alternately spliced WT1 antisense transcripts interact with WT1 sense RNA and show epigenetic and splicing defects in cancer.* RNA, 2007. **13**(12): p. 2287-99.
112. Niu, X., et al., *Role of MiR-3619-5p in beta-Catenin-Mediated Non-Small Cell Lung Cancer Growth and Invasion.* Cell Physiol Biochem, 2015. **37**(4): p. 1527-36.
113. Hunsberger, J.G., et al., *Novel integrative genomic tool for interrogating lithium response in bipolar disorder.* Transl Psychiatry, 2015. **5**: p. e504.
114. Zhang, J.F., et al., *MiR-637 maintains the balance between adipocytes and osteoblasts by directly targeting Osterix.* Molecular Biology of the Cell, 2011. **22**(21): p. 3955-3961.
115. Que, T., et al., *Decreased miRNA-637 is an unfavorable prognosis marker and promotes glioma cell growth, migration and invasion via direct targeting Akt1.* Oncogene, 2015. **34**(38): p. 4952-4963.
116. Xu, J., et al., *MiR-185-3p and miR-324-3p Predict Radiosensitivity of Nasopharyngeal Carcinoma and Modulate Cancer Cell Growth and Apoptosis by Targeting SMAD7.* Med Sci Monit, 2015. **21**: p. 2828-36.
117. Macconi, D., et al., *MicroRNA-324-3p Promotes Renal Fibrosis and Is a Target of ACE Inhibition.* Journal of the American Society of Nephrology, 2012. **23**(9): p. 1496-1505.
118. Staedel, C. and F. Darfeuille, *MicroRNAs and bacterial infection.* Cellular Microbiology, 2013. **15**(9): p. 1496-1507.
119. Ghorpade, D.S., et al., *MicroRNA-155 Is Required for Mycobacterium bovis BCG-Mediated Apoptosis of Macrophages.* Molecular and Cellular Biology, 2012. **32**(12): p. 2239-2253.
120. Wang, J.L., et al., *MicroRNA-155 induction by Mycobacterium bovis BCG enhances ROS production through targeting SHIP1.* Molecular Immunology, 2014. **62**(1): p. 29-36.

121. Driessen, C., et al., *Cathepsin S controls the trafficking and maturation of MHC class II molecules in dendritic cells.* J Cell Biol, 1999. **147**(4): p. 775-90.
122. Conus, S. and H.U. Simon, *Cathepsins and their involvement in immune responses.* Swiss Medical Weekly, 2010. **140**: p. 4-11.
123. Zhang, L., et al., *Inhibition of cathepsin S induces autophagy and apoptosis in human glioblastoma cell lines through ROS-mediated PI3K/AKT/mTOR/p70S6K and JNK signaling pathways.* Toxicol Lett, 2014. **228**(3): p. 248-59.
124. Zullo, A.J. and S. Lee, *Mycobacterial induction of autophagy varies by species and occurs independently of mammalian target of rapamycin inhibition.* J Biol Chem, 2012. **287**(16): p. 12668-78.
125. Shin, D.M., et al., *Mycobacterium tuberculosis eis regulates autophagy, inflammation, and cell death through redox-dependent signaling.* PLoS Pathog, 2010. **6**(12): p. e1001230.
126. Deretic, V., *Autophagy, an immunologic magic bullet: Mycobacterium tuberculosis phagosome maturation block and how to bypass it.* Future Microbiol, 2008. **3**(5): p. 517-24.
127. Fabri, M., et al., *Vitamin D Is Required for IFN-gamma-Mediated Antimicrobial Activity of Human Macrophages.* Science Translational Medicine, 2011. **3**(104).
128. Wang, J., et al., *MicroRNA-155 promotes autophagy to eliminate intracellular mycobacteria by targeting Rheb.* PLoS Pathog, 2013. **9**(10): p. e1003697.
129. Huang, Y.P., R. Guerrero-Preston, and E.A. Ratovitski, *Phospho-Delta Np63 alpha-dependent regulation of autophagic signaling through transcription and micro-RNA modulation.* Cell Cycle, 2012. **11**(6): p. 1247-1259.
130. Yang, X., et al., *mir-30d Regulates multiple genes in the autophagy pathway and impairs autophagy process in human cancer cells.* Biochem Biophys Res Commun, 2013. **431**(3): p. 617-22.
131. Yu, Y., et al., *Targeting microRNA-30a-mediated autophagy enhances imatinib activity against human chronic myeloid leukemia cells.* Leukemia, 2012. **26**(8): p. 1752-60.
132. Kimmey, J.M., et al., *Unique role for ATG5 in neutrophil-mediated immunopathology during M. tuberculosis infection.* Nature, 2015. **528**(7583): p. 565-+.

9. Publications and scientific activities

Peer reviewed articles:

Kamlesh Pawar, Carlos Hanisch, Sergio Eliseo Palma Vera, Ralf Einspanier, Soroush Sharbati. Down regulated lncRNA MEG3 eliminates mycobacteria in macrophages via autophagy. Scientific Reports.
Impact Factor: 5.578

Kamlesh Pawar, Jutta Sharbati, Ralf Einspanier, Soroush Sharbati. Mycobacterium bovis BCG interferes with miR-3619-5p control of Cathepsin S in the process of autophagy. Frontiers in cellular and infection microbiology.
Impact Factor: 3.7

L Hoeke, J Sharbati, **Kamlesh Pawar**, A Keller, R Einspanier, S Sharbati Intestinal Salmonella typhimurium infection leads to miR-29a induced caveolin 2 regulation. PloS one 8 (6), e67300 (2013).
Impact Factor: 3.234

Kamlesh Pawar and Kaul G Toxicity of titanium oxide nanoparticles cause functionality and DNA damage in buffalo (*Bubalus bubalis*) sperm in vitro. Toxicol Ind Health. 30(6):520-33 (2014).
Impact Factor: 1.859

Kamlesh Pawar and Kaul G. Assessment of buffalo (*Bubalus bubalis*) sperm DNA fragmentation using a sperm chromatin dispersion (SCD) test. Reproduction in Domestic Animals. 46(6), 964-969 (2011).
Impact Factor: 1.356

Kamlesh Pawar and Kaul G. Toxicity of Europium Oxide Nanoparticles on the Buffalo (*Bubalus bubalis*) Spermatozoa DNA Damage. Advanced Science, Engineering and Medicine, 5(1), 11-17 (2013)

Conference and talks:

Pawar K, J. Sharbati, R. Einspanier, S. Sharbati. miR-3619-5p mediates Cathepsin S control of autophagy in mycobacterial infection of macrophages. Tagung der DVG-Fachgruppe "Physiologie und Biochemie". Berlin, Germany. (2016). 30 March – 1 April 2016: (Short talk)

Pawar K, C. Hanisch, S. Eliseo Palma Vera, R. Einspanier, S. Sharbati. Mycobacteria escape autophagy by counter regulating long non-coding RNA MEG3. DGZ, Cologne, Germany (2015). 24 March - 27 March, 2015: (Short talk and poster presentation)

Pawar K, Sharbati J, Einspanier R, Sharbati S. A novel approach towards finding regulatory microRNAs during phagosomal maturation in infection. FEBS EMBO, Paris, France (2014). 30 August - 4 September 2014: (Poster presentation)

Summer schools:

September 21 - September 25, 2015:
Summer school modern methods in infection biology 2015. Julius-Maximilians-Universität Würzburg, Würzburg, Germany.
Course content: Microbiology, Parasitology, Systems Biology, lectures and lab courses

September 07 - September 18, 2015:
SYNMarburg 2015, 3rd Summer school: From microbial cell biology to complex communities, Marburg, Germany.
Course content: Hands on experience of cutting edge scientific methods and provide exciting insights into synthetic microbiology projects existing in Marburg.

June 20 - July 4, 2015:
The international summer school 2015 on Pathogen-Host Interplay. ZIBI summer school, Berlin, Germany.
Course content: One week lecture course and two days scientific symposium on "New concepts in pathogen recognition and host defense**"**

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11. Declaration

Hiermit bestätige ich, **Kamlesh Ganesh Pawar**, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe

Berlin, dated: 18.01.2017 **Kamlesh Ganesh Pawar**