UNIVERSITY OF CRETE
DEPARTMENT OF SCIENCES OF HEALTH
FUCULTY OF MEDICINE

The role of LC3-associated phagocytosis pathway in Group B Streptococcus (GBS) elimination in adult and neonatal macrophages

Ο ρόλος του μονοπατιού της LC3-associated phagocytosis στην καταπολέμηση του Στρεπτοκόκκου Ομάδας Β σε ενήλικα και νεογνικά μακροφάγα

Lapi Ioanna

ΛΑΠΙ ΙΩΑΝΝΑ

(1060158)

Laboratory of Clinical Chemistry

School of Medicine, University of Crete

Supervisors:

Tsatsanis Christos, Professor

Vergadi Eleni, Post-Doctoral Fellow

Kolliniati Ourania, PhD student

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ABSTRACT

Group B Streptococcus (GBS) or *Streptococcus agalactiae* is a Gram-positive commensal bacterium that colonizes the gastrointestinal and genitourinary tract. This unharmful microorganism is able to transit from being part of the normal flora to becoming a highly invasive pathogen, afflicting immunocompromised, elderly and pregnant adults as well as infants and neonates. Since 1970, GBS remains a leading cause of neonatal sepsis, pneumonia and meningitis, accompanied by high morbidity and mortality rates. Although the widespread screening of pregnant women for this pathogen and the subsequent antibiotic prophylaxis has reduced the risk of transmission to the baby, yet no vaccine has been developed to protect from all strains of GBS. Neonates possess a naive immune system that is constantly developing. In particular, the adaptive immune responses are inadequate due to limited exposures to antigens *in utero* in combination with the short-lived protection transplacentally transferred maternal antibodies provide. Therefore, the innate immunity plays a crucial role during the neonatal period. Macrophages, key components of the innate immune system, are required for controlling bacterial infections, including GBS. Canonical autophagy is a vital mechanism that engulfs and degrades a variety of intracellular cargos to maintain cellular homeostasis. However, this highly conserved mechanism has been also implicated in pathogen elimination. Over the past decade, another molecular pathway involved in microbial clearance has emerged, LC3-associated phagocytosis. Upon receptor engagement, LAP utilizes members of the canonical autophagy machinery to sequester cargos, such as intracellular bacterial pathogens, in single-membrane vesicles, delivering them to lysosomes for degradation.

Since newborns are considered more vulnerable to GBS infection, we sought to identify potential differences between adult and neonatal macrophages that account for the increased neonatal susceptibility to the disease. Hence, we investigated the molecular mechanisms utilized by neonatal and adult cells in response to GBS infection. First, we verified that neonatal macrophages have reduced bactericidal capacity compared to adult ones. Apart from eliminating GBS more efficiently, adult macrophages also presented enhanced LC3-II co-localization with GBS in contrast to the corresponding neonatal cells. The involvement of LC3-II led us to explore distinct components of canonical and non-canonical autophagy using bacterial survival assays and confocal microscopy. Our findings demonstrated that LC3-associated phagocytosis pathway is activated in adult macrophages to facilitate GBS clearance, but in neonatal cells is dysfunctional. We further explored factors, such as cytokines, that may have an impact on the antimicrobial activity of neonates. Interestingly, neonates were capable of producing pro-inflammatory cytokines, IL-6 and TNFα, as well as the anti-inflammatory cytokine IL-10, at higher levels compared to adults upon infection.

In conclusion, we identified a molecular pathway, LC3-associated phagocytosis (LAP), which is involved in GBS clearance in adult macrophages, but seems to be dysfunctional in neonatal ones. Neonatal macrophages were not deficient in their cytokine responses, but simply distinct from adult like immune responses. We observed that neonates produced higher levels of IL-6 and IL-10 compared to adults, which may contribute to their deficit in the LAP pathway and the subsequent reduced bactericidal capacity. Understanding the molecular mechanisms and the factors implicated in GBS elimination would assist in developing new therapeutics for this highly versatile and invasive pathogen.

ΠΕΡΙΛΗΨΗ

Ο Στρεπτόκοκκος ομάδας Β ή *Streptococcus agalactiae* πρόκειται για ένα Gram-θετικό συμβιωτικό βακτήριο της γαστρεντερικής και ουρογεννητικής οδού. Αυτός ο συνήθως αβλαβής μικροοργανισμός έχει την ικανότητα να μετατρέπεται από βακτήριο της φυσικής χλωρίδας σε άκρως επιθετικό παθογόνο, πλήττοντας ενήλικα άτομα με ανοσοανεπάρκεια, έγκυες γυναίκες, ηλικιωμένους, καθώς και νεογνά. Από το 1970, το συγκεκριμένο παθογόνο παραμένει κύρια αιτία νεογνικής σήψης, πνευμονίας, και μηνιγγίτιδας, συνοδευόμενο από υψηλά ποσοστά νοσηρότητας και θνησιμότητας. Παρά το γεγονός ότι ο εκτεταμένος έλεγχος των εγκύων σε συνδυασμό με την επικείμενη χορήγηση αντιβιοτικής θεραπείας έχουν μείωση τον κίνδυνο μετάδοσης του βακτηρίου στο νεογνό, δεν έχει αναπτυχθεί ακόμη εμβόλιο που να προστατεύει από όλα τα στελέχη. Τα νεογνά έχουν ένα αδύναμο ανοσοποιητικό σύστημα, το οποία αναπτύσσεται συνεχώς. Πιο συγκεκριμένα, οι αποκρίσεις της επίκτητης ανοσίας είναι ανεπαρκείς εξαιτίας της περιορισμένης έκθεσης σε αντιγόνα στη μήτρα και της βραχύχρονης προστασίας που παρέχουν τα μητρικά αντισώματα. Επομένως, η έμφυτη ανοσία έχει καθοριστικό ρόλο κατά τη νεογνική ηλικία. Τα μακροφάγα είναι κύτταρα της έμφυτης ανοσίας, τα οποία είναι ζωτικής σημασίας για την ανάπτυξη των μη-ειδικών μηχανισμών άμυνας, αλλά και για την αντιμετώπιση βακτηριακών μολύνσεων, συμπεριλαμβανομένου του στρεπτοκόκκου ομάδας Β. Η κανονική αυτοφαγία αποτελεί έναν απαραίτητο μηχανισμό που εγκολπώνει και καταστρέφει ποικιλία ενδοκυττάριων φορτίων, διατηρώντας τη κυτταρική ομοιοστασία. Ωστόσο, αυτός ο καλά συντηρημένος μηχανισμός συμμετέχει και στην καταπολέμηση παθογόνων. Την τελευταία δεκαετία ανακαλύφθηκε ένα καινούργιο μοριακό μονοπάτι που εμπλέκεται στην εκκαθάριση μικροβίων, το μονοπάτι της LC3-associated phagocytosis (LAP). Μετά από πρόσδεση στον υποδοχέα, το μονοπάτι της LAP χρησιμοποιεί πρωτεΐνες της κανονικής αυτοφαγίας για τη δέσμευση φορτίων, όπως για παράδειγμα ενδοκυττάριων βακτηριακών παθογόνων, σε κυστίδια μονής λιπιδιακής μεμβράνης, μεταφέροντας τα στα λυσοσώματα για αποικοδόμηση.

Επειδή λοιπόν τα νεογνά θεωρούνται πιο ευάλωτα σε λοιμώξεις από στρεπτοκόκκο ομάδας Β, προσπαθήσαμε μέσα από τη συγκεκριμένη πτυχιακή εργασία να ταυτοποιήσουμε πιθανές διαφορές μεταξύ ενήλικων και νεογνικών μακροφάγων στις οποίες θα μπορούσε να οφείλεται η αυξημένη ευαισθησία των νεογνών σε αυτό το παθογόνο. Συνεπώς, διερευνήσαμε τους μοριακούς μηχανισμούς που χρησιμοποιούνται από τα ενήλικα και τα νεογνικά κύτταρα έπειτα από μόλυνση με το βακτήριο. Αρχικά, επιβεβαιώσαμε ότι τα νεογνικά μακροφάγα έχουν μειωμένη βακτηριοκτόνο ικανότητα συγκριτικά με τα αντίστοιχα ενήλικα. Εκτός του ότι τα ενήλικα κύτταρα αντιμετώπισαν πιο αποτελεσματικά το βακτήριο, παρουσίασαν υψηλότερα ποσοστά συνεντοπισμού της πρωτεΐνης LC3 με το βακτήριο σε σύγκριση με τα νεογνικά. Η συμμετοχή της LC3 μας κατεύθυνε στην εξερεύνηση διακριτών συστατικών των μονοπατιών της κανονικής αυτοφαγίας και της LAP, χρησιμοποιώντας μεθόδους προσδιορισμού ενδοκυττάριου βακτηρίου και συνεστιακής μικροσκοπίας. Τα ευρήματά μας υποδεικνύουν την ενεργοποίηση του μονοπατιού της LAP στα ενήλικα μακροφάγα για την καταπολέμηση του στρεπτοκόκκου, ενώ στα νεογνικά κύτταρα ο μηχανισμός αυτός φαίνεται να μη λειτουργεί σωστά. Επιπροσθέτως, μελετήσαμε παράγοντες, όπως είναι οι κυτοκίνες, που πιθανώς να έχουν επίδραση στην αντιμικροβιακή δράση των νεογνικών μακροφάγων. Ενδιαφέρον παρουσιάζει ότι τα νεογνά είναι ικανά να παράγουν προ-φλεγμονώδεις κυτοκίνες, IL-6 και TNFα, καθώς και την αντι-φλεγμονώδη κυτοκίνη IL-10, σε υψηλότερα επίπεδα σε σύγκριση με τα ενήλικα.

Συμπερασματικά, στην παρούσα εργασία εντοπίσαμε ότι το μονοπάτι της LC3-associated phagocytosis ενεργοποιείται για την καταπολέμηση του στρεπτοκόκκου ομάδας Β στα ενήλικα μακροφάγα, ενώ στα νεογνικά κύτταρα είναι μη λειτουργικό. Τα νεογνικά μακροφάγα δεν είναι ανεπαρκή όσον αφορά της αποκρίσεις τους σε κυτοκίνες, απλά διαφέρουν συγκριτικά με τα ενήλικα. Παρατηρήσαμε ότι τα νεογνά παρήγαγαν πολύ υψηλά επίπεδα IL-6 και IL-10 σε σύγκριση με τα ενήλικα, γεγονός που μπορεί να σχετίζεται με τη μη σωστή λειτουργία του μονοπατιού της LAP και την συνακόλουθη μειωμένη βακτηριοκτόνο ικανότητα των νεογνών. Η κατανόηση των μοριακών μηχανισμών και παραγόντων που συμμετέχουν στην

καταπολέμηση του στρεπτοκόκκου ομάδας Β θα συμβάλλει στην ανάπτυξη νέων θεραπειών για αυτό το άκρως επιθετικό παθογόνο.

INTRODUCTION

Streptococcus agalactiae: a leading pathogen in neonatal age

History

The pathogen *Streptococcus agalactiae*, commonly known as Group B Streptococcus (GBS), was first recognized by Nocard and Mollereau in 1887 as a source of bovine mastitis that resulted in agalactia (from the Greek: a-, no; galactos, milk), namely lack of milk (Le Doare & Kampmann, 2014). In the 1930s, Lancefield group described GBS as a human pathogen, colonizing the vaginal tract of asymptomatic women (Lancefield & Hare, 1934). However, the pathogenicity of this organism was not discovered until 1938 when three reports of fatal post-partum infection were published (Fry, 1938). Thereafter, numerous neonatal infection cases led to its classification as the predominant organism causing bacteremia and meningitis in newborns and young infants less than three months old. Invasive GBS disease is responsible for high morbidity and mortality rates among the aforementioned groups as well as the pregnant women and the elderly. In addition, increased risk for invasive GBS infection have also the immunocompromised adults with an underlying disease, such as malignancy, diabetes mellitus or a pre-existing infection, like HIV. Considering the spleen's important role in neutralizing encapsulated organisms, adults with either a splenectomy or functional asplenia, such as those with sickle cell disease, have also heightened susceptibility to GBS disease (Hanna & Noor, 2020).

Pathogen characteristics

GBS is a gram-positive, catalase-negative organism with a tendency to form chains as reflected by the genus name *Streptococcus*. The term group B streptococcus is based on a specific cell wall carbohydrate antigen, which is common to all GBS strains. When cultivated on blood agar, GBS forms small colorless colonies that cause beta-hemolysis or complete hemolysis due to a toxin produced, known as beta-hemolysin, which elicits complete lysis of the hemoglobin in red blood cells (Hanna M & Noor A, 2020).

GBS can be categorized by using serotyping and multilocus sequence typing (MLST). Serotyping divides *S. agalactiae* into types Ia, Ib, II, III, IV, V, VI, VII, VIII and the newly described IX serotype, taking into account a surface capsular polysaccharide (CPS). According to a pan-European study, GBS serotypes Ia, III, and V together accounted for 88%, 96%, and 67% of strains isolated from neonates with early-onset disease, neonates with late-onset disease (two subgroups of invasive neonatal GBS disease), and vaginal-rectal swabs of colonized pregnant women who delivered healthy babies, respectively (Fabbrini et al., 2016). However, the dominant serotypes causing disease differ regionally (Raabe & Shane, 2019). MLST is based on the allelic profile of seven conserved genes in order to group GBS strains into sequence types (STs), which can be clustered into clonal complexes (CCs) (Korir et al., 2017).

Group B Streptococcal colonization in pregnant and nonpregnant adults

GBS can colonize the gastro-intestinal and genitourinary tract in up to one-third of healthy asymptomatic women, while GBS carriage rate rises in pregnancy, when around 1 in 5 pregnant women are colonized worldwide. The incidence of invasive disease due to GBS is also higher in pregnant women, estimated at 0.38 cases per 1000 pregnancies, with a case fatality rate (proportion of cases that die) of 0.2% (Hall et al., 2017). In regard to pregnant women colonized with this pathogen, GBS is able to be vertically (mother-to-child) transmitted to the newborn via inhalation of contaminated vaginal or amniotic fluid during parturition. This fluid inhalation provides GBS with direct access to the respiratory tract, where it can adhere to lung epithelial cells and establish an infection. Furthermore, GBS is also able to spread *in utero* through the placental membranes of the mother, causing membranes to rupture and subsequently premature delivery (Melin, 2011). GBS colonization and persistence in host tissues is facilitated by bacterial adhesins, including the fibrinogen-binding proteins (Fbs), the laminin-binding protein (Lmb), the group B streptococcal C5a peptidase (ScpB), the streptococcal fibronectin binding protein A (SfbA), the GBS immunogenic bacterial adhesin (BibA) and the hypervirulent adhesion (HvgA), while global virulence regulators play a crucial part in the transition to invasive infections (Shabayek & Spellerberg, 2018).

An increasing incidence of invasive disease has been also observed in nonpregnant adults; particularly from 3.6 cases/100,000 people in 1990 to 7.3 cases/100,000 people in 2007, based on surveillance in 10 U.S. states. Typical manifestations of GBS disease in adults include skin and/or soft-tissue infection, bacteremia without focus, pneumonia, and osteomyelitis, whereas serious clinical syndromes, such as meningitis, streptococcal toxic shock syndrome and endocarditis, are rare. In terms of nonpregnant adults, invasive GBS disease afflicts mainly adults over 65 years of age, African American, and adults with diabetes. Thus, a plausible explanation for the observed doubling in the incidence of adult GBS disease, is the increasing prevalence of adults with chronic medical conditions (Skoff et al., 2009).

Neonatal infection

Nowadays, GBS is considered the leading etiology of culture-confirmed neonatal bacterial infection in the United States accompanied with high mortality and morbidity. According to the World Health Organization (WHO), reported incidence of GBS disease varies geographically, but the vast majority of the disease burden lies in low-and-middle-income countries, with estimates around 3 cases per 1000 live births in some areas, excluding stillbirth. Invasive neonatal GBS disease may be divided into three subgroups based on the age of presentation. The early-onset disease defines as the onset of the infection in the first six days of life, but in most neonates (61% to 95%) symptoms begin within the first 24 hours. About 80% of early-onset GBS cases present with bacteremia, whereas pneumonia and meningitis are less likely presentations, accounting for 15% and 5% to 10%, respectively (Hanna & Noor, 2020). Late-onset disease, which refers to GBS infection from day 7 to day 89 of life, is attributed to horizontal acquisition of GBS. However, transmission of this pathogen has been infrequently ascribed to human milk among women with GBS mastitis (Arias-Camison, 2003). The most common manifestations of late-onset disease are bacteremia as well as meningitis, accounting for nearly 25% of cases (Melin, 2011). Late, late-onset GBS disease, also known as later-onset or very late-onset disease, defines as GBS infection in infants three months of age or older and has been associated with HIV infection or an immunodeficiency (Hanna & Noor, 2020). A recent meta-analysis of serotype prevalence showed that serotypes I to V account for 97% of invasive neonatal GBS disease, with serotype III accounting for nearly half (43%) of early-onset and 73% of late-onset disease (Madrid et al., 2017). Moreover, several studies indicate a

highly virulent clone of GBS serotype III, ST-17, as the main sequence type causing the most neonatal invasive infections and almost all cases of meningitis (Poyart et al., 2008). In general, neonatal invasive GBS disease is responsible for fatality rates ranging from 1 - 8.4% in full-term infants to 5 - 20% in preterm infants (Madrid et al., 2017).

Treatment/Management

In randomized and controlled clinical trials during the mid-1980, intrapartum administration of intravenous penicillin or ampicillin to GBS carriers protected their newborns from developing early-onset disease (Boyer et al., 1984). Since then, guidelines have been constantly updated with intrapartum parenteral antibiotic prophylaxis being the key intervention. Pregnant women, who had a positive maternal GBS culture in the weeks before delivery or possess maternal characteristics that increase the risk of early-onset GBS disease in their offspring, are subjected to this treatment. There are medical organizations in countries that agree with the culture-based United States approach (eg, France) and others (eg, United Kingdom) that prefer the risk-based identification of women suitable for intrapartum antibiotic prophylaxis (Madrid et al., 2017). The first-line therapy for intrapartum antibiotic prophylaxis is penicillin or ampicillin administration prior to delivery. Alternatively, cefazolin may be used for penicillin-allergic patients, while in case of patients with severe penicillin allergy, clindamycin is recommended for susceptible GBS isolates and vancomycin for resistant isolates. However, a large number of studies have reported GBS resistance to clindamycin, erythromycin, and fluoroquinolones as well as vancomycin, and thereby, these antibiotics should only be used when absolutely necessary (Raabe & Shane, 2019). Overall, intrapartum antibiotic prophylaxis has reduced the rate of early-onset GBS disease among infants born to GBS-colonized women by 86 to 89% (Raabe & Shane, 2019; Verani et al., 2010). Nevertheless, maternal intrapartum antibiotic prophylaxis has not changed the incidence of late-onset GBS disease, remaining at approximately 0.27 per 1000 live births (Hanna & Noor, 2020).

Vaccination of mothers against GBS during the third trimester is an alternative strategy under development; yet, no licensed vaccines are available. Although monovalent CPS-conjugate vaccines (predominantly using tetanus toxoid as the conjugate protein) were approved by Phase I and II of clinical trials, this type of vaccine is not sufficient to provide protection against different GBS serotypes that are found in invasive infections (Shabayek & Spellerberg, 2018). On the other hand, surface protein target vaccines, such as Rib and Alpha C, seem to have a broad distribution among GBS isolates. Multiple GBS vaccine candidates, including a trivalent protein-polysaccharide conjugate vaccine targeting serotypes Ia, Ib, and III, are currently in development (Heath, 2016). This trivalent GBS conjugate vaccine has been evaluated in Phases I and II trials for its safety and immunogenicity and there are considerations for a Phase III trial (Shabayek & Spellerberg, 2018).

Neonatal Immunity and Responses to Infection

The immune system: innate and adaptive immunity

The human body has been masterfully designed to control the resident colonizing microflora, but also to fight pathogens by acquiring a variety of defense mechanisms that in most cases protect against the development of invasive microbial diseases. These defense mechanisms include physical (eg, skin and mucosal lining), mechanical (ciliated cells from the respiratory tracts) and biochemical barriers (tears or saliva containing antimicrobial lysozyme) as well as two inducible immune defense systems, the innate and the adaptive immune systems (Albiger et al., 2007). Innate immunity is considered the first line of defense against

pathogens, while the adaptive immune responses are slower processes, mediated by T and B cells. These types of lymphocytes are able to respond to a great number of antigens due to the fact that express highly diverse antigen receptors, generated through DNA rearrangement (Akira & Takeda, 2004). The development of this intricate immune system initiates during the early embryonic stages and keeps evolving in a complicated step-wise manner in order to gain the ability to target unknown future pathogens, while remaining tolerant to self and maternal antigens as well as to some future commensal microbial organisms (Yu et al., 2018).

Neonatal immunity

Worldwide, infections such as sepsis, meningitis and pneumonia, are responsible for a large number of neonatal deaths (Hug et al., 2019). According to estimates by WHO and the Maternal and Child Epidemiology Estimation group, 14% of all neonatal deaths in 2017 were due to sepsis or meningitis. It is not surprising that neonates are highly susceptible to infections in comparison with the adults, taking into account the transition from a semi-allogeneic (half of the antigens being of paternal, and thus of foreign origin) sterile environment to a microbial-rich surrounding. Limited exposures of neonates to antigens *in utero*, in combination with the short-lived protection acquired through the transplacentally transferred maternal antibodies, result in a deficient adaptive immune response (Basha et al., 2014; Korir et al., 2017). Hence, the innate immunity, an inbuilt defense mechanism that does not require prior immunologic experience, plays a vital role in providing protection during the neonatal period.

Innate immunity in neonates: The role of macrophages

Given their inadequate adaptive immune system, newborns rely principally on the innate immune cells in order to respond to pathogens. At the end of the 19th century, Elie Metchnikoff was the first to introduce the term 'macrophage' that means "macro = big and phage = eater" (Moghaddam et al., 2018). Macrophages are large phagocytic mononuclear cells, with extremely plastic and heterogeneous phenotypes, found in all tissues (Atri, 2018). The origin of macrophages comes from the yolk sac and fetal liver, in which heterogenous long-lived tissue resident macrophages are generated and distributed in different tissue and organs. These include Kupffer cells in the liver, microglial cells in the central nervous system, and alveolar macrophages in the lung (Transl et al., 2020). However, macrophages development also continues in adult life; they derive from bone marrow stem cells in response to monocyte colony stimulating factor (M-CSF) to form monocytes, circulating in the blood. Upon inflammation, circulating monocytes are recruited in inflammatory tissues, where they differentiate into macrophages and perform their function (Gordon & Taylor, 2005). Macrophages are considered a very important cell type due to their necessary role both in innate and in adaptive immune responses. Being the first line of defense against pathogens and other noxious stimuli, such as cell debris, macrophages drive a protective inflammatory response. Upon activation by signalling cascades downstream of TLR and cytokine receptors, macrophages produce pro-inflammatory cytokines and chemokines. In addition to their anti-pathogenic role, they are also involved in the resolution of inflammation, prohibiting prolonged inflammatory responses that may be harmful (Vergadi et al., 2017).

Macrophage polarization

As previously mentioned, macrophages are able to acquire distinct phenotypes due to their plasticity, in other words they obtain different polarization states. A polarization state refers to a macrophage activation state at a given point in space and time. This concept of macrophage polarization is an attempt to cluster the different

immune responses of macrophages according to the activation stimuli in addition to surface markers, production of specific factors, and biological activities. The stimuli could be either intrinsic or extrinsic from the tissue environment, including cytokines, growth factors, fatty acids, prostaglandins and pathogen-derived molecules. All these diverse activation states are grouped into two major polarization programs, classically activated macrophages or M1, and alternatively activated macrophages or M2 (Murray, 2016). M1 macrophages are activated by Toll-like receptor (TLR) ligands or cytokines secreted by Th-1 lymphocytes, such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNFα). These cells promote Th1 polarization of CD4 T lymphocytes and kill ingested microbes through activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system and the subsequent generation of reactive oxygen species (ROS) (Moghaddam et al., 2018). The detrimental effects of a prolonged inflammatory response are inhibited by regulatory mechanisms driven by the anti-inflammatory function of M2 macrophages. Based on *in vitro* experiments, the M2 spectrum is much broader, consisting of four distinct subtypes, M2a, M2b, M2c and M2d, dependent on the nature of the signal. These M2-like subtypes, induced by Th2 responses, constitute anti-inflammatory macrophages. M2a macrophages, activated by IL-4, IL-13 and M-CSF, promote fibrosis and wound healing. M2b macrophages are stimulated by exposure to both immune complexes (ICs) and Toll-like receptor (TLR) ligands or IL-1 receptor agonists. This subtype is the only one that secrets pro-inflammatory cytokines, IL-1β, IL-6 and TNFα, but only low levels of IL-12 as well as high levels of anti-inflammatory IL-10, and thereby performing immune-regulatory functions. M2c term refers to macrophages, which are stimulated by IL-10, glucocorticoids and Transforming growth factor beta (TGF-β), and perform efferocytosis. Finally, M2d macrophages are induced by combined exposure to TLR with adenosine A2A receptor ligands, or by IL-6, and contribute to angiogenesis and tumour growth (Transl et al., 2020).

Studies using knock-out mice have identified a number of signature genes as markers for the discrimination between the M1-like and M2-like polarization states. However, these murine markers are of limited use in human studies due to important disparities between the mouse and the human immune system biology (Sly, 2015). For instance, *in vitro* studies mostly use monocytes as the source of macrophages in humans, whereas bone marrow-derived or peritoneal cavity macrophages are mainly used in rodents (Murray, 2016). M1-like polarized macrophages are best characterized by surface markers, such as CD80, CD86, major histocompatibility complex II (MHC-II) as well as the MHC class II transactivator (CIITA) and the inducible nitric oxide synthase (iNOS). Additionally, they are able to stimulate an acute inflammatory response with the production of pro-inflammatory cytokines, such as TNFα, IL-1α, IL-1β, IL-6, IL-12, IL-23 and cyclooxygenase-2 (COX-2), and reduced production of IL-10. M2a-like macrophages express high levels of CD206, CD36, CD163, MHC-II, decoy receptor IL-1 receptor II (IL-RII) in addition to interleukin-1 receptor antagonist (IL-1RA), Arginase-1 (Arg1), chitinase-like 3 (Ym1), Resistin-like beta (FIZZ1) and TGF-β. M2b-like macrophages are characterized by high CD86 and MHC-II expression, pro-inflammatory cytokine production as well as anti-inflammatory IL-10, but low IL-12 secretion. M2c macrophages express high levels of CD206, CD163, TLR1, TLR8 and the Mer receptor tyrosine kinase (MerTK), while M2d macrophages secret high levels of vascular endothelial growth factor (VEGF) and IL-10, but low IL-12 and TNFα (Roszer, 2015; Viola et al., 2019). Nevertheless, the M1/M2 polarization states have emerged from *in vitro* experiments, and therefore, may not fully describe macrophage polarization *in vivo*, in which the tissue microenvironment is more complex.

Innate immunity deficiencies in neonates

As already stated, GBS generally infect infants during the first 3 months of life. Since their immune system is not yet fully matured, newborns depend mainly on the innate immune cells, like neutrophils, monocytes,

macrophages and dendritic cells, to defend against invading pathogens (Simon et al., 2015). However, the function of these cells, involved in innate immunity, is weak at the beginning, and thereby, neonates are highly susceptible. Neutrophils, which are the first cells normally recruited to the site of infection, are reduced in number in neonates compared with the stored cells in adults (Korir et al., 2017). Neonatal neutrophils in the cord blood also show reduced levels of phagocytosis in the first 3 days after birth as well as poor ability to migrate into infection sites compared to adult ones in the peripheral blood (Filias et al., 2011; Korir et al., 2017). They also present weak bactericidal functions and poor responses to inflammatory stimuli (Nussbaum et al., 2013). Unlike neutrophils, monocytes in neonates, especially in preterm ones, are in greater number in contrast to adults. In addition, studies have shown that neonatal monocytes have a comparable phagocytic ability compared to adult ones, but reduced levels of major histocompatibility complex (MHC) class II expression on their surface, suggesting a diminished capacity for antigen presentation (Korir et al., 2017). In 2014, Valero and colleagues infected monocyte/macrophage (Mo/MΦ) cultures from neonatal, adult, and elderly subjects with four dengue virus types and defined the concentrations of three major pro-inflammatory cytokines, TNFα, IL-6, and IL-1β. The observed decreased IL-1β, IL-6, and TNFα production in neonatal Mo/MΦ was indicative of a diminished inflammatory response (Valero et al., 2014). Moreover, a study that compared the adult peripheral with the cord blood monocytes after GBS infection, found no difference in phagocytic uptake, bacterial degradation, and reactive oxygen species (ROS) production (Gille et al., 2009). Another study, which examined the differences between peritoneal neonatal and adult macrophages, showed a reduction in MHC class II, CD11b, CD14, CD80, CD86, TLR2, TLR4 and TLR9 expression levels in neonatal macrophages. The diminished expression of these genes suggests a poor ability in presenting antigens, with a corresponding reduction in the ability to induce T-cell proliferation (Winterberg et al., 2015). In regard to dendritic cells (DCs), cord blood ones are considered immature owing to a reduction in the expression of specific genes-markers, such as MHC classes I and II, ICAM-1/CD54, CD80 and CD86, relative to adults (Willems et al., 2009). Cord blood DCs have been also shown incapable of stimulating either adult or cord blood mononuclear or T cells, in contrast to adult DCs (Hunt et al., 1994). Interestingly, it has been also demonstrated that neonatal peripheral blood immune cells display distinct expression of inhibitory receptors compared to adult cells, which could play a role in the regulation of the neonatal immune system (Walk et al., 2012). Overall, the naïve neonatal immune system, which rely mostly on the innate immune cells, is partially deficient relative to the adult one, contributing to the heightened susceptibility to infection and perpetuated inflammation in term and preterm newborns.

Pathogen recognition: Toll-like receptors (TLRs) in Innate Immunity

TLR family

The innate immune cells possess pattern recognition receptors (PRRs), soluble or cell associated, which recognize evolutionarily conserved pathogen associated molecular patterns (PAMPs). This interaction evokes an immune response through the activation of different signalling pathways, triggering anti-microbial gene induction and pro-inflammatory cytokine production (Kumar & Bhat, 2016). Among PRRs, Toll-like receptors (TLRs) have been studied most extensively. These receptors function as sensors, recognising invasive pathogenic microorganisms through PAMPS that are present in microbial components (Akira & Takeda, 2004). The interaction of a PAMP with a TLR receptor, present at the cell surface or intracellularly, stimulates distinct adaptor molecules that trigger intracellular signalling pathways, resulting in pro-inflammatory and antimicrobial responses. Cytokines, chemokines, cell adhesion molecules, and immunoreceptors together act

as orchestrators of the early host response to infection, while simultaneously set the ground for the subsequent activation of antigen-specific acquired immunity (Mogensen, 2009). The toll receptor was initially discovered in Drosophila melanogaster for playing a role in embryonic development and later an additional function was showed on pathogen sensing and immunity in the fly (Fresno et al., 2011). Up to now, 13 members of the TLR family have been identified in mammals, 10 of them in humans and 12 in mice, with TLR-1–TLR-9 being conserved in both species (Dunston & Griffiths, 2010; Kawai & Akira, 2010). A TLR receptor, which is a type I transmembrane protein, consist of an ectodomain containing leucine-rich repeats (LRR) that mediate the recognition of PAMPs, a transmembrane domain, and an intracellular Toll–interleukin 1 (IL-1) receptor (TIR) domain required for downstream signal transduction. In addition to the cell surface TLRs, intracellular TLRs also exist, with a luminal ligand-binding LRR domain and a cytoplasmic signalling TIR domain (Kawai & Akira, 2010). TLRs and interleukin-1 receptor (IL-1R) are closely related, having a cytoplasmic TIR domain in common, and an extracellular LRR and three immunoglobulin (Ig)G-like domains, respectively (Akira & Takeda, 2004). TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are cell surface receptors, while TLR3, TLR7, TLR8 and TLR9 are expressed in intracellular compartments, such as endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes (Fresno et al., 2011). TLR-2 forms heterodimer complexes with TLR-1 and TLR-6. Studies have shown that the TLR2-TLR1 heterodimer recognizes triacylated lipopeptides from Gram-negative bacteria and mycoplasma, whereas the TLR2-TLR6 heterodimer recognizes diacylated lipopeptides from Gram-positive bacteria and mycoplasma. TLR-3, TLR-7, TLR-8, and TLR-9 are involved in the recognition of nucleic-acid-like structures. Specifically, TLR-3, TLR-7, TLR-8 recognizes single-stranded and double-stranded RNA, while TLR-9 recognizes motifs, such as hypomethylated CpG motifs, present in prokaryotic DNA. TLR-4 is mostly known for detecting the endotoxin lipopolysaccharide (LPS) from Gram-negative bacteria. However, this receptor recognizes a variety of ligands, such as mannan from yeast, host heat shock proteins, the plant-derived cytostatic drug paclitaxel, the fusion protein of respiratory syncytial virus (RSV), fibrinogen, envelope proteins from virus and the cytotoxin pneumolysin from *Streptococcus pneumoniae*. Finally, TLR-5 detects conserved domains on bacterial flagella, which are bacterial motor organelles responsible for chemotaxis, adhesion and invasion of host surfaces (Akira et al., 2006; Albiger et al., 2007).

TLR signalling pathways

Upon ligand recognition and binding, a number of distinct adaptor proteins are recruited, initiating signal transduction via two main pathways; the myeloid differentiation primary response gene 88 (MyD88)-dependent pathway, and the MyD88-independent pathway (**Fig.1**) (Fresno et al., 2011). The presence of a bacterial infection activates the MyD88-dependent pathway, which induces bactericidal compound production, such as reactive nitrogen species (RNS), nitric oxide (NO) via inducible nitric oxide synthase (iNOS) activation, and proinflammatory cytokine production (Dunston & Griffiths, 2010). All TLRs, except TLR-3, signal through MyD88, activating the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinases (MAPKs). In the alternative MyD88-independent pathway, TLR-3 and TLR-4 uses TIR domain-containing adaptor inducing interferons (TRIF), instead of MyD88, to activate the transcription factors Interferon regulatory factor 3 (IRF3) and NF-κB, inducing the production of type I interferon (IFN) and inflammatory cytokines. TRIF-related adaptor molecule (TRAM) and TIR domain-containing adaptor protein (TIRAP) are considered sorting adaptors; TRAM recruits TRIF to TLR-4, whereas TIRAP binds MyD88 to TLR-2 and TLR4 (Kawai & Akira, 2010).

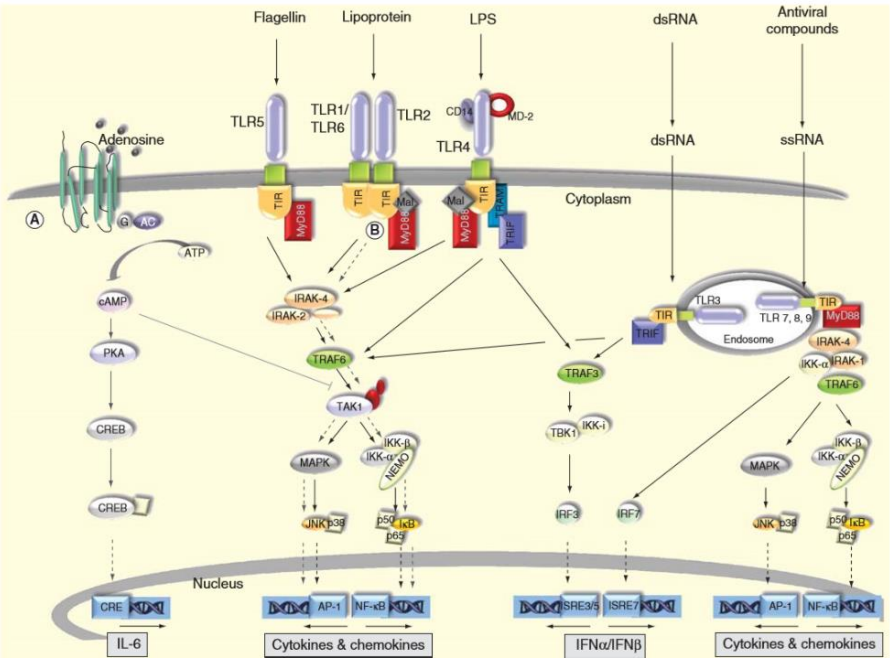

Figure 1. MyD88-dependent and TRIF-dependent TLR signalling pathways. MyD88, which is common to all TLR signalling pathways except TLR3, leads to the production of pro-inflammatory cytokines and chemokines. By contrast, TLR3- and TLR4-mediated TRIF-dependent signalling activates NF-kB and IRF3, resulting in the induction of pro-inflammatory genes and Type I IFN (Glaser & Speer, 2013).

GBS recognition via TLR signalling

TLR members, which are present in host innate immune cells, have a crucial role in recognizing foreign invaders, including GBS. Upon GBS phagocytosis, TLR-2 and TLR-6 interact with GBS cell wall components, such as extracellular lipoproteins. This interaction results in the activation of the MyD88-depedent signalling pathway and the subsequent production of pro-inflammatory cytokines (Korir et al., 2017). A recent study using human primary monocytes and monocyte-derived macrophages demonstrated that GBS is sensed through TLR-8, which activates IRF-5, leading to IFNβ and IL-12 production as well as TNF induction. As TLR-8 is a major endosomal sensor of degraded RNA of different bacterial species including streptococci, it is likely that GBS RNA is the ligand responsible for the TLR-8 activation (Ehrnström et al., 2017). Another study showed that live GBS stimulates the activation of a serine/threonine kinase, protein kinase D1 (PKD1), in macrophages. PKD1 activation by GBS, which is MyD88-dependent, seems to play an indispensable role in GBS-mediated induction of MAPKs and NF-κB, and pro-inflammatory gene expression (K. Upadhyay et al., 2017). In 2008, Charrel-Dennis M. and colleagues first descripted that GBS destroys the phagolysosomal membrane owing to its pore-forming toxins, releasing bacterial DNA into the cytosol of murine macrophages. GBS DNA is able to activate type I IFN production via TBK1-mediated phosphorylation of IRF3 (Charrel-dennis et al., 2008). After almost a decade, Andrade et al. showed that cyclic-di-AMP, produced by GBS in infected macrophages, induces type I IFN in a cGAS/STING-dependent and TLR-independent manner. However, GBS expresses a cell wall-anchored ectonucleotidase, CdnP, which hydrolyzes extracellular bacterial c-di-AMP, keeping c-di-AMP levels low and thereby IFN-β levels reduced (Andrade et al., 2017). Moreover, GBS single-stranded RNA (ssRNA) is recognized by monocytes and macrophages via a complex comprising MyD88 and UNC-93B, but this interaction occurs independently of known nucleotide-sensing TLRs. In general, TLR signalling contributes to GBS recognition, leading to the production of pro-inflammatory mediators in order to limit the early proliferation and spread of GBS.

Pathogen elimination: Canonical and Non-canonical Autophagy

Canonical autophagy/Xenophagy

Specialized immune cells, such as macrophages, utilize phagocytosis as a mechanism for the elimination of invading microbes. The reported phagocytotic uptake of *Bacillus anthracis* in 1884 paved the way for other pathogenic bacteria as well as fungi and parasites that were shown to be taken up by receptor-mediated phagocytosis (Schille et al., 2018). After cytoskeletal rearrangements, the phagocytosed microorganism is engulfed into a vesicle called phagosome and delivered into lysosomes (phagolysosome). In the resulting phagolysosome, acid hydrolases such as cathepsins, glycosidases, DNases or lipases accompanied by proton influx, destroy the pathogenic invader. In contrast to phagocytosis, macroautophagy has evolved as a survival pathway for ingestion of intracellular structures. Specifically, this pathway is one of the three subtypes of canonical autophagy (macro-, micro-, and chaperone-mediated autophagy); however, the term "autophagy" is mostly used for macroautophagy. Like the phagosome, the autophagosome is formed through the sequestration of the target structure into a double-membrane vesicle, and matures after the fusion with a lysosome (aytolysosomes), leading to the degradation of the cargo (Herb et al., 2019). Studies, using Saccharomyces cerevisiae as a model organism, have identified key autophagy-related genes (ATGs) that participate in the activation of the pathway and the subsequent engulfment and degradation of the intracellular cargos (Ohsumi, 2014). Upon nutrient deprivation or other stress signal, this pathway is triggered in order to degrade damaged or unnecessary components and recycle them into basic building blocks for future usage, maintaining cellular homeostasis (Martinez, 2018). Additionally, autophagy contributes to microbicidal immunity as cytosolic microbes and altered or damaged pathogen-containing vacuoles can be targeted. This process, which targets intracellular structures of nonself origin, is referred as xenophagy.

Non-canonical autophagy: LC3-associated phagocytosis (LAP)

LC3-associated phagocytosis (LAP), a newly discovered pathway, is considered a non-canonical autophagy pathway. Although this pathway share many ATG components with the canonical autophagy, LAP uses them in conjunction with distinct components for conjugation of the family of microtubule-associated proteins 1A/1B light chain 3 (MAP1LC3A, MAP1LC3B, MAP1LC3C, referred to collectively here as LC3) to single-membrane phagosomes (LAPosome) (Martinez, 2018). To date, the molecular mechanisms regarding LAP induction are not completely understood. However, a great number of cargos including pathogens, dying cells, soluble ligands and protein aggregates have been shown to mediate LC3 recruitment to LAPosomes. Cargo recognition is mediated by distinct surface receptors; pathogen recognition receptors such as TLRs (in particular, TLR1–TLR2 heterodimer, TLR2–TLR6 heterodimer, and TLR4), immunoglobulin (Ig) receptors recognizing opsonized foreign particles as well as receptors that drive the clearance of cell corpses, like T cell immunoglobulin mucin receptor 4 (TIM4) (Heckmann & Green, 2019). Moreover, recent work demonstrated that an intracellular TLR receptor, TLR-9, induces LC3 recruitment in a LAP-dependent manner after stimulation by CpG oligonucleotides (Hayashi et al., 2018) or DNA-containing immune complexes (Henault et al., 2013).

Although studies have shown that ligation of the aforementioned receptors engage LAP, it is still unclear how these ligation events lead to the recruitment of LAP components to the phagosome. Following activation and phagosome formation, the phosphotidylinositol 3-kinase complex (PI3KC3), composed of BECN1, VPS15, and

VPS34, is the first multi-protein complex recruited. VPS34 is the catalytic subunit of the complex, functioning by phosphorylating the inositol ring of the phosphotidylinositides (PtdIns) to produce phosphatidylinositol 3-phosphate (PI3P) (Heckmann & Green, 2019). The PI3K complex requires two additional subunits to be fully functional, UVRAG and Rubicon (Heckmann et al., 2018). Rubicon, a RUN-domain containing protein, has been characterized as a negative regulator of PI3P production and LC3 lipidation during canonical autophagy (Matsunaga et al., 2009; Zhong et al., 2009). By contrast, LAP depends on Rubicon due to its dual role. First, Rubicon stabilizes the UVRAG-PI3KC3 complex at the phagosome, facilitating the generation of PI3P by VPS34. Second, Rubicon is required for the assembly and function of NADPH oxidase 2 (NOX2) (Martinez, 2018). NOX2, which is the primary NADPH oxidase in phagocytic cells, consists of the membrane protein subunit, $p22^{phox}$, and the catalytic subunit $gp91^{phox}$ (refered also as NOX2), together with the regulatory subunits $p47^{phox}$, $p40^{phox}$, $p67^{phox}$ and the small GTPase Rac (Yang et al., 2012). NOX2 is essential for ROS production in the phagosomal lumen during phagosome maturation. In particular, a superoxide anion (O_2^-) is produced and rapidly dismutated to hydrogen peroxide (H_2O_2) in the phagosome lumen (Heckmann et al., 2018). NOX2 is indispensable for LAP in macrophages, but the reason why Nox2-derived ROS are so important for LAP is not completely understood. ROS produced in the phagosomal lumen can diffuse to the cytosol where they might activate cytosolic enzymes that are required for LAP (Heckmann & Green, 2019). Another function of ROS production is the oxidation of membrane lipids, but it is not clear whether this effect of ROS in required for LAP (Martinez et al., 2015). Both PI3P and ROS are critical for the recruitment and activation of ubiquitin-like conjugation systems, Atg7-Atg3 and Atg12-Atg5-Atg16, at the phagosome. In the cytosol, (pro-) LC3 is cleaved by the autophagy-related protein (Atg) 4 to LC3-I, revealing the glycine residue required for lipidation. Subsequently, the autophagic conjugation systems covalently link LC3-I to phosphatidylethanolamine (PE), generating LC3-II on the phagosomal surface (now termed LAPosome) (Schille et al., 2018). The conjugation of LC3-II on the LAPosome is required for fusion to the lysosomal, which is the last step in LAP, resulting in successful processing of the engulfed cargo. In summary, LAP can be delineated into three stages; (i) induction by surface proteins and phagosome cup formation, (ii) assembly of the multimeric Rubicon-UVRAG PI3KC3 on the phagosome for PI3P and ROS production, (iii) recruitment of the ubiquitin-like conjugation systems for processing and ligation of LC3 to the phagosome (LAPosome) (**Fig.2**).

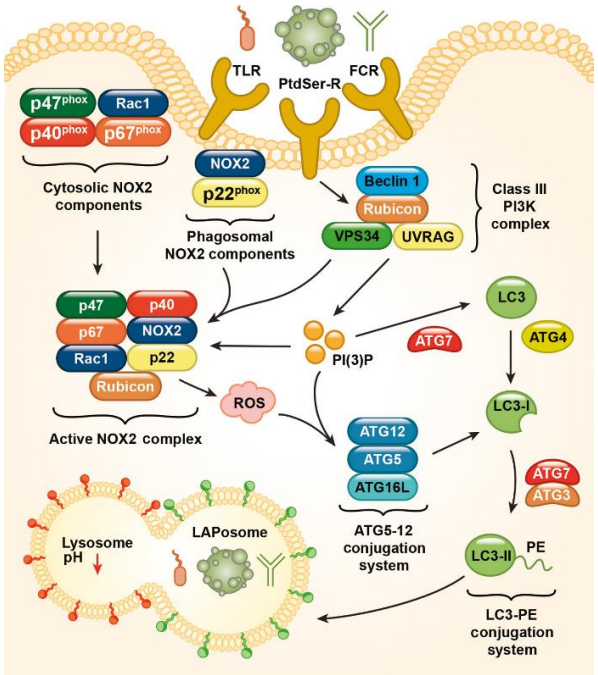

Figure 2. LC3-associated phagocytosis (LAP) pathway. Upon receptor engagement (TLR, PtdSer-R, or Fc receptors), components of the LAP pathway are recruited to the cargo-containing phagosome. The Class III PI3K complex assembles and associates with the vesicle and is responsible for the PI3P production. PI3P recruits the downstream conjugation systems (ATG 5-12 Conjugation System and LC3-PE Conjugation System), while stabilizes the NOX2 complex for the production of ROS. The active NOX2 complex is composed of the cytosolic NOX2 components ($p47^{phox}$, $p40^{phox}$, $p67^{phox}$, and Rac1) and the phagosomal NOX2 components (NOX2 and $p22^{phox}$) at the phagosome. Both ROS and PI3P are required for the subsequent lipidation and translocation of LC3-II to the single membrane of the phagosome (LAPosome), and LC3-II is required for fusion of the LAPosome with the lysosome (Wong et al., 2018).

LAP and anti-microbial immunity

Among other functions, LAP mainly promotes the fusion of lysosomes with phagosomes, facilitating phagosomal maturation (Romao & Münz, 2014). This is the reason why LAP is essential for anti-microbial immunity. By favoring phagosomal maturation, LAP enhances the degradation of live microbes, such as bacteria, fungi and parasites killing and subsequently controls the infection. Several studies have provided evidence regarding the role of LAP in restricting the growth of intracellular pathogens. For instance, Masud and colleagues showed that the LAP pathway is responsible for macrophage defense against *Salmonella typhimerium* during systemic infection using *in vivo* imaging of GFP-LC3 transgenic zebrafish embryos (Masud et al., 2019). Another pathogenic bacterium targeted by LAP is *Listeria monocytogenes*. In 2007, *L. monocytogenes* was shown to establish intracellular proliferating niches, known as SLAPs, in RAW264.7 macrophages by exploiting the LAP pathway (Lam et al., 2013). However, a recent work demonstrated that LAP, and not canonical autophagy, is required for controlling *L. monocytogenes* infection *in vivo* (Gluschko et al., 2018). In the case of *Mycobacterium tuberculosis*, survival within macrophages is achieved by avoiding its own delivery to the phagolysosomal compartment in order to replicate (Stanley & Cox, 2013). To prevent targeting and killing by LAP, *M. tuberculosis* possess a virulence factor, CpsA. The mechanism underlying the evasion of this antimicrobial pathway is prevention of recruitment of NOX2 to *M. tuberculosis*-containing phagosomes by CpsA, leading to impaired ROS production and, thereby, inhibiting the induction of LAP. In contrast, LAP can successfully eliminate the cpsA mutant in both macrophages and mice (Koster et al., 2017). Apart from bacteria, LAP has a crucial role also in immunity to fungal infections. For example, engulfed *Aspergillus fumigatus* conidia (spores of fungi) are targeted by the LAP pathway for degradation (Akoumianaki et al., 2016; Martinez et al., 2015; Sprenkeler et al., 2016). However, *A. fumigatus* cell wall component melanin, an important virulence factor, impedes LAP induction by selectively excluding $p22^{phox}$ subunit from the phagosome membrane. Consequently, melanin removal, either caused by genetic deficiency, chemical removal or swelling during germination, is required to expose the cell wall PAMPs, resulting in LAP activation and, subsequently, *A. fumigatus* killing (Akoumianaki et al., 2016). In addition, parasites have been also shown to be targeted by LAP (Herb et al., 2019). For example, internalization of *Leishmania major* promastigotes by macrophages promotes LC3 lipidation, and accordingly, activation of the LAP pathway. However, *L. major* interferes with macrophage antimicrobial activity by evading LAP. Mechanistically, *L. major* possesses a surface metalloprotease, known as GP63, which impedes NOX2 recruitment to phagosomes (Matte et al., 2016). In particular, GP63 directly cleaves VAMP8, a SNARE protein that regulates phagosomal assembly of NOX2 (Matheoud et al., 2013). In contrast to viable parasites, apoptotic-phosphatidylserine expressing promastigotes are degraded by LAP (Crauwels et al., 2015).

Elimination pathways and *Streptococcus agalactiae*

On the one hand, LAP is considered an important elimination pathway against various microbial pathogens, but on the other hand many pathogens have evolved diverse strategies to circumvent LAP (S. Upadhyay &

Philips, 2019). Several phagocytosed pathogens prevent or delay the phagosome maturation process, or even escape from the phagosome into the cytosol to replicate by lysing the membrane (Thi et al., 2012). In the case of GBS, multiple mechanisms are employed in order for GBS to stay concealed from immune cells or inhibit phagocytosis, and thereby, persist within the host (Korir et al., 2017). The major virulence factor of GBS is the secreted β-hemolysin/cytolysin toxin that is encoded by the cyl operon. This pore-forming toxin is expressed in more than 99% of GBS strains (Axel Ring et al., 2002). Unluckily, there are many unanswered questions regarding the molecular mechanisms involved in GBS elimination. In 2014, Cutting and colleagues demonstrated that the autophagy pathway is activated in human brain microvascular endothelial cells during GBS infection *in vitro*. However, GBS was detected within single-membrane structures using electron microscopy (Cutting et al., 2014). Another study sought to determine whether GBS regulates autophagy within cells in the female genital tract. According to this prospective cohort study conducted in GBS-positive and GBS-negative pregnant women, the concentration of p62, which is a cytoplasmic protein consumed during autophagy, as well as the autophagy inhibitor hsp70, were higher in vaginal epithelial cells obtained from GBS-positive compared to GBS-negative women, suggesting hsp70-induced autophagy inhibition (Scholl et al., 2016).

The aim of the study

Group B Streptococcus (GBS) can be the cause of highly invasive neonatal disease. On the contrary, immunocompetent adults are able to cope with this pathogen. The objective of the current study is to determine the mechanism utilized by neonatal and adult macrophages in response to GBS infection and identify potential differences that account for the increased susceptibility of neonates to the disease. Therefore, we investigated the molecular pathways implicated in GBS clearance and our results indicate the involvement of the LC3-associated phagocytosis (LAP) after exploring distinct components of this pathway. In addition, we further addressed the ability of neonatal macrophages to produce pro- and anti-inflammatory mediators compared to those produced by adult ones.

MATERIALS AND METHODS

Bacterial strain and growth conditions

For experimental infection, *Streptococcus agalactiae* (GBS) strain COH-1 was utilized in this study. COH-1 strain, a well-characterized human serotype III, was isolated from cases of human neonatal meningitis.

Bacteria were grown in Todd Hewitt Broth (THB) medium. The liquid culture medium was prepared by suspending 37 g THB and 5 g yeast extract in 1L distilled water on a slightly heated stirrer. The medium was then autoclaved at 121°C for 20 minutes (min), cooled down, and supplemented with antibiotics; 5 ug/mL Colistin-Sulphate and 0.5 ug/mL Oxalinic acid (Streptococcus Selective Supplement, Oxoid) was added under aseptic conditions (1:1 ethanol/sterile water). After antibiotic supplementation, the liquid THB medium was stored at 4°C. For the intracellular killing experiments, solid THB medium was used. The THB medium, supplemented with 15 g agar, was distributed in sterile Petri dishes in the presence of fire and stored at 8°C.

For the experiments, single bacterial colonies were dissolved in 50 ml-falcon containing 7 ml THB liquid medium. The bacterial culture was placed at 37°C for around an hour with shaking (270-280 turns/minute) in order for the bacteria to propagate. After the appropriate amount of time, when GBS reached mid-exponential growth phase, namely an optical density ($O.D_{600}$) ranging from 0.5 to 0.7, the bacterial proliferation was halted by placing the culture into ice.

Elicitation of primary macrophages

Thioglycollate was diluted in 4% w/v normal saline and heated for 1-2 min to facilitate dissolving. The solution was then autoclaved, aliquoted, and stored at 4 ^{o}C in a dark place (covered with aluminium foil) for at least a 1 to 2-month period before use.

For the purpose of this study, male and female mice of C57BL/6 background were used. In particular, adult and neonatal (3 to 4-day-old) mice were immobilized and cleaned with 70% ethanol on the abdominal area. Peritonitis was induced by intraperitoneal injection of thioglycolate solution, 1.5 ml for adults and 0.2 ml for neonates. Four days after the thioglycollate injection, macrophages concentrated in the peritoneal cavity were harvested by performing peritoneal lavage.

Isolation and culture of primary macrophages

Thioglycollate-injected mice were sacrificed on the 4th day after the initial injection. The skin of the abdominal area was carefully removed, leaving the peritoneal membrane intact and conspicuous. In contrast to adult mice, newborns were anesthetized using a gas anesthetic, sevoflurane.

An injection of 8-10 ml of Low glucose 1 g/L Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (P/S) was administrated intraperitoneally in adult mice, whereas neonates were injected with 4-5 ml of the same cell medium. After the injection, the abdomen was gently palpated in order the fluid to be equally distributed. The syringe was then disconnected from the needle, and subsequently, the gushing cell containing fluid was collected in a 50 ml-falcon. This procedure was repeated twice. The peritoneal exudate was centrifugated 5 min at 1000 rpm (revolutions per minute) and the cell pellet was resuspended in fresh culture medium. The resulting thioglycolate-elicited peritoneal macrophage (TGEM) cell suspension was seeded appropriately for experimentation.

Intracellular killing assay in wild type adult and neonatal TGEMs

The isolated TGEMs obtained from adult and neonatal mice were plated in 96 well plates at a density of 80000-100000 cells/well. Pentuiplicate/sextuplicate wells were seeded for each condition. The day of the

experiment, GBS was cultured as previously described until reaching the desired OD. According to the estimated concentration, the appropriate number of bacteria was centrifuged at 5000 rpm for 5 min and resuspended in Low glucose 1 g/L DMEM with 10% FBS. TGEMs were washed carefully three times with Dulbecco's phosphate-buffered saline (DPBS) and infected with the medium containing GBS at an MOI (multiplicity of infection) of 10 (100 ul/well). Two hours later, GBS was removed, followed by multiple washes with DPBS for the elimination of the extracellular and surface bound bacteria. Low glucose 1 g/L DMEM supplemented with 10% FBS and 1% P/S was added (100 ul/well). After passing desired time, the supernatants were discarded, while the adherent TGEMs were washed twice with DPBS and lysed with Triton-X 0.02% diluted in sterilized water (100 ul/well). Each sample-well was serially diluted and plated in THB agar petri dishes in the presence of fire. The plates were placed at 37°C overnight and the quantification of the intracellular bacterial load in each inoculum was estimated through colony-forming unit (cfu) counting.

- Wt adult and neonatal macrophages: 0, 2, 4, 6, 9, 12, and 15 hpi (hours post infection)

For the transfection assays, one day after seeding, cells were washed twice with DPBS and the cell medium was changed to Low glucose 1 g/L DMEM supplemented with 10% Fetal Bovine Serum (FBS). TGEMs were transfected with siRNAs against Rubicon, NOX2 or ULK1 by using the transfection reagent Lipofectamine RNAiMAX. In particular, 0.3 ul 10 uM siRNA per well was mixed with 5 ul opti-MEM per well. The siRNA:opti-MEM solution was then mixed with 0.3 ul Lipofectamine RNAiMAX diluted in 5 ul opti-MEM. This mixture was incubated for 5 min at room temperature (RT) to form RNA-lipid complexes, followed by equally distribution in the 96-wells (10 ul/well).
Three days after the transfection, the cells were infected with GBS with the aforementioned protocol.

- Wt adult and neonatal macrophages transfected with siRubicon and siScramble (control): 4 hpi.
- Wt adult and neonatal macrophages transfected with siNOX2 and siScramble (control): 4 hpi.
- Wt adult and neonatal macrophages transfected with siULK1 and siScramble (control): 4 hpi.

RNA isolation, cDNA synthesis and real time quantitative (rt-PCR)
TGEMs from adult and neonatal mice were seeded in 24-well plates at a density of 450000-500000 cells per well. Total RNA was extracted using Trizol (TRI Reagent/ Sigma-Aldrich). After solubilization, the addition of chloroform (1/5 of V_{Trizol}) caused phase separation. After a 15 minute-centrifugation at 12000 rcf (relative centrifugal force) 4°C, the total RNA from each sample remained in the upper aqueous phase and was transferred to a new sterile eppendorf. Isopropanol was then added in each sample (1/2 V_{Trizol}) to assist the precipitation of RNA, followed by a centrifugation at 12000 rcf for 10 min (4°C). The supernatants were discarded and the tubes were filled with 75% ethanol (V_{Trizol}), followed again by a centrifugation (7500 rcf, 5 min, 4°C). After repeating this process, the air-drying pellets (10 min at RT) were diluted in 20 ul RNAse-free water (TaKaRa®) and heated for 5 min at 55°C. The RNA concentration was quantified using the Nanodrop micro-volume spectrophotometer.
The cDNA synthesis was performed using the TaKaRa® PrimeScript^{TM}RT (Perfect Real Time), following manufacturer instructions. Template RNA of each sample (800 ng/ul) was combined with a mixture of 1X PrimeScript Buffer, PrimeScript Reverse Transcriptase, Oligo dT Primer (25 pmol), Random 6 mers (200 pmol), and RNAse-free water. This reaction mixture was incubated using the following conditions; 10 min at 30°C, 45 min at 50°C, and 5 min at 95°C (enzyme inactivation). Samples were then diluted in distilled water (1:2) and stored at -20°C.

The aforementioned synthesized cDNAs were utilised for quantitative PCR (rt-PCR) analysis. According to the instructions of the KAPA SYBR® FAST qPCR kit Master Mix (2X) Universal, 2 ul cDNA was used as the template of the reaction together with the forward and reverse primer (10 uM) in a final reaction volume of 10 ul. Each sample was analysed in duplicates. Ribosomal Protein S9 (RPS9) was chosen as the reference gene and the expression of IL-6, TNFα and IL-12b was quantified. These reactions were performed in the Applied Biosystems-qPCR machine, while the mRNA transcription analysis via StepOne software v2.3 and GraphPad Prism 7.0 software.

IL-6: F: 5'-CAAAGCCAGAGTCCTTCAGAG-3' & R: 5'-CACTCCTTCTGTGACTCCAGC-3'
IL-12b: F: 5'–GGAGGGGTGTAACCAGAAAGGTGC–3' & R: 5–CCTGCAGGGAACACATGCCCAC–3'
RSP9: F: 5'-GCTAGACGAGAAGGATCCCC-3' & R: 5'- CAGGCCCAGCTTAAAGACCT-3'

Immunocytochemistry and Confocal Imaging

For the immunocytochemistry assays, 24-wells were covered with round glass coverslips of 12mm diameter. TGEMs, diluted in Low glucose 1 g/L DMEM with 10% FBS and 1% P/S, were plated in these 24-well plates at a density of 400000 cells per well and placed in an incubator (37°C, 5% CO_2) overnight to adhere on coverslips.

1) The next day, TGEMs were transfected with siRNAs (siRubicon and siULK1) as previously described and GBS inoculation was conducted three days later.
2) For the lysotracker staining, TGMEs were pre-stained with lysotracker (100 uM) for 1 hour on the day of the experiment.

In both experiments, the wells were washed with DPBS twice and the cell medium was changed to Low glucose 1 g/L DMEM, supplemented with 10% FBS. As previously described, GBS was propagated in a THB liquid culture until reaching mid-exponential phase, an OD between 0.5 and 07. TGEMs were infected with GBS at an MOI of 4. After 2 hours of infection, GBS was removed, followed by DPBS washes and fresh cell medium, Low glucose 1 g/L DMEM, 10% FBS, 1% P/S, was added to the wells. After the desired time-points, TGEMs on coverslips were washed with DPBS, fixed with ice-cold methanol for 15 min at -20°C, washed again with DPBS and stored at 4°C. Non-infected TGMEs were used as negative controls.
For the first experiment, coverslips were transferred to a clean surface, which was covered with parafilm to be stable, and incubated for 15 min at RT on a rocker-shaker with Blocking solution that consists of 5% FBS, 1% Bovine Serum Albumin (BSA-Sigma/Aldrich) and 0.05% Triton-X 100 in PBS. After repeating the process twice, blocking solution was aspirated and the primary antibody rabbit anti-LC3B (Sigma/Aldrich, 1/800 dilution in Blocking solution) was added for 1 hour at RT on a rocker-shaker. Coverslips were then washed with Blocking solution for three times (5 min each wash) and incubated with the secondary antibody CF555 (Biotium, 1/500 dilution in Blocking solution) for 40 min at RT on a rocker-shaker in a dark place. Following that, cells were washed again with Blocking solution and for blockage, 10 ug/ml anti-rabbit antibody raised in goat, was added. This step is required due to the fact that the second primary antibody that was used was raised in the same species as the first one. After a 40 min-incubation, the second primary antibody, namely rabbit anti-GBS (Abcam, 1/500 dilution), was added for 1 hour at RT on a rocker-shaker. Three 5 min-washes with Blocking solution followed and cells were then incubated with the secondary antibody CF488A (Biotium, 1/500 dilution in Blocking solution). All coverslips were then washed thoroughly with Blocking solution and stained with DAPI (1/1000 dilution) for 3 min at RT. After the nuclear staining, coverslips were washed again with DPBS, mixed with mounting medium, sealed, and stored at 4°C. The observation of the coverslips was

performed via Leica SP8 Confocal Microscope. The images were analysed in Leica LAS AF Lite and GraphPad Prism 7.0 software was used for the statistical analysis.
For the second experiment, cells were pre-stained with lysotracker (100 uM) for 1 hour on the day of the experiment. GBS infection was conducted as previously described. After 3 and 4 hours of infection, coverslips were fixed and stained with the aforementioned immunostaining procedure. The main difference was that the coverslips were incubated only with the primary antibody, rabbit anti-GBS (Abcam, 1/500 dilution), and the secondary CFS488 (Biotium, 1/500 dilution in Blocking solution). DAPI (1/1000 dilution) was utilized to stain the nucleus. The observation of the coverslips was performed via Leica SP8 Confocal Microscope and the images were analysed via Leica LAS AF Lite. For the statistical analysis, GraphPad Prism 7.0 software was used.

Enzyme-linked immunosorbent assay (ELISA)

TGEMs from adult and neonatal mice were cultured in 24-well plates at a density of 1000000 per well. GBS was cultured until reaching the desired OD, and accordingly, the cells were infected at an MOI of 10. After two hours of infection, the supernatants were centrifugated at 5000 rpm for 5 min in order for GBS to be removed. The supernatants corresponding to the 2hpi-time point (2 hours post infection) were collected, while the rest (4hpi and 6 hpi) were returned to the wells, supplemented with 1% P/S. The remaining supernatants were collected at the desired time points (4hpi and 6 hpi). The supernatants of uninfected TGEMs of both adult and neonates were utilized as negative controls (0 hpi).
The levels of TNF-α, IL-6 and IL-10 in the culture supernatants were determined by ELISA using Biolegend kits. According to the manufacturers' instructions, 96-well plates were coated with Capture Antibody (diluted in 1X Coating Buffer) on day 1 (100 ul/well), sealed and incubated at 4°C overnight. The next day, the plate was washed (300 ul/well) four times with Wash buffer (0.05% Tween-20 in PBS) and 1X Assay Diluent A was added (200 ul/well) for 1 hour at RT with shaking, to block non-specific binding and reduce background. The plate was washed again with Wash buffer (4 times) and then the standards and samples were added to the wells with the appropriate dilutions required, at RT for 2 hours on a rocker-shaker. After 4 washes with Wash buffer, the wells were incubated with Detection Antibody (diluted in 1X Assay Diluent A) for 1 hour at RT with shaking (100 ul/well). The plate was washed again and Avidin-HRP solution (diluted in 1X Assay Diluent A) was added for 30 min at RT (100 ul/well). Next, the plate was washed again for 5 final washes with Wash buffer and incubated with freshly mixed TMB Substrate Solution (1:1; Substrate Solution A and Substrate Solution B) for 20 min in the dark. Finally, the reaction was stopped by adding 100 ul of Stop Solution (acid solution, eg 2N H_2SO_4) to each well. The microwell absorbance was read at 450 and 570 nm using a microplate reader (TECAN) and the results were analyzed via Microsoft Excel and GraphPad Prism 7.0 software. The protein concentration of each sample was measured by Bicinchoninic assay (BCA), as explained below, and used for normalization.

Protein harvest and quantification

TGEMs from adult and neonatal mice were cultured in a 24 well-plate at a density of 1000000 cells per well in cell medium, containing low glucose 1 g/L DMEM, 10% FBS, and 1% P/S. The next day, the medium was changed with fresh one and the following day, supernatants were removed, cells were then washed twice with sterile DPBS and infected with GBS at an MOI of 10 (0, 2, 4, and 6 hpi). GBS proliferation and the subsequent infection of cells were performed as previously described. Thereafter, the 24 well-plate was placed on ice and a mixture of RIPA, 1x Protease inhibitors and 1x Phosphatase inhibitors, was added to each well. After 5 minutes, TGEMs were thoroughly scrapped and each cell suspension was harvested in a sterile eppendorf tube. Samples were then centrifuged at maximum speed (12.000 rpm) for 5 min at 4°C; cell debris remained in the pellet and the protein extract in the supernatant. Protein extracts (supernatants) were transferred to new sterile eppendorf tubes and stored at -80 ^{o}C.

The Bicinchoninic assay (BCA) was utilized for the quantification of the total protein concentration of our samples. This method is based on the principle that proteins can reduce Cu^{+2} to Cu^{+1} in an alkaline solution (the biuret reaction), resulting in a purple color formation by bicinchoninic acid. A standard curve was created by using serial dilutions of bovine serum albumin (BSA) solution of known concentration; 2000ug/ml, 1000ug/ml, 500ug/ml, 250ug/ml, 125ug/ml, and 62.5ug/ml. The BSA standards (5 ul of each dilution) were mixed with 20 ul H20 in duplicates, whereas the unknown protein samples (3 ul of each sample) were also mixed with H20 (22 ul) in duplicates. For the samples, 5 ul of RIPA (plus Protease/Phosphatase Inhibitors) was mixed with 22 ul H20 and used as a control. BCA Reagents A and B in proportion 50:1 was added to each well (200 ul). The plate was then sealed and incubated at 37°C in the dark for 30 minutes. After the 30-minute period, the absorbance was measured at 569 nm using a microplate reader (TECAN). Data were analyzed via Microsoft Excel and Graph Pad Prism 7.0, following a linear regression model.

Immunoblotting

Immunoblotting or Western blotting was utilized to identify the proteins LC3-II and LC3-I, separated based on size by gel electrophoresis. After the quantification of the protein concentration for each cell lysate, the appropriate volume of lysate (15 ug) was mixed with 1x Laemmli sample buffer and heated at 95°C for 5 min, spinned down and kept on ice until loading. To prepare the 12% polyacrylamide gel (1.5 mm thick), acrylamide (40%) was mixed with the appropriate amount of separating gel buffer (1M Tris pH 8.8, 10% SDS, and H20) and stalking gel buffer (1M Tris pH 6.8, 10% SDS, and H20), respectively. The polymerization was induced by adding 10% ammonium persulfate (APS) and TEMED® reagents. The samples were then loaded along with a molecular weight marker (4 ul). Electrophoresis of samples was performed at 70V in reservoir buffer (0.025M Tris, 0.192M Glycine, 0.1% SDS, and H20) until the proteins passed the stalking phase, followed by 120V during the separating phase of the gel. After an hour, the electrophoresis was stopped and the proteins were transferred from the gel to an activated PVDF membrane (1-2-minute methanol activation). The device was filled with transfer buffer (0.025M Tris, 0.192M Glycine, 20% methanol, and H20) and placed on a magnetic stirrer at a constant current of 400mA under cold conditions. After transferring the proteins, the membrane was blocked with 5% skimmed milk diluted in PBS-T (phosphate buffered saline supplemented with 0.1% Tween® 20) for an hour at RT on a rocker. The membrane was then washed with PBS-T (three times) and incubated with the primary antibody overnight at 4°C. The next day, the membrane was washed again (three times) with PBS-T and incubated at RT with the secondary antibody for 1 hour. After three washes with PBS-T, the membrane was rinsed with enhanced chemiluminescent (ECL) peroxidase substrate and exposed to trans-UV light in a fluorescent imager (BioRad Laboratories, Inc.). The fluorescence signals were digitalized and analyzed using the manufacturer corresponding software (ImageLab).

RESULTS

Neonatal macrophages have reduced bactericidal capacity.

Since the neonatal immune system is relatively immature due to limited antigenic experience, newborns depend mainly on the innate immune cells in order to respond to pathogenic invaders (Kumar & Bhat, 2016). Mouse peritoneal macrophages, a well-characterized macrophage population, have a vital role in the control of infections and inflammatory pathologies, and thereby, are widely used in immunological studies (Cassado et al., 2015). Hence, we utilized peritoneal macrophages, which were collected under elicited conditions, for all our experiments. First, we sought to determine whether there is any difference in microbial clearance between wild-type (wt) adult and neonatal macrophages. For this reason, we performed an *in vitro* assay of intracellular bacterial killing that allows the estimation of the number of phagocytosed bacteria that survived intracellularly. Accordingly, wt adult and neonatal macrophages were infected with GBS at an MOI of 10 for 2 hours. Bacterial intracellular survival was determined by CFU counting on THB agar plates at 0, 2, 4, 6, 9, 12, and 15 hours post infection (hpi) (**Fig.3**).

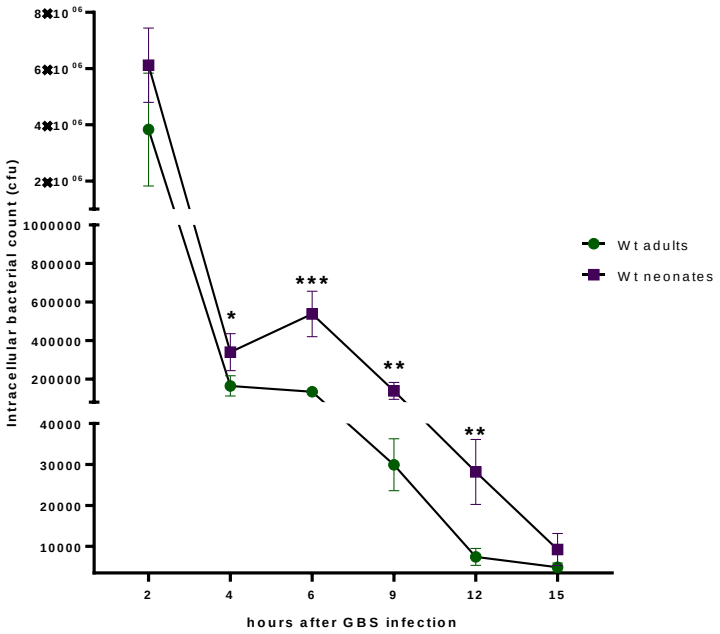

Figure 3. Survival of GBS. Wt adult and neonatal macrophages were infected with GBS at an MOI of 10 and intracellular bacteria were measured by CFU counting at the time points indicated. The mean ±SD of one experiment in pentuiplicate wells and duplicate colony counts is shown. ***p<0.001, **p<0.01, *p<0.05 .

As depicted in diagram 3, both groups followed a similar pattern of CFU counts. Specifically, intracellular survived bacteria were initially high at 2 hpi, reduced sharply at 4 hpi and then gradually decreased at the later time points. GBS infection was resolved at 15 hpi for both groups. Neonatal macrophages had intracellularly an elevated number of bacteria during most of the time points analyzed in comparison with adult cells. A significant increase in CFU counts was observed at 4, 6, 9, and 12 hpi. In particular, the intracellular bacterial load of neonatal cells showed a 4-fold rise at 6 hpi compared with adult ones. Preliminary data from our lab support these findings. Another bacterial survival assay (**Fig.4**) as well as a two-color flow cytometry and sequential gating analysis demonstrated no difference in phagocytic capacity between adult and neonatal

macrophages. Therefore, neonatal cells have clearly reduced bactericidal capacity compared to the corresponding adult cells, which were more efficient at microbial clearance.

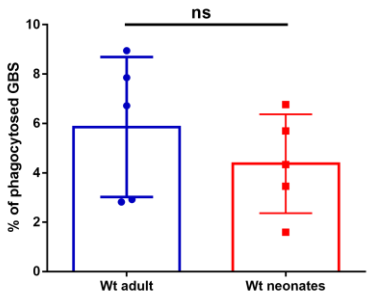

Figure 4. Percentage of phagocytosed bacteria. The percentage was estimated by the ratio of the total number of phagocytosed bacteria (killed and survived inside macrophages) to the initial number of inoculated bacteria.

Co-localization of LC3-II with GBS is increased in adult macrophages.

After confirming that the peritoneal macrophages of newborns cannot eliminate GBS in an efficient manner, we continued investigating whether microtubule-associated protein 1A/1B-light chain 3 (LC3) participates in GBS clearance. The cytosolic form of LC3, LC3-I, is conjugated to phosphatidylethanolamine (PE) to create a lipidated form (LC3-II). LC3-II is a crucial member of canonical and non-canonical autophagy, both of which are considered pathways for pathogen elimination. To determine LC3-II co-localization with GBS upon infection, we used wt adult and neonatal macrophages challenged with GBS at an MOI of 4 for 2 hours. For confocal microscopy, cells were fixed at 3 hpi and stained for LC3-II and GBS. DAPI was used for nuclear staining (**Fig.5**).

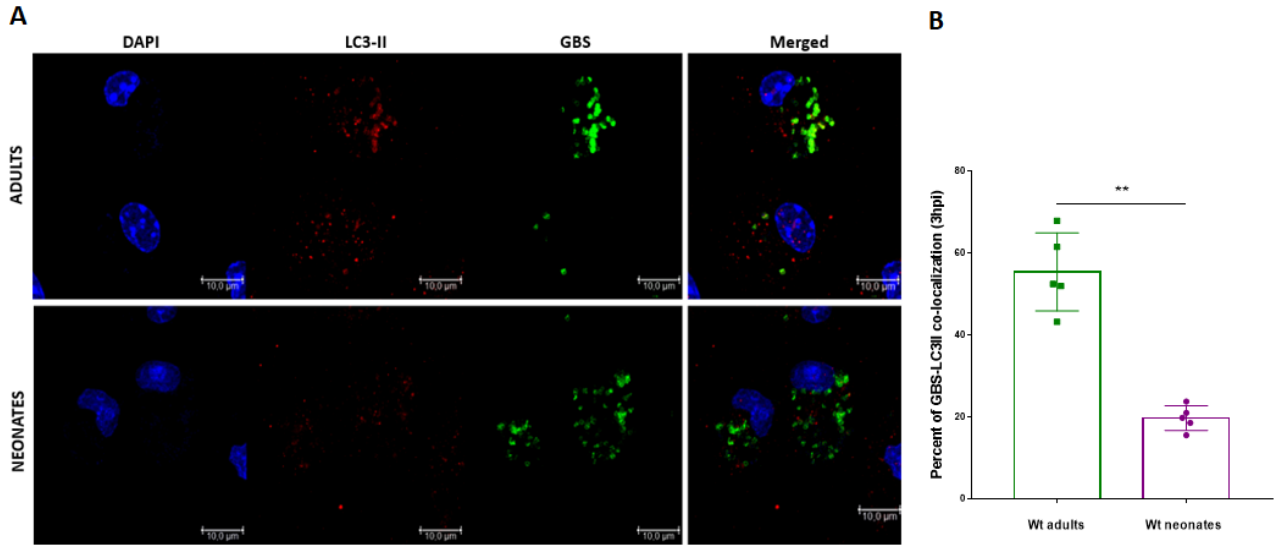

Figure 5. Co-localization (yellow signal) of LC3-II (red signal) with GBS (green signal) assayed by confocal microscopy. Nuclei were stained with DAPI. (A): Confocal microscopy images of wt adult and neonatal macrophages 3 hours after GBS infection. (B): Percentage of LC3-II/GBS co-localization at 3 hpi. Mean value ±SD is depicted. ** $p<0.01$. Scale bar corresponds to 10 um.

We observed that LC3-II concentration is high in both groups, which can be attributed to GBS infection. However, neonatal macrophages had an increased number of intracellular bacteria (green particles) compared with the corresponding adult cells as representatively shown in Figure 5A. LC3-II (red signal) co-localizes with GBS (green signal), forming ring-shaped structures. By the co-localization analysis of confocal microscope (yellow fluorescence), it is evident that the LC3-II/GBS co-localization events are elevated in adult

macrophages (3-fold increase), which is indicative of an improved clearance capacity. The ring-shaped structures, created by LC3-II/GBS co-localization, might be vacuoles decorated with LC3-II that surround the pathogen. This is evidence that can be linked to an autophagic pathway activated upon GBS infection.

Co-localization of GBS with lysosomes is enhanced at 4 hpi in adult macrophages.

After verifying that LC3-II co-localization with GBS is increased at 3 hpi in wt adult macrophages compared to neonatal ones, we decided to ascertain whether GBS co-localizes also with lysosomes, suggesting bacterial delivery to lysosomes for degradation. Hence, wt adult macrophages were pre-stained with lysotracker and then infected with GBS at an MOI of 4. The cells were fixed at 3 and 4 hpi and stained for GBS and nuclear staining DAPI. Images were taken using confocal microscopy. A representative image is shown in Figure 6 in which adult macrophages displayed more co-localization events (yellow signal) at the later time point, namely at 4 hpi. Therefore, it seems that GBS is delivered to lysosomes 4 hours after GBS inoculation in adult cells. Since adult macrophages have improved bactericidal capacity and increased co-localization of LC3-II with GBS, the delivery of GBS to this acidic compartment may indicate its subsequent destruction. This experiment was conducted to standardize the lysotracker staining conditions and should be repeated with both adult and neonatal cells.

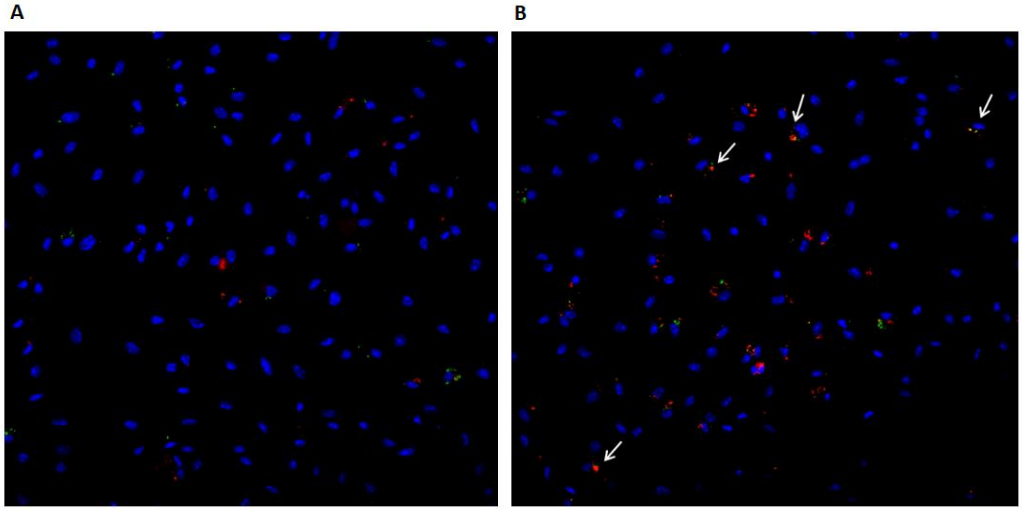

Figure 6. Co-localization of GBS with lysosomes (yellow signal) in wt adult macrophages. After lysotracker pre-treatment (red), the cells were challenged with GBS at an MOI of 4. Afterwards, fixation at 3hpi (A) and 4 hpi (B) followed. Immunostaining was performed with anti-GBS (green) and nuclear staining DAPI (blue). The cells designated by the white arrows were both GBS and lysotracker positive.

LC3-II/LC3-I ratio is elevated in neonatal macrophages.

The aforementioned data suggest the possible involvement of an autophagic pathway in GBS clearance. LC3 constitutes the only protein that is associated with all types of autophagic membranes, including the phagosome and the autophagosome. In the cytosol, proLC3 is cleaved at its C terminal by ATG4 to form LC3-I, which is then conjugated to PE by ATG7 (E1) and ATG3 (E2) ubiquitination systems. This lipidated form binds to both inner and outer membrane of phagosome/autophagosome. The inner membrane bound LC3-II is degraded after fusion with lysosomes, whereas LC3-II on the outer membrane is deconjugated by ATG4 and returns to the cytosol (Zhang et al., 2016). Therefore, we sought to monitor changes in LC3 amount using immunoblotting. Protein samples were harvested from adult and neonatal macrophages (1000000 cells/well) at 0, 2, 4, and 6 hours after GBS infection. As both canonical and non-canonical autophagy are highly dynamic

processes, the number of autophagosomes/phagosomes detected at any time point results from the rate of their generation and degradation after fusion with lysosomes (Zhang et al., 2016). According to the immunoblotting assay, the LC3-II/LC3-I ratio remained stable upon GBS infection in adult macrophages. However, a slight increase was observed 4 hours post infection. As regards neonates, the ratio was extremely elevated upon bacterial infection, especially at 4 hpi, suggesting either upregulation of autophagosome/phagosome formation or blockage of degradation (**Fig.7**).

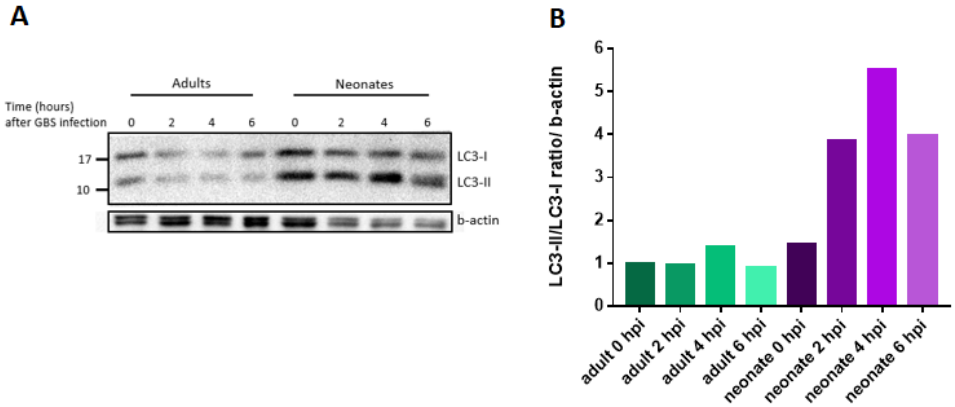

Figure 7. Determination of the LC3-I and LC3-II protein levels in wt adult and neonatal macrophages at 0, 2, 4, and 6 hours after GBS infection (hpi). Panel (A) represents immunoblot bands for LC3-I and LC3-II, and b-actin as internal loading control. Panel (B) shows the quantitative analysis of band intensity using Image Lab analysis software; data are presented as LC3-II/LC3-I ratio/ b-actin.

Rubicon and NOX2, but not ULK1, are required for GBS clearance in adult macrophages.

Confocal microscopy and immunoblotting analysis showed the involvement of LC3-II in GBS clearance. However, LC3-II is an important constitute of both autophagy and LC3-associated phagocytosis (LAP). To discern which elimination pathway is induced after GBS infection, we transfected wt adult and neonatal macrophages with siRNAs against two essential proteins involved in the aforesaid pathways, ULK1 and Rubicon, respectively. ULK1, a serine/threonine protein, forms a complex with at least three proteins, ATG13, FIP200 and ATG101, and is involved in autophagy initiation (Zachari & Ganley, 2017). On the other hand, Rubicon is recruited to the pathogen-containing phagosome during LAP, where stabilizes the UVRAG-PI3KC3 complex for PI3P generation and facilitates the assembly and function of NOX2 (Martinez, 2018). It is interesting to note that Rubicon is also an inhibitor of autophagy (Matsunaga et al., 2009; Zhong et al., 2009). Three days after transfection, the cells were infected with GBS at an MOI of 10 for 2 hours. Each sample-well was serially diluted and plated on THB agar petri dishes, while the intracellular bacterial load in each inoculum was estimated through colony-forming unit (CFU) counting. Cells transfected with siScramble were utilized as controls.

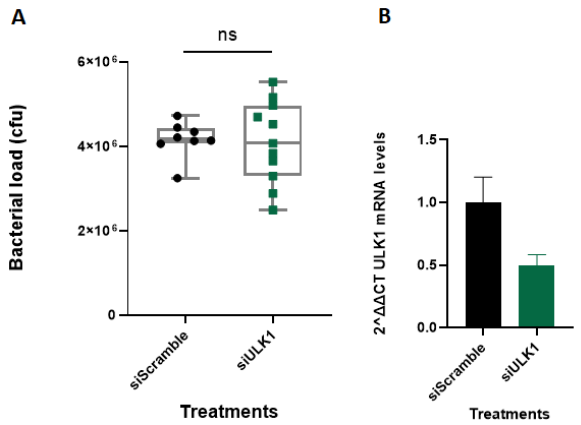

Figure 8. ULK1 is not required for GBS clearance in wt adult macrophages. (A): Intracellular killing capacity of wt adult macrophages after knocking down ULK1 at 4 hpi (MOI 1:10). (B): The efficiency of ULK1 knockdown was assayed using real-time PCR. Mean value ±SD of two experiments in pentuiplicate wells and triplicate colony counts is shown.

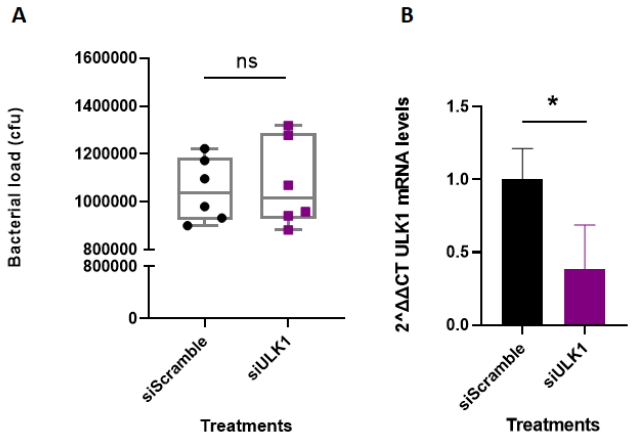

Figure 9. ULK1 is dispensable for GBS clearance in wt neonatal macrophages. (A): Intracellular killing capacity of wt neonatal macrophages after knocking down ULK1 at 4 hpi (MOI 1:10). (B): The efficiency of ULK1 knockdown was assayed using real-time PCR. Mean value ±SD of one experiment in sextuplicate wells and triplicate colony counts is depicted. *p<0.05 .

According to the intracellular killing assays, the intracellular bacterial load did not change significantly when ULK1 was knocked down in both adult and neonatal macrophages (**Fig.8** and **Fig.9**). Specifically, the CFU counts did not vary between the cells transfected with siULK1 and those with siScramble, which was utilized as the control. These findings indicated that ULK1 is dispensable for GBS elimination in both groups. The same experiment was conducted by knocking down Rubicon this time. Rubicon knockdown led to an increased intracellular bacterial load in adult cells (2.5-fold increase) (**Fig.10**). In regard to neonates, the bacterial burden of siRubicon-transfected cells remained high as in the corresponding control cells (**Fig.11**). Hence, it seems that neither ULK1 nor Rubicon has an impact on the elimination of GBS in neonatal macrophages. Interestingly, these bacterial survival assays showed that the adult cells have increased bactericidal capacity in the presence of Rubicon, which may be indicative of LC3-associated phagocytosis (LAP) pathway activation.

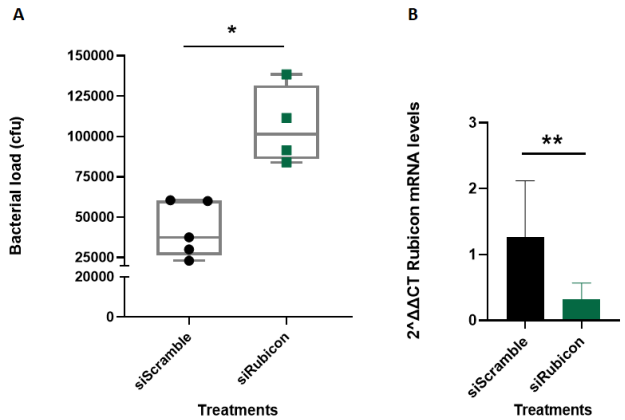

Figure 10. Rubicon is required for GBS elimination in wt adult macrophages. (A): Intracellular killing capacity of wt adult macrophages after knocking down Rubicon at 4 hpi (MOI 1:10). (B): The efficiency of Rubicon knockdown was assayed using real-time PCR. Mean value ±SD of one experiment in pentuiplicate wells and duplicate colony counts is displayed. * $p<0.05$, ** $p<0.01$.

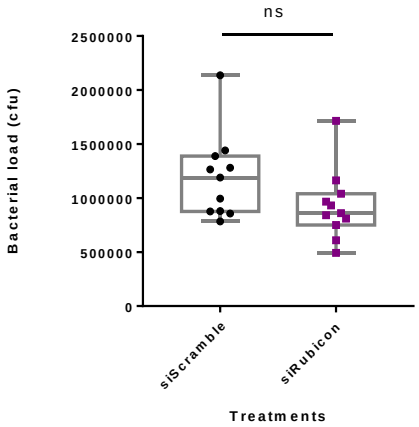

Figure 11. Rubicon is dispensable for GBS elimination in wt neonatal macrophages. (A): Intracellular killing capacity of wt neonatal macrophages after knocking down Rubicon at 4 hpi (MOI 1:10). (B): The efficiency of Rubicon knockdown was assayed using real-time PCR. Mean value ±SD of two experiments in pentuiplicate wells and duplicate colony counts is depicted.

To confirm that, we performed another intracellular killing assay following the same conditions by knocking down NOX2. Specifically, wt adult and neonatal macrophages were transfected with siNOX2 and siScramble to determine the importance of this constituent in GBS clearance. NOX2 is responsible for ROS generation during LAP; ROS are toxic to microbes and are also required for activating the ATG-conjugation systems (S. Upadhyay & Philips, 2019). As shown in Figure 12, the CFU counts rose dramatically to 10.245.833 from 7.275.556, suggesting an important role of NOX2 in the bactericidal capacity of adult macrophages. In contrast to adult cells, neonates exhibited a downward trend in CFU counts when NOX2 was knocked down, but it was not statistically significant. Consequently, NOX2 and Rubicon, two essential components of LAP, are required for GBS degradation in wt adult macrophages.

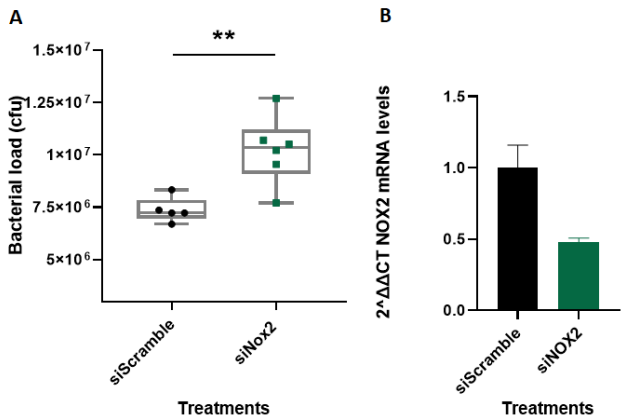

Figure 12. NOX2 is essential for GBS elimination in wt adult macrophages. (A): Intracellular killing capacity of wt adult macrophages after knocking down NOX2 at 4 hpi (MOI 1:10). (B): The efficiency of NOX2 knockdown was assayed using real-time PCR. Mean value ±SD of one experiment in sextuplicate wells and triplicate colony counts is shown. ** $p<0.01$.

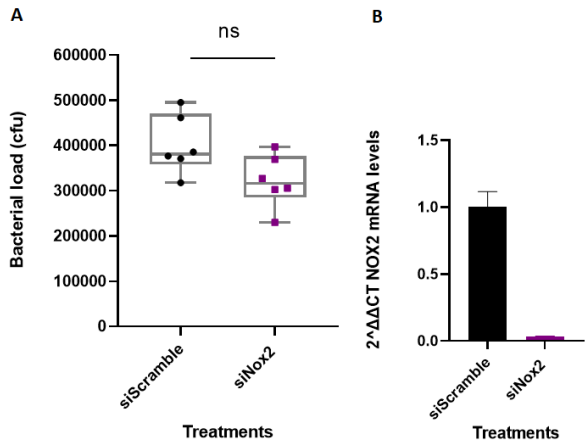

Figure 13. NOX2 is not required for GBS elimination in wt neonatal macrophages. Intracellular killing capacity of wt neonatal macrophages after knocking down NOX2 at 4 hpi (MOI 1:10). Mean value ±SD of one experiment in sextuplicate wells and triplicate colony counts is displayed.

The engagement of LAP in adult macrophages upon GBS infection.

The bacterial survival assays demonstrated the importance of Rubicon and NOX2 in GBS clearance in adult macrophages. Thus, we knocked down again Rubicon, the critical component of LAP, as well as ULK1, an essential protein of canonical autophagy, to confirm the involvement of the LAP pathway. Accordingly, we transfected adult and neonatal macrophages with siRNAs against Rubicon, ULK1, and siScramble as the control. After the transfection, the cells were infected with GBS at an MOI of 4 and fixed 3 hours after the infection. The cells were then stained with fluorescent antibodies against GBS and LC3-II for the detection of the intracellular bacterial load and the engagement of LC3-II, respectively (**Fig.14** and **Fig.16**). For the immunocytochemical staining, we utilized as negative controls transfected (siScramble) and non-transfected cells not infected with GBS.

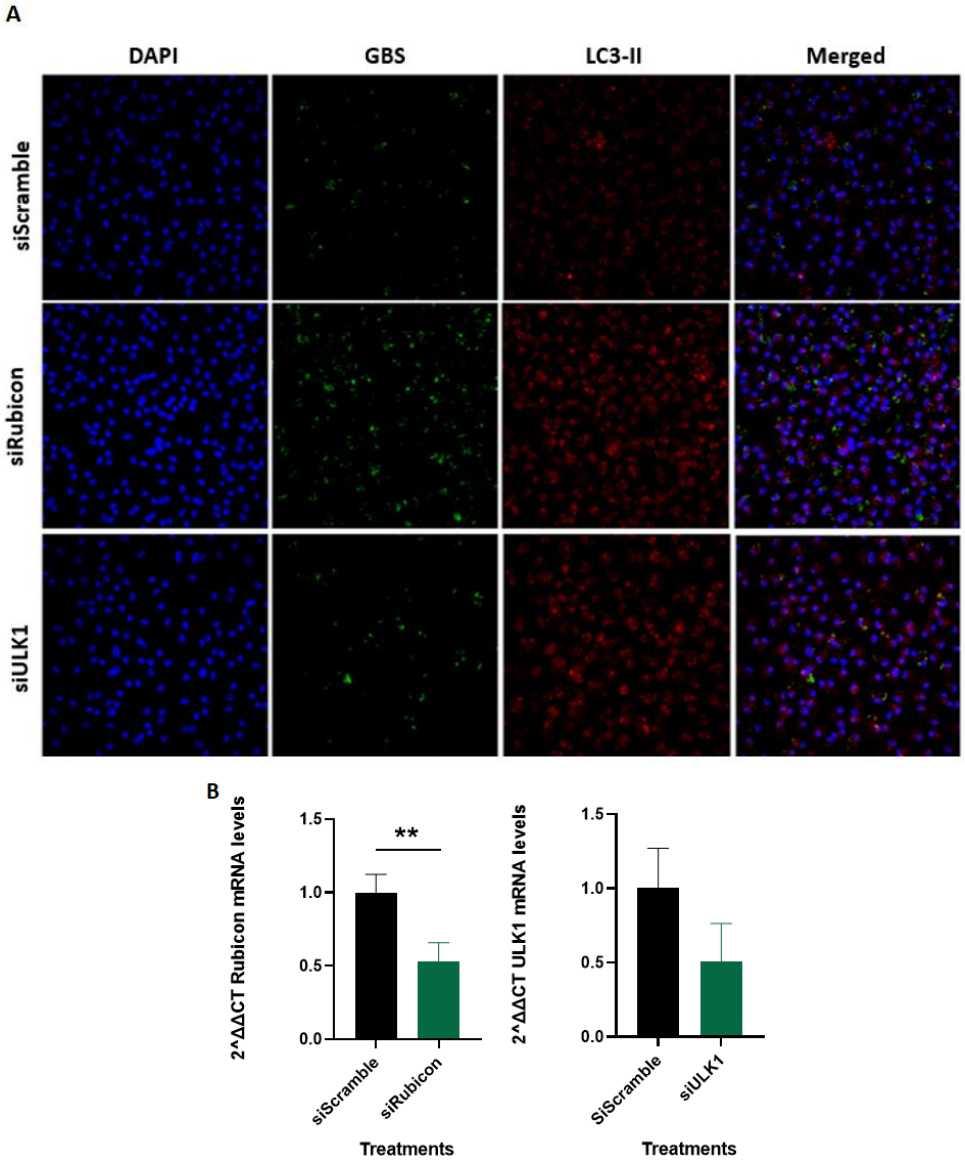

Figure 14. The effect of Rubicon and ULK1 knockdown in GBS clearance. (A): Confocal microscopy on wt adult macrophages transfected with siRubicon, siULK1, and siScramble as the control. The cells were infected with GBS at an MOI of 4 and stained for GBS and LC3-II at 3hpi. Cell nuclei were stained with DAPI. (B): The efficiency of Rubicon and ULK1 knockdown was assayed using real-time PCR. **$p<0.01$.

As shown in Figure 15, the bacterial load was greatly increased at 3hpi in siRubicon-transfected adult cells in comparison with the control. By contrast, no difference was identified in GBS number between adult macrophages treated with siULK1 and those with siScramble. These findings are in accordance with the aforesaid bacterial survival assays, suggesting an essential role of Rubicon in GBS degradation in adult cells. Both siRubicon- and siULK1- transfected adult macrophages presented higher levels of LC3-II at 3 hpi compared with siScramble-treated cells.

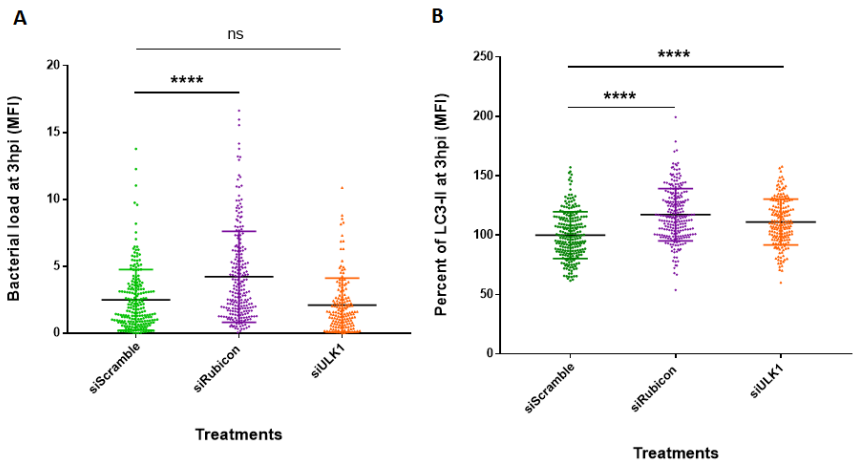

Figure 15. The effect of Rubicon and ULK1 knockdown in GBS clearance. Adult macrophages were infected with GBS (MOI 1:4) and stained for GBS and LC3-II at 3hpi. (A): Intracellular killing capacity. (B): The LC3-II percentage. Each dot represents the mean fluorescence intensity of GBS (A) or LC3 (B) at specific regions of interest. Confocal microscopy analysis was performed using Leica LAS AF Lite and the statistical analysis via GraphPad Prism 7.0. MFI: Mean Fluorescence intensity. ****p<0.0001 .

We performed the same transfection assays on wt neonatal macrophages. After knocking down Rubicon and ULK1, we challenged the cells with GBS and stained them at 3 hpi for monitoring GBS number as well as LC3-II level changes during these treatments. Representative images are displayed in Figure 16. The bacterial load did not change significantly when Rubicon was knocked down. In contrast to siRubicon-treated cells, siULK1-transfected neonatal macrophages exhibited a little increase in GBS number when compared to the control. As regards LC3-II levels, a significant rise was observed exclusively in cells transfected with siULK1 (**Fig.17**). Overall, the aforementioned data indicate the crucial role of Rubicon and the subsequent involvement of LAP regarding GBS elimination in adult macrophages. On the contrary, Rubicon knockdown did not have any effect on the bacterial number of neonatal cells. Consequently, the LAP pathway may be dysfunctional in neonatal macrophages, which could account for the reduced bactericidal capacity that we have previously observed. However, according to this experiment, we cannot rule out the engagement of canonical autophagy in GBS clearance in neonatal cells.

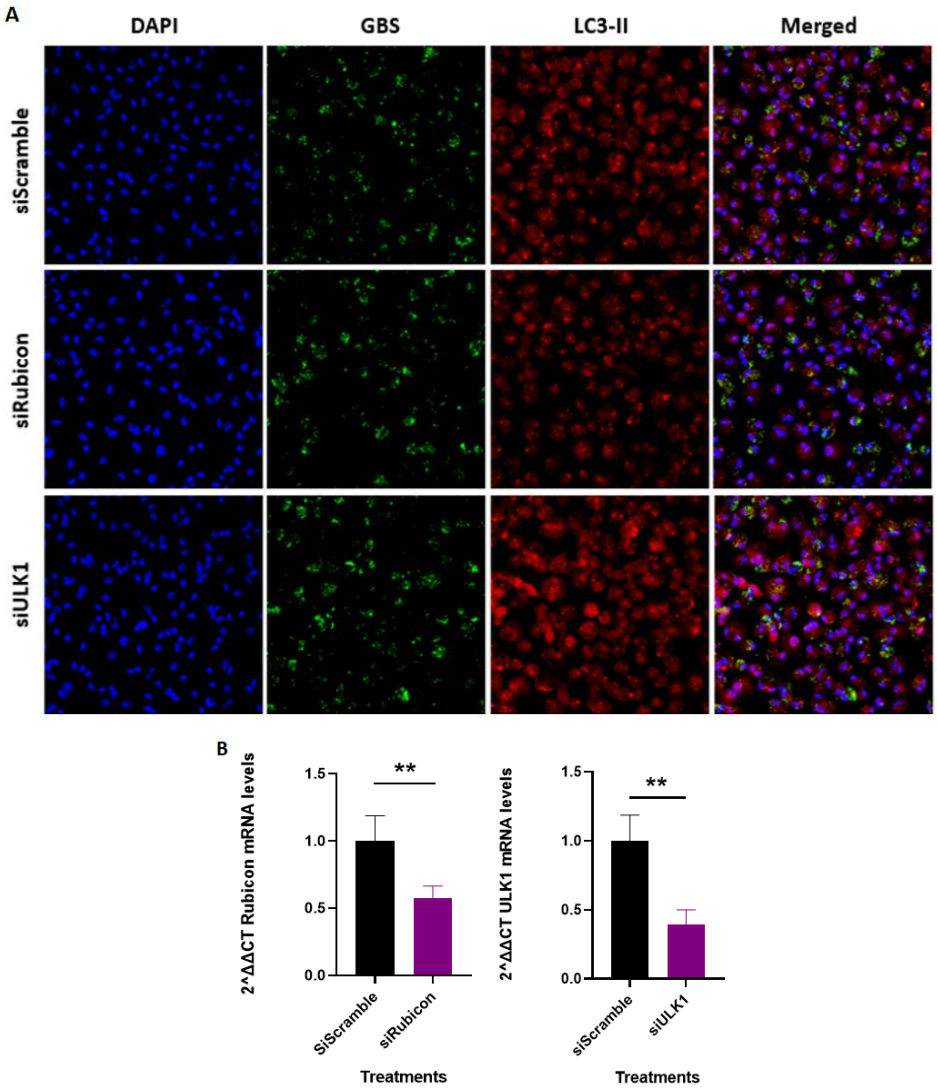

Figure 16. The effect of Rubicon and ULK1 knockdown in GBS clearance. (A): Confocal microscopy on wt neonatal macrophages transfected with siRubicon, siULK1, and siScramble as the control. The cells were infected with GBS at an MOI of 4 and stained for GBS and LC3-II at 3hpi. Cell nuclei were stained with DAPI. (B) The efficiency of Rubicon and ULK1 knockdown was assayed using real-time PCR. **p<0.01 .

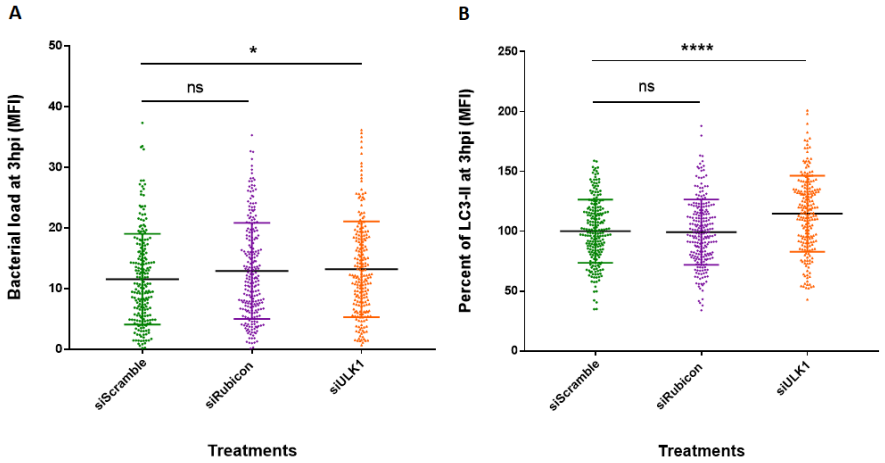

Figure 17. The effect of Rubicon and ULK1 knockdown in GBS clearance. Neonatal macrophages were infected with GBS (MOI 1:4) and stained for GBS and LC3-II at 3hpi. (A): Intracellular killing capacity. (B): The LC3-II percentage. Each dot represents the mean fluorescence intensity of GBS (A) or LC3 (B) at specific regions of interest. Confocal microscopy analysis was performed using Leica LAS AF Lite and the statistical analysis via GraphPad Prism 7.00. MFI: Mean Fluorescence intensity. *p<0.05, ****p<0.0001 .

Neonatal macrophages show an elevated pro-inflammatory cytokine profile after GBS infection.

The aforesaid results showed that newborns are dysfunctional in eliminating GBS in comparison with the corresponding adults although both groups have similar phagocytic capacity. Moreover, transfection assays against constitutive components of LAP confirmed the involvement of this pathway in the bacterial clearance. As it is well established that GBS induces a strong host inflammatory response, we sought to clarify whether neonatal macrophages are able to produce pro-inflammatory cytokines at levels compared to those produced by adult macrophages. First, we evaluated the expression of the inflammatory cytokines IL-6, TNFα, and IL-12b (**Fig.18**).

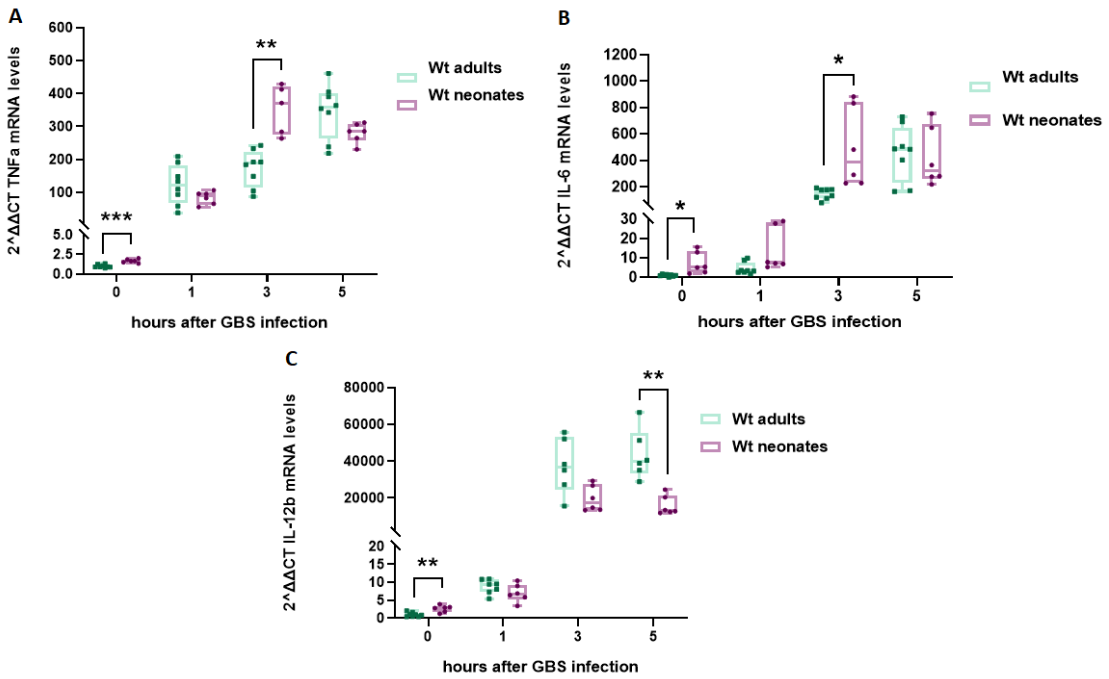

Figure 18. mRNA expression of IL-6 (A), TNFα (B), and IL-12b (C) was analyzed by q-PCR. Data are presented as mean ± SD of one experiment. Statistical significance was assessed using the Holm-Sidak method (multiple t tests). Horizontal bars indicate median values. *p<0.05, **p<0.01, ***p<0.001 .

Measurement of these mediators by qRT-PCR showed that neonates were not barren of pro-inflammatory cytokine expression following GBS infection. Neonatal macrophages expressed under basal conditions higher IL-12B mRNA levels compared to adult ones. Although the expression of this cytokine was progressively enhanced for both groups, adult cells exhibited an increase in the number of IL-12B mRNA transcripts at 3 and 5 hours following GBS infection in comparison with the corresponding neonatal cells (**Fig.18C**). In respect of TNFα, neonates showed elevated expression levels basally compared to adults. TNFα mRNA transcripts increased rapidly at 3 hpi in neonates and declined at the adult level 5 hours post infection (**Fig.18B**). Additionally, IL-6 mRNA expression levels were higher in neonatal macrophages compared to those expressed by adult cells during almost all time points, apart from the last one at which the difference leveled out (**Fig.18A**).

We also studied the secretion of TNFα and IL-6, which are considered early response cytokines in response to Toll-like receptor (TLR)-activating microbial stimuli, in the supernatants of adult and neonatal macrophages via ELISA. Apropos TNFα levels, both cell types produced low levels under basal conditions. TNFα production was increased at 2 hpi for both groups, reaching similar levels. At the late time points, 4 and 6 hpi, neonates

secreted higher levels of this pro-inflammatory cytokine (**Fig.19A**). Interestingly, IL-6 production was far more distinct between these two groups. As shown in diagram 19B, newborns produced elevated levels of this cytokine both basally and post infection. In particular, IL-6 levels rose progressively 2 hours after bacterial infection and remained significantly increased until 6 hpi. According to the Figure 19C, a high ratio of GBS-induced IL-6/TNFα production was observed in neonatal macrophages in comparison with adult cells during all time points. Consequently, these immunoassays illustrated that neonates exhibit clearly higher IL-6, but also TNFα, production after GBS infection compared to adults.

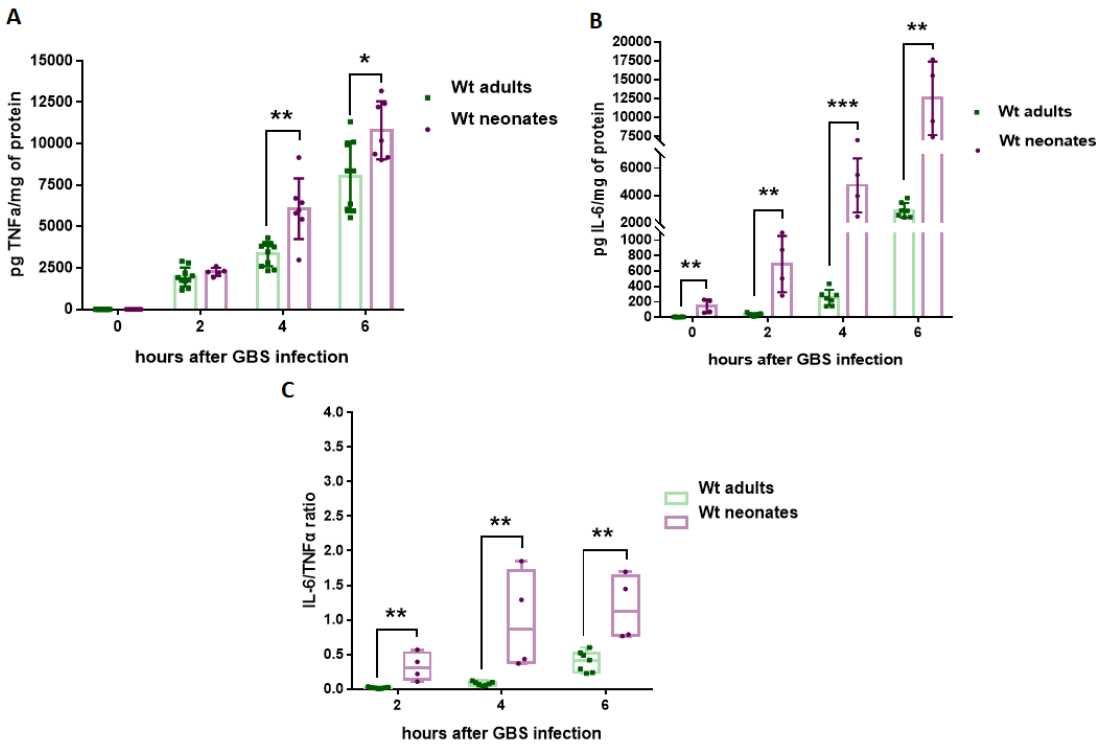

Figure 19. Pro-inflammatory cytokine production by adult and neonatal macrophages. Cytokine levels, TNFα (A) and IL-6 (B), were quantified by ELISA in the supernatants of adult and neonatal cells at 0, 2, 4, and 6 hours after GBS inoculation. (C): The ratio of IL-6/TNFα. The data were calculated with GraphPad Prism 7.0-based curve-fitting software using a 4-parameter logistics curve-fitting algorithm. Protein concentration of each sample measured by BCA was used for normalization. Data are presented as mean ± SD of three (TNFα) and two (IL-6) experiments. Statistical significance was assessed using the Holm-Sidak method (multiple t tests). *p<0.05, **p<0.01, ***p<0.001.

Neonatal macrophages display increased anti-inflammatory IL-10 production upon GBS infection.

According to previous work in our laboratory, neonatal macrophages express high IL-10 mRNA levels upon GBS challenge compared to the corresponding adult cells (**Fig.20**). Specifically, the basal transcriptional levels of this gene are relatively low in both groups in the absence of bacterial stimulus. Upon GBS stimulation, IL10 mRNA expression levels became immediately upregulated in both groups. However, neonatal macrophages had statistically significant increased mRNA transcript levels during all time points. Hence, we performed protein quantification using ELISA for determining the levels of this anti-inflammatory cytokine in the supernatants of adult and neonatal macrophages after GBS infection.

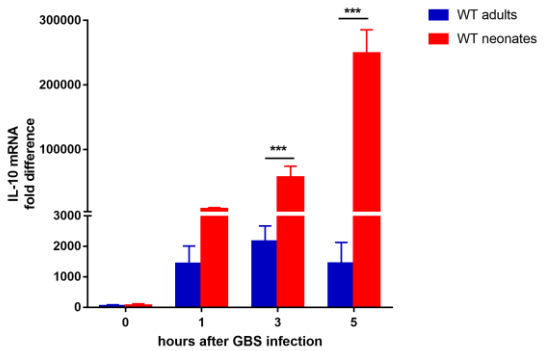

Figure 20. Real-time PCR analysis of the IL-10 mRNA expression levels in wt adult and neonatal macrophages upon challenge with GBS. ***p<0.001 .

Protein quantification analysis regarding IL-10 support the aforementioned data (**Fig. 21**). Although the secretion of this anti-inflammatory cytokine was low under basal conditions, it was partially enhanced at 2 hpi in both groups. After 2 hours, IL-10 production levels continued to increase in neonatal cells and rose sharply in a span of 2 hours, namely 6 hours following GBS infection, in comparison with adult macrophages.

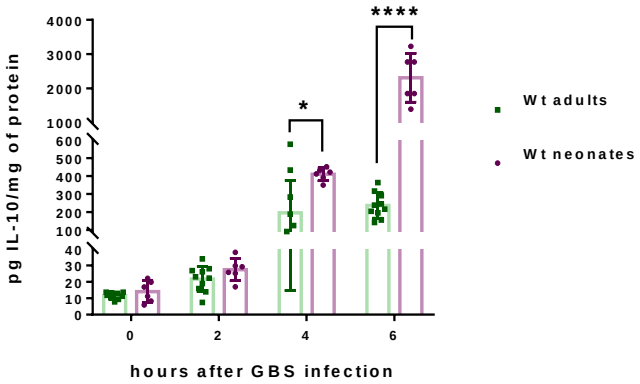

Figure 21. Anti-inflammatory cytokine production by adult and neonatal macrophages. Both groups were infected with GBS and the IL-10 production levels were quantified via ELISA. The data were calculated with GraphPad Prism 7.0-based curve-fitting software using a 4-parameter logistics curve-fitting algorithm. Protein concentration of each sample measured by BCA was used for normalization. Data are presented as mean ± SD of three experiments. Statistical significance was evaluated using Holm-Sidak method (multiple t tests). * p<0.05, ****p<0.0001 .

DISCUSSION

In the present study, we aimed to identify the differences between neonatal and adult macrophages regarding the elimination of the invasive pathogen, GBS. First, we verified that neonatal macrophages infected with GBS have reduced bactericidal capacity compared to adult ones using bacterial survival assay. Despite the fact that CFU counts were gradually diminished in both groups, neonatal macrophages had an elevated intracellular number of bacteria during all time points, especially at 4, 6, 9, and 12 hpi. Hence, neonates seem not capable of eliminating GBS as efficiently as adults. Although this incompetence could be easily attributed to a difference in phagocytic capacity between adult and neonatal macrophages, previous data from our lab did not identify any difference. Furthermore, another research group have reported that the phagocytic ability of neonatal monocytes is the same as that of adult monocytes (Filias et al., 2011). Therefore, our data are in accordance with existing bibliography, supporting the notion that newborns do not have incapability in phagocytic function.

As LC3 constitutes an essential member of both canonical and non-canonical autophagy, we continued investigating its implication in GBS clearance. Confocal microscopy analysis showed that LC3-II co-localization with GBS was greatly enhanced 3 hours following GBS inoculation in adult cells compared with neonatal ones. This co-localization of LC3-II with GBS, which was more pronounced in adult macrophages, appeared as ring-shaped structures that could be vacuoles containing GBS decorated with LC3-II. Co-localization of GBS was observed also with lysosomes after lysotracker pre-staining in adult cells, especially at 4 hpi, suggesting delivery of GBS to lysosomes for degradation. It remains to be established whether this phenomenon occurs also in neonatal macrophages.

The involvement of LC3-II indicates a possible role for either canonical or non-canonical autophagy. Immunoblotting analysis showed that neonatal macrophages had increased LC3-II/LC3-I ratio upon infection compared to adult cells. This increase in LC3-II levels implies the accumulation of autophagosomes/phagosomes, but does not guarantee degradation. To distinguish if there is enhancement of the autophagic flux or blockage of autophagosome/phagosome-lysosome fusion, a common approach is the use of drugs that inhibit autophagic flux by targeting lysosome function. For example, intralysosomal pH neutralizing agents, like chloroquine, or drugs that inhibit the vacuolar type H^+-ATPase complex necessary for lysosomal acidification, such as bafilomycin A1 (Juhász, 2012). Nevertheless, a wide range of these autophagy inhibitors have been reported to activate a non-canonical autophagy pathway that drives LC3 lipidation on single-membrane endolysosomal compartments independently of the canonical autophagy pathway (Florey et al., 2015; Jacquin et al., 2017). This raises a question about the interpretation of LC3 lipidation data in the absence and presence of these drugs using the immunoblotting assay.

To elucidate which pathway plays a part in GBS elimination, we continued by knocking down with siRNAs two essential proteins, ULK1 and Rubicon, involved in canonical autophagy and LC3-associated phagocytosis (LAP), respectively. Intracellular killing assays demonstrated that ULK1 is dispensable for both adult and neonatal macrophages, whereas Rubicon was required mostly in adult cells for GBS clearance. Similar results were obtained with NOX2, another crucial member of LAP, with respect to adult macrophages. However, neonates displayed a downward trend in CFU counts when NOX2 was knocked down, which may indicate that treatment with siNOX2 alleviated ROS production and thereby assisted neonatal cells to cope slightly better with the pathogen. It is therefore necessary to study further the ability of neonates to produce ROS. Moreover, previous lab data acquired by Transmission electron microscopy (TEM) have shown that GBS is encircled by a

single-membrane structure, a characteristic feature of LAP. Overall, these findings suggest that the LAP pathway is activated in adult macrophages to facilitate GBS degradation, whereas in neonatal cells this pathway appears to be dysfunctional.

The engagement of LAP for GBS clearance in adult cells was also confirmed through confocal microscopy analysis. Rubicon knockdown, but not ULK1, increased the bacterial burden of adult cells. On the other hand, the bacterial load of neonatal macrophages did not change when Rubicon was knocked down, only slightly after ULK1 knockdown. Consequently, we shall not rule out the involvement of canonical autophagy in GBS clearance in neonatal cells although this finding objects to the previous bacterial survival experiment. It could possibly be a time-related issue as neonatal macrophages initially can't eliminate intracellular GBS due to LAP deficiency, but soon afterwards, the antibacterial autophagy (xenophagy) takes the helm. For instance, a recent publication demonstrated that internalized *Salmonella Typhimurium* cause damage to the residing vacuole, which is sensed by the v-ATPase, triggering xenophagy initiation. Thus, repetition of the bacterial survival experiment using siULK1 is required. As far as the LC3-II levels concerned, both Rubicon and ULK1 knockdown resulted in LC3-II accumulation in adult cells. Upon GBS infection, the induced LAP pathway is disrupted when Rubicon is knocked down, and thereby, lipidated LC3 levels are increased as well as the aforesaid bacterial burden. Likewise, ULK1 knockdown disrupted the basal level of autophagy, leading to elevated LC3-II levels, but did not have any impact on the bacterial load. In contrast to adult macrophages, the amount of LC3-II did not change when Rubicon was knocked down, verifying the LAP deficit of neonatal cells.

The disability of neonatal macrophages to eliminate intracellular GBS due to LAP deficiency led us to study the expression as well as the secretion of pro- and anti-inflammatory cytokines. We sought to determine whether neonates are capable of producing cytokines at levels compared to those produced by adults. Neonatal macrophages expressed under basal conditions higher mRNA levels of the pro-inflammatory genes, TNFα, IL-6 and IL-12b, compared to adult cells. Upon GBS infection, IL-6 and TNFα mRNA transcripts were increased in neonatal cells in comparison with the corresponding adult ones, whereas the opposite was observed regarding IL-12B expression levels. However, cytokine production is also controlled post-transcriptionally, and therefore, we also examined the secretion of cytokines at the protein level using the ELISA immunoassay. TNFα and IL-6 constitute early response cytokines produced in response to TLR-activating microbial stimuli, and indeed, GBS recognition in phagocytes is mediated via interactions with TLRs (Henneke et al., 2001; Mancuso et al., 2004). Interestingly, neonatal macrophages secreted extremely high levels of IL-6 both basally and following GBS infection. Additionally, TNFα production was also higher in neonatal cells compared with adult ones mostly at later time points. TNFα is characterized as a pro-inflammatory cytokine, while IL-6 is considered a multifunctional cytokine that induces the synthesis of acute phase proteins like lipopolysaccharide binding protein (LBP) and C-reactive protein (CRP), but also has anti-inflammatory and Th2-polarizing properties (Angelone et al., 2006). Neonatal macrophages had elevated IL-6/TNFα ratio during all time points after GBS infection compared to adult cells. Angelone D. and colleagues (2006) showed that the innate immune responses of human neonatal monocytes to microbial TLR agonists are also biased toward a high IL-6/TNF-α ratio *in vitro* as well as *in vivo* (Angelone et al., 2006). IL-6 production was high even in uninfected neonatal cells, which could be attributed to the initial exposure of the newborn at birth to environmental TLR agonists, including colonizing bacteria. IL-6, which is a Th2-inducer, may contribute to the disability of neonates regarding GBS elimination, rendering the newborn more susceptible to microbial infection. IL-10 production was also increased in neonatal cells compared to adult ones at later time points, especially at 6 hours post infection. According to existing bibliography, the newborn immune system produces higher levels of anti-inflammatory cytokines than pro-inflammatory cytokines (Korir et al., 2017; Kumar & Bhat, 2016). Our findings

are partially contradictory since neonatal macrophages seem capable of producing high levels of pro-inflammatory mediators upon GBS infection. Nevertheless, the innate immune responses of neonates against infectious agents is a developing field of study that should be further be explored.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

In conclusion, the data presented in this thesis identified a molecular pathway, LC3-associated phagocytosis, which is involved in GBS clearance in adult macrophages, but seems to be dysfunctional in neonatal ones. Although both cell types have similar phagocytic ability, our findings suggest that neonatal cells have reduced bactericidal capacity owing to this deficit in the LAP pathway.

ULK1 knockdown in neonatal cells showed the possible engagement of autophagy for GBS degradation. It is well established that some invading bacteria, which are restricted within vacuoles in mammalian cells, are able to disrupt the vacuole and escape into the cytoplasm. These escaped bacteria can be targeted by xenophagy (Kwon & Song, 2018). As previously stated, recent lab data acquired by electron microscopy have reported that GBS is encircled by single-membrane vacuoles, the majority of which seem to be ruptured in neonatal macrophages. Consequently, it is essential to study further whether GBS is able to escape and replicate within the cell cytosol. This has been the case for other bacteria, such *as Mycobacterium Tuberculosis*, *Listeria Monocytogenes*, *Rickettsia prowazekii* (Pizarro-Cerdá et al., 2012; Simeone et al., 2012; Whitworth et al., 2005). Understanding if GBS is capable of escaping in the host cytosol would explain the increased bacterial burden observed in neonatal macrophages. The escape could be possibly mediated via a specific toxin produced by GBS, such as β-hemolysin/cytolysin. This toxin promotes invasion by surpassing cell membrane barriers due to its cytolytic properties (A Ring et al., 2000). According to different studies, GBS β-haemolysin has immunomodulatory properties that facilitate intracellular survival of GBS (Bebien et al., 2012; Rosa-Fraile et al., 2014). In addition, the two-component system CovS/CovR, which is a major acid response regulator in GBS, has been also implicated in intracellular survival of this pathogen (Cumley et al., 2012). Apart from confirming the escape of GBS, we should also clarify the role of antibacterial autophagy regarding GBS elimination.

Furthermore, we showed that IL-6 production was greatly elevated both basally and after GBS infection in neonatal cells. IL-6 induces the transcription of STAT-3, which has been reported to upregulate the transcription of NOX2 subunits, $p47^{phox}$ and $gp91^{phox}$, promoting directly NOX2 activation (Gabrilovich & Hurwitz, 2008). As IL-6 is produced at the very beginning of the infection, the involvement of this cytokine in the efficacy of LAP should be further investigated. Nevertheless, the production of the anti-inflammatory cytokine IL-10 was also increased in neonatal cells compared to adult ones. A previous study has also shown that this immunosuppressive cytokine is produced at high levels in neonatal immune cells (Belderbos et al., 2009). This increase in IL-10 production may prime neonatal macrophages to respond in a different way to GBS infection compared to adult cells. Therefore, we should study the implication of IL-6 or IL-10 in the LAP pathway by blocking their signaling pathways in neonates and observe how this blockade affects GBS elimination.

GBS is considered a highly invasive pathogen that causes invasive disease in neonates in addition to elderly and immunocompromised adults. Currently, there are no available vaccines to provide protection against all serotypes of this pathogen. Since GBS is a leading cause of neonatal sepsis and meningitis, there is an urgent need for developing new therapeutics as well as preventatives. Therefore, a better understanding of the molecular mechanisms utilized by neonatal and adult macrophages in response to GBS infection may improve our ability to prevent and treat this primarily neonatal disease.

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