Juncal García García

Saliva-mediated killing of intracellular mycobacteria

TREBALL DE FI DE GRAU

Dirigit per Antoni Romeu Figuerola

Grau Bioquímica i Biologia Molecular

UNIVERSITAT ROVIRA I VIRGILI

Tarragona

2014

This Project has been carried out in the Department of Infection Medicine at Biomedical Centrum (BMC), Lund University (Sweden) under the supervision of Dr. Ole Sørensen.

Table of contents

ABSTRACT

Mycobacterium tuberculosis is able to infect and survive in macrophages and this is pivotal for development of tuberculosis. Clearing of infection is difficult, which consequently leads to persistent infection. However, a novel process recently in macrophages has been discovered where macrophages form macrophages Extracellular Traps (METs), which is a mechanism similar to apoptosis that possible, could help to eliminate the pathogen. This process has also been observed in neutrophils (NETs), activated by a sialic-acid containing salivary component, mucins (data non-published). This process involves a process called autophagy, which results in the breakdown of structural proteins of the nuclear and plasma membrane that in turn facilitates the release of DNA. Previous studies also indicate that intracellular *Mycobacterium tuberculosis* is subject to elimination through autophagy of the infected cell. Surprisingly, we observed a similar effect wherein, peripheral blood monocytes demonstrated increased killing of intracellular mycobacteria after exposure to saliva. In this work, through different experiments, which we have been optimizing continuously, we exposed mycobacteria-infected monocytes (cell lines and peripheral blood monocytes) to saliva and examined the intracellular survival rate of the mycobacteria. Our result indicated that saliva stimulation mediated killing of intracellular mycobacteria but that free sialic acid did not show any apparent effect. Nevertheless, there are indications that the active component in saliva encompasses a sialic acid containing moiety. Though the results were not conclusive, promising preliminary results make it feasible to further explore the possibility that saliva could contain component(s) stimulating monocytes to kill mycobacteria and other intracellular pathogens.

INTRODUCTION

Immune system

Human body has developed a really complex system to defend itself against the constant attacks from both inside and outside. Thus the immune response mediated by the immune system is the process by which the body recognizes itself from non-self (foreign matter). For that, human body has different components which try to destroy or neutralize foreign matter (living and nonliving). This capacity to distinguish self from non-self serves to protect against infection by pathogens including viruses, bacteria, fungi, parasites such as some protozoa, isolate or remove non-microbial foreign substances that go inside the body, and also to destroy cancer cells that arise in the body.

Barriers of immune system

The human body has developed different mechanisms to remove possible infections. Those mechanisms are formed for cells and a diversity of chemical devices such as the complement system which respond to the infection. They are grouped in three barriers of defense according to their role and localization. The three barriers of defense of the human body are: The skin, the cellular counterattack or non-specific response and the immune response as the third line of defense. The first two barriers are non-specific and they form the innate immune system. Most of the times, the possible pathogens don't pass the first line, it gives an idea about how effective this system can be. The third line of defense, the immune response, corresponds to the acquired or adaptive immune system which is able to recognize reinfections and kill the pathogen. (Johnson, Raven, Singer, & Losos, 2002)

Immune cells

Both innate and adaptive immune systems are formed for a range of cells, which interact with each other and with the protein defense for giving the immune response. All those cells come from the same precursor, stem cells located in the bone marrow. Those cells are: Phagocytic cells (Neutrophils and macrophages that kill mostly by phagocytosis), natural killer cells (NK cells, which kill by making pores in cells); basophils (which contain granules of toxic chemicals which can digest foreign microorganisms and are involved in the allergic response); mast cells (contain a diversity of inflammatory chemicals, which cause blood vessels constriction near the wound); eosinophiles (which secrete enzymes to kill parasitic worms and other pathogens) and platelets. The receptors of these cells are pattern recognition receptors (PPRRs) which recognize broad molecular patterns found on pathogens called pathogen associated molecular patterns (PAMPs). (Janeway, Travers, & Walport, 2001)

Macrophages

Microorganisms such as bacteria which penetrate the epithelial surfaces of the body for the first time are recognized immediately by cells and molecules which are able to carry out the innate immune response. Macrophages with other professional phagocytic cells including neutrophils and monocytes are in charge of the recognition and removal of invading pathogens, playing an essential role in the host-defense system. Macrophages, also called "big-eaters" are able to eliminate pathogens directly or indirectly via innate and

adaptive immune responses, respectively. Phagocytic macrophages carry out the defense against pathogenic bacteria by means of surface receptors which are capable to recognize and bind common constituents of numerous bacterial surfaces. The direct bactericidal functions of macrophages include the phagocytosis process (shown in figure 1) and the generation of reactive oxygen species (ROS). Bacterial molecules binding to the macrophages' receptors trigger the phagocytic cell to engulf the bacterium, involving it into phagosomes. The bacteria-containing phagosomes fuse with lysosomes in a process of ''maturation'' which leads to the consequent degradation of the bacteria.

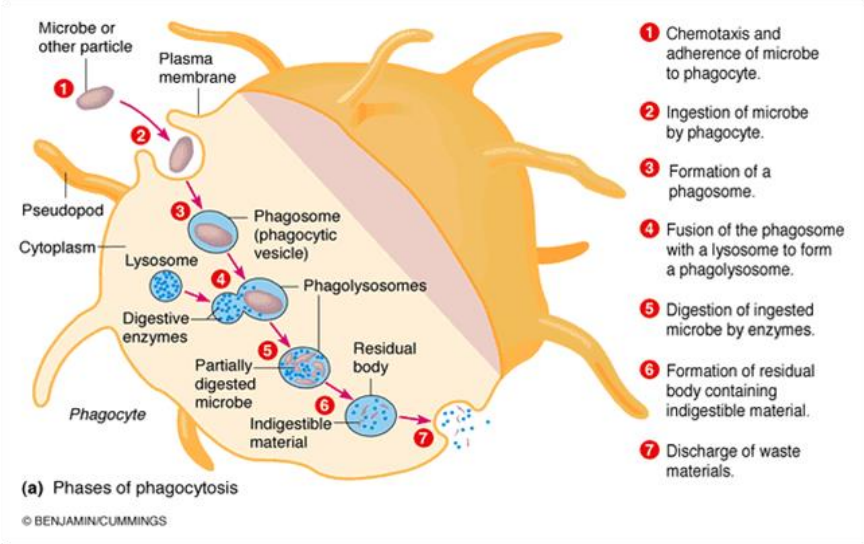


Figure 1.Process of macrophages' phagocytosis and digestion of a microbe. Steps: (1) Detection of a microbe or other particle. (2) Engulfment of the microbe through the plasma membrane. (3) Phagosome formation (phagocytic vesicle). (4) Phagolysosome formation (fusion with lysosome). (5) Digestion of microbe in the phagolysosome. (6) End of the digestion, formation of residual body. (7) Discharge of waste material.

Another indirect function of activated macrophages immune response is the secretion of biologically active molecules, which contributes to the inflammation process, characterized by the increased production of many inflammatory molecules such as cytokines and chemokines. Thus macrophages together with neutrophils are also known as inflammatory cells. The cytokines and chemokines released by macrophages in response to bacterial components, together, promote the recruitment of other blood cells such as leukocytes to the site of infection and the activation of additional immune cells. (Janeway, Travers, & Walport, 2001)

In certain cases, the host's protecting responses are overcome by the invading pathogen, and the death of activated macrophages can be triggered in order to survive. (Gong & Rodney J. Devenish, 2012)

Neutrophil "the polymorphonuclear granulocyte"

Neutrophil granulocytes constitute by far the largest population of white blood cells in mammals. As part of the acute phase of the innate immune system, the neutrophils, when needed, leave the blood vessels and migrate towards sites of ongoing infection. (Mayadas, Cullere, & Lowell, 2013) Prior to activation, neutrophils display a round shape with the nucleus divided into three to five lobules. This, along with a set of four distinct types of granules, gives the neutrophils a very specific character, and constitutes part of the polymorphonuclear cell family along with basophils and eosinophils.

Resting on the neutrophil surface are a large array of receptor molecules, that target various pathogenic factors. These include G-protein coupled chemokine and chemoattractant receptors, Fc receptors for antibody recognition, selectin and selectin ligands and integrins for adhesion to activated epithelial cells, but also Toll-like receptors and lectins. Upon reaching the sites and subsequent exposure to inflammatory cues such as cytokines and chemokines from activated epitheilial cells, mast cells and macrophages, the neutrophil undergoes a radical change in morphology, initially to enable migration to the site of inflammation. If in direct contact with microbes, the neutrophil has the ability to act as a phagocyte if the target is coated with opsonins, where it attaches to and engulfs the foreign invader in a phagosome, which fuses with internal granules containing proteases, reactive oxygen species and antimicrobial peptides to degrade the pathogen. A different mode of disabling pathogens in direct contact with the cell is extracellularly. This involves exocytosis of the granules, which are secreted directly onto the aggressor, causing membrane ruptures, pathogen apoptotic signals and eventual disabling. (Hickey & Kubes, 2009) The third and recently discovered approach of neutrophils is another extracellular method of clearance, and indeed a completely new way for a cell to die that involves neither distinct apoptosis nor necrosis, but something inbetween - NETosis. (Hickey & Kubes, 2009) (Zawrotniak & Rapala-Kozik, 2013) This involves mobilization of large parts of the cellular content including the nucleosome (nuclear DNA and histones), granular content (one enzyme heavily implicated is the neutrophil elastase) and to a lesser extent cytosolic proteins, following activation by inflammatory cues. As the neutrophil deconstructs itself and homogenizes its content, the membrane integrity becomes increasingly compromised, which eventually culminates in its rupture and content spilling out. The now homogenous cell mass rapidly forms a webwork that acts as an efficient trap for capturing and, to an extent, disabling or destroying foreign material. (Brinkmann & Zychlinsky, 2012) Other studies have demonstrated a type of NETosis that does not lead to the death of the cell, but instead functions as a discreet secretion without disrupting cell vitality. (Yipp & Kubes, 2013)

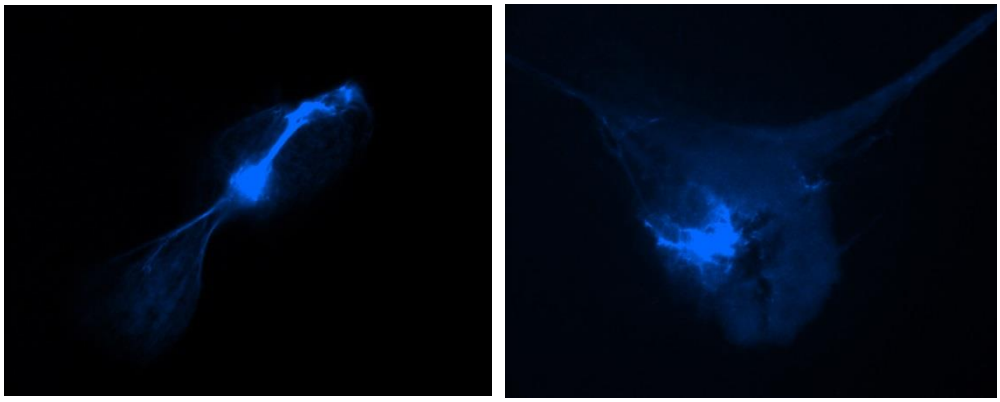


Figure 2. NETs formation stimulated by saliva. Neutrophils were exposed to saliva. After stimulation (1h), cells were dyed with DAPI staining (fluorescent stain that binds to A-T rich regions in DNA), and images were acquired from the samples.

Autophagy

What is the autophagy process?

In a nutrients stress situation and in some critical moments of development, cells do not have enough sources of energy and they are in a situation of cellular starvation. In this case, cells have developed a self-degradative process in which they are able to digest self-material and obtain energy from its degradation. (Glick, Barth, & Macleod, 2010) Cells can also use this process for recycling unwanted and damaged cellular components including proteins and organelles. Thus this process, called autophagy can be defined as a cellular homeostatic process through cells can control their cytoplasmic biomass for different purposes. Autophagy is present in mammals' cells, plants and yeast which mean it has been conserves over time among eukaryotes and it can be considered a survival mechanism. (Deretic, 2008) (Gong & Rodney J. Devenish, 2012)

The term 'autophagy' which derives from the Greek meaning 'eating of self', was called this way because it was first based on the observed degradation of mitochondria and other intra-cellular structures within lysosomes. In recent years, this process has been 'rediscovered', finding more functions of it and consequently understanding and appreciating its physiological significance. Besides of being important in response to cellular starvation, autophagy plays an important role in many physiological and pathological pathways. This process promotes cellular senescence and cell surface antigen presentation, also protects against genome instability and prevents necrosis, giving it a key role in the prevention of some diseases such as cancer, cardiomyopathy, neurodegeneration, diabetes, liver disease, autoimmune diseases and infections. (Glick, Barth, & Macleod, 2010) More recently, this autophagy process has been described as a key component of host immune defense against intracellular microorganisms, being responsible for eliminating intracellular pathogens including bacteria, viruses, fungi, and parasitic protozoa in a process called xenophagy (autophagy of invading microorganisms). (Gong & Rodney J. Devenish, 2012)

Autophagy comes in several forms depending on the way they fuse with the lysosome. The degradation of cytoplasmic components including proteins and whole organelles through an intermediary of a double membrane-bound vesicle, denominated autophagosome, which fuses with the lysosome to form an autolysosome, is called macroautophagy. A morphologically distinct form of autophagy in which cytosolic components are directly taken up by the lysosome itself through invagination of the lysosomal membrane is called microautophagy. And another form of autophagy called chaperone-mediated autophagy (CMA) consists in the translocation of the individual targeted proteins across the lysosomal membrane in a complex with chaperone proteins that are recognized by the lysosomal membrane receptor lysosomal-associated membrane protein (LAMP), resulting in their unfolding and degradation. (Glick, Barth, & Macleod, 2010) Although all of them have been described in mammalian cells, only macroautophagy has to-date been associated with the elimination of intracellular bacteria. (Gong & Rodney J. Devenish, 2012)

In the past, the autophagy process was assumed to be a wholly nonselective process. According to this view, nutrients deficiency leads to engulfment and digestion of portion of

the cytoplasm (called cargo) randomly, including the organelles of the cell. However, as has been mentioned above, autophagy represents also an intracellular quality control pathway of the damaged cellular material. Thus, the autophagy machinery needs a mechanism to distinguish between normal and anomalous/damaged cellular components and target them for degradation. Consequently, the molecular mechanisms of cargo recognition are being studied and autophagy receptors are one of the targets for those studies, taking a pivotal place in selective autophagy. (Gomes & Dikic, 2014)

How does this process work?

Macroautophagy (henceforth referred to as autophagy) is regulated by a number of autophagy-related genes (ATGs) including those which encode proteins required for signaling (such as Beclin 1) and autophagosome formation/cargo recognition such as LC3, which is used as an autophagosome formation marker. Fluorescent microscopy has made it possible to use LC3 (microtubule-associated protein 1 light chain 3) to label the autophagosome. (Gomes & Dikic, 2014)

During autophagy, sequestration of a portion of cytoplasm (cargo) starts with the formation of an isolation membrane called phagophore, originated from endoplasmic reticulum and mitochondrial membranes, particularly, mitochondria-associated endoplasmic reticulum membranes (MAMs). At the same time ATG proteins are recruited to this phagophore. This phagophore expands to engulf intra-cellular material, thereby sequestering the cargo in a double-membraned, becoming an autophagosome. This autophagosome matures through fusion with the lysosome, forming an autolysosome. The enzymes in the resulting compartment (autolysosome) break down the inner membrane from the autophagosome and degrade the cargo through lysosomal acid proteases. (Castro-Obregon, 2010) The resulting macromolecules are released to the cytoplasm through lysosomal permeases, transporters export amino acids and other by-products of degradation, where they can be re-used for building macromolecules and for metabolism. All this process is represented in figure 3. (Glick, Barth, & Macleod, 2010)

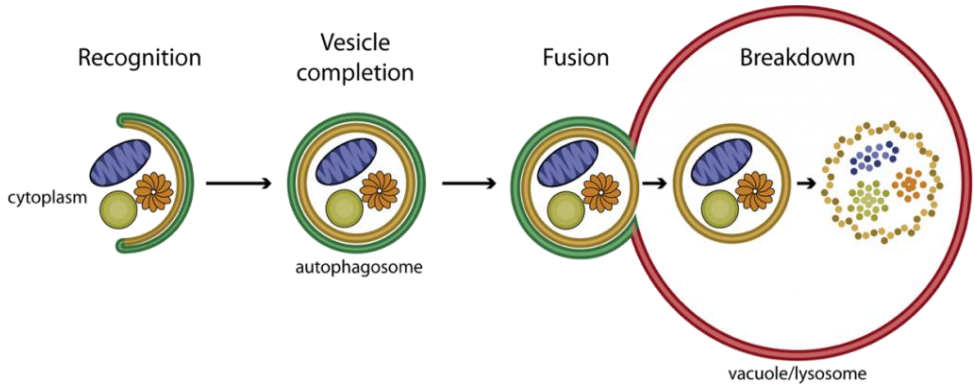


Figure 3.Autopagy process of cytoplasmatic material. The phagofore (isolated membrane) recognizes elements of the cytomplasm and expands to engulf the intra-cellular material. The cargo is sequestrated on a double-membraned called autophagosome. This autophagosome matures through fusion with the lysosome, forming an autolysosome. The enzymes in the resulting compartment (autolysosome) break down the inner membrane from the autophagosome and degrade the cargo through lysosomal acid proteases.

The autophagosome may fuse with an endosome (the product of endocytosis), which is a form of heterophagy (process in which material originated from outside of the cell is

internalized and degraded). Process showed in figure 4. The product of the endosome-autophagosome fusion is called an amphisome. The completed autophagosome or amphisome fuses with a lysosome and follows the same degradative process as the autophagosome, releasing the degraded material to the cytoplasm. (Castro-Obregon, 2010)

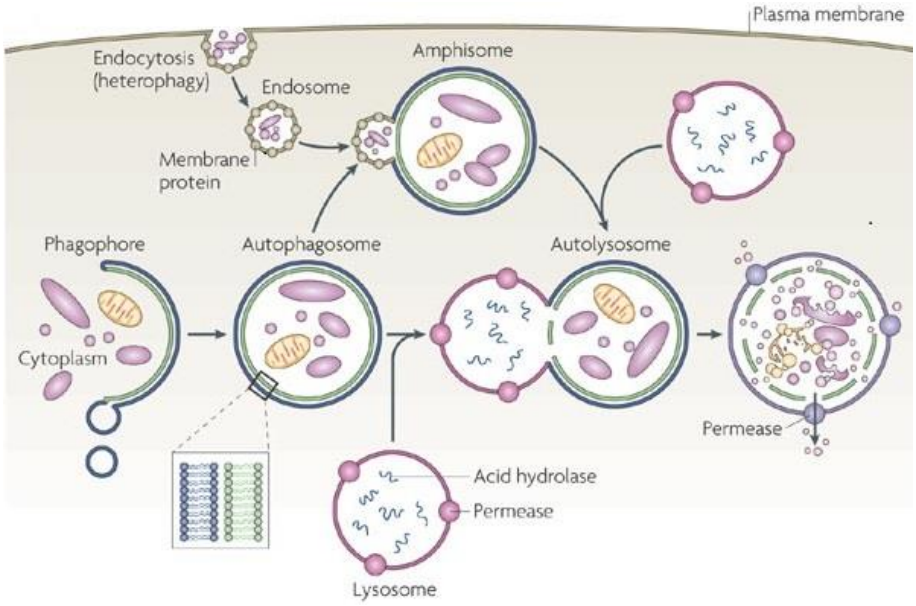


Figure 4.Formation of an autolysosome from an endosome. Above the phagofore (isolated membrane) recognizes elements of the cytomplasm and expands to engulf the intra-cellular material. The cargo is sequestrated on a double-membraned called autophagosome. This autophagosome matures through fusion with the lysosome, forming an autolysosome. The enzymes in the resulting compartment (autolysosome) break down the inner membrane from the autophagosome and degrade the cargo through lysosomal acid proteases. Bellow foreign particles are engulfed by endocytosis forming an endosome, which can fuse to an autophagosome forming an amphisome. This amphisome can fuse a lysosome, forming an autolysosome which degrade the contained material. Both processes end in the degradation of the engulfed material in small particles.

Autophagy and the immune response

As mentioned previously, it has been demonstrated that autophagy is a crucial weapon in the fight against pathogens such as intracellular bacteria, playing a role in both innate and adaptive immunity. Autophagy is implicated in processes such as regulation of inflammasome activation, cytosolic antigen processing for major histocompatibility complex (MHC) class II presentation, thymic selection (selection of T-cells) or lymphocyte homeostasis. Importantly, the autophagy machinery cannot be assumed as exclusive machinery for the elimination of intracellular pathogens, inasmuch as the process contributes to MHC class II presentation. Therefore, host defense against pathogens requires coordination of multiple innate immune signaling pathways. (Gomes & Dikic, 2014)

During infection, host cells are able to recognize the pathogen-associated molecular patterns (PAMPs) of a diversity of microbes through the expression of several pattern recognition receptors (PRRs). The recognition of foreign or danger material in the cell by those innate receptors can trigger an intracellular signaling cascade, leading to activation of antimicrobial effector mechanisms to promote the cleaning of the infection. Thus, this process is considered one of the effector mechanisms downstream of those receptors. Autophagy also can produce a crosstalk with intracellular signaling molecules and effectors. Overall, through those interactions, autophagy carries out not only direct

microbial degradation through the lysosomal degradative pathway, but also other protective mechanisms, such as the ubiquitin-mediated pathway, lysozyme secretion, and antigen presentation. Moreover, some studies have provided evidence that autophagy acts as a 'tuning module' in the regulation of innate immunity through the prevention of excessive inflammatory responses and inflammasome signaling. In addition, it is thought that reactive oxygen species (ROS) of cellular and mitochondrial origin may play a role in the regulation of autophagy, thereby influencing innate defense. (Yuk, Yoshimori, & Jo, 2012)

Macrophages and autophagy

All cells in our bodies are capable of undergoing autophagy, thus macrophages are able to carry out the autophagic process and eliminate intracellular infections. (Deretic, 2008) However, numerous microorganisms have developed strategies to evade or take control of the autophagic pathway of the cell as a survival strategy, causing a persistent infection. (Yuk, Yoshimori, & Jo, 2012) This is the case of Mycobacterium tuberculosis, which is able to infect macrophages and interact with their autophagic pathway in order to survive. This intracellular bacterium is capable to block the phagosomal maturation and also interacts with the host cell system to recruit nutrients for its replication. Notwithstanding, the induction of autophagy results in the fusion of the mycobacterial phagosome with lysosomes, leading to the degradation of the pathogen. (Gomes & Dikic, 2014)

The identification of the mechanisms or virulence factors related with the evasion of autophagy may provide new strategies and perspectives for therapeutic intervention in infection diseases caused by intracellular pathogens such as Mycobacterium tuberculosis. (Yuk, Yoshimori, & Jo, 2012)

Tuberculosis: "Captain among these Men of Death"

Mycobacterium tuberculosis (Mtb) is the causative agent of the global human tuberculosis epidemic. (Wong & Jr, 2013) *M. tuberculosis* may have killed more persons than any other microbial pathogen, infecting one third of the human world population and kills someone every 15 seconds following a cycle of infection, latency, reactivation and transmission. (Vergne, et al., 2006) Thus, it is a great example of what we could call a successful human pathogen.

Some recent genome wide studies of the genetic variation of different Mtb strains have dated the first human infecting ancestor to have arisen in Africa approximately 35.000-20.000 years ago. Since that moment, Mtb has followed the human population as they wandered across the continents and settled in throughout Europe, Asia and Indonesia. Then, it is believed that the separated strains of Mtb developed over the course of history and, as the age of discoveries and trading by sea begun during the 15th century, the different strains of Mtb spread and mixed throughout the rest of the world. (Hershberg, et al., 2008) As a consequence of its spreading, finally it reached epidemic proportions in Europe and North America during the 18th and 19th centuries, when it earned the sobriquet of "Captain Among these Men of Death". (Daniel, 2006)

Even if there has been some progresses since its bacterial cause was discovered, the global tuberculosis (TB) epidemic is still a continuous problem because a range of reasons. First, its capacity to cause latent disease leads to that an estimated 1-2 billion people worldwide is infected with the bacteria. Second, immunodeficiency caused by malnutrition, old age or HIV infection enhances development of active disease, either from a primary infection or by the reactivation of a latent infection. Moreover, the TB epidemic is greatly exacerbated by insufficient public health measures to detect, prevent, and treat it.

Treatment of TB is based in the use of more than one drug (normally isoniazide, rifampicin, streptomucin, ethambutol and pyrazinamide), usually during longer periods of time. (Norbis, 2013) Some random chromosomal mutations can make Mtb resistant to every drug used to treat the disease. Fortunately, these mutations are infrequent, thus acquired drug resistance for tuberculosis is almost always caused by an inadequate treatment. (Chan & Iseman, 2002) The incorrect use of anti-tuberculosis drugs (anti-TB), either due to errors in medical prescription, intermittent drug use, poor patient compliance, or the low quality of drugs, led to emergence of *Mycobacterium tuberculosis* strains with an expanding spectrum of resistance. Some strains showed to be resistant to more than one anti-TB drug (resistant to isoniazid and rifampicin), which were called multidrug-resistant (MDR) strains. Subsequently, other MDR strains showed new resistant to more anti-TB drugs, which were called extensively drug-resistance TB (XDR-TB). The emergence of these last strains is the result of a mismanagement of MDR cases and consequently the efficacy loss of the treatment. (Matteelli, Roggi, & Carvalho, 2014) The emergence and growth of XDR-TB make the current treatment for TB completely insufficient for fighting against those new strains and demands the development of new drugs and rapid diagnostics for tuberculosis. (Dheda, et al., 2014)

The present vaccine, *M. bovis* Bacillus Calmette-Guérin (BCG) provides limited protection against childhood TB and does not protect against adult pulmonary TB. BCG vaccine is prepared from the attenuated *M. bovis* and it is commonly used as a candidate for *M. tuberculosis* in research.

Outside of the Gram spectrum

Mycobacterium tuberculosis (Mtb) belongs to the family of mycobacteria, which notably include the well-known *Mycobacterium bovis* (Causative agent behind tuberculosis in several animals) and *Mycobacterium leprae* (Causative agent behind Leprosy). Despite of it is not technically defined as Gram-positive bacteria (as they do not retain crystal violet, characteristic which defined this group), mycobacteria is still classified as such, due to higher structural resemblance compared with Gram-negative bacteria. (Russell, 2001)

Surviving as a highly immunogenic pathogen

Despite being highly immunogenic, *Mycobacterium tuberculosis (Mtb)* is highly capable of surviving in the human host. Mtb express numerous Pathogen Associated Molecular Patterns (PAMPs), which can be recognized by Pattern Recognition Receptors (PRPs), such as TLRs and NODs. Interestingly, during an Mtb infection, the response of the adaptive immune system is severely delayed, despite of the fact that the main reservoir of Mtb is Macrophages. Also, recent evidence indicates, unlike most other pathogens, there is little to no selection against immunogenicity. The bacteria lack hypervariable epitopes on important structural components, a common feature on other pathogens that uses it to avoid targeting of the adaptive immune response. Being highly immunogenic and unwilling to change surface markers to protect against antibodies, one would expect Mtb to be easily cleared by the immune system, but as it has been demonstrated, this is not the case. (Russell, 2013)

Strategies of survival and pathogesis

A small number of airborne *Mycobacterium tuberculosis* can be sufficient to start an infection in the lungs. Thus the infection is produced through the airways, uptake into tissue resident macrophages, formation of granulomas and transmission of released bacteria.

At the site of infection the bacteria are rapidly phagocytosed by alveolar macrophages. These cells are programmed to eliminate pathogens and have therefore different receptors that can be utilized by the bacteria to facilitate their entrance. Once it has been taken up, Mtb prevents the maturation of the phagosome, which include keeping the pH at a reasonable level (pH 6.4-6.6) and inhibiting the fusion of lysosomes which contain a variety of enzymes and antimicrobial substances that have the ability to degrade pathogens in an acidified environment. In addition, Mtb modifies the endosome in a way that it enters the biosynthetic vesicle transport system. Taken together, these strategies create a protected compartment inside the macrophages, where nutrients are automatically delivered to the bacteria by the host cell vesicle system. Thus with all these mechanisms, the bacteria are case are protected from being killed.

Mtb has the ability to delay the initiation of adaptive immune response and continue replication inside phagosomes. This delay may lead to a high increase in bacterial number that is difficult to control later on. However, the infected macrophages manage to migrate from the infected lung to the lymph nodes and present mycobacteria antigens that can be

recognized by antigen-specific T_H1 cells. Activated T_H1 cells in turn, secrete cytokines that increase macrophage intracellular killing ability to destroy the bacteria. Th1-cells secrete IFN-γ and TNF cytokines, which are critical in establishment of a protective immunity against tuberculosis. (Ottenhoff, 2012) Some *M.tuberculosis* can be eliminated and others continue to survive in the resting macrophages. When Mtb effectively resist the destructive effect of macrophages, chronic inflammation can be developed. This often has a characteristic pattern called Granulomas. Granulomas are well-organized aggregates of fused immune cells consisting of macrophages surrounded by activated lymphocytes, fibroblasts and giant multinucleated cells. Their main function is to localize the infection that resists destruction to a limited area. The cells in the center of large granulomas can become isolated and die from lack of oxygen and the effects of activated macrophages. Thus there is a balance between pro-inflammatory and anti-inflammatory immune responses that control the Mtb proliferation within granulomas and destruction of these granulomas over time. However, dysregulation in the immune response leads to granuloma progression and dissemination of bacteria into the airways, which may cause a deadly infection. (Guirado, 2013)

Primary TB infection can either be removed by host immune defense, develop to active TB or become latent. In most cases the infected persons have an asymptomatic latent infection and less than 10% develop active TB during their lifetime. It is difficult to predict who will develop an active TB and who will remain healthy. However, the risk of active TB increases in immunosuppressed people such as during anti-tumor therapy or co-infection with human immunodeficiency virus (HIV). Other risk factors that play an important role, involve genetics of both host and TB pathogen. (Gengenbacher, 2012)

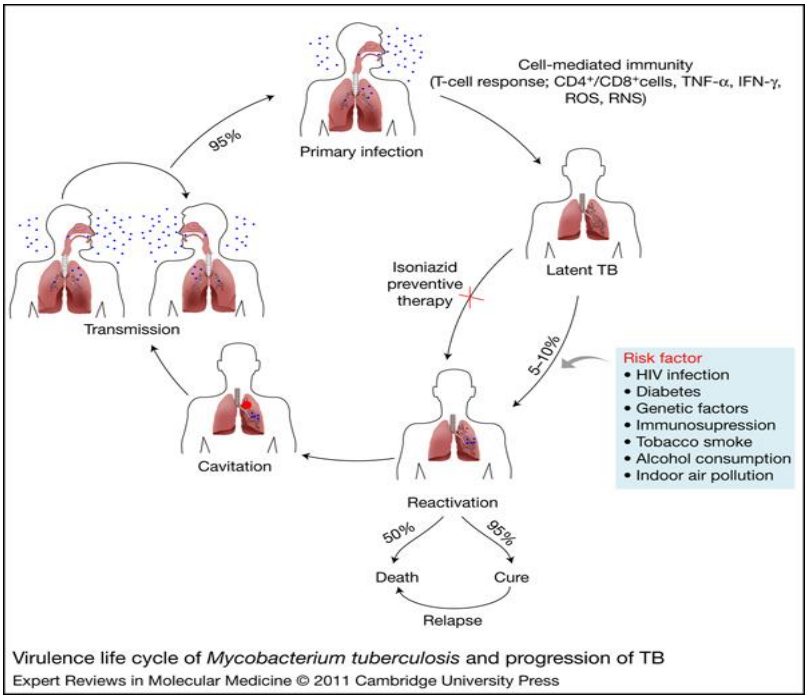


Figure 5.Virulence life cycle of Mycobacterium tuberculosis and progression of Tuberculosis. The mycobacterium is transmitted through airways. Primary TB infection can either be removed by host immune defense, develop to active TB or become latent. In most cases the infected persons have an asymptomatic latent infection and less than 10% develop active TB during their lifetime (often caused by risk factors). The reactivation leads to a cavitation in which state the mycobacterium is allowed to spread again (transmission).

Mycobacterial models: A useful tool for studying M.tuberculosis

The direct study of *Mycobacterium tuberculosis* is essential for understanding its pathogenesis. However, the use of this pathogen in the laboratory is labor-intensive for the following reasons. First, *M. tuberculosis* is a Category III human pathogen, it means that its manipulation has to fill some requirements. Dedicated biosafety level three laboratories (BSL-3) and animal facilities are required. Also substantial training is needed before handling, and carries with it a risk of accidental exposure. Second, *M. tuberculosis* is a slow growth bacterium which doubles every 22 hours in liquid culture. Accordingly, colony formation requires two to three weeks, making the experiments normally too lengthy.

For these reasons, there is a necessity of finding out other methods to study the pathogenesis of Mtb. The possibility is the study of this mycobacterium using mycobacterial model systems to gain insight into Mtb virulence mechanisms. Since some laboratories successfully studied Mtb through these models, its use is becoming more common.

Multiple mycobacterial species have been harnessed in the past to understand Mtb virulence, but three of them are predominating as model systems. These species are: *Mycobacterium bovis* (BCG strain), *Mycobacterium smegmatis*, and *Mycobacterium marinum.*

Table 1

A comparison of three mycobacterial models to *M. tuberculosis*. In this table, we highlight the similarities and differences among *M. smegmatis*, *M. marinum*, BCG, and *M. tuberculosis*

Species	Genome size (MB)	Growth rate	Primary host	Category	Major advantages	Major disadvantages
M. smegmatis	7.0	4 hours liquid 2–3 days colony	n/a	1	Rapid growth Ease of genetics	Genomic divergence Nonpathogenic
M. marinum	6.6	10–12 hours liquid 1–2 weeks colony	Ectotherms	2	Faster growth than TB TB-like disease in a large host range	Distinct intracellular lifestyle (cytosolic)
M. bovis (*BCG*)	4.3	22 hours liquid 2–3 weeks colony	Cattle humans	2	Conserved genes 99% identical to TB Attenuated	Lacks virulence factors because of deletions
M. tuberculosis	4.4	22 hours liquid 2–3 weeks colony	Humans	3	Causative agent of TB Results directly applicable	Highly infectious Category 3 pathogen

Table 1. A comparison of three mycobacterial models to *M. tuberculosis*. In this table are shown the similarities and differences among the different models (*M.smegmatis, M.marinum and M.bovis BCG*) and *M.tuberculosis*. The characteristics which differenciate them are the size of the genome, the growth rate, the primary host and the category (risk).

M. Bovis (BCG) is a member of the TB complex that attenuated by serial passage in the laboratory. Even if it grows slowly like Mtb, it is category 2 organisms, making its use easier and less risky. *M. Smegmatis* is a soil dwelling saprophytic mycobacterial species that is a distant relative of M. tuberculosis. However, *M. smegmatis* is a fast growth bacterium, with a doubling time of approximately four hours and colony generation in two to three days. Moreover, this avirulent mycobacterial is easy to manipulate genetically, fact that makes it a particularly convenient model. *M. marinum* is an occasional human pathogen which causes a TB-like infection in ectotherms. It doubles every 10–12 hours, resulting in colony formation from one to two weeks. Also it is amenable to similar genetic manipulations as *M. smegmatis* which is the high interest for genetic researching. Importantly, *M. marinum* causes caseating granulomas in zebrafish, which resemble those formed by *M. tuberculosis* in humans which makes it a good reference. Moreover, some

known virulence determinants are conserved between *M. marinum* and *M. tuberculosis* such that genes from *M. tuberculosis* can complement mutations in orthologous *M. marinum* genes, which is a really useful tool. (Shiloh & Champion, 2010) In this project the chosen model has been *M.smegmatis*, mainly for the fast growing rate.

The role of saliva and relation to pathogens

Saliva is a multifactorial body fluid which has an important role in the human immune system. On one side it must facilitate the taste and detection of foods nutritious to the body, but at the same time it serves to defend the body from possible infections. This makes the composition of saliva very complex, both in terms of composition and also physical proprieties.

Saliva is a clear, slightly acidic mucoserous exocrine secretion consisting of solution that is approximately 99% water and some key components, both organic and inorganic. The inorganic constituents are a range of electrolytes (sodium, potassium, calcium, chloride, magnesium, bicarbonate and phosphates). The organic constituents are a wide variety of proteins represented by enzymes (such as α-amylase and lysozyme), immunoglobulins (sIgA), mucins, other antimicrobial factors (such as Statherins and Cystatins), mucosal glycoproteins (Extra-Parotid Glycoprotein (EP-GP)), traces of albumin and some polypeptides (Histatins), oligopeptides and other proteins of importance to oral health (such as Proline-rich proteins (PRPs)). There are also glucose and nitrogenous products such as urea and ammonia. (Almeida, Grégio, Machad, Naval, Lima, & Azevedo, 2008) Salivary components, particularly proteins, are multifunctional (carrying out more than one function), redundant (performing similar functions but to different grades) and amphifunctional (acting both and against the host). (Humphrey & Williamson, february 2001) Some of these proteins are involved in both innate and acquired immunity. Notwithstanding these defense proteins which are founded in saliva are present in rather low concentrations, their effects globally results in an efficient molecular defense network of the oral cavity. (Fábián, Hermann, Beck, Fejérdy , & Fábián , 2012)

Various components of saliva and their functions[9]

Salivary component	Function
Amylase	Digestive
Bicarbonate	Buffering
Calcium	Remineralization
Histatins	Antimicrobial
IgA	Antimicrobial; inhibits microbial adherence
IgM	Antimicrobial; inhibits microbial adherence
Lactoferrin	Antimicrobial; hydrolysis of cell membrane
Lactoperoxidase	Antibacterial; hydrolysis of cell membrane
Lysozyme	Antimicrobial; hydrolysis of cell membrane
Mucins	Antibacterial, digestion, lubrication, pellicle formation
Protease	Digestive
Phosphates	Buffering
Proline-rich proteins	Antimicrobial, lubrication, remineralization
Statherin	Antimicrobial, remineralization
Water	Cleansing, digestion, lubrication, mucosal integrity
Urea	Buffering

Table 2. Main component of saliva and their functions. In this table different salivary components of saliva are shown with their respective functions, encompassing digestion, buffering, remineralization, antimicrobial functions, lubrication and integrity.

Saliva is produced by three pairs of major salivary glands (parotid, submandibular and sublingual) plus numerous minor salivary glands founded in lower lips, tongue, palate, cheeks, and pharynx. The types of cells of these glands are acinar cells, various duct system cells, and myoepithelial cells. Secretion of saliva by these glands can be classified in three types depending on which glands secrete it. As serous (mainly produced by the parotid gland), mucous (secreted from minor glands), or mixed (serous and mucous secretions from the sublingual and submandibular glands). (Humphrey & Williamson, february 2001)

Besides of the particular components of saliva, total or whole saliva refers to the complex mixture from the salivary glands, the gingival fold, oral mucosa transudate, in addition to mucous or the nasal cavity and pharynx, non-adherent oral bacterial (plaque), food remainders, desquamated epithelial and blood cells, as well as traces of medications or chemical products. Therefore saliva has to be considered as a really complex solution in charge of important functions related with the oral health and operation. (Almeida, Grégio, Machad, Naval, Lima, & Azevedo, 2008)

Functions

Functions of saliva can be organized into five major categories that serve to maintain oral health: Lubrication and protection, buffering action and clearance, maintenance of tooth integrity, antibacterial activity, taste and digestion.

Lubrication and protection

Saliva forms a seromucosal covering that lubricates and protects the oral tissues acting as a barrier against irritants such as protolithic and hydrolytic enzymes produced in plaque and potential carcinogens from smoking and exogenous chemicals. (Humphrey & Williamson, february 2001) This occurs due to mucins, which are complex protein molecules responsible for lubrication, protection against dehydration, and maintenance of salivary viscoelasticity. (Almeida, Grégio, Machad, Naval, Lima, & Azevedo, 2008)

Buffering action and clearance

Buffering action and clearance are a function of saliva that is carried out through the following components: water, bicarbonate, phosphate, calcium, urea, amphoteric proteins such as staterin and proline-rich anionic proteins and enzymes. Bicarbonate is the most important buffering system. It diffuses into plaque and acts as a buffer by neutralizing acids. It also generates ammonia to form amines, which serve as a buffer by neutralizing acids as well. (Humphrey & Williamson, february 2001)

Maintenance of tooth integrity

Maintaining tooth integrity is another function of saliva that facilitates de demineralization and remineralization process of tooth, essential for oral health. It protects the mouth preventing the colonization by potentially pathogenic microorganisms by denting them optimization of environmental conditions. It also neutralizes and cleans the acids produced by acidogenic microorganisms, thus, preventing enamel demineralization. (Humphrey & Williamson, february 2001)

Antibacterial activity

Another function of saliva is its antibacterial activity. Saliva contains a spectrum of immunologic and non-immunologic proteins with antibacterial properties which provide protection for teeth and mucosal surfaces. The defense factors of saliva include both immune and non-immune systems. Amongst the compounds of salivary non-immune system, mucins are founded as a predominant factor. (Doddsa, Johnsonb, & Yehc, 2004)
Immunologic contents of saliva include secretory IgA, IgG, and IgM. Among the non-immunologic salivary components, there are selected enzymes (lysozyme, lactoferrin, and peroxidase), mucins, peptides, and other proteins such as statherins. Secretory IgA is the largest immunologic component of saliva. It is active on mucosal surfaces and it can also

neutralize viruses, bacteria and enzyme toxins. Against bacteria, it serves as an antibody to bacterial antigens and works to aggregate or clump bacteria, hence inhibiting bacterial attachment to host tissues. (Humphrey & Williamson, february 2001)

Taste and digestion

Obviously, another function of saliva is involved in taste and the beginning of the digestive process in the mouth. The hypotonicity of saliva enhances the tasting capacity of salty foods and nutrient sources. This enhanced tasting capability depends on the presence of a salivary protein called gustin.

Saliva has an early, limited role in total digestion by beginning the breakdown of starch with α-amylase (ptyalin), a major component of parotid saliva that initially dissolves sugar. Salivary enzymes also initiate fat digestion. However, an important role of the saliva in this function is that it serves to lubricate the food bolus, which aids in swallowing. (Humphrey & Williamson, february 2001)

Mucins, essential glycoproteins in saliva

Mucins are highly glycosylated proteins with a content of sugars from 50% - 90% of the dry weight of the molecule. As it is shown in the picture (B), they are constituted for a protein part as a "trunk" and the rest are glycan ramifications. Those oligosaccharide side-chains vary in length from 1 to more than 20 sugars residues, mostly attached by o-glicosidic linkages of n-acetil galacosanine to serine or threonine. The biochemical and functional properties of mucins are mostly determined by the terminal residues, particularly, sialic acid, sulphate or fucose residues (Figure 6). (Schenkels, Veerman, & Amerongen, 1995)

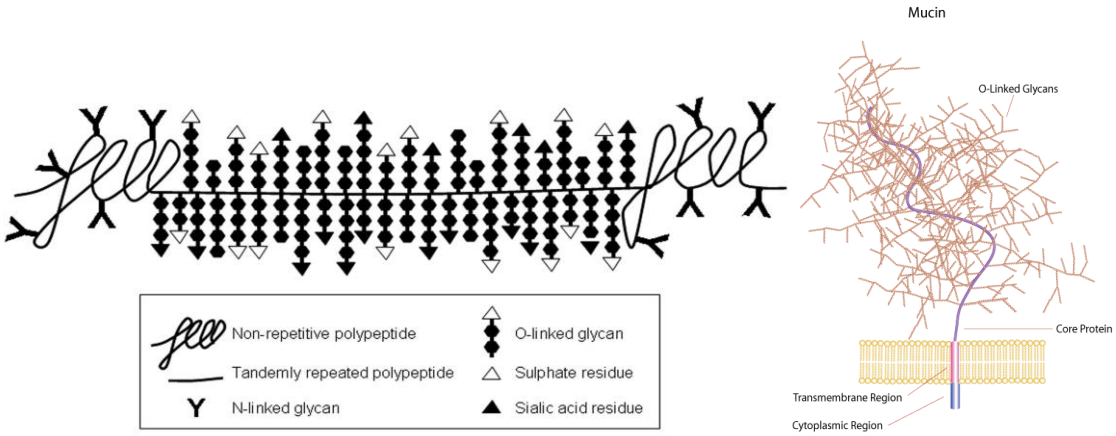


Figure 6.Mucins' structure. At the right there is a expanded mucin's structure and the different residues it can have (mainly sulphate or sialic acid residues). The different linkages (N-linked and O-linked glycans) are shown. At the left, a whole mucin (cytoplasmic region, transmembrane region, core protein and different glycan residues).

Their high degree of glycosylation and potential for hydration prevent desiccation and their viscoelastic properties provide lubrication. (Doddsa, Johnsonb, & Yehc, 2004) Importantly, mucins are the main protein compounds of the mucous layer that coats epithelial surfaces in the respiratory, gastrointestinal, and reproductive tracts as well as in the oral cavity. (Bruno, et al., 2005) Besides of these essential functions, mucins also perform an important antibacterial function by selectively modulating the adhesion of

microorganisms to oral tissue surfaces, which contributes to the control of bacterial and fungal colonization. (Humphrey & Williamson, february 2001) In addition, mucins protect the oral tissue surfaces against proteolytic attacks by microorganisms. (Almeida, Grégio, Machad, Naval, Lima, & Azevedo, 2008) For carrying out these functions, they train a protective coating covering tooth enamel and oral mucosa, which act as a functional barrier capable of modulating the adverse effects of the oral environment, being important within the epithelial perimeter of mucosal defense. (Slomiany, Murty, Piotrowski, & A.Slomiany, 1996) Thus, mucins are well recognized as an important factor in the conservation of the health of the oral cavity, forming part of the non-immune host defense system.

Human saliva contains two saliva-specific types of mucins called low-molecular weight mucin glycoproteins (103 kDa) and high-molecular weight mucin glycoproteins (130-150 kDa), MG2 or MUC7 and MG1 or MUC5B respectively. (Doddsa, Johnsonb, & Yehc, 2004) They are synthesized and secreted by submandibular, sublingual and minor salivary glands and are the product of two different genes. (Slomiany, Murty, Piotrowski, & A.Slomiany, 1996) These two types of mucins are structurally and functionally distinct, differing with respect to bacterial clearance ability. MG2 or MUC5B contributes to the viscoelasticity of saliva, exhibits a high affinity for hydroxyapatite (component of the enamel pellicle) and binds to certain strains of bacteria. (Bruno, et al., 2005) Otherwise, MG1 or MUC7 is more efficient in bacterial aggregation and clearance, binding and agglutinating a variety of oral microbes. This low molecular weight form predominates in saliva and oral mucosal mucus coat of caries-resistant individuals, while the level of the MG2 is higher in caries-susceptible subjects. The caries-resistant individuals also exhibit in their saliva a major activity of protease which is capable of the conversion of the high molecular weight mucin to the low molecular weight form. (Slomiany, Murty, Piotrowski, & A.Slomiany, 1996) MG1 also exhibits affinity for cementum but not for hydroxyapatite surfaces. (Bruno, et al., 2005).

It is thought that the bacterial aggregating activity of salivary mucins may be associated with sulfomucins (mostly sulfate residues) rather than sialomucins (mostly sialic acid residues). The removal of sialic acid in the saliva causes only a partial loss in the aggregation activity of mucins. In contrast, the desulfaction causes a complete loss in the bacterial aggregation activity, showing the importance of the sulfation process in the processing of salivaty mucins. It is, thus, conceivable that the major determinant of the extent of bacterial aggregating activity of saliva could be sulfomucins and not sualomucins. (Slomiany, Murty, Piotrowski, & A.Slomiany, 1996).

Among all the above functions already mentioned, they also preserve mucosal integrity regulating mucosal calcium homeostasis, process that is important in the buffering capacity of the saliva.
As a result, it is becoming apparent that salivary mucins perform a multitude of functions pivotal for the maintenance of the integrity and health of oral cavity against possible pathogens. (Slomiany, Murty, Piotrowski, & A.Slomiany, 1996)

Mucins' Bindings

The mucins in oral mucous interact with the epithelial surfaces in the oral cavity through specific membrane receptors. Carbohydrate structures present in mucins are greatly diverse and they are involved in this interaction. Consequently, mucins offer a large range of binding sites for microbial adhesion which could render the vulnerability to disruption by those opportunistic microbies (pathogens and commensals) colonizing the oral mucosa. (Slomiany, Murty, Piotrowski, & A.Slomiany, 1996) However, by offering binding sites similar to those of epithelial cells, mucin can prevent pathogen adhesion to the underlying epithelial cells, and further translocation into the mucosa. (Derrien, Passel, Bovenkamp, Schipper, Vos, & Dekker, 2010) This is the case of MG2 which binds to several strains of bacteria including oral *Streptococci*, *Actinobacillus actinomycetemcomitans* and *Pseudomonas aeruginosa,* avoiding their interaction with the epithelial surface. (Bruno, et al., 2005)

Also mucins can intectact with non-mucin proteins present in saliva. This interaction modulates the biological activity of complex proteins and also protects them from proteolysis. It can also serve as a deliverance system for distribution of secretory salivary proteins along the oral cavity. (Bruno, et al., 2005)

Taken together, reductions in the output of mucins could have prejudicial effects on oral and systemic health, as well as quality of life. (Doddsa, Johnsonb, & Yehc, 2004)

AIM AND OBJECTIVES

Among last years, the role of saliva has been rediscovered, finding new functions of this potential body fluid. Also the functions of macrophages are being renewed, proving that they have more than one strategy for cleaning a possible infection, such as the formation of METs (Macrophages Extracellular Traps). Since saliva is able to activate the NETosis process (Neutrophil Extracellular Traps), we investigated if saliva has any effect in infected macrophages, and if it could lead to the killing of intracellular bacteria.

RESULTS

Saliva and sialic acid cause nuclear swelling

Unpublished data from the research group of Ole Sørensen (Lund University) demonstrated that saliva induces NET formation (Neutrophils Extracellular Traps) in purified blood neutrophils (PMNs). Particularly, a sialic acid-containing part of salivary mucins (silayl lewis^x), is able to activate this process. This interesting observation led us to investigate if saliva and sialic acid exert similar effects on other cells form the myeloid line, such as macrophages (PBMCs). In a previous experiment, PBMCs were exposed to saliva, saliva buffer (buffer isotonic in composition to saliva) and 2,3 bound sialic acid (25µg/ml) samples. A control sample was taken with medium. After stimulation (1h), cells were dyed with DAPI staining (fluorescent stain that binds to A-T rich regions in DNA), and images were acquired from samples.

The results indicated that there was a shift in the nuclear morphology in cells exposed saliva and sialic acid compared to the controls (figure 7). Nuclear swelling and changes in morphology may indicate nuclear descondensation and nuclear envelope breakdown. The observed phenomenon was very similar to neutrophil extracellular trap generation. However, extracellular traps in PBMCs look different than in PMNs, since the DNA is not similarly condensed in the nuclei. This prompted us to investigate the matter further.

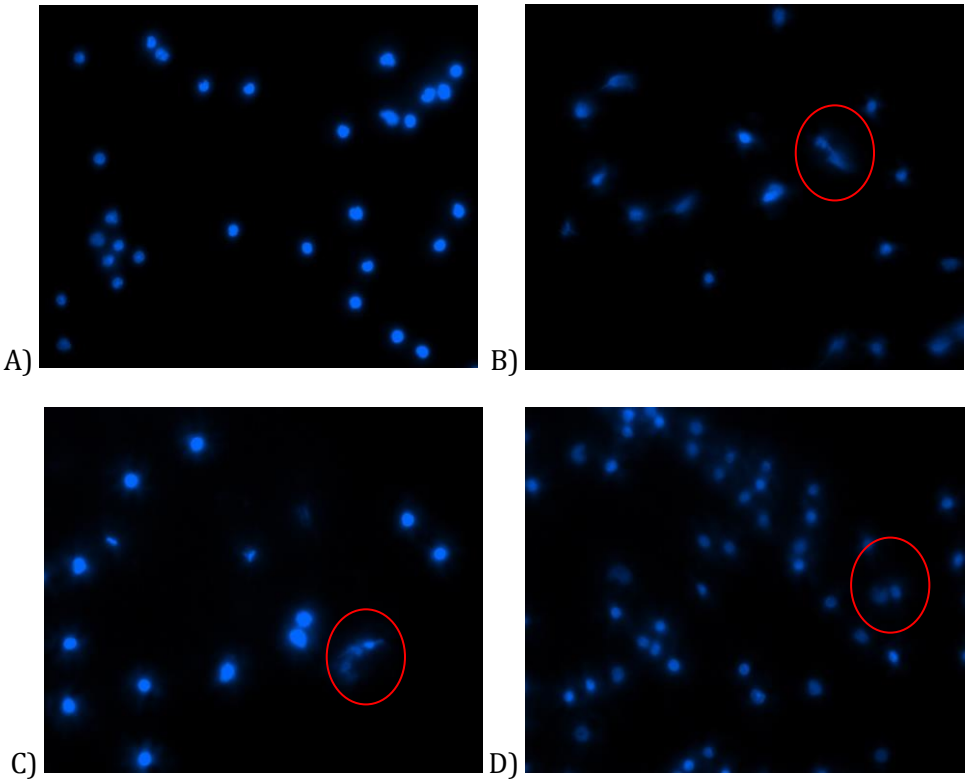


Figure 7.Effect of saliva, saliva buffer and sialic acid in PBMCs. PBMCs were exposed to saliva, saliva buffer, sialic acid (25µg/ml) and medium as a control. After stimulation (1h), cells were dyed with DAPI staining (fluorescent stain that binds to A-T rich regions in DNA), and images were acquired from the samples. The different pictures, from A-D represents: (a) Control in medium. (b) saliva buffer (c) saliva (d) 2,3 bound sialic acid. METs are encircled in red.

Saliva may induce bacteria killing in *M.smegmatis* infected THP-1 cells

NET formation involves the process of autophagy, which results in the breakdown of structural proteins of the nuclear and plasma membrane that in turn facilitates the release of DNA form the semi-digested cell. Previous studies also indicate that intracellular mycobacterium tuberculosis is subject to elimination through autophagy in the infected cells.

To try to prove the possible effect of the saliva in infected macrophages with *M.smegmatis*, a macrophage cell line (THP-1 cells) was used. Cells were infected with mycobacteria and afterwards exposed to saliva and free sialic acid (2,3 bound sialic acid). After stimulation, cells were permealibized and the bacteria which were alive inside the cells were plated out on LB plates for colony counting. The results four independent experiment demonstrated a possible increased killing of the bacteria by the saliva-stimulated macrophages. However, free sialic acid did not seem to have a very significant effect on the killing of the internalized bacteria. After some experiments, it was decided to check the cells during the stimulation and see how they looked like by microscopy. THP-1 cells looked vacuolized and semidetached after saliva stimulation, which leads us to believe that the observed killing effect was due to cell death either by apoptosis or autophagy, since these modes of cell death are known to promote killing of intracellular mycobacteria.

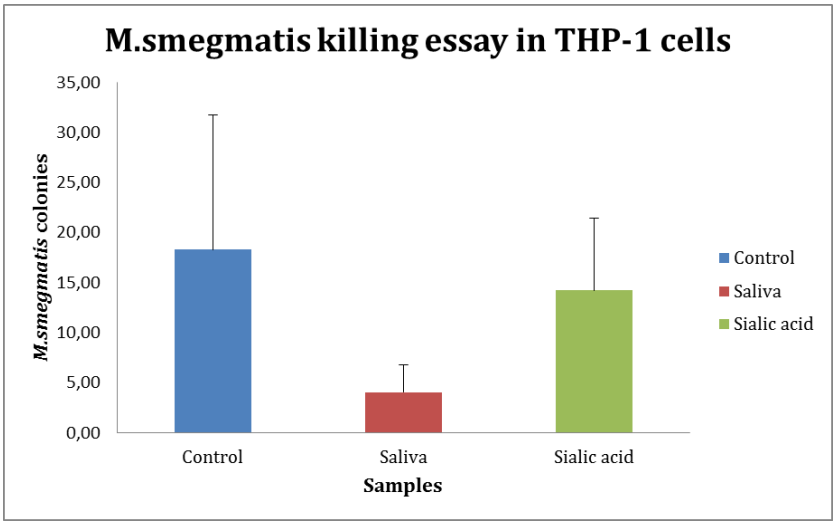


Table 3.Colony counting of *M.smegmatis* killing essay in THP-1 cells. Cells were infected with *M.smegmatis* and exposed to saliva (100%) and sialic acid (25µg/ml). After stimulation, cells were permealibized with triton X-100 and the bacteria which were alive inside the cells were plated out in LB plates for colony counting.

Saliva lacks direct antimicrobial activity against *M.smegmatis*

Saliva is known to have antimicrobial activity towards several Gram-positive and Gram-negative bacteria. To rule out direct killing of *M.smegmatis* by saliva we incubated bacteria with saliva, 2,3 bound sialic acid, 7H9 medium (as control for normal growing of bacteria), saliva buffer and gentamycin (antibiotic which shows the killing). The bacteria was incubated with the different samples for 2 hours, diluted and plated on LB plates. After 3 days, colonies were counted. The results of three independent experiments surprisingly indicated that bacterial growth actually flourished in presence of saliva when compared to growth medium. As expected, gentamycin inhibited bacterial growth. Saliva buffer with BSA also had no effect on the bacterial killing. Interestingly, sialic acid had a mild antimicrobial effect when compared to control, which is previously undocumented.

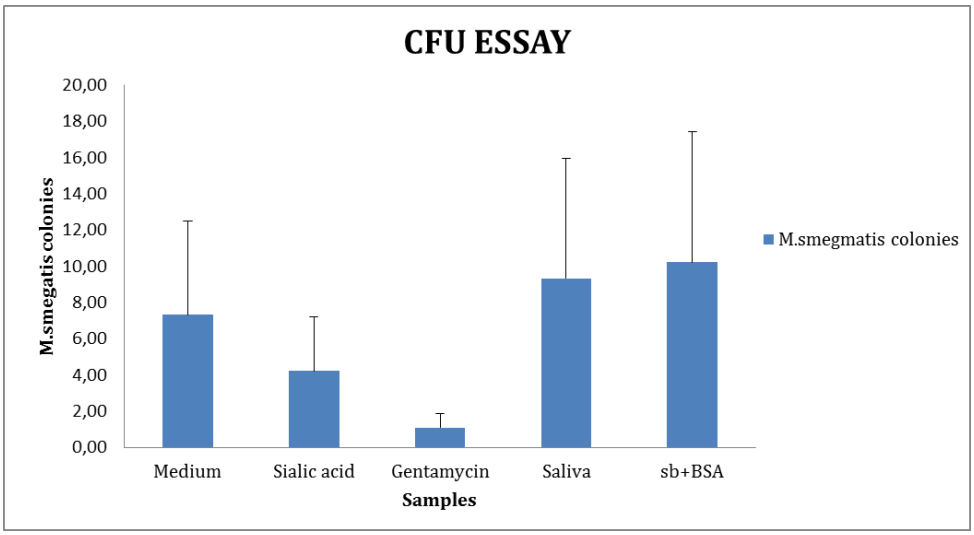


Table 4. Saliva doesn't kill *M.smegmatis* by itself. Samples of *M.smegmatis* were exposed directly to saliva, sialic acid, gentamycin, saliva buffer + BSA and 7H9 medium as control and incubated for 2 hours. Bacteria were plated out on LB plates for colony counting.

Saliva may induce bacteria killing in *M.smegmatis* U937 infected cells

After the experiments with the macrophages cell line THP-1 cells, which indicated that the cell line were not completely viable after saliva treatment, we decided to test another macrophages cell line (U937 cells). This cell line seemed to be more resistant to saliva. The same killing assay described above was performed with U937 cells. Since we observed no effect of free sialic acid (2.3 bound) we tested a 2,6 bound sialic acid and sialyl lewis^X (which is a bigger glycan which contains 2.3 sialic acid and is present in mucins). The sialyl lewis^X is the physiological relevant form of sialic acid bound to mucins (free 2.3 sialic acid is not present in saliva). Additionally the saliva was treated with neuraminidase, which is known to cleave off sialic acid residues from mucins. The colony counts indicated a possible effect of saliva-sham treated, which had not been treated with neuraminidase, demonstrating the possible effects of sialic acid on mucins while neuraminidase-treated saliva had no effect. The 2.6 bound sialic acid also seemed to have resulted in the intracellular killing (data not shown). This was only done once and warrant further investigation.

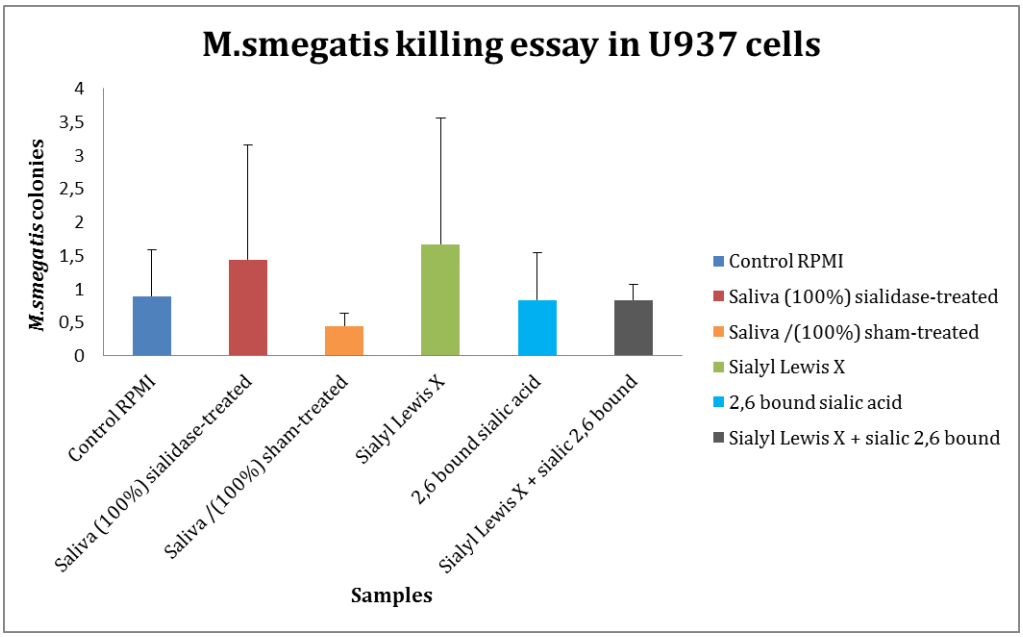


Table 5. Cells were infected with *M.smegmatis* and exposed to saliva 100% sialidase-treated, saliva 100% sham-treated, Sialyl Lewis^X (10µg/ml), 2,6 bound sialic acid (25µg/ml), Sialyl Lewis^X (10µg/ml) + 2,6 bound sialic acid. After stimulation, cells were permealibized with triton X-100 and the bacteria which were alive inside the cells were plated out on LB plates for colony counting.

Saliva may induce killing of mycobacteria by autophagy in PBMCs infected cells with *M.smegmatis* through LC3 marker

After a promising preliminary experiment in U937 cells, we thought it might be interesting to check the status of autophagy in cells exposed to saliva post infection. This experiment was based in immunocytochemistry, using 2 different antibodies as markers. The used marker for the autophagy process was LC3 as a marker of autophagosome formation. In addition, a membrane lysosome marker called LAMP-1 was used. Interestingly, we wanted to check if LAMP-1 could be found together with the bacteria after saliva stimulation, which would indicate fusion of the phagosome with the lysosome.

First cells were infected with *M.smegmatis*, stimulated with saliva and its components for 2 hours and permealibized with triton X-100. Immunochemestry was performed using the antibodies mentioned previously. Samples were mounted with DAPI and kept overnight. Images were then acquired. The images suggested the activation of the autophagy in the cells through the LC3 marker. They also suggest there could be a phagosome-lyososome fusion after saliva stimulation. Additionally, 2,6 bound sialic acid could have the same effect as saliva (figure 3 and 4).

Control

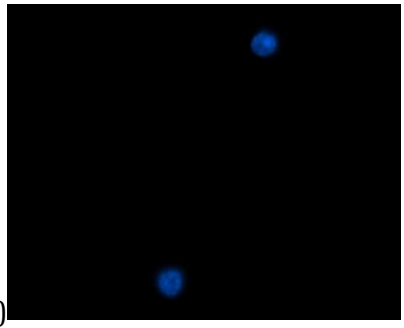
A)  B)  C) 

Figure 8. Saliva may activate autophagy in PBMCs infected cells with *M.smegmatis* through LC3 and LAMP-1 markers. Control sample. First cells were infected with *M.smegmatis*, stimulated with saliva for 2 hours and permealibized with triton X-100. Immunochemestry performed using LC3 (autophagosome formation marker) and LAMP-1 (lysosome fusion marker) antobodies. Samples were mounted with DAPI and kept overnight. (a) DAPI staining (b) LC3 (c) LAMP-1.

Saliva buffer

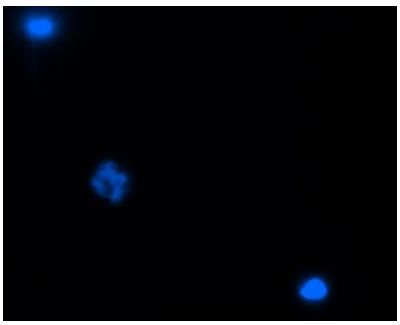
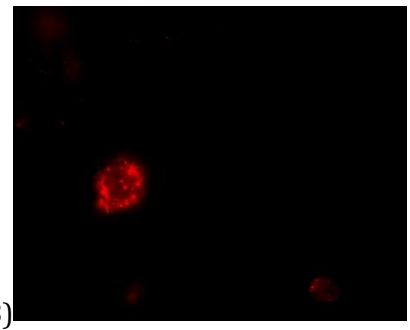
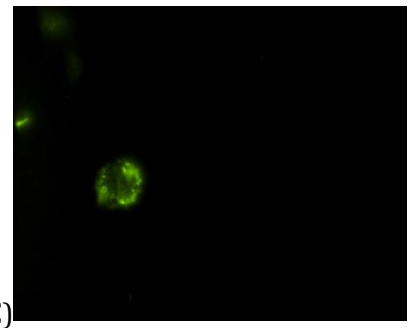
A)  B)  C) 

Figure 9. Saliva may activate autophagy in PBMCs infected cells with *M.smegmatis* through LC3 and LAMP-1 markers. Saliva buffer sample. First cells were infected with *M.smegmatis*, stimulated with saliva for 2 hours and permealibized with triton X-100. Immunochemestry performed using LC3 (autophagosome formation marker) and LAMP-1 (lysosome fusion marker) antobodies. Samples were mounted with DAPI and kept overnight. (a) DAPI staining (b) LC3 (c) LAMP-1.

Sialic acid 5µg/ml

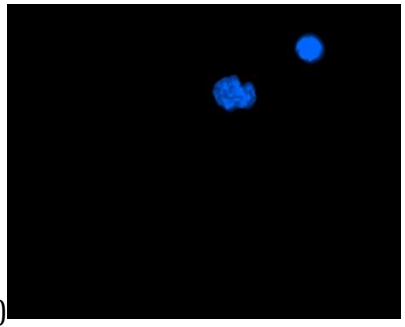
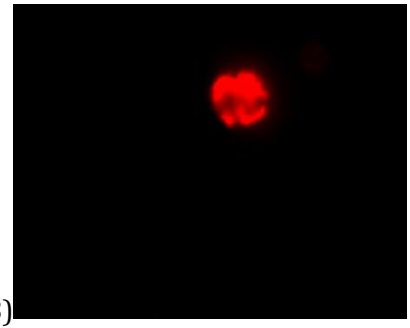
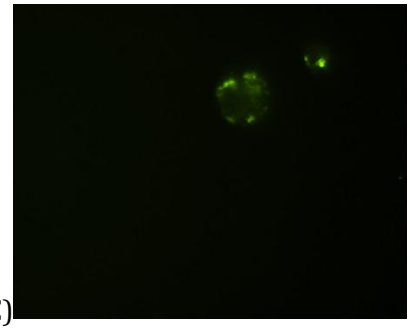
A)  B)  C) 

Figure 10. Saliva may activate autophagy in PBMCs infected cells with *M.smegmatis* through LC3 and LAMP-1 markers. Sialic acid 5 µg/ml sample. First cells were infected with *M.smegmatis*, stimulated with saliva for 2 hours and permealibized with triton X-100. Immunochemestry performed using LC3 (autophagosome formation marker) and LAMP-1 (lysosome fusion marker) antobodies. Samples were mounted with DAPI and kept overnight. (a) DAPI staining (b) LC3 (c) LAMP-1.

Sialic acid 50 µg/ml

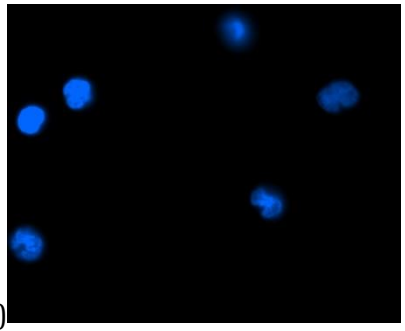
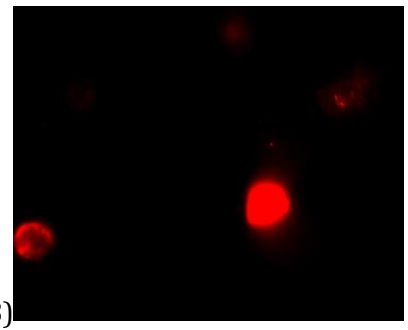
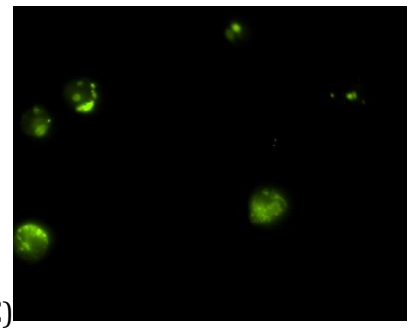
A)  B)  C) 

Figure 11. Saliva may activate autophagy in PBMCs infected cells with *M.smegmatis* through LC3 and LAMP-1 markers. Sialic acid 50 µg/ml sample. First cells were infected with *M.smegmatis*, stimulated with saliva for 2 hours and permealibized with triton X-100. Immunochemestry performed using LC3 (autophagosome formation marker) and LAMP-1 (lysosome fusion marker) antobodies. Samples were mounted with DAPI and kept overnight. (a) DAPI staining (b) LC3 (c) LAMP-1.

Saliva

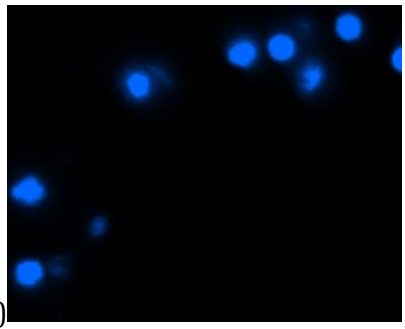
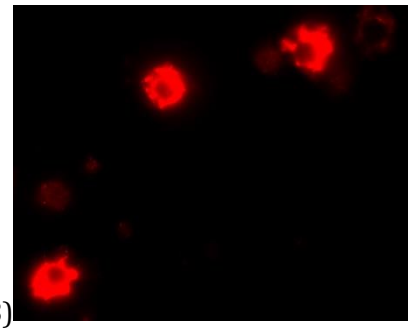
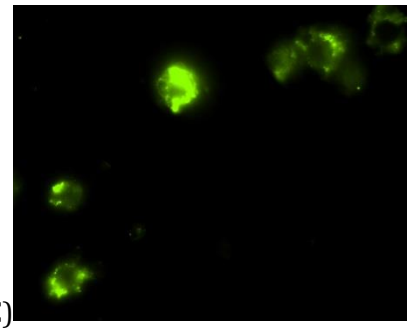
A)  B)  C) 

Figure 12. Saliva may activate autophagy in PBMCs infected cells with *M.smegmatis* through LC3 and LAMP-1 markers. Saliva 100% sample. First cells were infected with *M.smegmatis*, stimulated with saliva for 2 hours and permealibized with triton X-100. Immunochemestry performed using LC3 (autophagosome formation marker) and LAMP-1 (lysosome fusion marker) antobodies. Samples were mounted with DAPI and kept overnight. (a) DAPI staining (b) LC3 (c) LAMP-1.

DISCUSSION

NET formation induced by saliva is newly described process. The component responsible to activate the process of NET formation is sialyl lewis^{x}, a tetrasaccharide found in mucins (Data unpublished). Also, only a few studies describe the process of extracellular trap formation in monocytes. Therefore, we investigated if extracellular trap formation was induced in monocytes by the same salivary component. We observed that peripheral blood monocytes after exposure to saliva underwent extracellular trap formation.

Interestingly NETs formation involves the process of autophagy, which results in the breakdown of structural proteins of the nuclear and plasma membrane that in turn facilitates the release of DNA form the semi-digested cell. At the same time, several studies have observed that intracellular bacteria, as *Mycobacterium tuberculosis*, are subject to elimination using this process in an infected cell. Accordingly, we investigated whether saliva could stimulate the killing of intracellular mycobacteria in monocytes. Inasmuch as *M.tuberculosis* is a class III pathogen and time consuming to culture, we decided to use a mycobacterium model with *M.smegmatis*. The infection of a macrophages cell line (THP-1 cells) with the *M. smegmatis* followed by stimulation of the cells with saliva and sialic acid showed a promising preliminary results, where saliva stimulation induced killing of intracellular mycobacteria in macrophages. In contrast stimulation of mycobacteria-infected macrophages with free sialic acid did not reduce the number of intracellular bacteria. However, judging from the strong effect of saliva, we suspected that saliva might be toxic towards the cells. It leads us to check the cells by microscopy at every step and especially after stimulation with saliva. Thus, we observed that monocytes did not look viable in saliva samples, meaning that the killing of the bacteria was not produced by direct activation of macrophages by saliva. The samples were almost void of living bacteria, which lead us to think that possibly the THP-1 cells in presence of saliva, went through a process of apoptosis, causing the death of both the cells and the intracellular bacteria.

To discard the possibility that saliva could kill the bacteria directly through its antimicrobial activity, we decided to expose directly bacteria to saliva and sialic acid samples. We observed that saliva did not kill mycobacteria. However, a small killing effect was observed in the sialic acid samples, which may have been caused by some technical problems and not from the exposure of the bacteria to the free sialic acid molecule. This indicate that decreased bacteria survival in mycobacteria-infected monoscytes was caused by a cellular process activated by saliva possibly autophagy.

As a consequence of the experiments with THP-1 cells, we decided to test U937 cells. Both THP-1 cells and U937 cells are different macrophage cells lines and consequently may have different responses to saliva. In contrast to the THP-1 cells, the U937 cells looked viable in presence of whole saliva. The sialic acid is found with a 2.3 and 2.6 bound in mucins. This gives a different conformation of the sialic acid compared to free sialic acid. This confirmation of the sialic acid is an important determinant for binding to siglecs (Sialic acid-binding immunoglobulin-type lectins), the receptors that may facilitate the effect of saliva. Additionally, to prove that the ligand was found in the sialic acid residues of the salivary mucins, we treated the saliva sialidase (neuraminidase) which resulted in the cleaving off sialic acid residues from mucins. The neuraminase-treated saliva did not

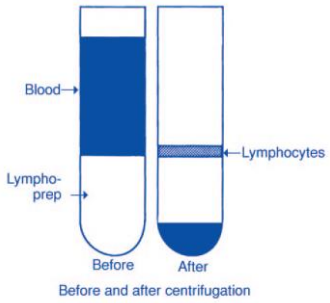
increase intracellular killing of mycobacteria in monocytes, while the non-treated saliva showed a considerable effect, which indicate that sialic acid are indeed. In addition, the 2.6 bound sialic acid seemed to have some effect, however not as potent as saliva. Finally the sialyl lewis^{x} (with a 2.3 bound sialic acid) had no effect. However, further more conclusive experiments are needed.

This discovery not just could contribute to the knowledge of the autophagy process, it could also open new ways to develop this component as a drug against infectious diseases which affect macrophages directly, such as *M.tuberculosis*. This mechanism could be a natural activation of the immune system against the infection, completely different with the current treatments based mainly in antibiotics.

MATERIALS AND METHODS

NETs experiment

PMNs and PBMCs were isolated from blood of a healthy donor which was subjected to dextran sedimentation. The supernatant was aspired and centrifuged at 200 g for 10 min at 4ºC. The pellet was resuspended in NaCl and carefully layered on top of an equal volume of Lymphoprep solution, a density gradient solution (Axis-shield, Oslo, NO). The sample was centrifuged at 400 g for 30 min at 4 ºC.



Before and after centrifugation

The interface containing PBMCs was kept. The pelled was suspended in ice-cold water for 30 seconds to lyse the erythrocytes and the tonicity was restored by addition of equal volume if 1.8% saline. The sample was centrifuged and resuspended in 0,9% NaCl to a concentration of $1x10^6$ cells/ml. Cells were allowed to stand at room temperature for 15 min. To check if PMNs and PBMCs kill intracellular bacteria, isolated blood cells were seeded in 5 wells on coverslips and incubated for 30 min at 37ºC. Cells were stimulated to produce METs (Monocyte Extracellular Traps) for 1 hour in different conditions: Control in RPMI 0,2% HSA, Control in saliva buffer, Saliva (100%), Sialyl $lewis^x$ (5µg/ml) and 2,3 bound N-acetyl neuraminic acid (25µg/ml). Produced NETs were fixed with PFA (4x in PBS) during 20 minutes at 37ºC and 5% CO2. They were carefully washed in PBS twice during 5 min each time. NETs were mounted in mounting medium with DAPI in dark. The samples were kept overnight in the incubator at 37ºC and 5% CO_2. Images were acquired using a Nikon Eclipse TE200 equipped with a Hamamatsu C4742-95 CCD camera, using Plan Apochromat 20x, 40x and 100x objectives. NIS-elements 3.1 (Nikon) software was used for image acquisition and processing.

Infection of THP-1 cells with *M.smegmatis*

The THP-1 monocytic cells were maintained at 37ºC in 5% CO_2 in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (FBS,Gibco), 10mM HEPES (Gibco), 1mM sodium pyruvate, 2mm L-glutamate, and penicillin-streptomyin solution (Gibco). The cells were seeded onto 24-well culture dishes at a density of 5×10^5 cells/ml and treated overnight with 20 nM phorbol myristate acetate (PMA) from Sigma to differentiate THP- 1 monocyte cells to macrophages. Cells were then washed once in PBS and were grown in RPMI-1640 medium containing 5% FBS, 2 mM L-glutamate and without penicillin-streptomycin. Cells were infected with *M.smegmatis* 5 million bacteria pr. Well for 2 hours and 30 min (25 µl solution of OD_{590}=0.2) and centrifugeed at 500 g for 5 min. Medium was changed to medium (Gibco) with 50µg/ml gentamycin for 1 hour. Cells were washed once in PBS and incubated for stimulation for 1 hour and 30 min in the follow conditions: Control (RPMI), Saliva (100%) and both 2,3 bound N-acetyl neuraminic acid (50µg/ml). After stimulation medium from each condition is aspired and plated in LB plates with 1:100 dilution. Cells were lysed in TH medium with 0.5% Triton X-100 and

plated at a dilution of 1:100 and 1:1000 on LB plates. Plates were kept at 37 degrees for 2-3 days to facilitate colony formation. *M. smegmatis* colonies were counted.

PBMCs experiment (with PBMCs and keratinocytes)

PBMCs were isolated from blood of a healthy donor which was subjected to dextran sedimentation. The supernatant was aspired and centrifuged at 200 g for 10 min at 4ºC. The pellet was resuspended in NaCl and carefully layered on top of an equal volume of Lymphoprep solution (density gradient medium). The leukocyte rich supernatant was separated by density gradient centrifugation at 400 g for 30 min at 4 ºC using lymphoprep solution. The interphase rich in PBMCs was aspired, resuspended in NaCl to a concentration of 7,5 x 105 cells/ml. Cells stood at room temperature for 15 min. PBMCs were incubated for 30 min on coverslips in the incubator. Cells were stimulated for 1 hour and 30 min in the following conditions: Control in saliva buffer, control RPMI, saliva, RPMI+ 2,6 bound N-acetyl neuraminic acid (100µg/ml), RPMI+ 2,6 bound N-acetyl neuraminic acid (25µg/ml) and RPMI+ 2,6 bound N-acetyl neuraminic acid (5µg/ml). METs were fixed with PFA (Paraformaldehyde in PBS) for a period of 20 minutes at 37ºC and 5% CO2 and permeabilized with Triton 0,5% X-100 for 30 seconds. Cells were washed in PBS 3 times (5 minutes each time) in the (shaker). 5% Goat serum in PBS (blocking solution) was added to block unspecific binding. Cells were incubated for 35 min at 37ºC and 5% CO_2. Blocking solution was removed and ells were incubated with LAMP-1 and LC3 antibodies (primary antibodies) overnight. PBMCs were washed 4 times in x1 TTBS (10 minutes each time). Secondary antibodies were added (anti rabbit and anti mouse). Cells with antibodies were incubated for 2 hours in dark (in a box) at 37 degrees. Antibodies were removed and cells were washed on x1TTBS 3 times (10 minutes each time). Cells were mounted in mounting medium with DAPI (4',6-diamino-2-phenylindole) in dark conditions. The samples were kept overnight in the incubator at 37ºC and 5% CO_2. Keratinocytes were subjected to the same process as control to check that 2,6 bound N-acetyl neuraminic acid does not produce the same effect in them and it is not toxic either.

Neuraminidase treatment of saliva

A 0,1 M sodium acetate, pH 5 buffer was prepared. Saliva was treated in 2 different conditions in plastic vials: Vial A (1,7ml saliva+ 40µl neuraminidase+160µl buffer), Vial B (1,7ml saliva+ 200µl buffer). The two vials were incubated in 37ºC for 3 hours (vortexed every hour). Saliva was concentrated on micro spin to 300-400 µl 5 times (cutoff 10 kDa or less). Each time the volume was replenished with saliva buffer until the same volume the saliva was added at the beginning (1,7ml). The treated saliva was frozen down at -20ºC for further experiment.

CFU essay with *M.smegmatis*

To check the effect of saliva directly to mycobacteria to discard that saliva is not killing bacteria we performed the next essay called CFU essay. *M.smegmatis* bacteria were centrifuged at 2200 g for 10 minutes. The supernatant was removed and the pellet was washed with 10mM Tris-glucose. The sample was vortexed to dissolve the pellet and centrifuged at 2200 g for 10 min. The supernatant was removed again and the pellet was resuspended in a small volume Tris-glucose such as 2ml. Possible clumps were broken with a thin syringe (0.2 µm). Bacteria were resuspended in Tris-glucose until an O.D. (590nm)= 0,2 (the blanc was done with Tris-glucose). Those bacteria were diluted 1:10

for further experiment. Bacteria was incubated in different conditions: Medium 7H9, 2,3 bound N-acetyl neuraminic acid (100µg/ml), gentamycin (25µg/ml), saliva 100% and saliva buffer in 2 mg/ml BSA (dialyzed in saliva buffer). The samples were incubated for 2 hours at 37ºC and 5% CO2 in the incubator. Samples were diluted 1:100, 1:1.000, 1:10.000 and plated out on LB plates. The plated bacteria were kept in the incubator for 3 days. *M. smegmatis* colonies were counted.

Abbreviations

PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern recognition receptors
NETs	Neutrophil extracellular traps
METs	Macrophage extracellular traps
Mtb	Mycobacterium tuberculosis
TB	Tuberculosis
DAPI	4',6-diamidino-2-phenylindole
LC3	Microtubule-associated protein 1 light chain 3
LAMP-1	Lysosomal-associated membrane protein 1

REFERENCES

Johnson, G. B., Raven, D. P., Singer, D. S., & Losos, D. J. (2002). *Biology.* Washington: McGraw-Hill.

Janeway, J., Travers, P., & Walport, M. (2001). *Immunobiology: The immune system on health and disease.* (5. edition, Ed.) New York: Garland Science.

Gong, L., & Rodney J. Devenish, a. M. (2012). Autophagy as a Macrophage Response to Bacterial Infection. *Life*, 740-745.

Mayadas, T. N., Cullere, X., & Lowell, C. A. (2013). The multifaceted functions of neutrophils. *Annu Rev Pathol*, 181-218.

Hickey, M. J., & Kubes, P. (2009). Intravascular immunity: the host-pathogen encounter in blood vessels. *Nature Reviews Immunology*, 364-375.

Zawrotniak, M., & Rapala-Kozik, M. (2013). Neutrophil extracellular traps (NETs)-formation and implications. *Acta Biochim Pol*, 277–284.

Brinkmann, V., & Zychlinsky, A. (2012). Neutrophl extracellular traps: Is immunity the second function of chromatin? *The Journal of Cell Biology*, 773-783.

Yipp, B. G., & Kubes, P. (2013). NETosis: how vital is it? *Blood journal*.

Glick, D., Barth, S., & Macleod, K. F. (2010). Autophagy: cellular and molecular mechanisms. *J.. Pathol.*, 1-8.

Deretic, V. (2008). *Autophagosome and phagosome.* Albuquerque, NM, USA: Human press.

Gomes, L. C., & Dikic, I. (2014). Autophagy in Antimicrobial Immunity. *Molecular cell* , 224-233.

Castro-Obregon, S. (2010). The Discovery of Lysosomes and Autophagy. Nature Education, 1-5.

Yuk, J.-M., Yoshimori, T., & Jo, E.-K. (2012). Autophagy and bacterial infectious diseases. *Experimental and Molecular Medicine*, 99-108.

Wong, K.-W., & Jr, W. R. (2013). Mycobacterium tuberculosis exploids Interferon gamma to stimulate macrophage extracellular trap formation and necrosis. *The journal of Infectious diseases*, 109-118.

Vergne, I., Singh, S., Roberts, E., Kyei, G., Master, S., Harris, J., et al. (2006). Autophagy is Immune Defense against Mycobacterium tuberculosis. *Landes Bioscience*, 175-177.

Hershberg, R., M, L., Small, P., H.Sheffer, Niemann, S., Homolka, S., et al. (2008). High functional diversity in Mycobacterium tuberculosis driven by genetic drift and human demography. *PLos Biol*, e311.

Daniel, T. M. (2006). The history of tuberculosis. *Respiratory Medicine*, 1862-1870.

Norbis, L. (2013). Tuberculosis: lights and shadows in the current diagnostic landscape. *New microbiologica*, 111-122.

Chan, E. D., & Iseman, M. D. (2002). Current medical treatment for tuberculosis. *BMJ*, 1282-1286.

Matteelli, A., Roggi, A., & Carvalho, A. C. (2014). Extensively drug-resistant tuberculosis: epidemiology and management. *Clinical Epidemiology*, 111-118.

Dheda, K., Gumbo, T., Gandhi, N. R., Murray, M., Theron, G., Undwandia, Z., et al. (2014). Global control of tuberculosis: from extensively drug-resistant to untreatable tuberculosis. *The Lancet Respiratory Medicine*, 321-388.

Russell, D. (2001). Mycobacterium tuberculosis: here today, and here tomorrow. . *Nat Rev Mol Cell Biol* , 569-577.

Russell, D. (2013). The evolutionary pressures that have molded Mycobacterium tuberculosis into an infectious adjuvant. *Current opinion in microbiology*, 78-84.

Ottenhoff, T. H. (2012). THe knowns and unknows of the immunopathogenesis of tuberculosis. *International Journal Tuerculosis Lung Diseases*, 1424-1432.

Guirado, E. (2013). Modeling the Mycobacterium tuberculosis granuloma - the critical battlefield on host immunity and disease. *Frontiers in Immunology*, 1-7.

Gengenbacher, M. (2012). Mycobacterium tuberculosis: Succes through dormancy. *NIH Public Access*, 514-532.

Shiloh, M. U., & Champion, P. D. (2010). To catch a killer. What can mucobacterial models teach us about Mycobacterium tuberculosis? *Current Opinion of Microbiology*, 86-90.

Almeida, P. D., Grégio, A. M., Machad, Naval, M. A., Lima, A. A., & Azevedo, L. R. (2008). Saliva Composition and Functions: A comprenhensive Review. *The Journal if contemporany dental practice*, 1-8.

Humphrey, S. P., & Williamson, R. T. (february 2001). A review of saliva: Normal composition, flow, and function. *The journal of prosthetic dentistry*, 162-168.

Fábián, T. K., Hermann, P., Beck, A., Fejérdy , P., & Fábián , G. (2012). Salivary Defense Proteins: Their Network and Role in Innate. *International Journal of Molecular Sciences*, 4296-4311.

Doddsa, M. W., Johnsonb, D. A., & Yehc, C.-K. (2004). Health benefits of saliva: a review. *Journal of Dentistry (2005)*, 223-230.

Schenkels, L. C., Veerman, E. C., & Amerongen, A. V. (1995). Biochemical Composition of Human Saliva in Relation To Other Mucosal Fluids. *Critical Reviews in Oral Biology & Medicine*, 161-169.

Bruno, L. S., Li, X., Wang, L., Soares, R. V., Siqueira, C. C., Oppenheim, F. G., et al. (2005). Two-hybid analysis of human salivary mucin MUC7 interactions. *Biochimica et Biophysica Acta*, 65-72.

Slomiany, B. L., Murty, V., Piotrowski, J., & A.Slomiany. (1996). Salivary mucins in oral mucosal defense. *Gen. Pharmac.* , 761-771.

Derrien, M., Passel, M. W., Bovenkamp, J. H., Schipper, R. G., Vos, W. M., & Dekker, J. (2010). Mucin-bacterial interactions in the human oral cavity and digestive tract. *Landes Bioscience*, 254-268.