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The elimination pathways of Streptococcus Group Beta (GBS) in adult and neonatal macrophages and the role of Akt1 kinase

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Τα μονοπάτια εξάλειψης του στρεπτοκόκκου Βήτα (GBS) σε ενήλικα και νεογνικά μακραφάγα και ο ρόλος της κινάσης Akt1

Περίληψη

Ο *Streptococcus Agalactiae*, γνωστός και ως στρεπτόκοκκος Βήτα (GBS) είναι ένα σημαντικό παθογόνο με μεγάλο εύρος ξενιστών, συμπεριλαμβανομένων και των ανθρώπων. Θεωρείται ένα σημαντικό ανθρωπινο παθογόνο που προκαλέι πολλές επιπλοκές στην υγεία ατόμων που ανήκουν σε ομάδες υψηλού κινδύνου όπως οι ανοσοκατεσταλμένοι ασθενείς, οι εγκυμονούσες γυναίκες και τα νεογνά. Η σοβαρότητα των μολύνσεων του στρεπτοκόκκου Βήτα είναι ιδιαίτερα αυξημένη στα νεογνά, στα οποία το παθογόνο αυτό θεωρείται η κύρια αιτία πνευμονίας, σήψης και μηνιγγίτιδας, που χαρακτηρίζονται από υψηλά ποσοστά νοσηρότητας και θνησιμότητας. Ωστόσο, δεν έχουν αναπτυχθεί κατάλληλα εμβόλια ενάντια όλων των ορότυπων του παθογόνου και γι αυτό το λόγο η έρευνα για την έυρεση νέων μορίων-στόχων με προστατευτικές ιδιότητες έναντι αυτού κρίνεται πλεον επιτακτική ανάγκη.

Τα νεογνά χρησιμοποιούν σε ένα μεγάλο βαθμό τους μηχανισμούς αμυνας της έμφυτης ανοσίας και κατά κύριο λόγο βασίζονται στη δράση των μακροφάγων, δεδομένου ότι το σύστημα επίκτητης ανοσίας είναι ακόμα ανώριμο. Τα μακροφάγα είναι εξιδεικευμένα φαγοκυτταρα που χαρακτηρίζονται από σύνθετη προέλευση και μεγάλη ποικιλομορφία. Με τη βοήθεια ειδικών υποδοχέων μπορούν να αναγνωρίζουν και να φαγοκυτταρώνουν τους εξωτερικής προέλευσης κινδύνους. Βάσει της ενεργοποίησής τους τα μακροφάγα κατατάσσονται στα κλασικά ενεργοποιημένα Μ1 και τα εναλλακτικά ενεργοποιημένα Μ2 κύτταρα. Τα Μ1 μακροφάγα έχουν ένα προφλεγμονώδη φαινότυπο και χαρακτηρίζονται από αυξημένη παραγωγή προφλεγμονωδών κυτοκινών, συνθάσης νιτρικού οξέος (iNOS) και ενεργών ειδών οξυγόνου (ROS), ενώ τα Μ2 κύτταρα έχουν έναν αντιφλεγμονώδη φαινότυπο. Βασικά μόρια στην ενεργοποίηση αυτή των μακροφάγων είναι οι Akt κινάσες. Είναι γνωστό ότι η έλλειψη έκφρασης της Akt1κινάσης δημιουργεί έναν Μ1 φαινότυπο ενώ η έλλειψη της Akt2 κινάσης ενεργοποιεί εναλλακτικά τα μακροφάγα προς ένα Μ2 φαινότυπο. Παρουσία ευκαιριακών παθογόνων, τα μακροφάγα αποκτούν ένα Μ1 φαινότυπο και χαρακτηρίζονται από αυξημένη δυνατότητα καταστροφής μικροβίων που οδηγεί στην πιο αποτελεσματική καταπολέμισή τους.

Ένα σημαντικό μονοπάτι που συμμετάσχει στη καταστροφή παθογόνων μικροργανισμών είναι αυτό της αυτοφαγίας. Η διαδικασία αυτή περιλαμβάνει τη δημιουργία αυτοφαγοσωμάτων, οργανιδίων που περικλύονται από διπλή μεμβράνη, τα οποία οδηγούν το βλαβερο φορτίο στα λυσοσώματα, προς αποικοδόμηση. Ωστόσο, κατά τη τελευταία δεκαετία, ένα εναλλακτικό μονοπάτι, γνωστό ως LC3 Associated Phagocytosis (LAP), έχει αναδειχθέι ως άμεσα κινητοποιούμενος μηχανισμός κατά των παθογόνων. Αντίθετα από τη κλασική αυτοφαγία, η LAP απόκριση είναι ταχύτερη και υψηλά εξαρτώμενη από τη παρουσία ROS.

Παρά τη εκτεταμένη βιβλιογραφία σχετικά με τη καταπολέμιση παθογόνων μεσω της κλασικής αυτοφαγίας ή της LAP, δεν υπάρχουν διαθέσιμες πληροφορίες για τα μονοπάτια που αξιοποιούνται για τη καταπολέμιση του Στρεπτοκόκκου Βήτα και την

αποτελεσματικότητά τους κατά τα νεογνικά στάδια. Σε αυτή τη μελέτη προσπαθήσαμε να διερευνήσουμε τα μονοπάτια που αξιοποιούνται από τα ενηλικα και νεογνικά μακροφάγα κύτταρα για τη καταπολέμιση του Στρεπτοκόκκου Βήτα, παρουσία και απουσία της Akt1 κινάσης. Συγκεκριμένα θέλαμε να διευκρινήσουμε πως τα νεογνικά μακροφάγα ανταποκρίνονται σε μολύνσεις από GBS, εάν παρουσιάζουν διαφορές συγκριτικά με τα ενήλικα μακροφάγα και κατά πόσο η απουσία της Akt1 κινάσης είναι ευεργετική για τη καταπολέμιση του GBS. Τα δεδομένα μας αποτελούν μία αρχική ένδειξη ότι ο GBS καταπολεμάται καλύτερα από τα $Akt1^{-/-}$ μακροφάγα και ότι στη διαδικασία αυτή εμπλέκονται η παραγωγή ROS και η αυτοφαγία.

Abstract

Streptococcus Agalactiae, also known as Streptococcus Group Beta (GBS) is an important pathogen with a wide range of host organisms, including humans. It is considered as a serious human pathogen that creates several health complications in high-risk groups, like the immunosuppressed patients, postpartum women and infants. The severity of GBS infections is extremely increased in infants where GBS is considered as the leading cause of pneumonia, sepsis and meningitis, characterized by high morbidity and mortality rates. However, there are no available vaccines to protect from all serotypes of this pathogen and therefore research of new target molecules with protective effects against GBS infections are now a necessity.

Neonates heavily exploit the defense mechanisms of innate immune system and especially macrophage activity, since their adaptive immune system is still immature. Macrophages are professional phagocytic cells of complex origin and wide diversity. They recognize extrinsic dangers and perform phagocytosis by utilizing a variety of receptor molecules that specifically bind pathogenic patterns. Based on their activation state they are distinguished as classically M1 and alternatively M2 activated cells. M1 cells display a pro-inflammatory profile, characterized by increased production of pro-inflammatory cytokines, iNOS (inducible nitric oxide synthase) and reactive oxygen species (ROS), while M2 cells have mainly anti-inflammatory responses. Key effectors of polarization process are among others Akt kinases. It is established that Ablation of Akt1 kinase polarizes cells towards the classic activation pathway (M1), while the absence of Akt2 kinase leads to the alternative activation of macrophages (M2). However in the presence of opportunistic pathogens, macrophages acquire an M1 identity and exhibit increased bactericidal capacity that leads to the efficient elimination of pathogens.

An important pathway that contributes to pathogenic organism elimination is autophagy. This process leads to the formation of double membrane organelles named autophagosomes that sequestrate harmful cargo to the lysosomes for degradation. However, during the last decade, an alternative phagocytic pathway, LC3 Associated Phagocytosis (LAP), has arisen as an immediate mechanism against pathogen infections. Unlike canonical autophagy this pathway is more rapid, highly dependent on ROS and leads to the formation of single membrane vesicles.

Although there is extended bibliography on pathogens targeted by either canonical autophagy or LAP, there are no available data on the mechanisms utilized to fight GBS infections and their efficacy in a neonatal cell stage. In the current study we tried to shed light on the specific pathways utilized by adult macrophages in the presence and in the absence of Akt1 kinase. We also sought to address how neonatal macrophages respond to GBS infection, if they display differences compared to adult cells and whether the depletion of Akt1 kinase is beneficial in terms of eliminating GBS bacteria and preventing the severe consequences that GBS infection cause. Our data are a first indication that GBS is more efficiently eliminated by $Akt1^{-/-}$ macrophages, probably in a ROS dependent manner and this process is related to autophagy.

Introduction

1.1 Streptococcus agalactiae epidemiology & worldwide distribution

Streptococcus agalactiae, also known as Group Beta Streptococcus (GBS), is a gram positive, anaerobic, beta hemolytic bacterium that has the capacity to infect and parasitize on several cells and form characteristic chains of progeny bacteria (Figure 1). Its hemolytic activity enables GBS to cause the lysis of infected cells and release bacteria, that way perpetuating infection. It is a pathogen o paramount importance mainly due to the wide range of host organisms that it successfully infects and the serious complications complications that it causes.

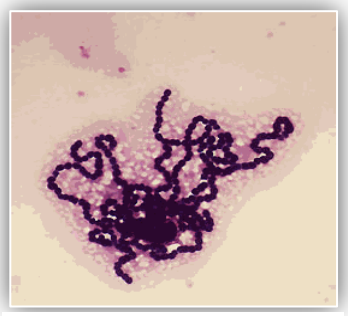


Figure 1: Morphology of Streptococcus Agalactiae

From amphibians and fish to cattle and humans, GBS has evolved in a way that manages to successfully submerge immune defenses and infect those organisms, causing numerous problems with severe health and socioeconomic consequences.[1] It is known that Streptococcal infections in fish species result in streptococcosis, septicemia and meningoencephalitis, whereas in bovines they are the leading cause of mastitis[2]. Although the transmission of this pathogen from one species to another via food consumption is a matter of great controversy, an increasing number of studies show that interspecies strains bare great genomic similarities.[3] Thus, Streptococcus agalactiae raises serious concerns for food industry as well as public health.[4]

Despite calves and fish, GBS is also a well known human pathogen that asymptomatically colonizes human body, but has the potential to cause severe infections with dreadful outcome. Based on pathogen's immune reactivity, scientists have managed to identify 10 distinguish GBS serotypes (Ia-IX) that are infectious for humans and differ in terms of virulence, infectivity and geographical distribution.[5] For instance, serotypes Ia, II, III & V are the ones identified in almost 80% of cases of adult infections while III, Ia & V are the most commonly identified among pregnant women, with frequencies of 25%, 23% and 19% respectively.[6, 7]

Serotype variation among different regions and populations, results in a subsequent variation in GBS disease incidence and colonization prevalence. According to recent studies, GBS colonization in non-pregnant adult individuals predominately depends on ethnicity and age, since disease rates are significantly higher in African dissented populations compared to Caucasians and tend to increase in older groups (≥65 y.o.). [8, 9] The likelihood of such diseases is also significantly elevated in immunocompromised individuals and people with serious underlying conditions that are in fact considered as high risk groups for developing life-threatening syndromes upon GBS infection. Other factors like sexual behavior, education and socioeconomic background also play a role upon disease rates, especially in pregnant population.[10] As far as pregnant are concerned, the incidence of asymptomatic colonization ranges from 6,5% to 43,6% and is particularly higher in African regions (22,4%),

like Jimma, Ethiopia where GBS colonization in women is 29,2%.[11] In Europe and America have a mean prevalence of 19-20% while in Asian countries the respective percentage is relatively low at 11,1%.[12, 13]

1.2 GBS transmission & related complications in adults and neonates

GBS is vertically transmitted from colonized mothers to fetuses and newborns during pregnancy and delivery, although in the majority of late onset diseases it is laterally transmitted from the surrounding environment. More specifically, GBS has the capacity to surpass placental membranes and spread through the amniotic cavity, infecting the fetus usually causing its premature death. However, in the majority of cases, bacteria transmitted from vaginal epithelium to the neonatal respiratory tract during delivery through the birth canal. Transmission depends heavily on maternal bacterial load and results in increased neonatal load which in turn affects disease progression and outcome.

As a part of human microflora, GBS is usually found colonizing the gastrointestinal, urinary and genital tract of individuals. In many cases, GBS infections are asymptomatic and do not bare any particular dangers for healthy adults. However, it can cause a wide spectrum of life-threatening diseases in pregnant, post-partum women, infants as well as other high risk groups, like immunocompromised individuals, the elderly and people suffering from other health complications. GBS born diseases vary from skin and urinary tract infections to bacteremia that gradually evolves to septic arthritis, pneumonia, endocarditis and meningitis. [14]In pregnant women, GBS infections can lead to severe endometritis, preterm birth or even stillbirths. [15]However, the group with the higher susceptibility and most severe symptoms upon GBS infection is infants.[15]

During the last 50 years, GBS has emerged as an important neonatal pathogen in Western world societies, characterized by high incidence of infection and increased morbidity and mortality rates. Depending on the time of infection, GBS can either cause early onset (EO) or late onset (LO) diseases. The former manifests during the first six days postpartum, accounts for 60% of GBS born diseases and is mainly preventable via use of antibiotics, while the later refers to diseases that occur during 7-90 days of infancy, is rare (0,34 per 1000 live births) and has no available means of prevention.[16] Successful prevention strategies have led to a remarkable decline in the incidence of EOGBSa, while LOGBS incidence has remained rather static. Both early and late onset GBS diseases can potentially lead to bacterial meningitis, pneumonia and sepsis. Neonatal meningitis has a 0.1-0.4 per 1000 births incidence and is mostly lethal although it can result in deafness and impairment of neurological, psychological and cognitive function in approximately 35% of survivors. [17]

The most known prevention strategies so far are screening of pregnant women and administration of intra-partum antibiotic prophylaxis. During the last decade, there have been made a lot of efforts for vaccine development against GBS and some of them have been successfully revaluated for phase II trials.[18] However, there are no available vaccines able to prevent GBS infections caused by all different serotypes. [19] [20]Taken together, the high mortality rates of Beta Streptococcal infections along with disease severity and the various subsequent socioeconomic consequences, highlight the importance of effective vaccine development against GBS.

1.3 Immune responses against GBS infections

1.3.1 GBS infection and host responses

Upon GBS infection, various inflammatory responses are triggered that result in the activation of innate and adaptive immune system. Innate immunity is the first line of defense mechanisms employed to protect host cells from opportunistic microorganisms such as bacteria, parasites, viruses and fungi and retain normal cell function and homeostasis when the later are challenged. Innate immune responses shape the successive adaptive immune responses that help to pathogen elimination process, homeostasis maintenance and also offer immune memory against the specific invading microorganism.

The GBS elimination process normally starts with the recognition of the pathogen from specific receptors in the surface of phagocytic cells. GBS is uptaken from these cells and several proinflammatory responses are triggered. This leads to the production of corresponding cytokines and several Reactive Oxygen/Nitrogen Species (ROS/RNS) that further facilitate this process. The degradation of GBS is followed by antigen presentation of pathogen components to several cells, leading to immune memory development against this pathogen.[21] Following the innate immune responses, Adaptive immunity is activated. More specifically, dendritic cells present GBS antigens to naïve T helpers and beta cells. T helpers proliferate and acquire a Th1 phenotype with acute bactericidal properties that help macrophage's and cytotoxic T cells' function, while beta lymphocytes proliferate and produce specific IgGs against GBS so that the host will resolve future GBS infections more efficiently.[22]

1.3.2 Neonatal immune responses to GBS

A typical GBS infection in neonates begins with the aspiration of the pathogen from the newborns and the subsequent transfer of the former through the respiratory tract. When GBS reaches the lower part of the airways, it manages to successfully surpass the enriched in anti-microbial factors mucus barrier and penetrate the epithelial cells. From there bacteria move toward the other layers of neonatal lungs and finally gain access to the blood stream. Through the bloodstream, bacteria have a widespread distribution in multiple neonatal organs causing organ failure, severe bacteremia and sepsis. When neonates suffer from prolonged bacteremia, bacteria manage to penetrate the blood–brain barrier (BBB) and infect neonatal brain eventually causing meningitis. [23]

Immune cells are the professional cells marshaled to kill extrinsic dangers. It is known that neonatal immune defenses are different compared to adult organism responses. Neonates have particularly underdeveloped innate and adaptive immune mechanisms. [24, 25]

As far as the former are concerned, neonates are characterized by an all in all reduced number of monocytes and alveolar macrophages that may account for their higher susceptibility to airway infections. Despite the reduced number of cells, neonates have impairments in TLR signaling, antigen presentation, chemotaxis and cytokine production. A characteristic example is that of IL12 and IFN-γ production that is particularly reduced in infants and contributes to their susceptibility to opportunistic infection. [26]

However, the landscape is even more different when it comes to adaptive immune system. When in utero, the exposures of fetuses to specific antigens are limited, as maternal antibodies protect them from external opportunistic pathogens. Consequently, the adaptive immune responses are limited and the system remains in a naïve state that does not allow for immune memory development. Neonatal organisms are also characterized by lymphopenia, since the number of lymphocytes is very limited. T helper cells mainly differentiate towards a Th2 route that induces tolerance to the pathogen and not efficient elimination, while Th1 and Th17 populations that exceed bactericidal activity are very limited. Moreover, dendritic cells do not have the ability to efficiently present the antigens to activate other cells and the total pro-inflammatory production is diminished. As far as beta cells are concerned, they suffer from poor signaling and delayed production of immature IgG. [24]

These deficiencies and immaturity of the adaptive immune system highlight the fact that neonates are highly dependent on their innate immune mechanisms for their protection from invading micro-organisms like GBS.

1.4 GBS infection & Innate Immunity: The role of macrophages

Important components of innate immune system are professional phagocytic cells, such as neutrophils and macrophages. Macrophages are cells that undergo many transcriptional, translational and metabolic adaptations in order to co-ordinate of the acute phase of inflammation and control its resolution. Though a complex cocktail of inflammatory factors that they release, they manage to abolish intrinsic and extrinsic threats and also restore the desired equilibrium.[21, 27]

It becomes clear that such cells that play a central role in wide range of functions are particularly important for neonates, since they comprise their most potent defense line against opportunistic pathogens. Neonatal adaptive immune system is quite immature and not sufficiently developed to assist host defense needs. Since adaptive immunity cannot be exploited upon infection, innate immune cells are utilized in cases where host homeostasis is perturbed and challenged by infectious pathogens like GBS. [27]Though GBS has the ability to manipulate innate immune responses and consequent inflammatory responses depending on the magnitude of infection, the final outcome is determined from the interaction between pathogen and immune cells.[28]

1.4.1 Heterogeneity within macrophages

A fundamental component of innate immune system is macrophage cells. This group of cells derives from hematopoietic cell lineages that reside in the bone marrow, is characterized by great heterogeneity, versatility and a wide spectrum of functions. Nowadays it is known that the three sources from where all macrophages arise are the yolk sac, fetal liver and the bone marrow.[29] In general, macrophages are categorized into tissue resident and circulating macrophages. The former are continuously produced by BM, released in bloodstream and infiltrate tissues, where they differentiate in order to replenish reservoirs, to fight and resolve inflammation. The latter reside into tissues, are able to self-renew and have a pivotal role in tissue morphology, defense and homeostasis and function maintenance. [30, 31] Despite their ontogenic complexity, Tissue distribution of macrophages also determines a

great part of their functions. For instance, brain and liver macrophages, also known as microglia and Kupffer cells alike, share some similarities but also have distinct characteristics so as to meet the needs of the corresponding organs.[32] However, functional diversity can also be found in macrophages that reside in the same tissue. Depending on microenvironment signals, tissue homeostatic state and downstream target cells, different macrophage subgroups arise within the same tissue.[29, 31]

1.4.2 M1 and M2 polarization of macrophages

Based on the activation state and response phenotype macrophages are classified into 2 distinct groups, the classically activated (M1) and the alternatively activated (M2) macrophages. M1 phenotype embraces signaling pathways that result in the induction of proinflammatory responses and thus it makes cells hyper-responsive to the necessary stimulation. Such stimuli include lipopolysachharides (LPS), interferon gamma (IFN-γ) and tumour necrosis factor (TNF) and their downstream signalling leads to transcriptional activation of genes like induced Nitrogen Oxide Synthase (iNOS), production and secretion of proinflammatory cytokines, such as IL1, IL6, IL12 and TNF and NADPH derived reactive oxygen and nitrogen species (ROS, RNS). Oxidative stress is important for initiating and maintaining a proinflammatory state that triggers subsequent responses for inflammation persistence. Moreover, they are characterized by downregulated production of anti-inflammatory cytokines like Consequently, M1 cells are related to Th1 pathologies, have microbicidal activity, control the acute phase of infection and have a role of protection against exogenous opportunistic pathogens. On the contrary, M2 polarization is induced by a wide variety of signals, such as IL10, IL13, IL4, immune-complexes and glycol-corticoids. [21] M2 cells bare immunomodulatory functions useful for inflammation resolution, but are often related to diseases like cancer, diabetes and arthritis where the above mentioned stimuli are abundant. [33, 34] Nevertheless, macrophage polarization is not a static but a rather dynamic and rapid process. Its plasticity is particularly orchestrated by epigenetic modifications and multiple signaling cascades, enables cells to adjust to host demands and creates a spectrum of phenotypes with distinguish functions, but also some overlapping characteristics.[35-37]

1.5 The PI3K-Akt kinase axis

1.5.1 PI-3K-Akt/PKB pathway and the role of Akt kinases in macrophage activation

A highly conserved, finely tuned and crucial in terms of macrophage activation pathway is phosphatidylinositol (PI-3K)-Akt/PKB pathway. Indeed, it has a vital role in cellular homeostasis since it participates in many different processes including, cell growth, proliferation, differentiation, metabolism, energy balance, survival and cell cycle progression.[38, 39]Due to its multifactorial contribution to cell function and maintenance, slight deregulations of its components can have detrimental consequences.[38] In fact, a vast majority of cancer types have a deregulated function of PI3K/Akt/mTORC1 pathways and have developed a type of “addiction” to it.

There are three classes of PI3K kinases, Class I, Class II and Class III that comprise of four, three and one member alike. It is important to mention that Class I kinases mostly localize

near the cellular membrane and interact with receptors mediating their signal transduction, while Class III kinase Vsp34 has specific intracellular location that allows it to participate immediately the autophagy pathway regulation as we are going to analyze on the following sections. Less information is available for PI3K II kinases, their role and the pathways that they participate in.[40, 41]

In general, PI3K is a heterodimeric kinase that consists of a catalytic and a regulatory subunit. It usually that interacts with proteins bound to certain transmembrane receptors. Upon stimulation of those receptors, PI3K becomes phosphorylated and is therefore activated. Thereafter, it catalyses the phosphorylation of lipids of the cellular membrane (PIP_2), producing second messenger molecules like phosphatidylinositol (3,4,5)-triphosphate PIP_3. These second messenger molecules are of critical importance for many cellular procedures and especially for canonical and non-canonical autophagic pathways as we are going to see in the following sections.[41, 42]Following that Akt becomes accumulated in membrane sites where PIP3 is lavish and interacts with it. Their interaction leads to their phosphorylation by PDK1. A second phosphorylation of Akt, mediated by a variety of other molecules leads to its complete activation. Activated Akt regulates many downstream molecules and distinct functions that contribute to the maintenance of cellular homeostasis. Termination of Akt activity is achieved via PTEN, which dephosphorylates PI3P and subsequently Akt activation. [42] The central role of this pathway in numerous cellular functions makes it of fundamental importance. The following picture summarizes the primordial role of this pathway in regulating countless subsequent pathways and adjusting cellular responses:

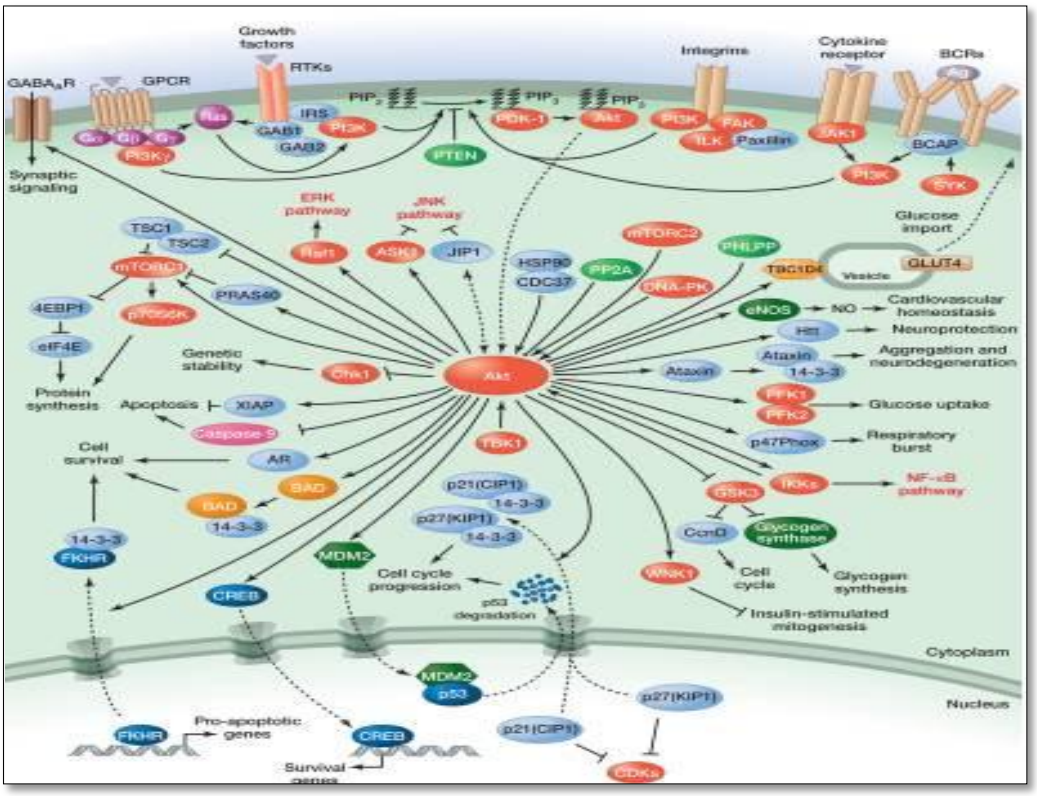


Figure 2: The central role of PI3K and Akt axis in cellular responses. (Hemmings et al, 2012)

1.5.2 Akt kinases in macrophage polarization

Akt kinases have a crucial role in PI3K/AKT/mTOR pathway. PKB/Akt protein family embraces three distinct serine/threonine kinase members: Akt1, Akt2 & Akt3. These 3 isoforms have an 80% identical aminoacid sequence. Their protein structure includes 3 domains

According to evidence arising from many studies, Akt kinases are expressed predominantly in a variety of innate immune cells like macrophages and neutrophils, since they have a critical role upon function, development and response adaptation. The most abundant forms of these kinases though are Akt1 and Akt2. In the cytoplasm, the most abundant isoform is Akt1 Independently of its kinase activity, akt1 has the ability to interfere with and successfully block NFkB signaling

Akt kinases are thought to be of paramount importance for macrophage M1/M2 polarization. Akt1 and Akt2 kinases have polar opposite role upon this phenomenon. Similarly to PI3K inhibition, abolishment of Akt1 gives rise to an M1 polarization of macrophages. Consequently, such cells tend to produce enhanced amounts of proinflammatory cytokines, reactive Oxygen and Nitrogen species (ROS, RNS) as well as having reinforced bactericidal capacity. Their anti-bacterial properties are confirmed by the hyper-responsiveness of Akt1-/- macrophages to lipopolysachharide (LPS) stimuli and the endotoxin tolerance that they present.[43] Akt1 deficient cells have a characteristically high expression of M1 signatures.

On the contrary, Akt2 deficiency creates an M2 like phenotype in macrophages. When stimulated with LPS, Akt2 deficient macrophages develop a hypo-responsiveness.[43] They have an increased expression of the classical M2 markers like IL10 and Arginase-1. IL10 is a critical cytokine especially for neonatal responses as it diminishes bactericidal capacity of cells and in that way it disarms innate immune system responses to extrinsic pathogens

PI3K pathway has major importance for macrophage function since it is implied in chemotaxis and pseudopod formation and therefore has an impact in phagocytosis. It is also implemented in TLR signaling as it mediates signal transduction but also leads to the formation of feedback loops that either upregulate or downregulate the initial signaling. It has a positive impact on mTORC1 signaling regulation

1.6 TLR signaling

1.6.1 The pathway of pathogen recognition: An insight into TLR signaling

Macrophages perform many different roles being professional sentinels, phagocytic and antigen-presenting cells. Their remarkable sensing ability can be attributed to a wide range of receptors that localize either on their surface or on the inside. These receptors are called Pattern Recognition Receptors (PRRs) and they mediate the recognition of conserved protein or nucleic acid Pathogen Associated Molecular Patterns (PAMPs) located on the surface of invading microorganisms. Except of professional phagocytes, like macrophages and dendritic cells, PRRs are also found in a variety of non-immune cells, in which they activate[44]

The most known and extensively described PRRs that initiate pathogen recognition and antimicrobial response signaling are Toll-like Receptors (TLRs). In both humans and mice, 13 members of TLR receptors have been described. As transmembrane proteins they have a characteristic structure that consists of a leukine rich extracellular domain that assists ligand recognition and an intracellular domain that mediates signal transduction to other molecules[45]. TLR localization is depended on the nature of their ligand. Therefore, TLR3, TLR7, TLR9 and TLR13 that specify in nucleic acid recognition reside in endocytic compartments, where foreign single or double stranded DNA/RNA is commonly released, while TLRs that bind specific ligands of microbial nature, like lipopolysachharides and/or lipoproteins, mostly localize on the extracellular membrane. After the initial recognition of the ligand, a signaling cascade is ignited which utilizes intermediate molecules like Myeloid Differentiation Factor 88 (MyD88), TRIFs, TRAM, TIRAM, SARM and IL-1R-associated kinase (IRAK). These molecules activate downstream pathways, like the MAPK and IKK pathway, promoting the nuclear localization of molecules, such as Nuclear factor kB (NFkB) and AP1 that have transcription factor activities.[46] This translocation assists the increased transcription of genes that encode for pro-inflammatory cytokines (TNF, IL6, IL12) and type I Interferons (IFNa).[47] These factors are then released in the surrounding environment and further occlude pathogen proliferation.

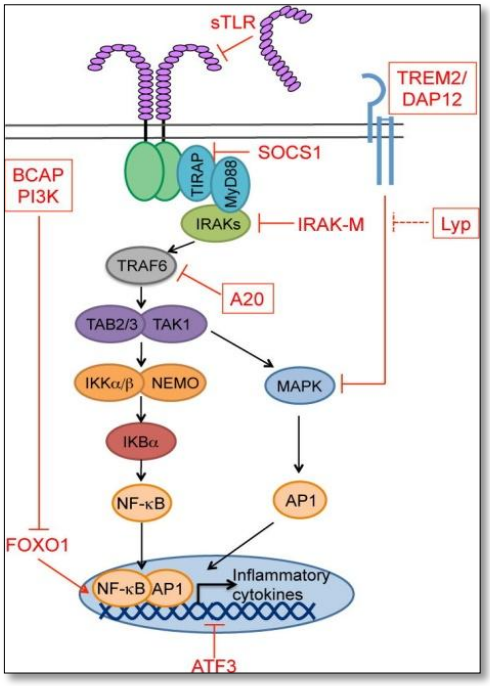


Figure 3: The TLR signaling pathway (Hamerman et al, 2017)

TLR signaling is a useful “alert signal” for the host organism for pathogen detection and elimination. On that note, it has also been linked to increased glycolysis that upregulates the production of proinflammatory cytokines. TLR signaling is also a stress signal for cells and leads to mitochondria increased ROS production.[46]

1.6.2 TLR signaling pathways in GBS infection

In case of Streptococcal infection, specific PAMPs are predominantly recognized via TLR6 and TLR2 receptors. TLR2 is indeed an extremely important molecule for GBS recognition and subsequent elimination. Studies in septic patients have shown that GBS infection initiates TLR2 signaling, activating PI3K pathway and resulting in increased secretion of adhesion molecules like CD62P, causing that way aggregation of platelets, a common manifestation of sepsis. [48]

The predominant virulent factors o GBS that initiate TLR2 signaling are Bacterial lipoproteins BLPs. BLPs are produced after the proteolytic cleavage and modification of their precursor molecules, pre-lipoproteins, by Lgt and Lsp bacterial enzymes. Studies in lgt and lsp mutant GBS strains have shown that this processing is a prerequisite to TLR2 signaling.[49] In the

case of human monocytes, TLR8 also participate in GBS recognition by binding its ssRNA. This molecule is released intracellularly after the endosomal processing of bacteria and serves in triggering potent inflammatory responses in mouse as well as in human macrophages, like TNF, IL6, IL12, Type I IFN and IL10 production.[50-52]

1.6.3 Akt1 kinase and TLR signaling

It is known that Akt1 kinase has a preeminent role in TLR signaling regulation. Previous studies have indicated that PI-3K-Akt axis is activated upon TLR4 signals, like LPS and that they create a negative feedback loop that impedes TLR4 activation upon either initial or further stimulation of the latter. [53] This is achieved via the regulation of many miRNAs that participate in this signaling cascade. A known miRNA is miRNA-155 that acts negatively upon Akt1 kinase.

1.7 The different pathways for pathogen elimination: Autophagy and LC3 Associated Phagocytosis

1.7.1 Autophagy in cellular homeostasis & balance: A small introduction

Autophagy is a pathway with many different critical functions tightly related to cellular homeostasis, metabolism, proliferation, nutrient uptake but also cellular defense. Etymologically, the word autophagy comes from the Greek term "αυτοφαγία" that describes self-devouring. This term describes a catabolic, quality and quantity control process, which begins with the engulfment of cytoplasmic constituents, organelles, proteins and/or foreign bodies, generally termed as cargo, and results in their degradation and release of protein and nucleic acid building blocks. The degradated materials are then recycled and re-used for synthesis of other cellular components. As a self maintenance pathway, autophagy takes place under basal conditions, in the absence of specific stimulus, although it is commonly triggered and up-regulated under conditions that create a state of stress to the cells. Such conditions are nutrient deprivation, pathogen infection, hypoxia and exercise.[54-56] Depending on the cargo and the purpose of this process, autophagy can be discriminated in different sub-pathways, like macro-autophagy, micro-autophagy, xenophagy, mitophagy, chaperone mediated autophagy etc.[57]

Many studies have provided evidence that autophagy plays a fundamental role in multiple cellular functions that regulate cell homeostasis, metabolism, ageing apoptosis and proliferation. This makes it is a highly conserved pathway encountered in many different organisms, from yeasts to humans. Disruptions of autophagic pathway normal function are closely related to many diseases like aggregopathies, neurodegenerative diseases, cardiovascular pathologies and even cancer progression. [58-60]

1.7.2 The autophagy pathway & its constituents

Generally, the autophagic pathway starts with phagocytosis of the cargo that is targeted towards degradation. This phagocytosis is commonly mediated by specific surface receptors that recognize cargo and cause actin polymerization and cytoskeletal rearrangements for cargo engulfment. However, canonical autophagy occurs also independently of specific receptor signaling. [46]

A vast majority of proteins participate in the process of autophagy. An indispensable component of the autophagy machinery is the Atg family of autophagy related proteins. This family consists of many members each of which has a special role upon this pathway and therefore are evolutionarily conserved in many different species. In yeast models, 41 different Atg proteins with distinct roles have been identified. [61] Their importance for cellular but also organism homeostasis is underlined by the fact that Knock Outs of most Atg genes prove to be lethal. [61] Some of the most important Atg members are Atg6/Beclin-1, Atg1/U1k1 and Atg8/LC3 that regulate the sequestration of intracellular contents into double-membrane autophagosomal vacuoles. [62]

Autophagy initiation demands the activation of a pre-initiation complex. This complex comprises of the factors ULK1/2, ATG13 & FIP200, it is important for phagophore nucleation and its role is to activate a downstream complex that bares PI3K Class III activity. This complex consists of VSP34 Beclin 1 and UVRAG or Atg13.[63, 64] The latter is a prerequisite for canonical autophagy but not for LAP as we are going to see in the following sections. Though this PI3K complex, the generation of PIP3 is assisted. The newly formed phagophore becomes gradually decorated with PI3P. This molecule serves as a signal for the activation of proteins with ubiquitin like activities, ATG5-12-16L and LC3-PE complexes, which help the stabilization and targeting of the elongating vesicle alike.[62] [65] The lipidated form of LC3 protein, known as LC3-PE or LC3II is crucial for this pathway as it sequestrates autophagosome towards fusion with the lysosome. The cytosolic form of LC3, named pro-LC3 is uptaken by atg4 and converted into LC3I. Then LC3I is immediately decorated with PE and forms LC3II that participates in autophagy. Ultimately, the autophagosome fuses with lysosome, forming the auto-lysosome.[63] This compartment is equipped with many hydrolytic molecules that degrade materials and release basic molecules like aminoacids and nucleotides. These materials are recycled and reused for the formation of other cellular structures. [63]

1.7.3 Autophagy as a mechanism of protection against pathogens: Xenophagy

Autophagy plays a really important role for immune system. It is a constituent component of immune-cell differentiation. There is a close relationship between autophagy and metabolism since they both share many common energy/nutrient sensing pathways. Therefore, autophagy dictates the metabolic reprogramming of immune cells that further controls their responses to certain stimuli. [66] An increasing amount of evidence are in support of the primordial role that autophagy has upon immunity. Today it is known that autophagic machinery is implied in 4 principle immune functions; pathogen elimination, inflammation control, immune modulator secretion and antigen presentation. These 4 functions are characteristic for macrophage cell activity. [67]

The autophagic pathway that is utilized upon opportunistic pathogen infections and assists their elimination is commonly described by the term *Xenophagy*. Xenophagy is regularly engaged by immune cells to isolate cytosolic replicating opportunistic pathogens in autophagosome lumens and deliver their genetic material to intracellular TLRs for recognition. This system demands the ubiquitination of pathogens for their upcoming engulfment and degradation.[63] Autophagic pathogen clearance requires ubiquitination of cargo for its subsequent clearance initiation.[65] The ubiquitination of foreign deleterious

micro-organisms is a specific signal that attracts autophagy initiation complex near the pathogenic cargo and sequestrates the upcoming pathogen elimination mediated by the lysosome.

1.7.4 Non-conventional autophagy and the paradigm of LC3 Associated Phagocytosis (LAP) pathway: An overview

Accept of classical autophagy, there is also a novel, distinct non-canonical autophagic pathway that is predominantly utilized for pathogen and apoptotic cell elimination and subsequent degradation. This pathway, known as LC3 Associated Phagocytosis (LAP), is a hybrid of phagocytosis and autophagy whose underlying mechanisms were first described almost a decade ago. As implied by its name, a critical component of this pathway is the microtubule-associated protein 1A/1B-light chain 3 (LC3) to phagosome membranes that serves the rapid clearance of dispensable material of self or foreign origin. This mechanism has a critical contribution to host defenses against a great variety of different pathogens.[68] Aside from its protective role against foreign microorganisms and it is also a major anti-inflammatory pathway the dysfunction of which has been related to auto-immune pathologies, like rheumatoid arthritis and lupus and other chronic diseases.[69] Hence, its multifactorial nature makes it elemental for many different organisms since it contributes to homeostasis maintenance.

Much like canonical autophagy, LAP begins with cargo engagement with specific receptors and the subsequent internalization of the former. In this case, internalization process is induced by receptors that also participate in triggering canonical autophagy like TLRs, FcR TIM, Dectin-1 complement receptors etc.[68] Some stimuli known to initiate signaling that activates LAP pathway are ß-glucan, mannose and lipoproteins and the majority of them act as signals of exogenous dangers. The engulfed exogenous material is now located in the cytosol inside phagosomes. The single membrane phagosomal compartments/organelles, created by cargo engulfment, are then rapidly decorated with PIP3. PI3P generation is performed by a PI3K Class III protein complex. Core constituents of this LAP initiation complex are the proteins VPS15, VPS34 and Beclin-1 (BECN-1), proteins that also participate in canonical autophagy. The localization of PI3K Class III complex is conducted by a protein named Rubicon.[63]

In the case of LAP pathway, initiation complex includes exclusively UVRAG, but not Atg14. [64]UVRAG containing PI3K complexes interact with Rubicon, to assist PIP3 generation and vacuole decoration. Rubicon is a protein that acts as inhibitor of canonical autophagy while it plays primordial role in LAP pathway. Multiple studies have indicated that Rubicon is a specific and essential component of LAP, since it facilitates and optimizes its progression via ROS production. This is performed via its interaction with the p22phox subunit of Nox2 protein, the major nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-2 of phagosomes. This interaction results in the translocation of the latter to the phagosomal membranes.[70] Along with p22phox, other proteins, such as p47phox, p67phox and Rac are also recruited to the phagosomal membranes. This shift is accompanied by an elevated production of Reactive Oxygen species (ROS) in the lumen of phagosome. [57] [70]Consequently, the cytoplasmic form of LC3, named pro-LC3, undergoes certain modifications to form LC3-I. This process is performed by Atg4. Simultaneously, autophagic

complexes Atg7/Atg3 and Atg12/Atg5/Atg16 drive the lipidation of LC3-I with the membrane phosphatidylethanolamine (PE), forming LC3-II and its localization in phagosome lumen respectively. [62] Unlike canonical autophagy this process does not require the participation of FIP200 and ULK1 molecules. [68]The LC3-II decorated single membrane vacuoles, known as LAPosomes, are then immediately fused with lysosomal vacuoles that contain a vast majority of enzyme with hydrolytic activities. The acidic pH of lysosomes allows hydrolytic enzymes to digest the incorporated materials. At this point cargo is degradated and a second line of immune responses are activated in order to terminate inflammation and restore balance. A synopsis of LC3 Associated Phagocytosis in pathogenic organism elimination is presented in the picture below (fig4):

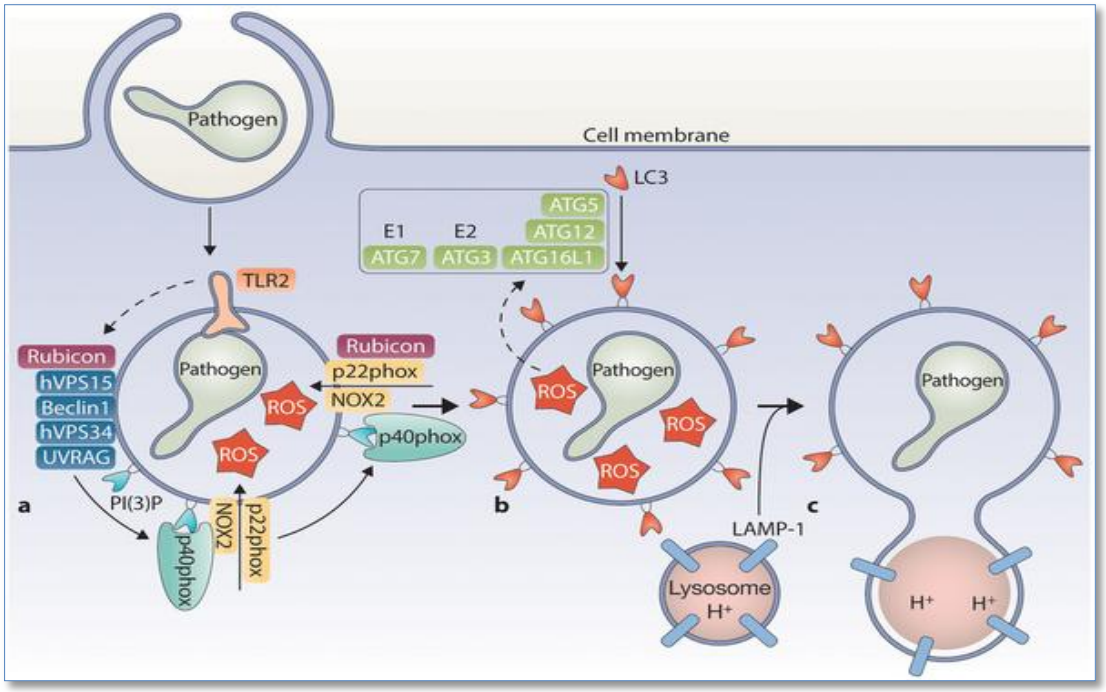


Figure 4: The LAP pathway of pathogen elimination (Boyle et al, Rubicon swaps autophagy for LAP. Nature Cell Biology. 2015)

1.7.5 The importance of LAP for innate immune responses

LC3 associated phagocytosis is a pathway predominantly induced in the presence of "danger signals", such as opportunist pathogens, apoptotic/necrotic cells and expendable, harmful debris.[68] Nevertheless, its defense activity is not restricted to pathogen destruction, but it also participates in triggering further immune responses via conducting antigen presentation.[71] More specifically, after degradation of the cargo its fragments are taken up for further processing and presentation via the major histocompatibility complex II (MHCII) while it suppresses presentation via MHCI.[69, 71]

In addition, it is known that LAP is indeed a mediator of immune-tolerant responses. Experiments conducted in LAP deficient mice (Rubicon$^{-/-}$) have demonstrated that upon pathogen infection, these animals have an extremely increased production of pro-inflammatory cytokines, such as IL6 and IL12 and inability to resolve inflammation via

production of anti-inflammatory cytokines.[57, 72] Though proinflammatory state of cells serves a more sufficient clearance of endogenous and exogenous dangers, after a certain point it can prove deleterious for the host. More specifically, many studies report that excess oxidative stress leads to damage of mitochondria and Endoplasmatic Recticullum (ER) stress.[73]Therefore, triggering of anti-inflammatory responses for achieving the resolution of inflammation is needed for restoring balance. This resolution is lost when the LAP pathway is dysfunctional.[74] When challenged with bacterial infection, these cells lack the ability to sufficiently clear them.[69] Moreover, LAP deficiency in mice leads to reduce ability to uptake and destroy apoptotic cells, increased auto-antigen presentation and auto-antibody production that makes mice prone to auto-immunity. Indeed many mice suffer from augmented proinflammatory cytokine production, decreased anti-inflammatory cytokine release and present kidney failure and a lupus-like phenotype. [57, 69, 72] [74]

Despite orchestrating the activation, LAP also shapes the outcome of immune responses. LAP is thought to have a major contribution to macrophage polarization. More specifically, LAP is in control of the macrophage metabolism since it participates in multiple metabolic pathways.[75] In order for macrophage cells to initiate signaling and subsequent responses to the corresponding stimuli, they demand a certain fluidity of energy that is via certain pathways. For instance, the pentose phosphate pathway, along with aerobic glycolysis, is the mechanism utilized by M1 cells for the generation of proinflammatory cytokines and Nitric Oxide (NO). On the other hand M2 cell responses initiated by signals like IL4 are significantly more mitochondrial depended. Such responses activate the fatty acid oxidation FAO pathway, lead to mitochondrial biogenesis, Arginase-1 and IL10 production. In the presence of the appropriate signal, both metabolic pathways can be induced by LAP. [75]

1.7.6 Canonical and LAP pathway in bacterial infections: Similarities and differences

During infection, numerous pathogens of bacterial, fungal or even parasitic origin are controlled and eliminated via the LAP pathway. Though canonical autophagy pathways like xenophagy are also utilized in the fight against foreign micro-organisms, LAP utilization is concerned as a more specific way to destroy pathogens and restore balance.

Autophagy and LAP phagocytosis have many similarities, but also a lot of differences. As far as its initiation is concerned, LAP a rapidly induced pathway that accelerates phagocytosis process and therefore it is ideal for pathogen elimination. Actually, a lot of results that derive from studies of the LAP pathway come in support of this notion. For instance, it has been shown that this pathway is initiated within the first 10 to 15 minutes in murine macrophages [76] On the contrary, xenophagy demands more time for achieving degradation of pathogens and significantly more enzymes and protein complexes that .

Unlike canonical autophagy, LAP pathway is not activated under basal conditions but mainly in the presence of specific stimuli. Its induction is highly depended on the nature of pathogenic stimulus, since not all the micro-organisms are cleared via LAP. In fact, many organisms are specifically targeted via xenophagy, others via LAP, while a number of them are targeted via both pathways. In both pathways, LC3 protein plays a major role in both pathways as it is the predominant protein that sequestrates the cargo containing vacuoles

towards fusion with lysosomes. Despite LC3, these two mechanisms of intruding pathogen eradication share a great number of common counterparts that assist the same role in both cases.

Their most distinguish characteristic, that helps scientists discriminate the two pathways is the morphology of vacuoles. Unlike xenophagy, where double membrane organelles are created, LAP is characterized by the formation of single membrane organelles surrounding the cargo. The membrane architecture along with the participation of different proteins like Rubicon and UVRAG for LAP and Atg14 for xenophagy helps us identify the pathway that is utilized in each case. Another important fact is that LAP is closely related to oxidative stress. As mentioned before, the increased presence of ROS is a prerequisite for this pathways induction and completion. The routes of the two pathways, the common participants and their differences are depicted in the following picture:

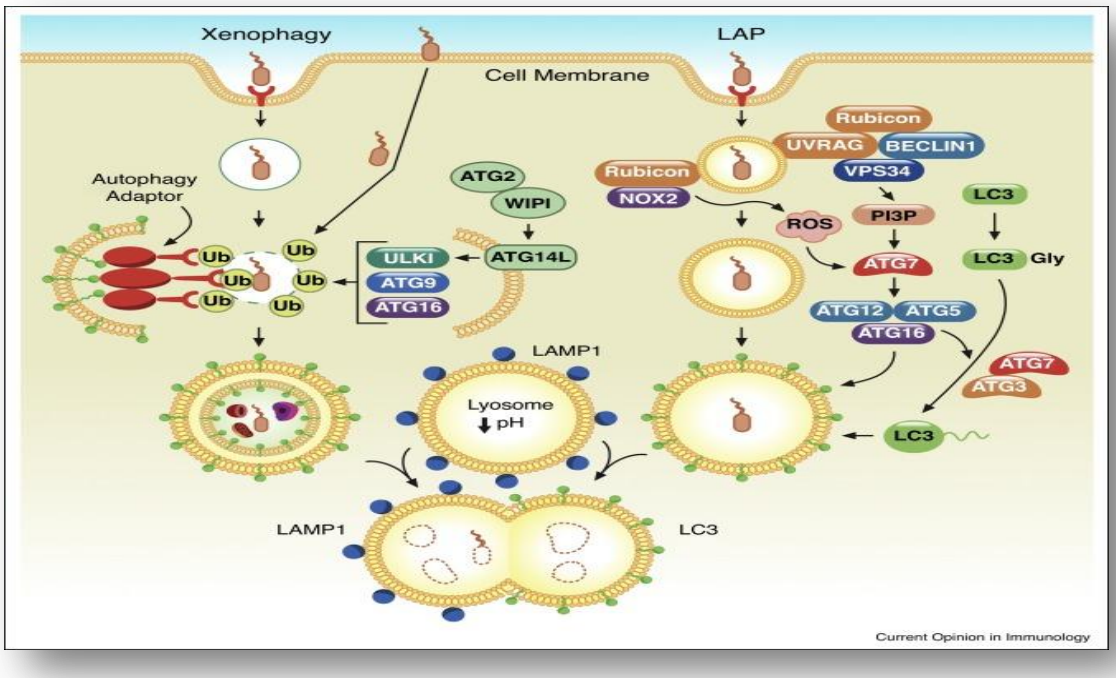


Figure 5: Differences of Xenophagy an LAP pathway (Sil et al. A ravenous defense: canonical and non-canonical autophagy in immunity, Current Opinion in Immunology, 2017)

1.8 Pathogen strategies for innate immune evasion

Despite their effectiveness in terms of pathogen clearance and equilibrium reestablishment, some opportunistic microorganisms have developed specific strategies to circumvent such immune burdens of the host and are able to evade degradation and escape in the cytosol. From the initial step of recognition, to the final step of lysosomal degradation, there are ingenious strategies that pathogens have developed to skew cellular responses.

Several pathogens have the ability to hide from recognition receptors by producing a biofilm that covers. As far as molecular cellular pathways are concerned, NFkB pathway is one of the potent targets of several bacteria since its manipulation leads to evasion of bactericidal immune responses and bacterial survival.[77] Many pathogens manage to drive immune

responses by repressing M1 activities and turning macrophage cell fate towards an M2 phenotype, characterized by wide production of IL10 and Arginase-1. Via this metabolic change of macrophage state, the bactericidal activity of macrophages is expunged. Another means of pathogen survival is the escape to the cytoplasm. Although the cytosolic environment of phagocytic monocytes like neutrophils and macrophages is not that hospitable, but rather hostile for invasive micro-organisms, many pathogens hijack the intracellular defense mechanisms and achieve the establishment of an intracellular niche. Upon their escape from lysosomes, pathogens have the capacity to multiply and produce progeny micro-organisms.[65] Furthermore, they exploit phagocytes as Trojan horses for their dissemination in various tissues through the bloodstream. This "trafficking" of pathogens contributes to further perpetuation of infection and development of serious, more complex complications. [78]

A well-studied example of such pathogen is *Staphylococcus Aureus*. This bacterium creates a biofilm that masks it and impedes recognition from phagocytic cells. Moreover, it modulates [79] especially strains that express excess amounts of certain virulence factors, which has the ability to impede phagocyte autophagic clearance, survive and spread.[80] Multiple infectious factors are targeted via canonical autophagy and LAP pathways. Such factors include viral pathogens like HIV and HSV, fungi such as *Aspergillus fumigates* and *Candida albicans*, but also bacteria like *Listeria, Leismania* and *Streptococci* to name but a few. [20, 80-86] These pathogens have developed mechanisms and characteristics that allow them to escape immune defenses and persist and proliferate inside host cells.

1.9 Elimination pathways in GBS infections

However, the mechanisms that trigger LAP targeting of specific pathogens, the means of pathogen escape from it as well as the LAP-pathogen interplay are not fully elucidated. **[87]** It is known that GBS bacteria have specific components like beta hemolysin that allows them to escape for the lysosomes to the cytosolic compartment.[88]That is not the only way that GBS circumvents defense mechanisms. It also has the ability to shift macrophage responses and upregulate anti-inflammatory cytokines that block cells' bactericidal activity, like IL10. It can also modulate macrophages to upregulate IL-10, an anti-inflammatory cytokine that blocks macrophages' bactericidal capacity. Induction of IL-10 is a key event in neonatal sepsis as it disarms neonatal immune response.[52, 89]

As far as Streptococcus agalactiae is concerned, the bibliography concerning the pathways that professional and non-professional cells utilize for its clearance is still limited. A quite recent study on human brain microvascular endothelial cells (hBMECs), demonstrated that GBS bacteria have the ability to activate autophagy machinery but they are mainly located inside single membrane organelles.[90] However, there is no other evidence on the specific mechanisms utilized upon GBS infections for pathogen elimination and their functionality in neonatal stages.

1. 10 Aims of the study

Based on preliminary data of our laboratory, we have seen that Akt1 deficient macrophages display a better and more acute response to pathogen infection and have an increased bactericidal activity against Streptococcus agalactiae (GBS). However, it remains unknown

which particular mechanisms are utilized in Akt1 deficient macrophages that endorse their responses against infections and whether this phenotype is established in a neonatal stage of macrophages. Therefore, in the current study we try to:

- Elucidate the elimination pathways of *Streptococcus Agalactiae* GBS in adult as well as neonatal murine macrophages.
- Explore the mechanisms and specific components of these pathways
- Address whether Akt1 deficiency further activates these mechanisms resulting in a more effective and accelerated clearance of GBS.

2. Materials and methods

Animals: Male and female mice of C57BL/6 background were used in this study. In detail, adult and neonate (≤10 days of age) C57BL/6 mice and mice that lacked the expression of Akt1 kinase specifically in macrophage/monocytes cells (LysM-Cre Akt1 $^{-/-}$ mice) were used for our experiments. Experiments in neonates were performed in 2-3 days-old animals.

Bacterial strains: For the purpose of this study, COH-1, a well-characterized human serotype III of Streptococcus Agalactiae (GBS), isolated from cases of human neonatal meningitis was used. Bacteria were propagated in Todd Hewitt Broth at 37^{o}C to mid-exponential (O.D.$_{600}$=0,5-0,7)

Medium Preparation for Group Beta Streptococcus (GBS): GBS was cultured in Todd Hewitt Broth Medium. For preparation of liquid culture medium, 37g TH Broth and 5g yeast extract were suspended in 1L of distilled water, while solid culture medium was also supplemented with 15g agar. Media were slightly heated and stirred to assist dissolving of powders. Solutions were autoclaved at 121^{o}C for 10-15 minutes and let to cool down to 50^{o}C before Streptococcus selective supplement, containing 5 ug/mL of Colistin-Sulphate and 0.5 ug/mL of Oxalinic acid (Streptococcus Selective Supplement, Oxoid) was added under aseptic conditions (2mL 1:1 ethanol/sterile water added in each vial). After supplementation with antibiotics, liquid TH Broth was stored at 4^{o}C, while TH Broth that contained agar was plated in sterile Petri dishes 96mm under the presence of fire. TH Broth containing plates were stored at 8^{o}C (cold room).

Streaking and liquid culture preparation of GBS: In order to isolate single bacterial colonies of GBS to further use it for other experimental procedures, a small proportion of bacteria were removed from glycerol stocks of GBS stored at -80^{o}C and was stroked on a TH Broth containing Petri dish using a plastic loop. This procedure took place under aseptic conditions and plates were placed at 37^{O}C for overnight incubation. Next day single colonies of GBS were formed on the surface of the medium.

In order to propagate bacteria, liquid cultures were set. For that reason, 6-7mL of liquid TH Broth was poured in 50ml falcons and many single colonies were dissolved in it. Falcons were tightly sealed and placed on a shaker in a hot room (37^{o}C) for a few hours. After the appropriate amount of time, cultures were placed on ice to stop bacterial proliferation and a certain amount of them 1mL was photometered for the estimation of the total number of bacteria inside each liquid culture. Cultures with an O.D. raging from 0,5 to 0,7 were used for

experiments. The verification of experimental inoculums was achieved via colony form unit (cfu) enumeration.

In vivo intranasal GBS infection model: For experiments that required the intranasal administration of GBS, neonates were thoroughly cleaned with 70% EtOH and 5uL of normal saline (NS), containing 20.000 GBS bacteria, were then administered into their nostrils. Bacteria dose of the inoculums was confirmed via colony form unit (cfu) counting after appropriate plating on TH plates.

In vivo intra-peritoneal GBS infection model: For experiments that required the administration of GBS intraperitoneally, neonates were cleaned with 70% EtOH in the abdominal area he desired amount of cultured GBS bacteria was isolated, spanned down and re-diluted in the appropriate amount of normal saline (NS). 40uL of this solution were injected in each neonate and the exact bacteria dose of it was estimated by plating a proportion of it and performing counting of viable colonies.

Survival assays: For monitoring neonate mouse survival after intra-peritoneal challenge with GBS, mice 2-5 days old were intravenously infected with several doses of GBS and observed for the following 5 days. The tails of dead animals were kept and used for genotyping purposes, in order to certify neonatal genotype. Mice were challenged with 500.000, 100.000 50.000 75.000 & 85.000 GBS.

In vivo tissue bacterial counting: After the intranasal/peritoneal administration of GBS, infected neonates were sacrificed 12 h post infection. Blood was collected via terminal cardiac puncture and stored in eppendorf tubes treated with heparin. Samples of lungs and brains were also collected aseptically after mice were euthanized. Blood was collected via terminal cardiac puncture. The left lung lobe was perfused with DPBS to remove excess blood, collected and homogenized in 500uL sterile water with a pestle. Neonatal cerebellums were also isolated, homogenized in 300uL sterile water.

Serial dilutions of each tissue (Blood, Brain and Lungs) were made and plated on THA plates under aseptic conditions. Plates were then placed in a hot room at 37^{o}C for overnight incubation. The next day, the bacterial load of each tissue was calculated via cfu enumeration.

Elicitation of peritoneal macrophages with Thioglycolate: Thioglycolate Broth (Brewer's Thioglycolate Medium Lot Number:) was diluted in normal saline 4%w/v and heated for 1-2 minutes to facilitate dissolving. The solution was then autoclaved, aliquoted and stored at 4^{o}C in dark place (light-sensitive solution) for at least 1-2 months before use.

Adult and neonatal mice were immobilized and cleaned with 70% EtOH in the abdominal area to prevent any infections. Then certain amount of thioglycolate solution (1,5mL for adults and 300uL for neonates) was injected intraperitoneally in each mouse. Four days after the initial injection, macrophages that concentrated in the peritoneal cavity were harvested by performing peritoneal lavage.

Peritoneal lavage for Macrophage isolation: Four days after the injection of Thioglycolate, mice were sacrificed and the skin of the abdominal area was removed carefully, leaving the

peritoneal membrane intact. Peritoneal lavage was performed by injecting 8-10mLs (1-3mLs for neonates) of cell medium (Dulbecco's Modified Eagle's Medium DMEM, Low Glucose 1g/L, supplemented with 10% Fetal Bovine Serum FBS and 1% penicillin/streptomycin P/S) intraperitoneally at the lower abdominal area, using a 10ml syringe. After the injection, the abdomen of the mouse was gently palpated to achieve an even distribution of the fluid in it. Syringe was disconnected from the needle and the cell containing fluid coming out of it, was collected in a 50mL falcon. This procedure was repeated 2-3 times in total. The solution was then centrifuged for 5 minutes at 1000rpm and the cell pellet was resuspended in fresh medium (DMEM, Low Glucose 10% FBS, 1% P/S). Neonate animals were slightly anesthetized by intra-peritoneal injection of anesthetics (ketamine/xylazine mixture 1mL/50uL, 10mg/mL and 20mg/mL alike).

Plate seeding of peritoneal macrophages: Following isolation, macrophages were seeded in cell culture plates for performing the appropriate processes. For intracellular killing assays, adult and neonatal wild type and LysCreAkt1$^{-/-}$ peritoneal macrophages were seeded in 96-well cells culture plates at a density of 100.000 cells per well. For Flow cytometric analysis experiments cells were seeded in 24 well plates at a density of 250.000 cells per well. For RNA extraction experiments cells were seeded in 24 well plates at a density of 350.000-400.000 cells/well. For protein extraction cells were seeded in 12 well plates at a density of 450.000-500.000 cells per well. For confocal microscopy assays cells were seeded in 24 well plates with glass coverslips at a density of 350.000 cells per well.

Intracellular killing assay in adult and neonatal wild type and Akt1$^{-/-}$ macrophages: The isolated macrophages from adult and neonatal mice were resuspended in fresh low glucose DMEM 10% FBS, 1% P/S and were seeded in 96 well culture plates at a density of 100.000 cells per well. Quadruplicates/centuplicates were created for each condition.

The day of the experiment, cell medium was changed to DMEM Low Glucose 10% FBS, 0,2% P/S one hour prior to GBS addition. GBS was propagated until reaching a desired OD_{600} and the proper amount of bacteria was transferred in sterile eppendorf tubes and centrifuged at 6500 rpm for 5 minutes (4^{o}C). The supernatant was discarded and bacterial pellet was resuspended in the appropriate amount of cell culture media (DMEM, Low Glucose 10%FBS). 100uL of medium containing GBS at an MOI 1:10 were administered in all wells. After 2hrs GBS stimulus was removed, cells were washed multiple times with DPBS to eliminate the extracellular and surface bound bacteria and 100uL of DMEM, Low Glucose 10% FBS, 1% P/S were added in each well. At the desired time-point, supernatant was removed; cells were washed twice with DPBS and lysed with 100uL Triton-X 0,025% (diluted in sterile water). Each sample was serially diluted and 100uL of the proper dilutions were plated in THA plates (sterile conditions). Plates were placed at 37^{o}C overnight. The bacterial load of each cell type was estimated via cfu counting. The time-points tested for neonates and adults were:

- Neonatal and Adult wt & LysMCre-Akt1$^{-/-}$ macrophages: 2, 4, 6, 8, 12 and 16 hpi

Based on results of the above mentioned experiment, the appropriate time-point was chosen for an intracellular killing assay after treatment of cells with autophagy inhibitors and/or inducers. The inhibitors used for this assay were: Rapamycin, Apocynine,

Chloroquine, 3-Methyladenine (3MA), Diphenyleneiodonium (DPI) at final concentrations of autophagy inhibitors DPI (20uM), 100Nm, 50uM, 10uM, 5mM and 20uM respectively.

Intracellular-Extracellular bacteria in adult and neonatal wild type and Akt1$^{-/-}$ macrophages: Following the concept if intracellular killing, intracellular-extracellular bacterial count was performed. For the purpose of this experiment, primary macrophages were seeded in 96 well plates at a density of 100.000 cells per well. Liquid cultures of GBS were placed in a hot room (37^{o}C, shaking required) for GBS propagation. After reaching the desired OD_{600}, bacteria were isolated and diluted in cell culture media (DMEM, Low Glucose 10% FBS, no P/S). Bacteria were added in cells at an MOI 1:10. After an hour incubation at 37^{o}C (5% CO_2), plates were removed from the incubator and placed on ice. The supernatants of each well were carefully collected, diluted and plated on THA plates. Cells were washed multiple times with DPBS to remove extracellular bacteria and then lysed with 100uL Triton-X 0,025% in water. The lysates were again diluted and plated on THA plates. For an accurate estimation of bacterial load, the bacteria-containing supernatant was also diluted and plated on THA plates.

Heat inactivation of GBS – FITC labeling: The desired amount of GBS was centrifuged (5minutes, 5.500 rpm, 4^{o}C) and bacterial pellet was resuspended in 1mL DPBS. The solution was heated for 1 hour at 56^{o}C. Thereafter, bacteria were re-centrifuged twice, the supernatant was discarded and bacterial pellet was resuspended in 1mL sodium bicarbonate (pH=7,2). FITC was added at a final concentration of 0,01ng/mL. The solution was heated for 1 hour at 37^{o}C in a dark place (FITC is light-sensitive). Stained bacterial were centrifuged and resuspended in DPBS, multiple times, in order to discard the unbound amount of FITC. Stained bacteria were diluted in cell medium (DMEM Low Glucose, 10% FBS, no P/S), thoroughly vortexed to break GBS chains and added in cell supernatants.

Immunostaining of macrophages & Confocal imaging: For confocal/immunostaining assays, cells, diluted in DMEM Low Glucose 10% FBS 1% P/S, were seeded in 24 well plates at a density of 300.000 cells per well. Before the addition of cells, round glass coverslips (12mm) were placed at the bottom of each well. After plating the cells, plates were placed in an incubator (37^{o}C, 5% CO_2) overnight for cells to adhere on coverslips. On the day of the experiment medium was removed and cells were washed with DPBS twice. Following that, cell medium (DMEM Low Glucose, 10% FBS, no P/S) that contained the FITC-labeled GBS was added in each well in a way to achieve an MOI 1:10 (3.000.000 bacteria per well). 2 hours after GBS addition, supernatant was discarded and fresh medium (DMEM Low Glucose, 10% FBS, 1% P/S) was added in the corresponding wells. After the appropriate amount of time, supernatant was discarded; cells were washed with DPBS, fixed with PFA (2% PFA in DPBS) and stored at 4^{o}C. Coverslips with cells that received no bacteria particles were used as negative controls in this process.

For immunostaining coverslips were transferred to a clean surface and washed with 100uL DPBS (x3) to remove excess PFA. Blocking solution (5% FBS, 1% BSA, 0,05% Triton-X 100 in PBS) was added in all coverslips and cells were incubated for 15 minutes at RT on shaker, This process was repeated twice. Following that, blocking solution was aspirated and 50uL of the primary antibody (Rabbit Ant1-LC3 Antibody, NOVUS, 1/200 dilution in Blocking Buffer

supplemented with 0,05% Triton X 100) were added in each coverslip for 1 hour at RT on a rotator. During this time, coverslips were kept at a dark place. The fluid was removed and coverslips were incubated twice with blocking solution for 5 minutes. After blocking, secondary antibody solution (Alexa Fluor 555 1/500 in Blocking Buffer) was added in coverslips for a 40- minute incubation at RT. All coverslips were washed thoroughly with DPBS (0,1% Triton-X) and TOPRO solution (1/1000) was added for 2 minutes, to assist nuclear staining. Coverslips were washed with DPBS, merged in mounting medium, sealed and Stored at 4^{o}C. Coverslips were observed at Leica Confocal Microscope and images were analyzed in Adobe Photoshop CS6.

FACS Immunostaining protocol: For flow cytometric analyses, 350.000 primary macrophage cells were seeded in 24 well plates and incubated with GBS at an MOI 1:10. The supernatant was discarded and cells were washed twice with DPBS. After DPBS removal, Trypan Blue 0,2% (0,4% Trypan Blue diluted in dH_2O 1:1) was added in all wells for 1-2 minutes to assist non specific signal blockage. Cells were washed properly with DPBS (x4) to remove the excess amount of Trypan Blue and cold FACS buffer was added (200uL per well). Cells were then scrapped and the cell containing supernatant was collected in sterile eppendorf tubes. Samples were centrifuged for 5 minutes at 500-650 rcf, 4^{o}C. Supernatants were discarded and 100uL of diluted antibodies specific for monocytes' cell surface marker **F480** were added in each cell pellet. Samples were vigorously pipetted to assist pellet re-suspension and incubated at room temperature (RT) for 20 minutes and for another 10 minutes on ice (4^{o}C). Incubations were performed in the absence of light (light-sensitive antibodies). Antibodies were diluted in cold FACS Buffer (APC-F480 dilution 1:400 and PE-F480 dilution 1:400). Cold FACS Buffer (900uL) was added in each tube and samples were re-centrifuged for cell pellet precipitation. Supernatants were discarded, cell pellets were re-suspended in cold FACS Buffer and re-centrifuged, a process repeated multiple times, to remove the excess amount of the antibody. Cell pellets were diluted in 2% paraformaldehyde (4% PFA diluted in FACS Buffer 1:1) and stored at 4^{o}C. Samples were analyzed in a Flow cytometer and the percentage of double positive cells was quantified.

Unstained macrophage cells, activated with unstained GBS were used for the elimination of auto-fluorescence signal. Single stained cells, such as unstained cells that received heat inactivated FITC labeled GBS or cells stained for the monocytes surface marker F480 were used for the adjustment of compensation and signal overlapping.

Dihydrorhodamine (DHR) assay: For the measurement of NADPH oxidase activity in peritoneal macrophages of Akt1$^{-/-}$ and wild type neonatal and adult macrophages, the dihydrorhodamine (DHR) flow cytometry assay was used. Cells were seeded in 24 well plates at a density of 250.000 per well. Cells were simultaneously stimulated with GBS at an MOI of 1:10 and 0,2ug/mL DHR (DMEM Low Glucose, 10% FBS). Cells were then incubated for the appropriate amount of time. After 2hrs, medium was changed to DMEM Low Glucose 1g/L, 10% FBS, 1% P/S supplemented with the right proportion of DHR. After the appropriate amount of time, wells were emptied and reactions were terminated by pouring cold PBS in each well. Cells were detached using a scrapper, centrifuged for 5 minutes at 500g at 4^{o}C and fixed with 200uL 2% PFA (4% PFA in PBS). Finally, the results of the oxidation of DHR to

a fluorescent compound, rhodamine 123 were measured by flow cytometry and analysed in GraphPad Prism v.7.

NO measurement assay in cell supernatants: In an uncoated 96 well plate (Bradford plate), 50uL of each experimental sample were added in duplicates or triplicates. For the generation of standard curve, serial ½ dilutions of Nitrite standard (0,1M sodium Nitrite in dH_2O) diluted in cells medium were made and pipetted in duplicates (100uM starting concentration). Using a multichannel pipette 50uL of Sulfanilamide solution (1% Sulphanilamide, 5% H_3PO_4)were added in all experimental samples and wells containing the dilution series for the Nitrate Standard Reference Curve. After a 5 minute incubation in a dark place at room temperature, 50uL of NED solution (0,1% N-1-napthylethylanediamine dihydrochloride in dH_2O) were added in all the wells and samples were again incubated at RT for 5-10minutes. After the formation of a purple/magenta color, the absorbance at 520-550nm was measured within 30minutes, using a microplate reader.

The resulting absorbance data were analyzed using Microsoft Excel and GraphPad Prism 5.0, following a linear regression model.

Protein harvest: For protein collection, primary macrophage cells were seeded in 12 well plates at a density of 450.000-500.000 cells per well. After the necessary treatments (Bafilomycin A1 100uM), supernatant was removed and cells were washes twice with 400uL sterile DPBS. Thereafter, plates were placed on ice and 400uL DPBS were added in each well. Cells were thoroughly scrapped and the cell containing solution was harvested in sterile eppendorf tubes. The samples were centrifuged for 10 minutes at 600 rcf (4^oC) and supernatant was discarded. Following that, 30uL of RIPA Buffer, supplemented with 1X Protease Inhibitors (Protease Inhibitor complete tablets, Roché, dissolved in distilled water to a stock concentration of 7X) and 1X Phosphatase inhibitors, were added in each cell pellet to assist cell lysis. After the re-suspension of cell pellets, samples were re-centrifuged at maximum speed (12.000g) for 15 minutes, at 4^oC, to discard cell debris and collect protein extract in the supernatant. Supernatants were collected, transferred to new eppendorf tubes and stored at -80^oC until use.

Protein quantification using Bicinchoninic Assay (BCA): The Bicinchoninic assay protocol was used for the determination of the total protein concentration of our samples. Serial dilutions of bovine serum albumin (BSA) solution of known concentration were used for the creation of a protein concentration standard curve. The standard curve was created by mixing 5uL of the corresponding standard with 20uL dH_2O. The protein standards used in this process were: 1000ug/ml, 500ug/ml, 250ug/ml, 125ug/ml, 62,5ug/ml, 31,25ug/ml and 15,625ug/ml. As controls, 5ul of PBS were used for BSA standards calibration, while 5ul of RIPA buffer (Protease/Phosphatase Inhibitors) was used as a control for our samples. The final volume of fluid in each well was 25uL. BCA Reagents A and B were mixed in proportion 50:1 and 200ul of the mix were pipetted to each well. The plate was sealed and incubated at 37^oC in a dark incubator for 30 minutes. Then, the absorbance at 550nm was measured using a microplate reader. Data were analyzed using Microsoft Excel and Graph Pad Prism 5.0, following a linear regression model.

Western Blot Analysis of protein samples: For Western Blot analysis 1.5mm thick, 13,3% polyacrylamide gels were used. Gels were prepared by mixing acrylamide (30%) with the appropriate amount of separating/stacking gel buffers and polymerization was induced after the addition of 10% ammonium persulfate (APS) and TEMED® reagents. 1.5M Tris (pH 8.8) and 1M Tris (pH 6.8) were used as the buffers for the separating and the stacking phase of the gel, respectively.

After quantitation of the protein concentration, 15 ug of each protein sample were used for western blot analysis. After the addition of Loading dye (at a final concentration 1x), samples were heated at 96^{o}C for 3 minutes, spanned down and loaded onto polyacrilamide gel. For the indication of molecular weight of proteins, 7uL of Benchmark protein ladder (Invitrogen®) were used. Electrophoresis of samples was performed at 80V while proteins moved through the stacking phase, and then at 100-120V in the separating phase of the gel. After approximately an hour, electrophoresis was stopped and proteins were transferred from gels to activated PVDF membranes (soaked in methanol for 1-2 minutes), in transfer buffer (2.9g Glycine, 5.8g Tris base, 0.37g SDS, 200ml methanol) at a constant current of 400mA. After transfer process, membranes were briefly washed in PBST buffer (phosphate buffered saline with Tween® 20, 0,1%) ,then blocked in 5% skimmed milk diluted in PBST for 1 hour. Membranes were washed again and incubated with the primary antibodies overnight at 4^{o}C. Afterwards, membranes were washed briefly in PBST and incubated at room temperature with the secondary antibody for 1 hour. Brief washes with PBST were performed to remove unbound secondary antibody molecules, and enhanced chemiluminescent (ECL) peroxidase substrate was used. Membranes were exposed to trans-UV light in a fluorescent imager (BioRad Laboratories, Inc.) and fluorescence signals were digitalized, and analyzed with manufacturer corresponding software (ImageLab).

Samples were normalized using antibodies for housekeeping genes (β-actin). Ratios of (target protein) / (housekeeping gene) were calculated for each sample after analyzing the digitally acquired signal (densitometry analysis). Differentiations in protein quantities at separate time-points/treatments were depicted in box plot graphs created with GraphPad prism.

The primary antibodies used in this study were:

LC3: LC3 Antibody (1712D), Novus Biological

Akt1: Akt1 (D9R8K) Rabbit mAb #75692, Cell Signaling

pAkt1: Phospho-Akt1 (Ser473) (D7F10) XP® Rabbit mAb (Akt1 Specific) #9018, Cell Signalling

Actin-b: β-Actin (8H10D10) Mouse mAb #3700, Cell Signaling

- Secondary Antibodies: 1) Goat anti-mouse IgG polyclonal antibody HRP conjugateBML-SA204-0100, 2) Goat anti-rabbit IgG polyclonal antibody HRP conjugate ADI-SAB-300-J

RNA isolation, cDNA synthesis and real time quantitative rt-PCR: For RNA isolation, primary macrophages of neonatal or adult mice were seeded in 24 well cell culture plates at a density of 350.000-400.000 cells per well. After the appropriate treatments, cells were

placed on ice and collected using 200ul of Trizol Reagent (Life Technologies). The cell containing supernatant was transferred to clean, sterile eppendorf tubes and 40uL (1/5 V) of chloroform were added in each sample. After brief vortexing, samples were centrifuged for 15 minutes at 12.000 rcf (4^{o}C). The upper aqueous phase was carefully collected and transferred to new sterile RNAse free eppendorf tubes and 100uL (1/2 V) of isopropanol were added in all samples. After a short incubation at room temperature to assist the precipitation of RNA, samples were centrifuged at 12.000g for 10 minutes (4^{o}C). Tubes were emptied, filled again with 250uL of 75% EtOH (Absolute EtOH diluted in dH_2O and centrifuged at 7.500g for 5 minutes (4^{o}C). This process was repeated twice, tubes were emptied and RNA pellets were left at room temperature for 10 minutes in order to dry. Pellets were re-diluted in 20uL of RNAse-free water (TaKaRa®) and heatblocked for 5 minutes at 65^{o}C. The RNA content of samples was verified using the Nanodrop micro-volume spectrophotometer.

Following RNA extraction, cDNA synthesis was performed using the TaKaRa® PrimeScript™ 1st strand cDNA synthesis kit, following manufacturer instructions. 1ug of Template RNA was used from each sample and after dNTP mixture (10mM), Random 6mers (50uM) and RNAse free H_2O addition. Samples were incubated at 65^{o}C for 5 minutes, and then cooled immediately on ice. 5ul of a mixture containing 5x PrimeScript Buffer, RNAse Inhibitor (40U/uL), PrimeScript RTase (200U/uL) and H_2O were then added to each sample. cDNA synthesis was performed at the following conditions: 1) 10 minutes at 30^{o}C, 2) 45 minutes at 50^{o}C, and 3) 5 minutes at 95^{o}C (enzyme inactivation). Samples were diluted at a final volume of 20uL and stored at -20^{o}C until use.

The cDNA produced by the above described procedure was used for rt-qPCR analysis. For this purpose, the FAST Green 2X qPCR Universal mix was used. Each reaction included 0,4The necessary volume (9uL) of master-mix (Forward and Reverse primer was used in all reactions along with 1ul of the corresponding cDNA. The final volume of each reaction was 10uL. Duplicates were made for each sample. RPS9 was used as the homeostatic gene of our samples and the expression of Atg5, Atg7, Nox2, IL10, IL12b and TNFa. RtPCRs were performed in Applied Biosystems-qPCR machine and the results of gene expression were analysed at StepOne software v2.3 and GraphPad Prismv7 software.

Atg5: F: 5'–ATGGACAGCTGCACACACTT–3' & R: 5'-CTGGGTAGCTCAGATGCTCG-3'
Atg7: F: 5'–ACCATGCAGGGAGCTAGAGA–3' & R: 5'-CCCATGCCTCCTTTCTGGTT–3'
Nox2: F: 5'–TCCTGGAGGAAGTGGGCCGAAG–3' & R: 5'–CCTCCACGGGCCCGGTACTC–3'
IL10: F: 5'–GCGCTGTCATCGATTTCT CCCCTG–3' & R: 5'–GGCCTTGTAGACACCTTGGTCTTG–3'
IL12b: F: 5'–GGAGGGGTGTAACCAGAAAGGTGC–3'& R: 5–CCTGCAGGGAACACATGCCCAC–3'
RSP9: F: 5'-GCTAGACGAGAAGGATCCCC-3' & R: 5'- CAGGCCCAGCTTAAAGACCT-3'

3. Results

3.1 Phagocytosis capacity of Wild Type & Akt1 deficient adult and neonatal macrophages

In order to determine the capacity of adult and neonatal macrophages to engulf GBS cells and whether this process is upregulated or reduced in the absence of Akt1 kinase we

conducted an experiment that determined the number of alive GBS bacteria localized in the extracellular environment and intracellularly. More specifically, all types of macrophages (wt adult, wt neonatal, Lys-Cre Akt1$^{-/-}$ adult and Lys-Cre Akt1$^{-/-}$ neonatal Mϕs) were incubated with GBS cells at an MOI of 1:10 for 1 hour. The extracellular bacteria-containing medium was diluted and plated, while intracellular bacterial load was estimated via lysis of cells, dilution and plating of the lysates. From cfu counting we managed to estimate the number of intracellular, extracellular bacteria and the initial number of bacteria introduced in each well, named as i, e and c respectively. Using these variants we created an equation for determining the number of bacteria that remained alive. The number of survived bacteria (s) is described by the following equation:

$$s=i+e$$

For the estimation of bacteria that were killed by macrophages (k) the equation that we used was the following:

$$k= c-(i+e)$$

Based on that we then proceeded in calculating the number of bacteria that were uptaken by macrophages (a). This number indicated the phagocytosed bacteria and it was calculated using the following equation:

$$a= k+i$$

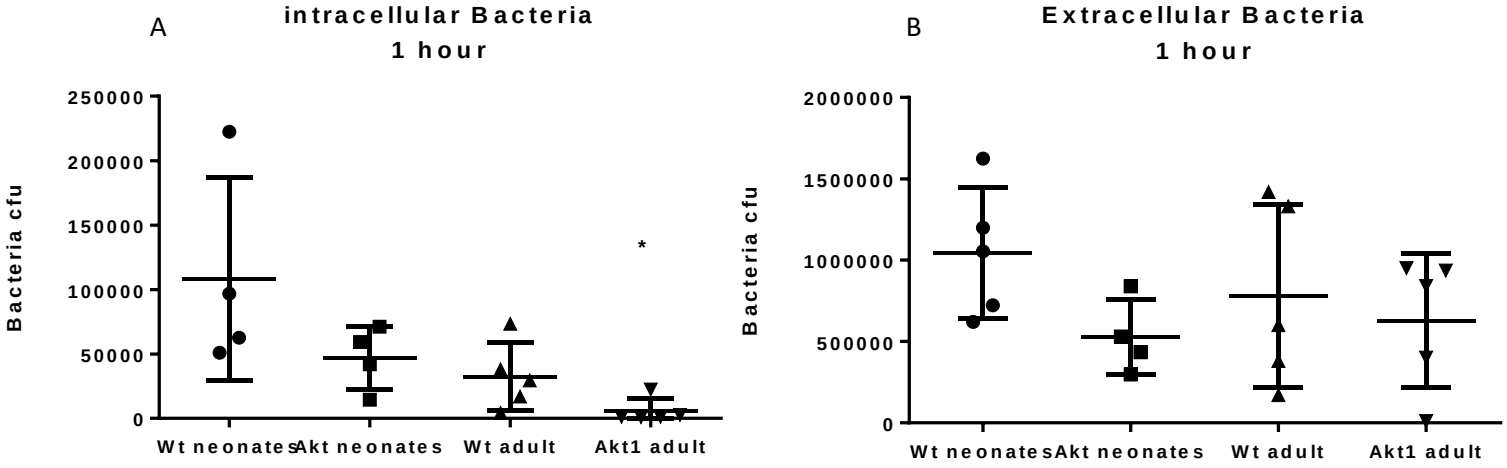
Finally the percentage of phagocytosed bacteria (P) was calculated by using the following equation, according to which:

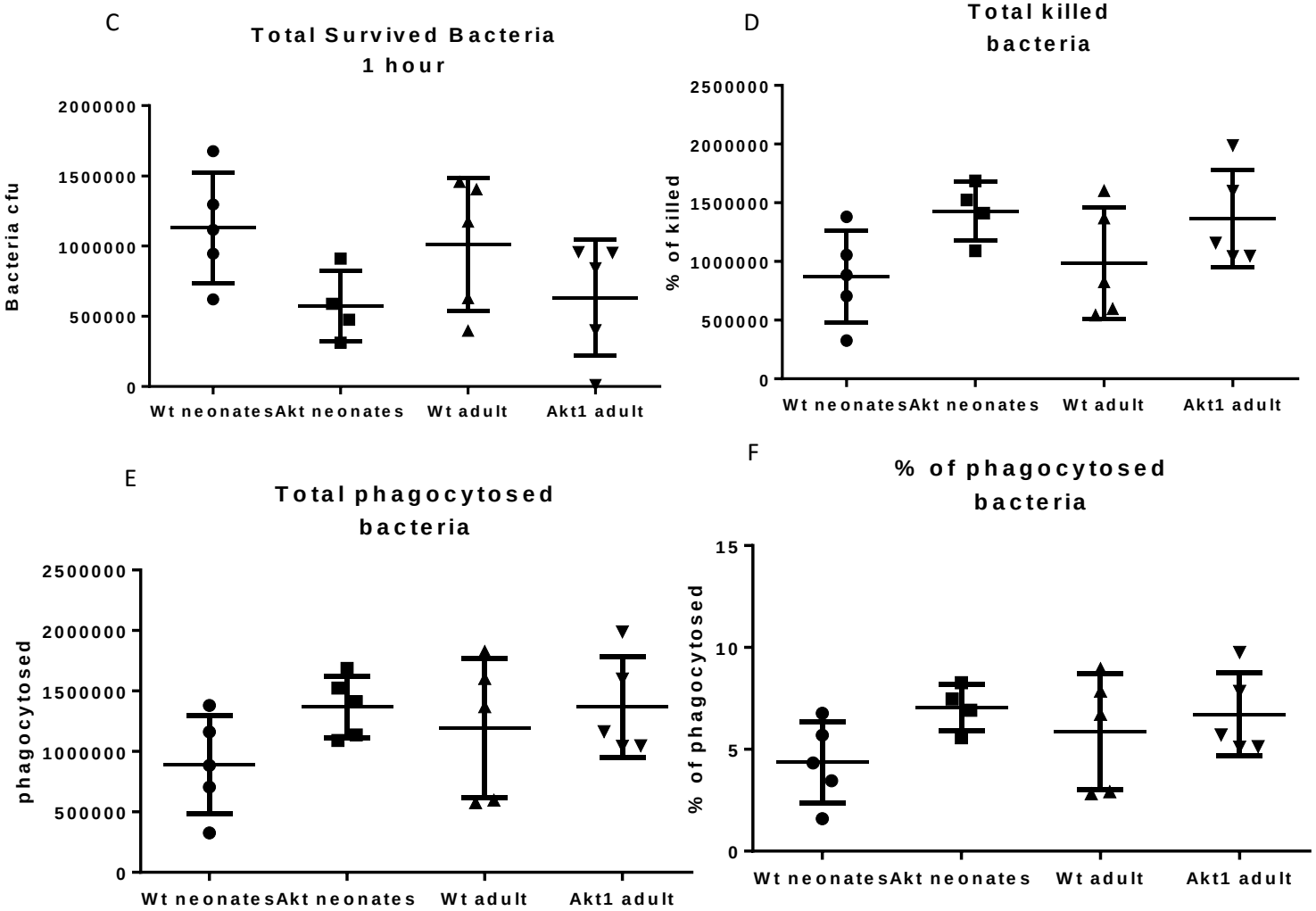
$$P=\frac{u}{c}*100\%$$

As shown in the data presented in diagram **6.A** the amount of intracellular bacteria is lower in Akt1 deficient adult and neonatal macrophages compared to the corresponding wild type cells. However, the corresponding amount of extracellular bacteria is also diminished in those cells compared to their wild type counterparts (Diagram **6.B**). The differences in intracellular and extracellular bacterial load are more distinguish when neonatal cells are compared, while adults have a slight but not statistically significant difference. In diagrams **6.C** and **6.D** we can see the number of survived and killed bacteria respectively. As we can see, Lys-Cre Akt1$^{-/-}$ Mϕs have less survived and more killed bacteria compared to wild type cells and that is the case for both adults and neonates. At this point it is important to determine the means of bacteria killing. Bacteria are either degradated intracellularly via the lysosome pathway or extracellularly via the production of nitric oxide NO by macrophages. In this case bacteria were killed intracellularly and not extracellularly and that was confirmed by performing measurement of NO levels in supernatants collected from macrophages that were treated with GBS at an MOI 1:10 for 1 hour. The NO levels of these supernatants were undetectable at time-points of 2, 4 and 6 hours after the initial GBS infection, with an exception of Akt1 neonatal macrophages 6hpi samples, where NO was detectable but the levels were significantly low. Therefore, we concluded that the number of killed bacteria was exclusively from bacteria that were phagocytosed and consequently degradated. As

demonstrated in diagrams **6.E** and **6.F** the number of phagocytosed bacteria and the percentage of phagocytosis are not significantly different between the different neonatal and adult macrophages. That led us to the conclusion that Akt1 ablation does not impact phagocytosis capacity of macrophages either in neonatal or later developmental stages.

In order to identify and determine the size of neonatal macrophage population that perform phagocytosis of GBS and whether this number is modified upon the absence of Akt1 kinase and thus has a negative or positive impact on phagocytosis capacity of macrophages, we performed a 2 color flow cytometry and sequential gating analysis. For that reason, macrophages isolated from wild type and Lys-Cre Akt1$^{-/-}$ infant mice were inoculated with heat inactivated FITC labeled GBS at an MOI 1/20 for 1 hour. Macrophage cells were stained with PE-F4/80 for this specific monocytes surface marker. After the exclusion of doublets and debris, macrophage cells were identified based on the expression of F4/80, the high side scatter and high auto-fluorescence. Despite these features, macrophage population that performed phagocytosis of GBS was also selected based on its FITC fluorescence. The results presented in the following diagram (Diagram **6.G**) indicate that neonatal macrophages do not have a significantly different phagocytosis capacity in the presence of in the absence of Akt1 kinase. More specifically, the double positive cells (PE+ and FITC+) represented in the upper right square R4 are the 17,9% of WT and the 18,8% of Lys-Cre Akt1$^{-/-}$ neonatal macrophages. Although there is a slight difference between these populations of the 2 distinct groups, it has no statistical significance. This comes in support of the previous data acquired by intracellular-extracellular killing assay and is a further confirmation that the initial bacterial phagocytosis is not affected by the lack of Akt1 expression. As a synopsis of the above mentioned results, diagram **6.H** demonstrates the phagocytosis capacity of all cell types which has no significant differences for adult or neonate cells that either express Akt1 kinase or not.





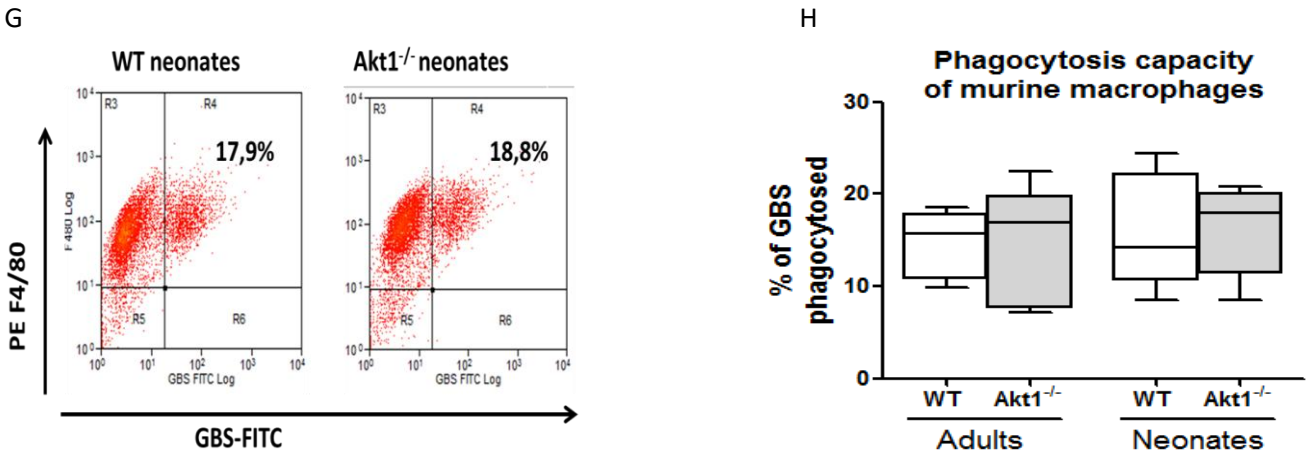


Figure 6

Phagocytosis capacity of Wild Type & Akt1 deficient adult and neonatal macrophages: Adult and neonatal murine macrophages were incubated with GBS particles at an MOI 1:10 for 1

hour. The amount of bacteria inside cells and in the extracellular medium was estimated by plating and cfu counting. (A) Total number of intracellular GBS i, (B) Total number of extracellular GBS e, (C) Total amount of survived bacteria s, (D) Total amount of killed bacteria k, (E) Total number of phagocytosed bacteria p (F) Percentage of phagocytosed bacteria P. (G) Neonatal murine macrophages were incubated with FITC labeled GBS (MOI 1:10) for 1 hour, stained with APC-F4/80 and the amount of double positive cells ($FITC^+$, APC-$F4/80^+$) was estimated via FACS analysis.(H) Phagocytosis capacity of murine macrophages. *, $P < 0.05$; **, $P < 0.05$; ***, $P < 0.005$

3.2 Lys-Cre $Akt1^{-/-}$ neonatal and adult macrophages have an increased capacity of GBS clearance compared to the corresponding wild type cells

After confirming that neonatal and adult cells have the same phagocytosis capacity whether they lack the expression of Akt1 kinase or not, we sought to address whether their efficiency of microbial clearance is influenced by the presence or the absence of Akt1 kinase. Therefore, we performed in vitro intracellular killing assays that allowed us to estimate the amount of phagocytosed bacteria that remained alive intracellularly. After challenging cells with GBS bacteria at an MOI of 1:10 for 2 hours, macrophages were lysed and their intracellular bacterial load was monitored over a time-course of 16 hours by performing cfu counting in cell lysates. As presented in the following diagrams, adult cells have an all-in-all better response in bacterial challenge, compared to the corresponding neonatal macrophages. That is depicted by their ability to clear bacteria. More specifically, as shown in diagrams **7.A** and **7.B** we can see that in a span of 2 and 4 hours post infection adult macrophages have managed to eliminate a greater number of bacteria compared to the respective neonatal macrophages. In detail, we saw that while wt adult macrophages have a bacterial load of 460.000 and 178.000, neonatal cells have 642.00 and 294.000 respectively. The picture is the same as far as LysCre $Akt1^{-/-}$ macrophages are concerned since adults display bacterial loads of 255.000 and 6.500 bacteria at 2 and 4 hours post infection, while neonates have 405.000 and 64.000 bacteria respectively.

However, the most interesting finding is that Akt1 macrophages have a better response in terms of clearance compared to wild type macrophages. As far as adult macrophages are concerned, in diagram **7.D** we see that LysCre $Akt1^{-/-}$ macrophages Although the initial bacterial load is the same for both wild type and LysCre $Akt1^{-/-}$ macrophages (560.000 bacteria), 2 hours after the initial infection, the intracellular load of LysCre $Akt1^{-/-}$ macrophages is 260.000 cfu. While LysCre $Akt1^{-/-}$ cells have degradated almost the two thirds of phagocytosed bacteria, this clearance efficiency is not observed in Wt cells. In a span of 2 hours, the corresponding intracellular bacterial load in wild type macrophages is 460.000 and cells have managed to kill 100.000 bacteria. The difference is more obvious at the time-points of 4 and 6 hours post infection in which WT cells have an intracellular load of 178.000 and 89.000, while the load in LysCre $Akt1^{-/-}$ cells is 6.500 and 2.500 respectively. This difference is significantly reduced at 8hpi as wt and LysCre $Akt1^{-/-}$ cells have 29.500 and 1.000 bacteria intracellularly and it is further reduced at 12hpi, while cells have managed to eliminate bacteria at 16hpi. These data demonstrate that Akt1 depletion in macrophages allows them to have a more acute and efficient response against GBS, which assist the immediate bacteria elimination.

This beneficial phenotype of Akt1 ablation is also replicable in neonatal macrophages as shown in diagram **7.C**. In a span of 2 hours after the initial challenge with GBS, the number phagocytosed bacteria has declined from 570.000 to 405.000 in LysCre Akt1$^{-/-}$ cells, while in wt cells bacterial load increased from 570.000 to 640.000. This increase is indicative of the escape and the consequent proliferation of GBS in the cytosolic compartment. As we can see, in the absence of Akt1 kinase, macrophages activate elimination mechanisms rapidly and therefore, bacteria do not manage to escape to the cytoplasm. At 4 and 6 hpi knock out neonatal cells have 64.000 and 10.550 bacteria, while wild type cells have 294.000 and 115.000 bacteria respectively. These differences have a statistical significance and are indicative of Akt1$^{-/-}$ cells efficiency during bacterial infection. At 8 hpi the difference in intracellular bacteria has reduced at 20.000 and 2.500 respectively and is further decreased at 12hpi. The phenomenon of GBS clearance is resolved at 16hpi for both neonatal cell types. These data indicate that ablation of Akt1 kinase in neonatal macrophages gives a bactericidal advantage in these cells, since they have the ability to immediately respond to GBS challenge, to possibly block bacterial escape from endosomes to the cytosol and lead to its rapid degradation.

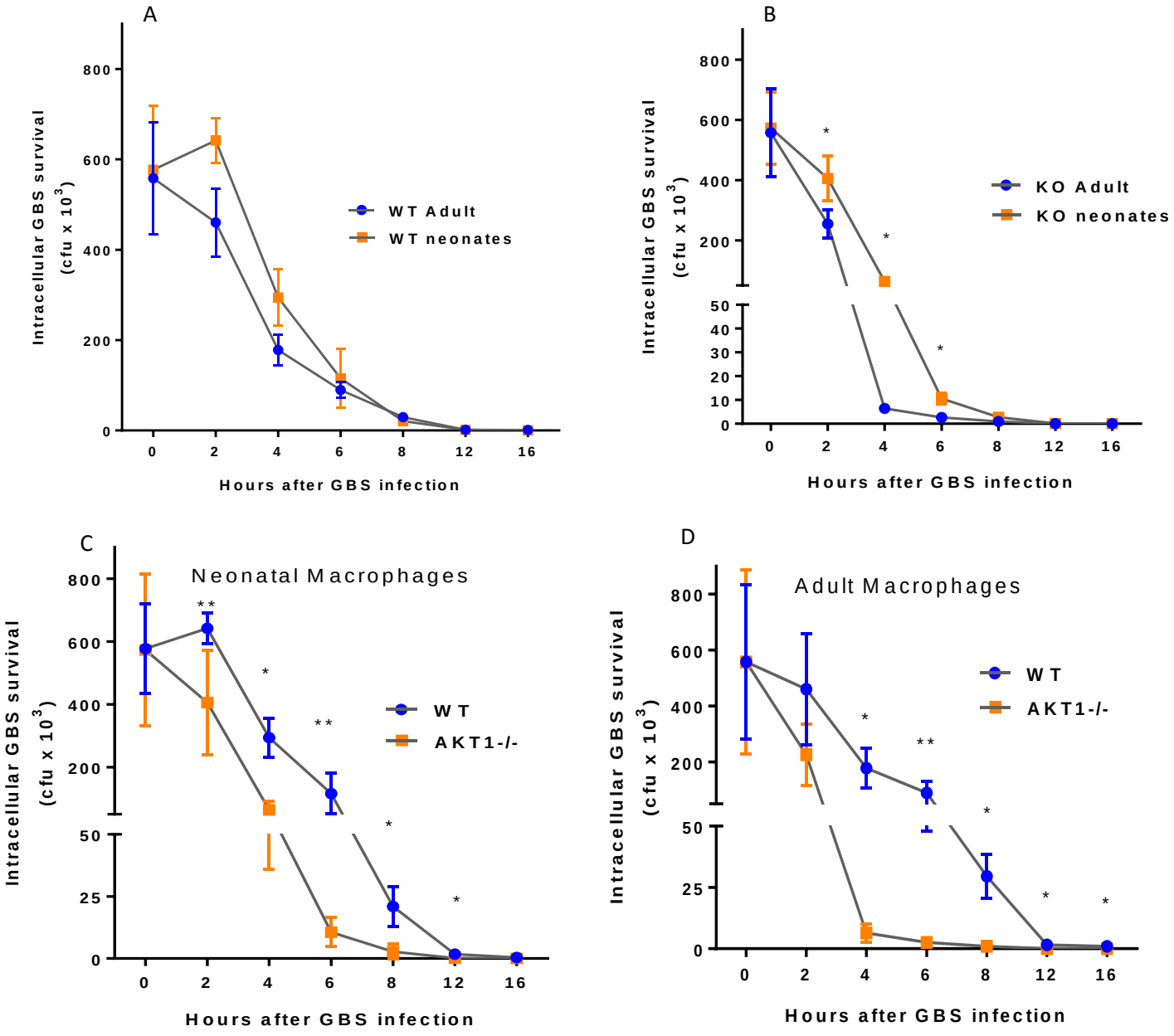


Figure 7

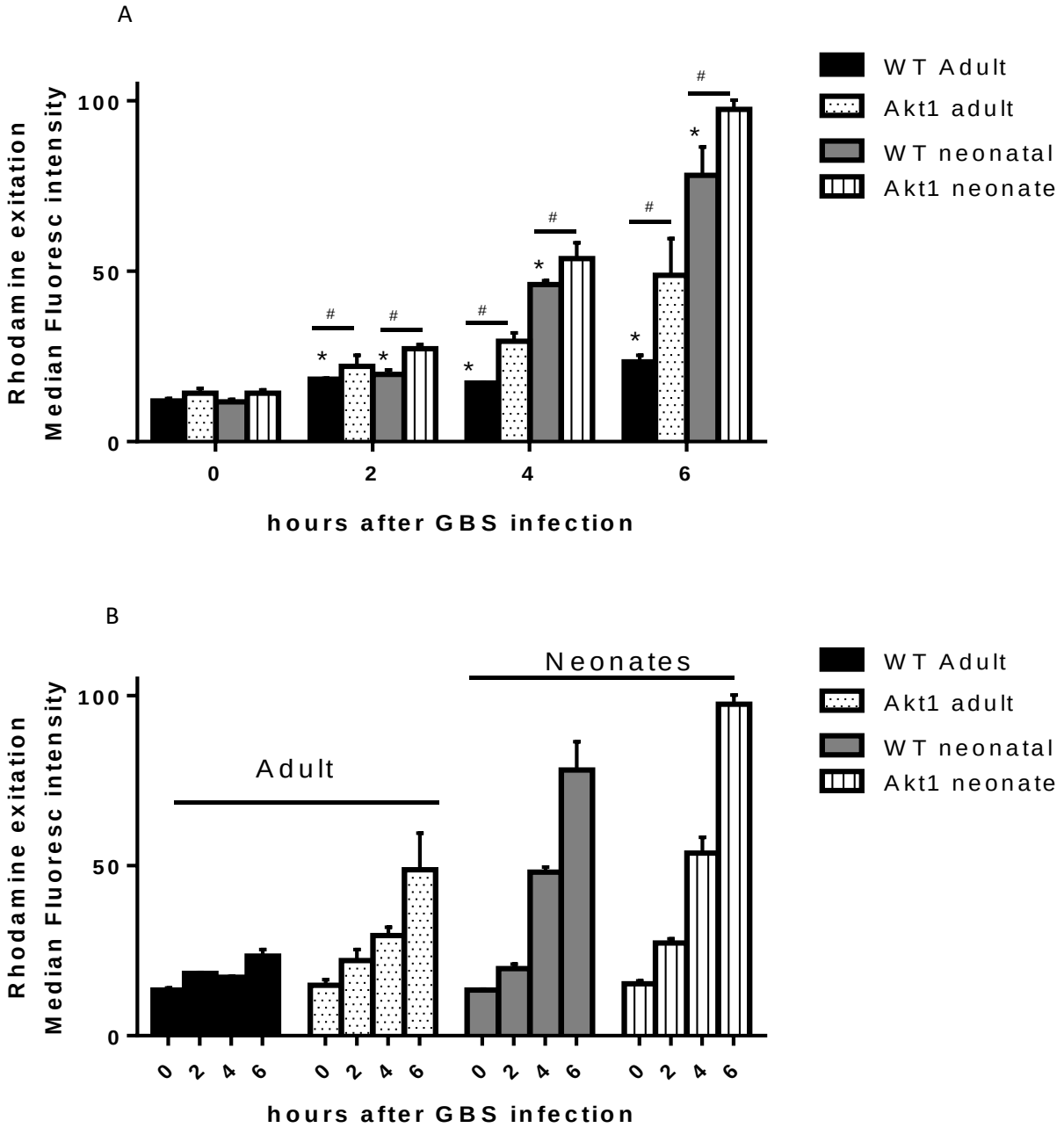
Lys-Cre Akt1$^{-/-}$ neonatal and adult macrophages have an increased capacity of GBS clearance compared to the corresponding wild type cells: Adult and neonatal murine macrophages were incubated with GBS bacteria (MOI 1:10) for 2 hours and at several time-points post infection (2-16hpi), cells were lysed and the intracellular bacterial load was estimated via plating and cfu counting. (A) Intracellular survival of GBS in Wild Type Adult and neonatal macrophages (B) Intracellular survival of GBS in LysCreAkt1$^{-/-}$ Adult and neonatal macrophages (C) Intracellular survival of GBS in Wild type and LysCreAkt1$^{-/-}$ Neonatal macrophages (D) Intracellular survival of GBS in Wild Type and LysCreAkt1$^{-/-}$ Adult macrophages. *, $P < 0.05$; **, $P < 0.05$; ***, $P < 0.005$

3.3 Akt1 deficient cells suffer from increased oxidative stress compared wt adult and neonatal macrophages upon GBS infection

After confirming the increased capacity of degradation and pathogen elimination that Akt $^{-/-}$ macrophages exhibit, next we sought to address whether these cells have an increased amount of Reactive Oxygen Species (ROS). Therefore, we performed the Dihydrorhodamine 123 assay. This substance is a non-fluorescent, free radical indicator that diffuses across membranes, where it oxidizes to rhodamine 123 that has a green fluorescence. Or the purpose of this experiment, neonatal and adult macrophages that either expressed Akt1 kinase or not were incubated with GBS bacteria At an MOI of 1:10 for 2 hours. Dihydrorhodamine 123 was added in each of our samples and at the time-points of 2, 4 and 6 hpi cells were fixed and sorted based on their fluorescence. Cells that received no GBS stimulus were used as negative controls. As presented in Diagrams **8.A** and **8.B**, we can see that ROS concentration, represented by the mean fluorescent intensity of Rhodamine, increases over time for all the macrophage populations tested and the peak of concentration is noticed at 6hpi. A really interesting fact is that although at basal conditions the amounts of ROS are the same for all cell types (10-15%), either adult or neonates, we can see that after 2 hours of stimulation with GBS, ROS levels have an almost 2 fold increase, which is further amplified in later time-points. At 2 hpi, we see that Akt1 cells have more ROS compared to their corresponding wild type cells and the greatest concentration of ROS among all cell types, have the LysCreAkt1$^{-/-}$ neonatal cells. At a span of 4 hpi the differences between the groups become more evident. Once again, the leading group in terms of ROS expression is Akt1 deficient neonatal macrophages which have a mean fluorescent intensity of 53,7%. Wt neonatal cells follow with a concentration of 46%, while adult Akt1 deficient and wt cells have the lowest concentrations of 29,5% and 17,15% respectively. Finally, at the timepoint of 6hpi, ROS concentrations are the highest for all samples. More specifically, for wt adult neonates we see a 5%increase compared to 4hpi timepoint, while Akt1 deficient adult cells have a 19% increase. For neonatal macrophages we observed that ROS concentration has a 32% increase in a span of 2 hours, while Akt1 deficient cells have a corresponding 42% increase. These data indicate that upon GBS challenge, Akt1 deficient cells display a dramatic increase in ROS generation and compared to wild type cells. Also, neonatal macrophages have extremely elevated levels of ROS compares to adult cells when challenged with GBS that tend to increase over time.

Another thing that we wanted to examine was the production of Nitrogen Monoxide (NO) by macrophages upon continuous challenge with GBS and how that is modified upon the absence of Akt1 kinase in adult mice. For that reason, adult wild type and LysCreAkt1$^{-/-}$

macrophages were continuously incubated with GBS bacteria at an MOI 1:10. Supernatants of each sample were collected and tested for their NO concentration. As presented in Diagram **8.C**, NO levels are low in basal conditions and they have a 2 fold change in the first 30 minutes and 1 hour of GBS infection. However there are no significant differences between the different macrophage types. The most interesting fact is that upon 2 and 3 hours after GBS challenge NO levels in Akt1 deficient macrophages have a 4 and 7 fold increase respectively compared to the basal levels. Another interesting thing is that in these later time-points wt and Akt1 deficient cells do have a statistically significant difference. After 2 hpi, NO levels of wt macrophages start to decrease from 3,5umol/L to 2,5umol/L. However that is not the case for LysCreAkt1$^{-/-}$ macrophages, which continue to rise from 4,5umol/L to 7umol/L. That data indicate that NO production is significantly increased in Akt1$^{-/-}$ cells compares to wt cells, when challenged with GBS.



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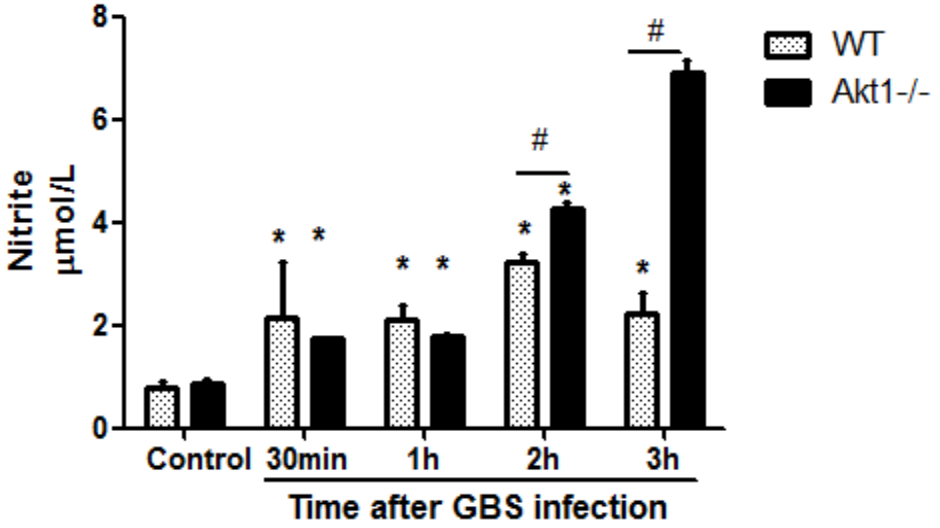

Figure 8:

Akt1 deficient cells suffer from increased oxidative stress compared wt adult and neonatal macrophages upon GBS infection: Adult and neonatal macrophages were infected with GBS particles (MOI 1:10) for 2 hours. (A) & (B) Adult wild type and LysCreAkt1$^{-/-}$ macrophages were exposed to dihydrorhodamine [(DHR) 0,2ug/mL] and challenged with GBS at an MOI 1:10 for 2 hours. The levels of Reactive Oxygen Species (ROS) at 2, 4 and 6 hpi were measured by performing the dihydrorhodamine (DHR) assay. The mean fluorescent intensity of samples was measured by FACS analysis. (C) Adult wild type and LysCreAkt1$^{-/-}$ macrophages where continuously challenged with GBS bacteria at an MOI 1:10 for 30 minutes, 1, 2 and 3 hours and the concentration of Nitrites (umol/mL) in cell supernatants, was estimated by NO measurement assay. *****is used to compare a column with the corresponding basal conditions, while **#** is used to compare the two different cell types. . */#, $P < 0.05$; **/##, $P < 0.005$; ***/###, $P < 0.0005$.

3.4 Akt1$^{-/-}$ macrophages display increased Nox2 and Atg5 expression while they have decreased levels of IL10 compared to wt cells upon GBS infection

After addressing ROS production in neonatal and adult cells treated with GBS we then sought to see the expression profile of these cells for a specific group of genes that participate in oxidative stress, autophagic machinery or to specific macrophage responses upon pathogen stimulus. For that reason, we treated cells with GBS bacteria (MOI 1:10) for 2 hours and we investigated the expression levels of the genes of interest at 1, 3 and 5 hpi. The genes we sought to study were Nox2, iNOS, IL12b, IL10, Atg5 and Atg7. More specifically, for IL10 (Diagram **9.A**), we can see that in the absence of bacterial stimulus the basal transcriptional levels of this gene are relatively low for all adult and neonatal cells, whether they express Akt1 kinase or not. However, when cells are stimulated with GBS for 1 hour, we notice that IL10 mRNA levels become immediately upregulated, especially in neonatal macrophages. In detail, the basal levels of IL10 in wt neonates are 60, 40 and after the 1 hour infection they increase to 7.655, having an 125-fold difference. At 3 hpi, the mRNA levels of IL10 have a further increase of 8-fold change at 56.532. At 5 hpi, IL10 transcriptional levels reach a pick of 248.798 (4.4-fold change). The picture is similar for

Akt1$^{-/-}$ macrophages, where basal levels are around 60,36 and after an hour of GBS stimulation the detected levels of IL10 are increased to 2.915. A rapid increase is noticed at 3hpi where IL10 levels reach 27.492having an almost 10-fold change. At 5hpi, IL10 levels reach a maximum of expression at 45.386. Comparing these two groups we can see that in each time point their IL10 expressions display a statistically significant difference ($p<0,05$), with wt macrophages having a far more abundant expression compared to their Akt1 deficient counterparts.

As far as adult macrophages are concerned, we see that under basal condition both cell types have a low expression of IL10 at around 36,6 and 38,8 for wt and Akt1$^{-/-}$ cells respectively. However, at 1hpi, IL10 levels in wt cells are rapidly increased to 1425 (39-fold change), while for Akt1$^{-/-}$ cells, this increase is significantly smaller at 474 (12-fold change). At the timepoint of 3hpi, we see that the corresponding levels of IL10 have risen at 2158 and 1133,7 respectively and the difference of expression between the two groups remains statistically significant. Finally, at 5hpi, IL10 levels decrease in both groups. More specifically, for wt adult cells we notice a decline from 2.158 to 1456 (1,5-fold change), while for Akt1$^{-/-}$ cells the corresponding levels of IL10 are 798 (1,4 fold-change). It is important to mention that the levels of IL10 have an increased statistical significance when wt groups are compared. Especially in late time points (5hpi) we notice that in wt neonatal cells, the levels of IL10 are around 248.798, while the corresponding levels in adult cells have decreased at 1456 ($P<0.0005$).

For Nox2, we see that the levels of expression increase gradually in all groups and have a pick of expression at 5hpi (Diagram **9.B**). More specifically, for neonatal cells we see that the basal levels of this gene in wt and Akt1$^{-/-}$ cells are relatively low and upon infection with GBS for 1 hour they increase by 2 and 3,2-folds respectively. At 3hpi, Nox2 expression levels experience a 5 fold and 5.7 fold change compared to basal conditions in wt an Akt1 deficient neonatal cells respectively, whereas at 5hpi the corresponding fold change is 5,9 and 4,3 respectively. It is important to mention that compared to their basal levels, the levels of Nox2 in all timepoints display a statistical significantly change. More specifically, for wt neonatal cells, we notice that at 1hpi and 3hpi the Nox2 levels display a statistically significant change compared to basal levels of this gene's expression ($p<0,005$), while at 5hpi this change has a lower significance $p<0,05$. In Akt1$^{-/-}$ neonatal cells, we see that at 1hpi and 3 hpi the corresponding difference has an increased significance ($p<0.0005$) and at 5hpi gene expression. These 2 cell groups do display important differences at time-points of 1hpi and 5 hpi. In adult cells the picture is quite different. Wt adult cells, during the 1st hour of GBS infection Nox2 levels exhibit a minor increase, which becomes more evident at 3hpi where we can see a 3,7-fold change compared to basal conditions. Nox2 production is further increased at 5pi where Nox2 levels exhibit a 7.2 fold change. On the other hand, LysCreAkt1$^{-/-}$ macrophages have a gradual increase of Nox2 levels at 1, 3 and 5 hpi that correspond to 2.3, 3.6 and 7.8 fold change respectively, compared to basal conditions. Both adult cell populations display statistically significant upregulation of Nox2 expression at during the later time-points (3 and 5hpi), while only Akt1$^{-/-}$ macrophages have such an acute increase at 1hpi.

For Atg5 we observe that in wild type neonatal cells the expression levels of this gene do not change during the early timepoints of 1hpi and 3 hpi, while at 5hpi they display a 2.9 fold change compared to the basal conditions (Diagram **9.C**). On the other hand, in LysCreAkt1$^{-/-}$ macrophages we see that at 3hpi, Atg5 levels have a 2.5 fold increase and at 5hpi expression of Atg5 is diminished at 1.38 fold difference compared to basal conditions. Moreover, adult cells have a pattern of expression similar to the neonatal cells. In detail, for wt adult macrophages we observed an increase of 1.8 fold change at 5hpi, while at earlier timepoints Atg5 expression does not change significantly. However, adult macrophages that lack the expression of Akt1, at 1hpi the mRNA levels of this gene have a 2 fold change increase, then at 3hpi they decrease by 0.5 fold change and at 5hpi they increase at 1.8 fold change compared to 0hpi timepoint.

Atg7 TNFa and IL12b were also tested for their expression levels at these specific time points but no significant differences were observed between the different groups (data not shown).

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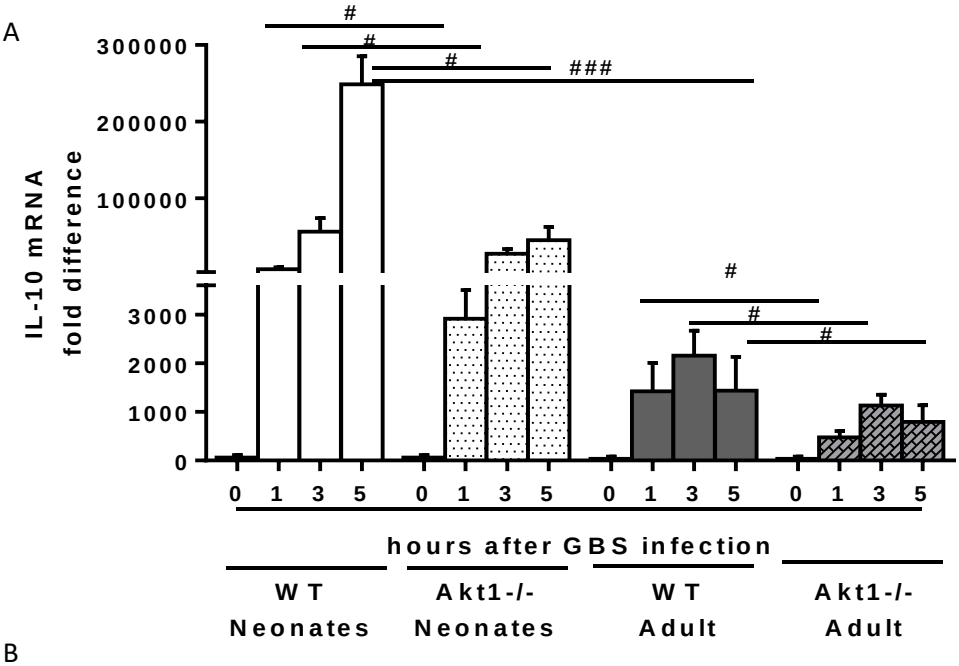

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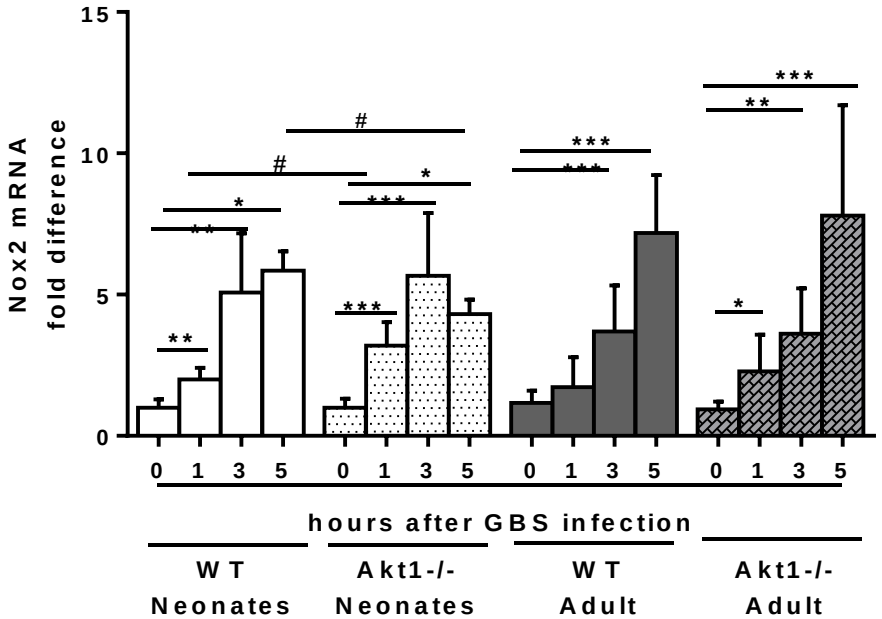

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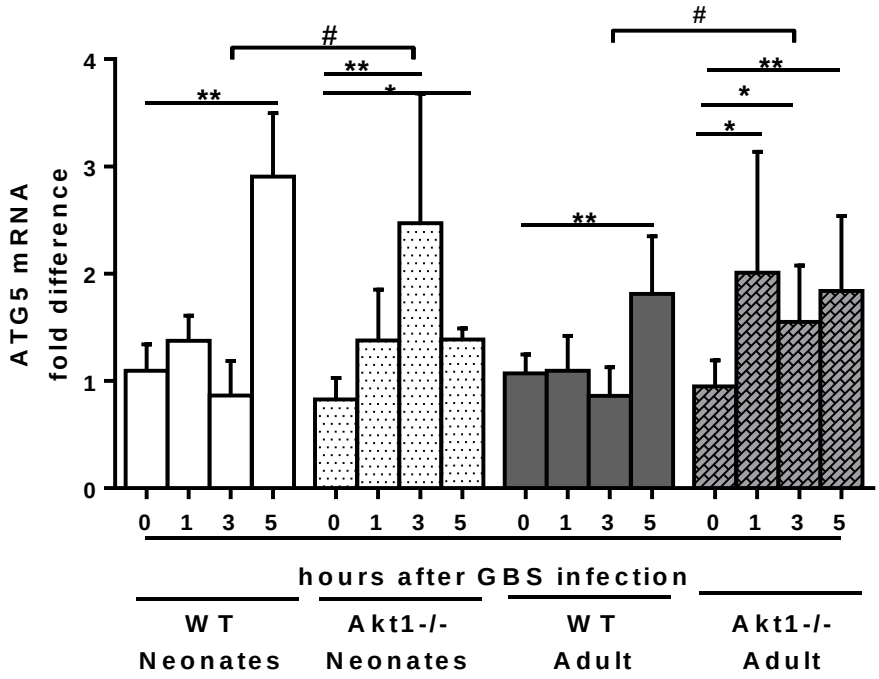

Figure 9:

Akt1$^{-/-}$ macrophages display increased Nox2 and Atg5 expression while they have decreased levels of IL10 compared to wt cells upon GBS infection: Adult and neonatal Wild Type and LysCreAkt1-/- macrophages were incubated with GBS particles for 2 hours. At 1, 3 and 5 hpi cells were collected, lysed and RNA was isolated. After cDNA synthesis the expression levels of genes were estimated by performing RT-qPCR. (A)The expression levels of IL10 in adult and neonatal wild type and LysCreAkt1$^{-/-}$ macrophages upon challenge with GBS (B) The expression levels of mNOX2 in adult and neonatal wild type and LysCreAkt1$^{-/-}$ macrophages upon challenge with GBS (C) The expression levels of Atg5 in adult and neonatal wild type and LysCreAkt1$^{-/-}$ macrophages upon challenge with GBS. ***** is used to compare a column with the corresponding basal conditions, while **#** is used to compare the two different cell types. . */#, P < 0.05; **/##, P < 0.005; ***/###, P < 0.0005.

3.5 Akt1$^{-/-}$ macrophages display more rapid increase in LC3II levels compared to wild type cells

Another thing we sought to address was whether the protein levels of LC3II change during GBS infection and whether this pattern is different in wild type and Akt1 deficient cells. For that reason adult macrophages were incubated with GBS bacteria at an MOI 1:10 and harvested at 1, 2 and 4 hpi. As shown in Figure **10.A**, in wild type cells LC3II levels start to increase at 2hpi and remain high at 4hpi, while in Akt1 deficient macrophages this increase is noticed at 1hpi and is maintained at 2 and 4 hpi. However, we noticed that upon treatment with Bafilomycin A1, LC3II levels appear to be even higher. This phenomenon is noticed in both cell types but is more prominent in Akt1-/- cells which may imply that the processes in which LC3II participat are more intense in these cells and thus their blockage increases the levels of LC3II so much.

Except of LC3II levels we sought to address the levels of total and activated Akt1 kinase in these macrophages. For that reason, cells were incubated with GBS at an MOI 1:10 for 30 minutes, 1 and 2 hours. Moreover, cells were also treated with Bafilomycin A1 that inhibits the fusion of autophagosomes with lysosomes. As shown in figure **10.B**, at the early timepoint of 30 minutes, wt cells have not any significant difference in terms of p-Akt1 expression compared to untreated wt cells. However, at 1 hpi levels of the phosphorylated form of Akt1 is increased, indicating that Akt1 activation takes place after 1 hour from the introduction of the triggering signal. An interesting fact is that the levels of total Akt1 seem to rise as early as 30 minutes after infection, increase more at 1hpi and remain steady at 2 and 3 hpi. However the phosphorylation increase seems to be abolished when cells are treated with Bafilomycin A1 that may imply a blockage of LC3 lipidation process when fusion of vesicles with lysosome is blocked.

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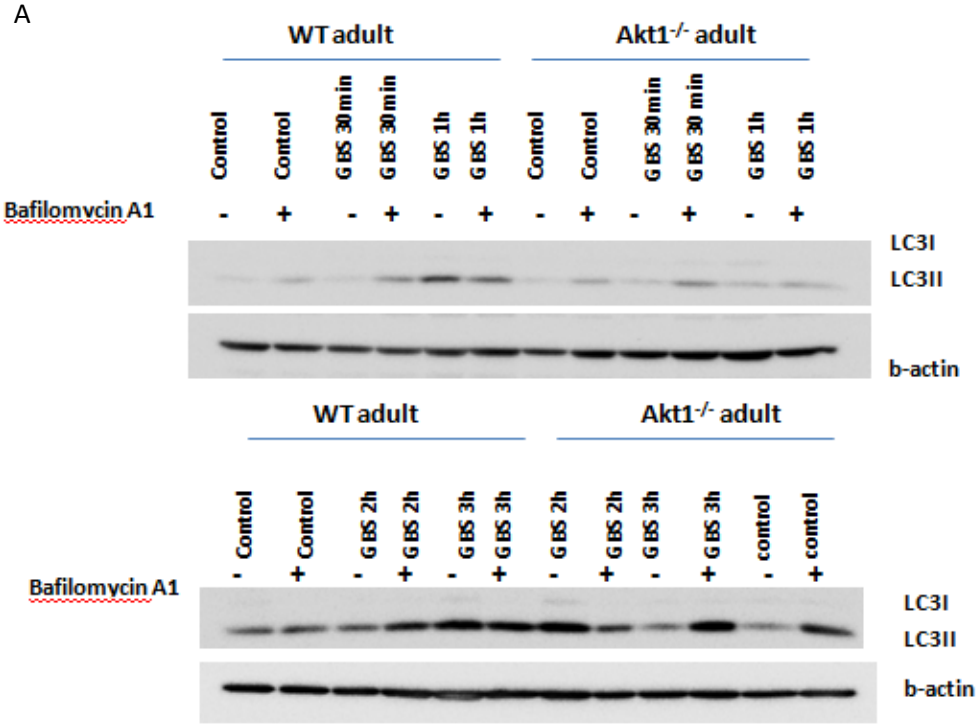

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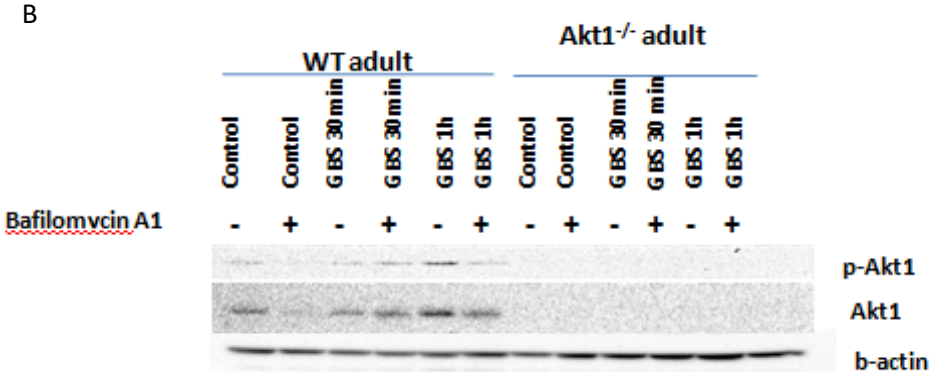

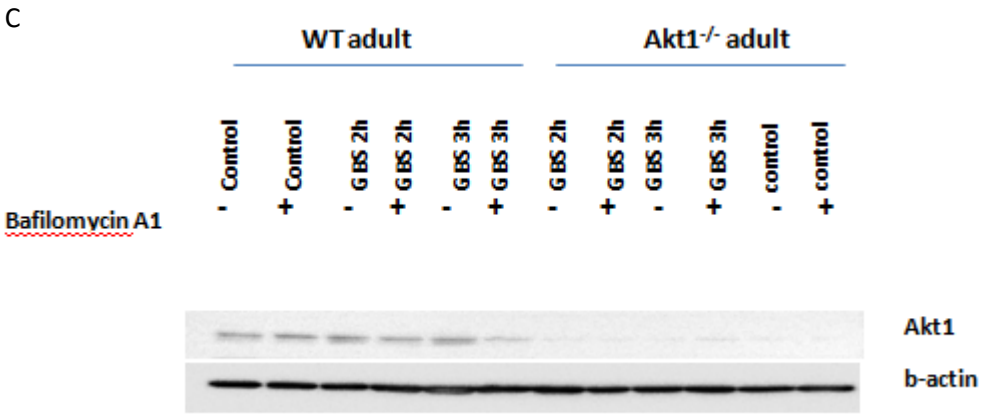

Figure 10:

Akt1$^{-/-}$ macrophages display more rapid increase in LC3II levels compared to wild type cells: Wild Type and LysCreAkt1$^{-/-}$ adult macrophages were treated with GBS bacteria at an MOI 1:10 for 2 hours. Cells were collected and lysed at the corresponding time-points after the initial infection for protein analysis. (A) The Levels of LC3II and actin in wt and LysCreAkt1$^{-/-}$ adult macrophages in the presence and absence of Bafilomycin A1 inhibitor (B) Akt1 and pAkt1 levels in wt and LysCreAkt1$^{-/-}$ adult macrophages at timepoints of 30 minutes and 1 hpi in the presence and absence of Bafilomycin A1 inhibitor (C) Akt1 levels in wt and LysCreAkt1$^{-/-}$ adult macrophages at time-points of 2 and 3 hpi in the presence and absence of Bafilomycin A1 inhibitor.

3.6 Inhibition of NO synthesis impairs Akt1$^{-/-}$ macrophage capacity to eliminate GBS

After confirming that ROS and especially NO levels of production are increased upon infection of wt and Akt1$^{-/-}$ macrophages with GBS and that the autophagic pathway may be implicated in the process of GBS killing, we sought to address whether inhibition of ROS production and/or autophagy has an impact on GBS clearance by wt or Akt1 adult macrophages. For that reason, we treated cells with different inhibitors and after infecting them with GBS bacteria at an MOI of 1:10. After 2 hours cells were lysed and the intracellular bacteria were estimated via the cfu counting method. The inhibitors that we used were DPI, which is a specific inhibitor of nitric oxide synthase, Apocynin, which is an inhibitor specific for NADPH Oxidase (NOX), Rapamycin, which is a specific mTOR inhibitor, 3MA which is a PI3K Class III inhibitor that impedes autophagosome formation and Chloroquine, which acts as a lysosomal pH regulator that delays lysosome acidification and therefore inhibits autophagosome-lysosome fusion and degradation. As presented in the following diagram (**11.A**) we can see that out of all the inhibitors the only one that had a negative impact in terms of bacterial killing was DPI. That is indicative of the fundamental role that iNOS and NO production play upon pathogen elimination process. In detail, what the diagram depicts is the fact that upon normal conditions, when cells are not treated with

any inhibitors, 2 hours after the initial infection with GBS, LysCreAkt1$^{-/-}$ macrophages display a lower intracellular bacterial load of 170*10^3 cfu compared to wt macrophages that have a respective load of 280*10^3 cfu. However, upon treatment with DPI, the respective bacterial loads change dramatically, especially for Akt1 deficient cells. In wt macrophages, DPI treatment causes a decrease of bacteria from 280.000 to 130.000 cfu, while in Akt1 deficient macrophages bacteria increase from 170.000 to 330.000 cfu.

A slight increase is also noticed when cells are treated with Apocynin, as in wt cells bacterial load slightly change from 280.000 to 260.000 but in LysCreAkt1$^{-/-}$ macrophages there is a rise from 170.000 to 240.000 cfu. However these changes do not have a statistical significance, but they are a good indication that NOX activity is also a prerequisite for pathogen elimination in LysCreAkt1$^{-/-}$ cells. As far as Rapamycin is concerned, we see that treatment of macrophages with this substance has minor effect on the intracellular bacteria. For wt cells we see that upon Rapamycin treatment bacterial load has a slight decline from 280.000 to 260.000 cfu, while the load of Akt1deficient macrophages does not display any changes. It is also evident that inhibition of PI3K III and autophagosome formation by 3MA treatment of macrophages has a similar effect with Apocynin as wild type macrophages display a change from 280.000 to 260.000, whereas LysCreAkt1$^{-/-}$ macrophages have an increase from 170.000 to 240.000 bacteria. Last but not least, Chloroquine treatment of macrophages leads to a decrease of bacterial load. More specifically, intracellular bacterial load is diminished from 280.000 to 210.000 and from 170.000 to 100.000 bacteria in wild type and LysCreAkt1$^{-/-}$ cells respectively. These results come in support of the notion that ROS production is important for triggering a quick response to pathogen elimination and that their absence impedes GBS elimination process.

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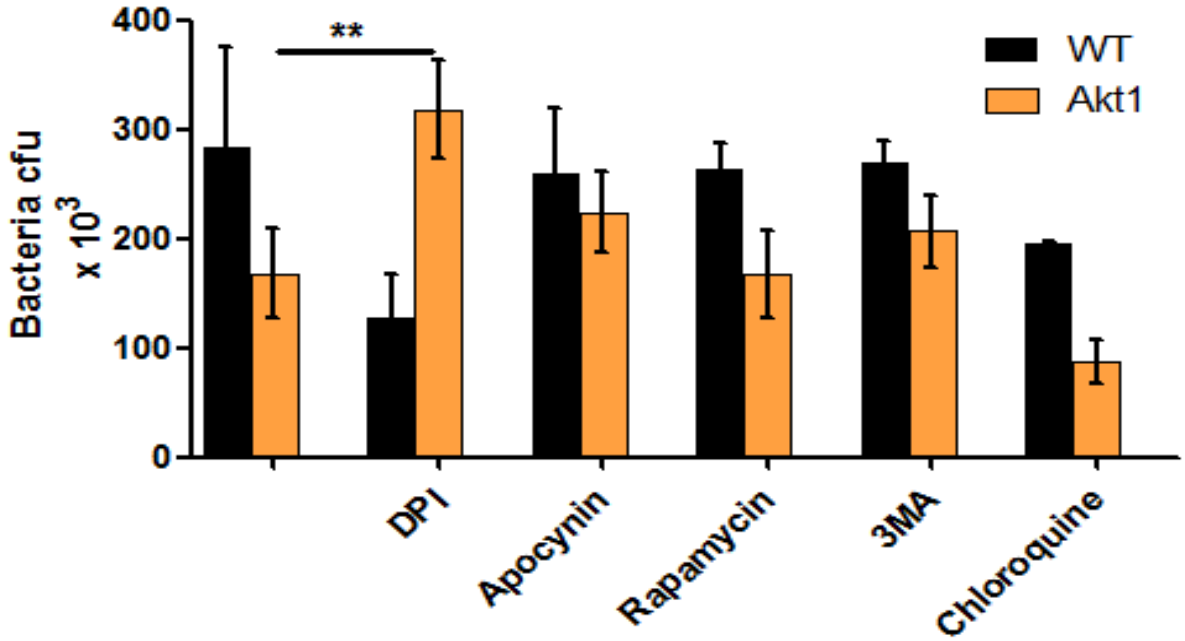


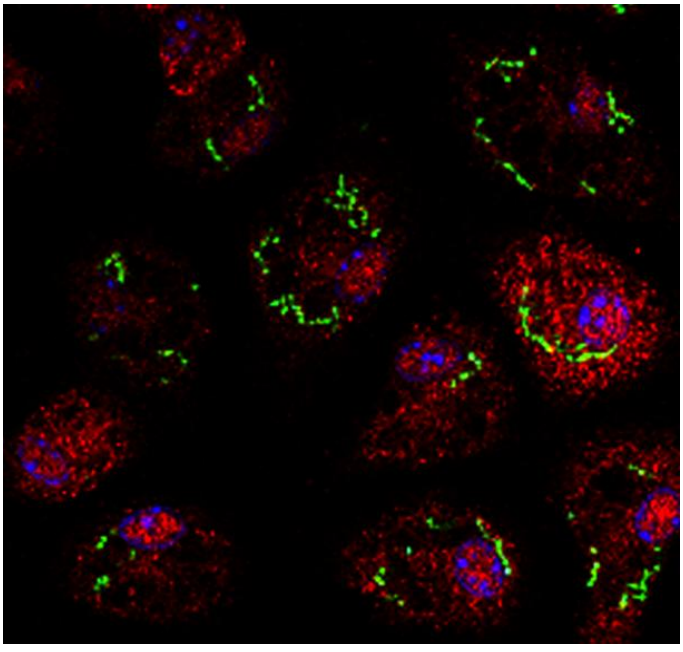
Figure 11:

Inhibition of NO synthesis impairs Akt1$^{-/-}$ macrophage capacity to eliminate GBS: (A) Adult and neonatal Wild Type and LysCreAkt1-/- macrophages were pre-treated with autophagy inhibitors DPI (20uM), Apocynin (50uM), Rapamycin(100nM), 3MA (5mM) and Chloroquine (10uM), 1h before adding GBS particles at an MOI 1:10. After 2 hours cells were lysed and lysates were diluted and plated. The intracellular bacterial load was calculated via cfu counting.

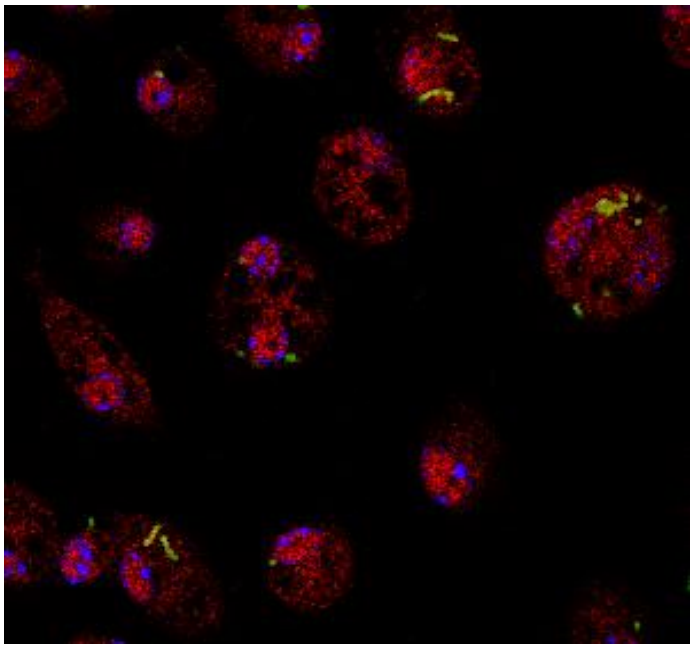
3.7 LC3 co-localization with GBS is more evident in LysCreAkt1$^{-/-}$ macrophages

After the observation that LC3II protein is increased upon GBS infection in wild type and LysCreAkt1$^{-/-}$ macrophages, we seized to address whether GBS co-localizes with the lipidated form of LC3 upon infection, which would be an evidence of autophagic pathway activation upon these conditions. For that reason, we performed confocal microscopy experiments, where we infected adult macrophages with heat inactivated and FITC labeled GBS bacteria at an MOI of 1:20 for 2 hours. Cells were fixed and stained for LC3II. TOPRO was used for nuclear staining. As we can see in the following pictures Fig.**12A** & **12B**, we observe that LC3II concentration is high in both cell types, which is mainly attributed to GBS infection. However, it is evident that in wild type adult macrophages the FITC signal is far more intense and many more GBS particles are found intracellularly. On the other hand, Akt1 depleted macrophages have significantly less GBS particles in their cytosolic area, which could in fact be attributed to their increased clearance capacity. Another interesting fact that can be seen in these pictures but also in Fig **12.C** is the fact that when colors are merged we can see that in LysCreAkt1$^{-/-}$ macrophages but not in wt cells, there is an intense co-localization of red and green color, which is interpreted as a yellow fluorescence of GBS bacteria. This could be translated as a similar pattern of LC3II and GBS distribution in the cytoplasmic area. Wild type cells have only a small number of yellow GBS particles while in Akt1 deficient cells the majority of GBS is co-localized with red LC3II. This co-localization may happen due to the formation of LC3II vacuoles that surround the pathogen, which is more pronounced in LysCreAkt1$^{-/-}$ macrophages.

A Wild Type macrophages



B LysCreAkt1$^{-/-}$ macrophages



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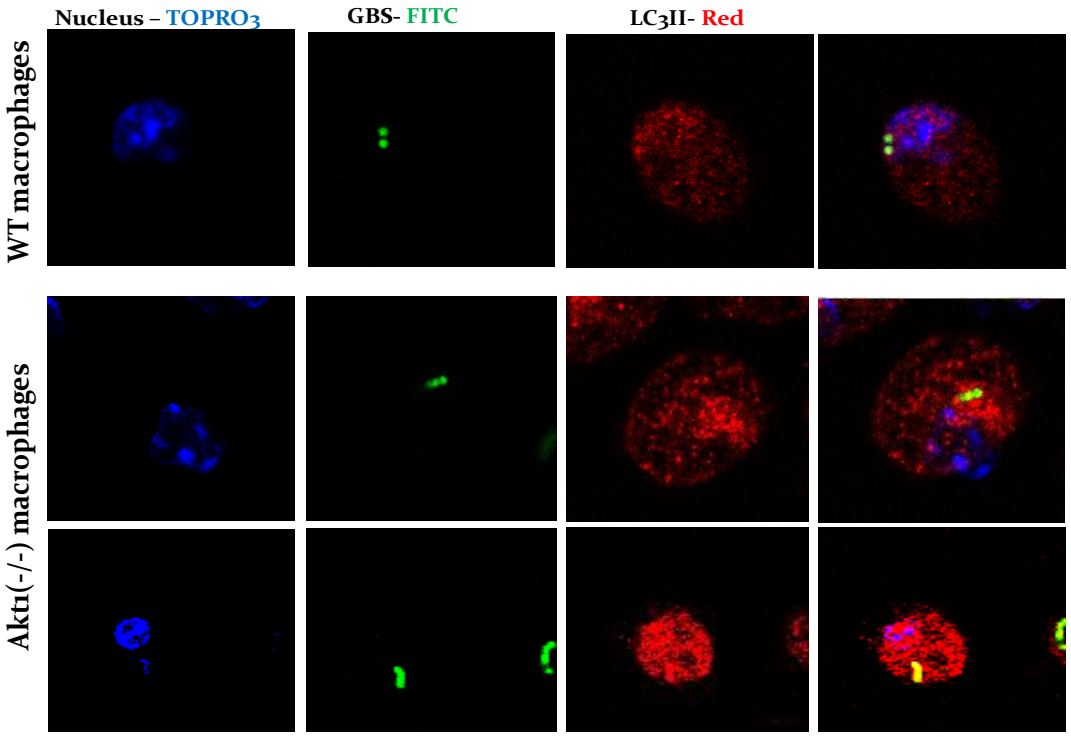

Figure 12:

LC3 co-localization with GBS is more evident in LysCreAkt1$^{-/-}$ macrophages: Adult LysCreAkt1$^{-/-}$ and WT macrophages were incubated with FITC labeled, heat inactivated GBS particles at an MOI of 1:10 for 2 hours. Cells were fixed with PFA 2% and stained red for LC3II (Rabbit Ant1-LC3 Antibody, NOVUS, 1/200 and Alexa Fluor 555 1/500. Nuclei were stained with TOPRO (1:1000) and coverslips were observed at Leica Confocal Microscope. (A)Confocal image of Wild Type adult macrophages after 1h incubation with GBS (FITC) (B) Confocal image of LysCreAkt1$^{-/-}$ adult macrophages after 1h incubation with GBS (FITC) (C) Images of nuclear staining, GBS staining and LC3II staining of Adult LysCreAkt1-/- and WT macrophages after 1 hour incubation with GBS.

3.8 LysCreAkt1-/- mice display a lower GBS bacterial load in their tissues compared to wild type animals

After confirming the benefit of Akt1 depletion in adult and neonatal macrophages upon GBS infection and showing that these macrophages have an increased ability of bacterial clearance compared to wild type macrophages, we decided to investigate whether this beneficial phenotype is also replicable in vivo. For that reason we administrated a standard proportion (5uL NS containing 50.000 bacteria) of GBS bacteria in both LysCreAkt1$^{-/-}$ and wt neonates intranasally, in order to replicate the means of neonatal infection and create a

model of GBS neonatal pneumonia. The aim of this experiment was to see whether neonates that lack Akt1 expression in their macrophages have a better response and lower bacterial load in their tissues compared to the wt neonates.

After 18 hours, neonates were sacrificed and certain tissues, specifically blood, lung and brain, were isolated. All tissues were homogenized, diluted and plated for bacterial cfu counting. This process leads to the results shown in diagram **13.A**, where each animal is represented as a dot. As we can see each group is characterized by a variation in values. However, we can see that the blood bacterial load is increased in some wt neonates while it remains relatively low for all Akt1$^{-/-}$ animals. In the case of wild type animals, blood has either an increased (200-300 cfu) or a decreased (<50 cfu) bacterial load. As far as brain tissues are concerned, we can see that there is no statistical significance between the two groups and the bacterial load is relatively low compare (<50) to the other tissues tested. Finally, lungs of LysCreAkt1$^{-/-}$ neonates do display a variation but they have relatively lower bacterial load compared to wt lungs. However, it is important to mention that this group of samples is characterized by great variation and therefore the picture is not clear about whether the bacteria that infiltrated lungs are increased in the case of wt animals. All in all these data indicate that LysCreAkt1$^{-/-}$ neonates have an increased ability of eliminating GBS and have an all in all lower bacterial load in their tissues but further experiment are needed for confirming this beneficial phenotype.

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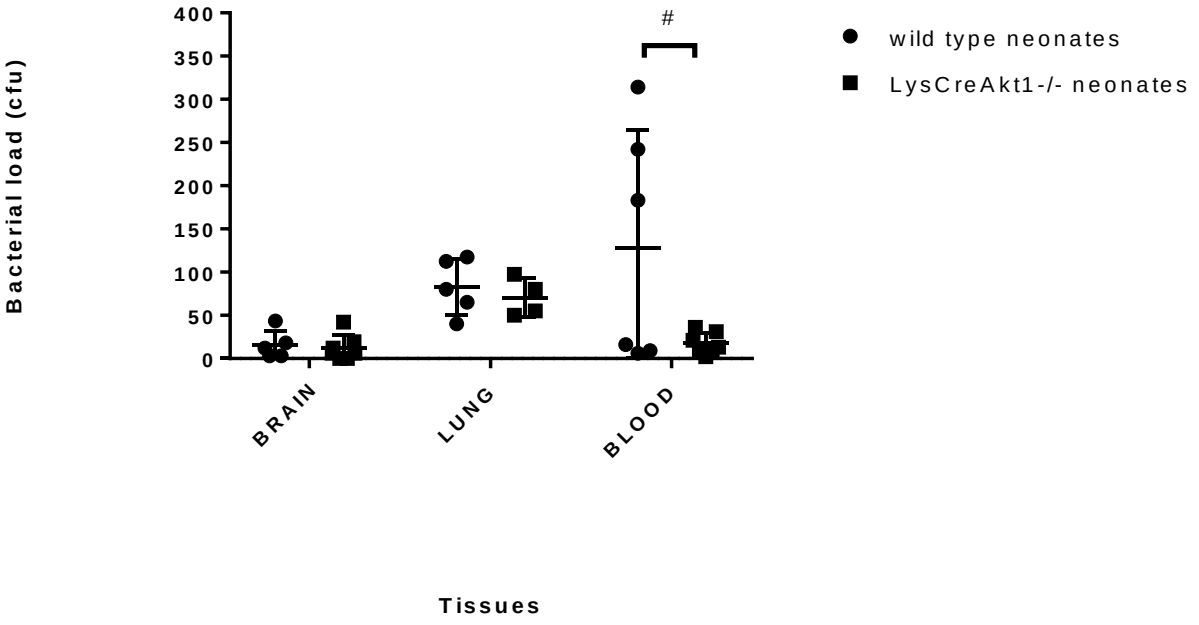


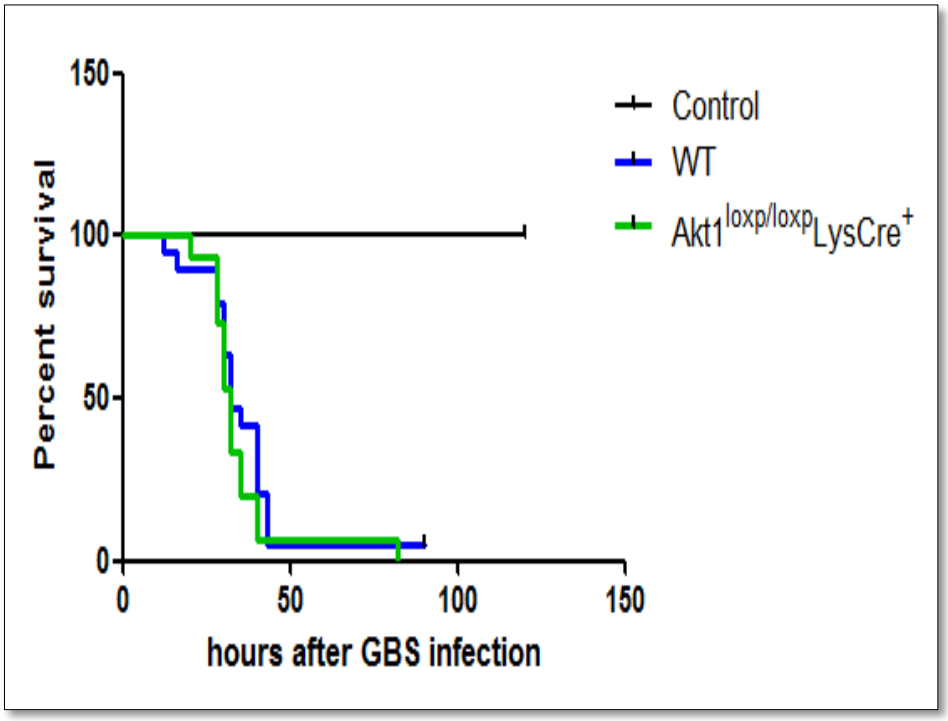
Figure 13

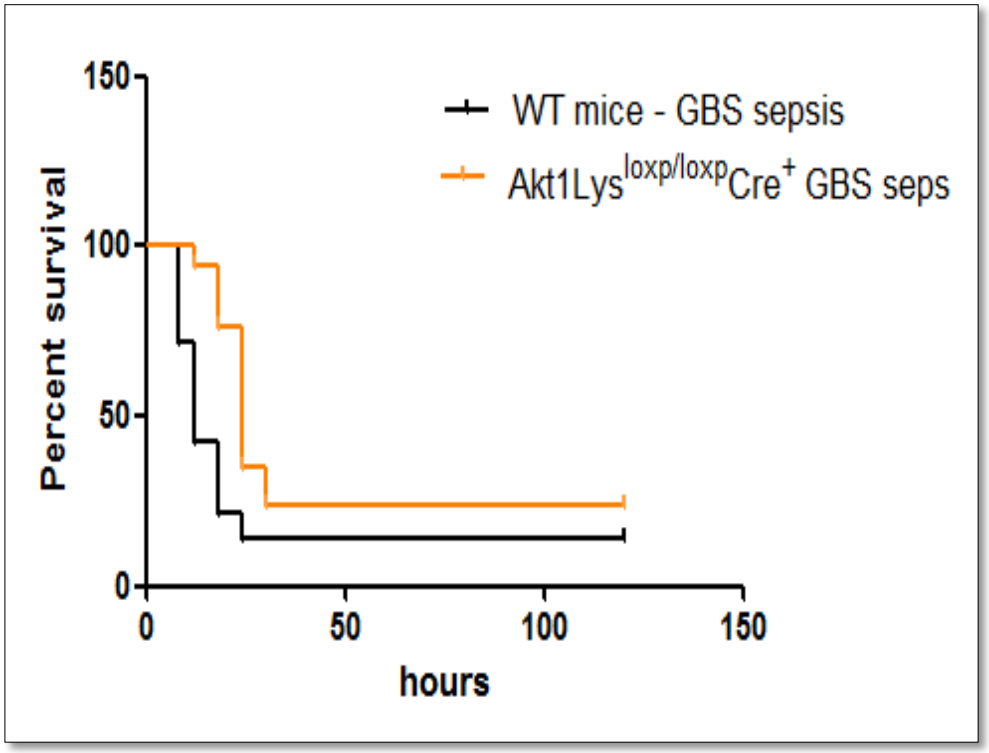
LysCreAkt1-/- mice display a lower GBS bacterial load in their tissues compared to wild type animals: Wild type and LysCreAkt1$^{-/-}$ murine neonates (<10d.o.) were intranasally challenged with 50.000 GBS particles and sacrificed after 18 hours. Blood, Brain and Lung tissues were removed

homogenized and plated for bacterial load estimation (cfu counting). (A) Bacterial load estimation in Brain, Lung and Blood tissues of murine neonates #, P < 0.05; ##, P < 0.005; ###, P < 0.0005.

3.9 Ablation of Akt1 kinase in macrophages does not affect mice survival upon GBS challenge

In order to address whether the beneficial phenotype of Akt1 depletion that is observed in vitro is also replicable in vivo and assists in a better survival of the corresponding mice, we performed survival assays in wt and Lys-Cre Akt1$^{-/-}$ neonate mice. For the purpose of this experiment, neonates were challenged with GBS that was injected intraperitoneally. Several doses of bacteria were used to monitor infant survival. Based on previous bibliography about GBS infections and survival assays performed in murine models we challenged neonates with a dose of 250.000 bacteria per animal (Diagram **14.A**) and we monitored their survival during the next days. As we can see in Fig.5a in such doses, both groups had a mortality rate of 95% and no differences in survival were observed. Assuming that this dose was immoderate for the pups, we decided to scale down the initial bacterial dose to 1/5 (100.000 bacteria per animal) in order to observe the desired survival difference. Though the mortality rate was significantly reduced to almost 80% (Diagram **14.B**), no statistically significant differences were observed between the two groups. Finally, a dose of 85.000 bacteria per animal was introduced to pups of the two groups. In spite of the fact that 30% of animals managed to survive, (70% mortality), no differences in survival rates of the two groups were noticed (Diagram **14.C**). Therefore, we concluded that pups with Akt1 deficiency in their macrophages do not display any benefit in terms of survival, compared to wild type animals, in higher or lower doses of GBS.





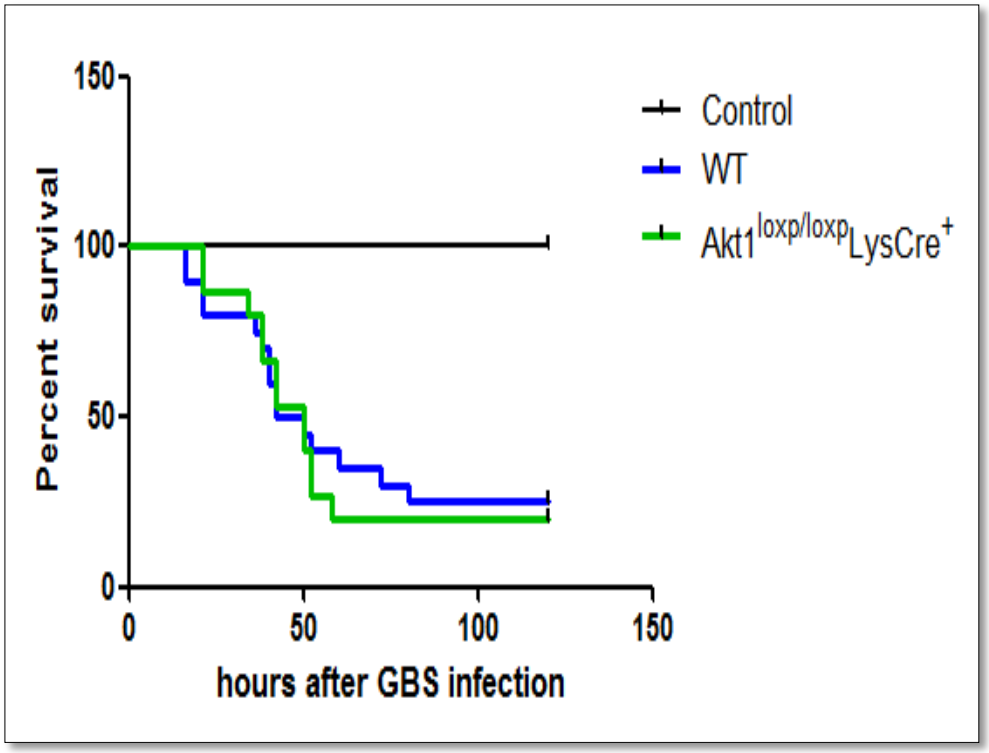


Figure 14:

Ablation of Akt1 kinase in macrophages does not affect mice survival upon GBS challenge: 3-7 days old wild type and LysCreAkt1$^{-/-}$ neonates were challenged with specific doses of GBS bacteria that

were administrate intraperitoneally. The mortality rate of neonates was documented every 3 hours. (A) Survival assay in murine neonates after the intraperitoneal administration of 250.000 GBS that resulted in 95% mortality, (B) Survival assay in murine neonates after the intraperitoneal administration of 100.000 BS bacteria that resulted in 80% mortality, (C) Survival assay in murine neonates with a dose of 85.000 GBS bacteria (70%mortality)

4. Discussion

Streptococcus agalactiae (GBS) is a beta hemolytic gram positive bacterium that asymptomatically colonizes human body. In spite of the fact that it is an important component of human bacterial flora and is found in almost 30% of adults it is considered as serious human pathogen that can cause a wide range of health complications.[8] High risk groups of developing such conditions are the elderly, immunosuppressed and other individuals that have underlying diseases, pregnant and post-partum women as well as neonates.[9] Especially in infants GBS has the capacity to cause serious complications, such as pneumonia, sepsis and meningitis, which are characterized by high morbidity and mortality rates. [15, 20]Multiple studies have indicated that GBS is the leading cause of neonatal meningitis worldwide.[20]

Innate immunity is a critical component of neonatal defense mechanisms against opportunistic infections, mainly because the adaptive immune system is neither mature, nor trained enough to successfully handle and eradicate them. Macrophages are professional phagocytes and antigen presenting cells that act both as sentinels to alarm the host organism and as “exterminators” of invading pathogens. When they recognise foreign stimuli, via the wide variety of receptors that they possess, they become activated and trigger downstream signalling pathways that initiate responses towards homeostasis restoration. Based on their activation state macrophages are categorised as classically activated M1 and alternatively activated M2 and Akt1 kinase is protein that has an important role in this process.

In the current study we tried to answer whether the absence of Akt1 kinase in adult and neonatal macrophages leads to more efficient responses and an overall better clearance of GBS, giving that way a survival benefit to neonates. Many studies have established the fact that Akt1 ablation leads to an M1 activation state that provides cells with the ability to better respond to bacterial infections.

In this context, we sought to investigate whether macrophages isolated from LysCreAkt1-/- adult and neonate mice have increased bactericidal capacity compared to wild type macrophages. We clearly demonstrated that macrophage phagocytosis ability is not modified in the presence or in the absence of Akt1 kinase, which means that all neonatal and all adult cells uptake similar amounts of bacteria, whether they express Akt1 or not. Based on that, we monitored the intracellular killing activity of macrophages upon challenge with GBS bacteria. Our results revealed that the absence of Akt1 expression is sufficient to confer increased capacity of intracellular clearance upon bacterial challenge. Neonatal cells do not clear bacteria as efficiently and rapidly as adult cells do. At any given timepoint

neonatal cells had more intracellular bacteria compared to their adult counterparts and this difference in bacterial load was more evident in early timepoints. A possible explanation for this could be the fact that neonatal macrophages are not as trained and alert as adult macrophages are and that is depicted in their responses against newly introduced stimuli, a notion that is supported by previous studies.[27]

Adult Akt1$^{-/-}$ macrophages are indeed much more efficient in eliminating the same load of intracellular GBS bacteria compared to wild type macrophages, which is attributed to their M1 like phenotype. [37] What is more, we can see that Akt1$^{-/-}$ neonatal cells also have a benefit in terms of GBS clearance compared to their wild type counterparts, since they manage to diminish bacterial load more rapidly. Though this difference between Akt1$^{-/-}$ and wild type cells is more prominent in the case of adult cells, it is also noticeable in neonatal cells. This means that even in early, neonatal stages, Akt1 ablation magnifies the antibacterial responses and mechanisms of the functionally immature macrophages, which assist in a more potent clearance of GBS. [37]

Treatment of Akt1-/- and wild type macrophages with specific inhibitors revealed which components are a prerequisite for GBS elimination. As mentioned above, we noticed that the inhibition of Nitric Oxide Synthase has a negative impact on Akt1$^{-/-}$ macrophage bactericidal activity. This is an indication that iNOS and NO play fundamental roles in pathogen elimination in these macrophages that display an M1 phenotype and that oxidative stress participates in the process of clearance.[21] Other inhibitors that had a quite similar pattern of effect were Apocynin and 3MA. Since, these compounds are blockades of NADPH Oxidase and PI3K Class III kinase respectively, their effect on Akt1$^{-/-}$ intracellular killing of GBS reveals that their targets may be indispensable components in clearance mechanisms that macrophages utilize to respond to opportunistic infection. Another important finding of this experiment is the fact that Rapamycin does not affect the elimination process of GBS. As an mTOR inhibitor this substance mainly impairs canonical autophagy but does not affect LAP. This result is an indication that may the latter but not the former pathway participates in GBS clearance process.

Previous studies have proven that M1 macrophages have increased oxidative stress compared to wild type cells, as they upregulate molecules that participate in production of ROS and RNS. In our study, we observed that neonatal cells suffered from increased levels of ROS production upon GBS infection. More specifically, at any given timepoint, LysCreAkt1$^{-/-}$ macrophages have increased ROS concentration compared to wild type neonatal macrophages and that is easily explained by their M1 profile. However we see that neonatal cells have all in all more elevated ROS levels than adult cells, which could be explained by their inability to successfully respond and eliminate GBS stimulus. As a result, neonatal cells are in a state of stress that leads to the eminent production of ROS that are also utilized in GBS elimination. Upon the presence of continuous GBS stimulus, Akt1 macrophages have produced great amounts of NO compared to wild type animals that can serve in elimination of extracellular bacteria. However, when the stimulus is not continuous we see that this NO overproduction is lost.

Expression analysis in wild type and Akt1$^{-/-}$ adult and neonatal macrophages demonstrated a really interesting finding; the fact that neonatal cells produce incredibly increased levels of IL10, unlike adult cells where IL10 production is relatively low. This fact is reported by other studies too.[27, 52, 89] GBS infection in neonates causes the upregulation of IL10 and in that way bacteria avoid destruction and subvert defence mechanisms. It was also revealed that Akt1$^{-/-}$ cells a show rapid and elevated increase in Nox2 and Atg5 levels. More specifically, we saw that when infected with GBS bacteria, Akt1$^{-/-}$ neonatal as well as adult cells display a quicker and higher elevation of these two factors compared to their wild type counterparts. This is an indication that NADPH oxidase and consequently ROS production is an indispensable part of GBS elimination process. Moreover, Atg5 upregulation in all cells shows the implication of an autophagic pathway in this process. This finding is in agreement with our western blot analysis data, which demonstrated that both wt and Akt1$^{-/-}$ macrophages increase their LC3II levels as a response to GBS infection. However, this phenomenon happens more rapidly in Akt1-/- macrophages. On the other hand, wild type macrophages display an gradual increase in Akt1 phosphorylation upon GBS infection, indicating that in these cells Akt1 activation is an important component for triggering pathogen elimination. Collectively, these data indicate that the pathway participating in GBS elimination process utilizes some component of autophagy and that it is rapidly activated in cells that lack Akt1 expression.

Our confocal microscopy data are a first indication that GBS bacteria are sequestered to the lysosomes via a pathway in which LC3II protein plays an important role. More specifically, we can see that in macrophages that lack Akt1 expression this mechanism is upregulated as less bacteria are found in the intracellular environment of these cells compared to wt macrophages at the same timepoint. That is indicative of the increased clearance capacity that Akt1 cells have compared to wt cells. Another interesting finding is that in these cells all streptococcal particles have characteristic yellow colours, which is due to the co-localization of the red fluorescence of LC3II and the green fluorescence of FITC-labelled GBS. This co-localization is a hint that Akt1$^{-/-}$ macrophages utilize an LC3II dependent mechanism for GBS elimination. On the other hand, in wild type cells LC3II and GBS co-localization is not that pronounce. Only a small number of the intracellular bacteria are yellow labelled. These data lead us towards the assumption that macrophages utilize either xenophagy or LAP pathway to achieve pathogen elimination and based on the fact that this mechanism is more pronounced in Akt1-/- cells that have increased ROS that mechanism may be LAP. Studies have revealed many pathogenic organisms that are targeted via these pathways.[81, 84, 86] However, the current study does not clarify the exact location of bacteria inside peritoneal macrophages, if they are free in the cytosolic compartment or inside specific vacuoles and whether these vacuoles have single or double membranes, indicative of xenophagy and LAP respectively. Therefore, in order to confirm our speculations further experiments, including Transmission Electron Microscopy have to be performed.

After noticing this difference in responses of wild type and Akt1$^{-/-}$ neonatal macrophages, we sought to see the effect of Akt kinase ablation in vivo. Specifically, we performed an intranasal administration of GBS bacteria in neonates of both genotypes and we estimated the bacterial load in lung, blood and brain tissues. Our data demonstrated that the blood bacterial load is higher in wild type neonates and importantly diminished in LysCreAkt1$^{-/-}$

neonates. However, this difference is not that obvious in lung and brains tissues. A possible explanation for that could be the fact that in the blood, circulating monocytes do not epress Akt1 kinase and their M1 bactericidal phenotype allows them to fight GBS infections in a more efficient way. On the contrary, in lungs, tissue resident macrophages are the first cells employed to fight against GBS. However, studies have shown that tissue resident macrophages and circulating monocytes differ a lot in terms of expression and thus alveolar macrophages may still express Akt kinase.[29, 30] Of course it is known that upon inflammation circulating monocytes infiltrate tissues in order to defend host organism against opportunistic bacteria, but it is possible that the exploitation of tissue resident macrophages in neonates that do not differ in the two groups may be the reason why we do not notice any significant differences in the number of GBS bacteria. As far as brain tissues are concerned, brain is a highly protected and difficult to penetrate tissue. The blood brain barrier impedes bacterial penetration and that may account for the relatively low bacterial load found in tissues of wild type and $Akt1^{-/-}$ neonates. Maybe a neonatal meningitis model could shed more light on whether LysCreAkt1-/- neonates have any GBS clearance benefit compared to their wild type counterparts.

Despite the clear benefit that Akt1 ablation offers to neonatal macrophages, our data revealed that upon challenge with GBS wild type and $LysCreAkt1^{-/-}$ neonates have the same mortality rates. This means that the depletion of Akt1 kinase in macrophages does not offer to neonates any benefit in terms of survival against GBS infections. Since many different doses of GBS bacteria were tested we concluded that the abolishment of Akt1-/- macrophage benefits in bacteria clearance is not due to the administration of high doses of GBS. Indeed, an overwhelming number of bacteria could lead to a saturation and dysfunction of clearance mechanism that macrophages utilize. Since that is not the case in our experiments, a possible explanation of this phenomenon could be the increased pro-inflammatory function of Akt1-/- macrophages. Upon challenge with bacterial stimuli, these cells produce immoderate amounts of interferons, ROS, TNFa, IL6 etc. [37]The overproduction of such factors could lead to the establishment of an uncontrollable, systemic state of inflammation that causes organ dysfunction that can prove lethal for neonates. To confirm whether this is the case, neonatal blood and serum have to be tested for the levels of these factors and whether they are increased in Akt1 mice.

Taken together, the data of the current study revealed that Akt1 macrophages handle GBS infections in a more efficient way compared to wild type cells. Akt1 ablation seems to be also beneficial for neonates as it makes macrophages exhibit an increased bactericidal activity, limits the disarming of responses that GBS causes and makes them more potent in fighting against opportunistic infections. The mechanisms by which GBS is eliminated are highly dependent on ROS production and pro-inflammatory cytokine upregulation. Our results also demonstrated that components of autophagic machinery are critical for GBS clearance process and that in Akt1 macrophages its initiation is rapid. Though our in vivo experiments indicated that this beneficial effect is not that evident in these processes, further experiments have to be performed to elucidate the phenomena that occur in vivo in wild type and $LysCreAkt1^{-/-}$ neonates after GBS infection.

As future perspectives, it will be essential to explore in detail the pathway utilized for GBS destruction and the exact components that participate in this process in adult as well as in neonatal macrophages. It is also important to clarify the mechanisms that make it GBS elimination more prominent upon Akt1 depletion and the pathways and the proteins that may have an increased activity under such conditions. Finally, we desire to shed light on whether these mechanisms are deficient in neonatal stages and the reason why this happens and based on that to address whether blockage of Akt1 kinase will offer any functional advantage to neonatal macrophages. The answers to these questions will address whether Akt1 kinase is a prominent target for the development of drug that will specifically inhibit this kinase and will manage to preven

5. Bibliography

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