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RENATA STIFANIC

O papel do receptor C5a em um modelo murino de doença dos Legionários.

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Dissertação apresentada ao Programa de Pós-Graduação em Imunologia Básica e Aplicada da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo para obtenção do título de Mestre em Ciências.

Área de concentração: Imunologia Básica e Aplicada.

Orientador: Prof. Dr. Dario Simões Zamboni.

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The role of the C5a receptor in a mouse model of Legionnaires' disease.

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RESUMO

STIFANIC, Renata. **O papel do receptor C5a em um modelo murino de doença dos Legionários.** 2016, 93f. Dissertação (Mestrado) – Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, 2016

Legionella longbeachae é uma espécie da família *Legionellaceae* que é comumente presente no solo em diversas regiões do globo. Uma infecção por *L. longbeachae* em indivíduos imunocomprometidos causa uma pneumonia severa, frequentemente levando a hospitalização e à morte. A prevalência destas bactérias como causa de pneumonia é grande, e certamente sub-estimada, uma vez que os métodos de diagnóstico convencionais detectam apenas as espécies de *Legionella pneumophila*. A anafilatoxina C5a è uma proteína inflamatória ativada pelo complemento, a qual é envolvida no recrutamento de células inflamatórias, um processo induzido pelas células da imunidade inata que leva a dano tecidual. Dados recentes gerados no nosso laboratório sugerem que a mortalidade de camundongos após a infecção por *L. longbeachae* é causada por uma falência pulmonar, associada a indução de um intenso processo inflamatório nos pulmões dos animais infectados. Nesse trabalho, nós investigamos papel do receptor de C5a (C5aR) na replicação bacteriana e na resistência de camundongos diante de uma infecção letal por *L. longbeachae*. Experimentos realizados com animais deficientes no receptor C5a indicam que os animais são protegidos durante uma infecção letal por *L. longbeachae* em comparação com animais selvagens, da linhagem BALB/c. De acordo com esses resultados, foi detectada uma menor carga bacteriana nos pulmões dos animais $C5a^{-/-}$ em comparação com animais selvagens. Experimentos realizados com animais controles da mesma linhagem demonstraram que $C5a^{-/-}$ diferem de animais $C5a^{+/-}$, o que suporta o papel desse receptor durante a infecção por *L. longbeachae.* Dessa forma, nossos dados sugerem que a sinalização via C5aR contribui para a patogênese da doença em modelo murino da infecção por *L. longbeachae*. Os mecanismos envolvidos na patogênese mediada pelo receptor C5a encontram-se sob investigação.

Palavras chaves: *Legionella longbeachae,* receptor de C5a, pneumonia.

ABSTRACT

STIFANIC, Renata The role of the C5a receptor in a mouse model of Legionnaires' disease. 2016, 93p. Dissertação (Mestrado) – Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, 2016

Legionella longbeachae is a species of the *Legionellaceae* family that is commonly present in the soil in various regions of the globe. Infections by *L. longbeachae* in immunocompromised individuals cause severe pneumonia, often leading to hospitalization and death. The prevalence of *L. longbeachae* as a cause of pneumonia is large, and certainly under-estimated, mainly because the conventional diagnostic methods only detect *Legionella pneumophila* species. The anaphylatoxin C5a is an inflammatory protein activated by the complement system, which is involved in the recruitment of inflammatory cells, a process induced by cells of the innate immunity, which leads to tissue damage. Recent data generated in our laboratory suggest that the mortality of mice after infection with *L. longbeachae* is caused by a lung failure, associated with the induction of an intense inflammatory process in the lungs of infected animals. In this study, we investigated the role of C5a receptor (C5aR) in bacterial replication and mice resistance on a lethal infection by *L. longbeachae*. Experiments with animals deficient in the C5a receptor indicate that the animals are protected during a lethal infection by *L. longbeachae* as compared with-wild type strain, BALB/c. According to these results, a lower bacterial load was detected in the lungs of $C5a^{-/-}$ animals compared with BALB/c animals. Experiments performed with control animals of the same strain demonstrated that $C5a^{-/-}$ differ from $C5a^{+/-}$ animals, which supports the role of this receptor during infection by *L. longbeachae*. Thus, our data suggest that C5aR signalling pathway contributes to the pathogenesis of the disease in a murine model of infection by *L. longbeachae*. The mechanisms involved in the pathogenesis mediated by C5a receptor are under investigation.

Keywords: *Legionella longbeachae*, C5a receptor, pneumonia.

SUMMARY

Introduction

1. INTRODUCTION

1.1 LEGIONELLA

Legionnaires' disease was first reported in 1976 at the meeting of the American Legion in Philadelphia, Pennsylvania. Among more than 2000 attendees of a Legionnaires' convention, 221 of them acquired the disease and 34 of them died. It took further six months for the identification of the causative agent; a Gram-negative bacterium, was named *Legionella pneumophila,* and inserted into a new family called *Legionellaceae*. The name *Legionella* was given due to the legionnaires who were infected at the convention and *pneumophila* because of the meaning "lung-loving". Since then, over 50 species of *Legionella* with 64 serotypes have been identified. (McDade, Shepard et al. 1977, Terranova, Cohen et al. 1978, McDade, Brenner et al. 1979, Cherry, Gorman et al. 1982, ALTMAN 2006).

1.1.1 Legionellosis – Types of disease

The Legionnaires' disease is also known as legionellosis. The generic term "legionellosis" is used to describe *Legionella* spp. infections, which can range in severity from a mild, febrile, self-limiting form (Pontiac fever) in to a rapid and potentially fatal pneumonia (Legionnaires' disease). Since their ability to give rise to sporadic cases, outbreaks and infections that needs hospitalization and can lead even to patients death, *Legionella* spp. are a major public health problem. Moreover, some atypical cases of legionellosis, such as cutaneous infection, endocarditis or ocular involvement were recently reported (Leggieri, Gouriet et al. 2012, Heriot, Mack et al. 2014, Grimstead, Tucker et al. 2015).

1.1.1.1 Legionnaires' disease

Patients affected with Legionnaires' disease lack characteristic symptoms or signs. Unfortunately, there are no typical syndromes that characterize the disease, and not everyone exposed to the organism will develop symptoms of the disease (Goldberg, Wrench et al. 1989, Cameron, Roder et al. 1991, Hirani and Macfarlane 1997, Ruiz, Villuendas et al. 2000, Gupta, Imperiale et al. 2001, Yu, Plouffe et al. 2002, Ragull, Luisa Pedro-Botet et al. 2006). However, several clinical signs are

classically associated with Legionnaires' disease, such as symptoms of pneumonia, cough and fever. It is also known, that immunosuppression and increased age are risk factors for development of the disease (Goldberg, Wrench et al. 1989).

The disease consists on a bronchopneumonia with an incubation period of 2 to 10 days, affecting from 2 to 7% of the people infected with *Legionella* spp. and presenting mortality rate ranging from 5 to 30% (Doebbeling and Wenzel 1987). Legionnaires' disease usually worsens during the first week and can be fatal, however appropriate early treatments of the illness usually result in full recovery. Complications related to legionellosis are mostly caused by respiratory failure, shock and acute renal and multi-organ failure.

Anorexia, malaise, lethargy and in some cases mild and unproductive cough are some of the initially symptoms of Legionnaires' disease. Around 30 to 50 % of the patients develop pus-forming sputum, blood-streaked sputum or cough up blood and chest pain. Prominent are also gastrointestinal symptoms, such as diarrhea, nausea, vomiting and abdominal pains, which are present in up to half of patients. Fever or fever associated with chills develops already in the first day in almost all the cases of Legionnaires' disease. In the first week of the illness, disorders related to the nervous system, such as confusion, delirium, depression, disorientation and hallucinations are present in almost 50% of the patients (Woodhead, Macfarlane et al. 1987, Stout and Yu 1997, Muder, Stout et al. 2000, Akbas and Yu 2001, Mulazimoglu and Yu 2001).

1.1.1.2 Pontiac fever

In the case of Pontiac fever the symptoms are similar to a common flu. Unlike Legionnaires' disease, where both immunosuppressed and/or patients with increased age are risk factors for infection, Pontiac fever does not appear to discriminate between adults and children, healthy or immunocompromised individuals (Goldberg, Wrench et al. 1989). The infection is milder, self-limiting, with an incubation period of 36 hours. Almost 100% of the people that enter in contact with the bacteria are affected, but the mortality rate is near by zero (Doebbeling and Wenzel 1987).

1.1.1.3 Atypical *Legionella* infections

As mentioned above, infections with *Legionella* spp usually appear in two distinct clinical manifestations, as Pontiac fever or Legionnaires' disease. Furthermore, different atypical manifestations of *Legionella* infection have been discovered. Cutaneous infection caused by *L. pneumophila* (Padrnos, Blair et al. 2014), prosthetic joint infection caused by *Legionella micdadei* (Fernandez-Cruz, Marin et al. 2011), septic foot infection, endocarditis, ocular involvement as well as cutaneous infection in immunosuppressed patient, were caused by *L. longbeachae* (Dugar, Rankin et al. 2009, Leggieri, Gouriet et al. 2012, Heriot, Mack et al. 2014, Grimstead, Tucker et al. 2015). Some autopsy researches showed that *Legionellae* spp. can spread from the respiratory system to the body. Agreeing with that finding, *Legionellae* have been detected already in different organs such as spleen, liver, kidney, myocardium, bone and bone marrow, joints, inguinal and intrathoracic lymph nodes and digestive tract (Lowry and Tompkins 1993).

The implication of several *Legionella* spp. in cases of sinusitis, cellulitis, pancreatitis, peritonitis and pyelonephritis was demonstrated. Most of these cases were registered in immune-compromised patients. Unfortunately, the extra pulmonary infections of *Legionella* spp. are often dramatic (Eitrem, Forsgren et al. 1987, Stout and Yu 1997).

1.1.2 *Legionella* spp. – Types of organism causing disease

In 1976, not just the term Legionnaires' disease was reported, but also a new bacteria genus was characterized. Strains isolated from patients have led to the identification of *Legionella*, a new bacterial genus, classified as gammaproteobacteria, which belongs to the family *Legionellaceae* (Brenner, Steigerwalt et al. 1979).

As mentioned before, bacteria of *Legionella* genus were first identified as dangerous agents for public health in 1976. The species responsible for the first identified outbreak was *Legionella pneumophila* (*L. pneumophila*) and it remains the most common infectious agent that cause legionellosis (McDade, Shepard et al. 1977,

Fields, Benson et al. 2002). Shortly thereafter, in 1980, a new species of *Legionella*, named *Legionella longbeachae (L. Longbeachae),* was isolated from a patient with pneumonia in Long Beach, California (McKinney, Porschen et al. 1981). A second serogroup of *L. longbeachae* was discovered during the same year (Bibb, Sorg et al. 1981), however none of these reported cases of *L. longbeachae* suggested a known source of infection. Currently, more than 50 species and 70 serogroups of *Legionella* genus were discovered, and the number continues to increase. Only *L. pneumophila* comprises more than 16 different serogroups. Two serogroups are found in *L. bozemanii, L. longbeachae, L. feeleii, L. hackeliae, L. sainthelensi, L. spiritensis, L. erythra,* and *L. quinlivanii*, and a unique serogroup in each of the remaining species (Boldur, Brill et al. 1985, Hookey, Saunders et al. 1996, Riffard, Vandenesch et al. 1996, Benson and Fields 1998, Fry and Harrison 1998, Fields, Benson et al. 2002, La Scola, Birtles et al. 2004). Beside the *L. pneumophila* and *L. longbeachae*, there are *Legionella micdadei, Legionella bozemanii, Legionella dumoffii* and another 19 species from the family of *Legionellaceae*, which are associated to a varying degree with human disease. In total, 40%-50% of *Legionella* spp. are able to induce the development of disease in humans (Fields, Benson et al. 2002, Muder and Yu 2002). However, many *Legionella* spp. are not common causative agents for Legionnaires' disease and some of them have been identified only once in clinical samples. *L. pneumophila* is responsible for more than 80% of human infection and *L. longbeachae* is the second most prevalent *Legionella* specie. The other most commonly isolated *Legionella* spp are *Legionella bozemanii* responsible for 2.4 % of human infection and *Legionella micdadei, Legionella dumoffii* and *Legionella feeleii*, which combined, are responsible for 2.2 % of human infection (Yu 2002).

1.1.3 Prevalence and distribution

An international-collaborative study revealed *L. pneumophila* as a causative agent for 91% of legionellosis. *Legionella longbeachae*, as the second most commonly isolated species, was responsible for 3.9 % of these cases (Yu VL, 2002). While, in the USA and Europe, *L.pneumophila* is the leading cause of Legionnaires' disease, *L. longbeachae* is responsible for approximately 30% of legionellosis cases in

Australia and New Zealand and nearly to 50% in South Australia and Thailand (Yu, Plouffe et al. 2002, Phares, Russell et al. 2007, Phares, Wangroongsarb et al. 2007). Despite the low prevalence worldwide, *L. longbeachae* plays a significant role as a causative agent of Legionnaires' disease in the southern hemisphere and 14 of the 20 *L. longbeachae* isolates came from Australia and New Zealand (Yu, Plouffe et al. 2002).

From 1996 to 2000, 42% of the cases reported in Southern Australia were attributed to *L. longbeachae* and 51% to *L. pneumophila* (Li, O'Brien et al. 2002). Moreover, between 1999 and 2010, in western Australia 87% of the diagnosed cases of Legionnaires' disease were caused by *L. longbeachae* and just 9% of cases by *L. pneumophila* (Commission for Occupational Safety and Health, 2010). In 2011, *L. longbeachae* was the causative agent of 42% of laboratory-reported cases of infection in Southern Australia and *L. pneumophila* for only 30% (Annual Report 2011, IESRNZ).

As mentioned before, United States, Japan and Thailand reported also cases where humans were infected with *L. Longbeachae* (Okazaki, Umeda et al. 1998, Beard, Carter et al. 2000, Phares, Russell et al. 2007, Phares, Wangroongsarb et al. 2007). In 2007 in Thailand was found that in a rural district *L. longbeachae* was responsible for 5% of clinically defined cases of pneumonia, whereas *L. pneumophila* was not reported. In Europe, there are few cases of pneumonia attributed to *L. longbeachae*, although the number has increased in the last decade (Whiley and Bentham 2011). In United Kingdom, the incidence of infection with *L. longbeachae* was reported in only 11 cases since 1984 (Lindsay, Brown et al. 2012). Nevertheless, from 2008 to 2012, 26 cases of pneumonia caused by *L. longbeachae* had been reported just in Scotland (Potts, Donaghy et al. 2013, Currie and Beattie 2015).

1.1.4 Taxonomy

Legionella is one genus of the family of *Legionellaceae* (Brenner, Steigerwalt et al. 1979, Birtles, Rowbotham et al. 1996). *Legionella* spp. are gram-negative, obligate aerobe bacteria which habits natural or man-made aquatic systems (Mampel,

Spirig et al. 2006) or in potting soil (Fields, Barbaree et al. 1990, Morrill, Barbaree et al. 1990, Fields, Benson et al. 2002). The mainly source of *L. pneumophila* is in biofilms of water systems and in natural sources like rivers and lakes (Fields, Benson et al. 2002, Lau and Ashbolt 2009). In contrast, since 1989 the infections with *L. longbeachae* were linked to gardening, when the bacteria was isolated from potting soils in South Australia after an outbreak which affected 23 people. Since then, Japan, Switzerland, Greece, Scotland and the United States reported cases where *L. longbeachae* has been isolated from compost and potting mixes, but not from water (Steele, Lanser et al. 1990, Koide, Arakaki et al. 2001, Den Boer, Verhoef et al. 2007, den Boer, Yzerman et al. 2007, Casati, Gioria-Martinoni et al. 2009, Cramp, Harte et al. 2010, Velonakis, Kiousi et al. 2010, Currie, Beattie et al. 2014). *Legionella* are coccobacilli, because its shape is intermediate between cocci (spherical bacteria) and bacilli (rod-shaped bacteria). Bacteria isolated from environmental sources measure 0.3-0.9 μm in width and 0.2-2μm in length, however in tissue and clinical specimens, the organisms are coccobacillary, measuring 1-2 μm. After growth on some culture media, elongated filamentous forms of the bacteria may be seen. In a recently performed genome analyses of *L. longbeachae*, two gene clusters encoding proteins that are assumed to be involved in production of lipopolysaccharide (LPS) and/or capsule were identified. Subsequently, the presence of a capsule-like structure surrounding the bacteria was demonstrated by electron microscopy analysis. Additionally, *L. longbeache* does not encode flagella and does not possess flagellar biosynthesis genes, which are present in *L. pneumophila* (Ren, Zamboni et al. 2006, Cazalet, Gomez-Valero et al. 2010).

1.1.5 Infection by *Legionella*

As already shown in various studies, there is an inherent relation between the pathogeneses end ecology of *Legionella* spp. In 1980 it was first demonstrated that *L. pneumophila* infects *Acanthamoeba castellanii* (*A. castellanii*) and human monocytes under lab conditions and the life cycle of *Legionella* in amoeba was characterized (Horwitz and Silverstein 1980, Rowbotham 1980). Subsequently, the ability of *L. pneumophila* to avoid phagosome–lysosome fusion and consequently to intracellularly multiply in human macrophages was discovered (Horwitz 1983).

Other *Legionella* spp., such as *L. longneachae*, are also able to infect and replicate in human and murine macrophages or protozoan hosts like *Dictyostelium discoideum* (Cirillo, Falkow et al. 1994, Fields 1996, Hagele, Kohler et al. 2000, Solomon, Rupper et al. 2000, Asare and Abu Kwaik 2007, Asare, Santic et al. 2007). Moreover, *L. longbeachae* is also capable to replicate in *A. castellanii* and in mice lung. Actually, *L. pneumophila* replicates just in A/J mice, while *L. longbeachae* replicate in in A/J, C57BL/6 and BALB/c mice. The reason for that seems to be the lack of flagellin in *L. longbeachae*. Flagellin induces naip 5 -dependent caspase-1 activation that triggers pyroptosis, a pro-inflammatory cell death. According to these, the lack of flagellin in *L. longbeachae* presumably fails to activate caspase-1, which is an enzyme responsible for the cleavage and secretion of IL-β (Diez, Lee et al. 2003, Wright, Goodart et al. 2003, Molofsky, Byrne et al. 2006, Ren, Zamboni et al. 2006, Zamboni, Kobayashi et al. 2006, Lamkanfi, Amer et al. 2007, Lightfield, Persson et al. 2008, Gobin, Susa et al. 2009, Cazalet, Gomez-Valero et al. 2010). This finding may explain the different mouse susceptibilities to *L. pneumophila* and *L. longbeachae* infection. Both *L. pneumophila* and *L. longbeachae* encode pilus-like structures. In addition, *L. longbeachae* encodes a chemotaxis system, a capsule-like structure, a long pilus-like structure, but no flagella. In contrast, *L. pneumophila* encodes flagella but no capsule-like structure, no long pilus-like structure, and no chemotaxis system. Moreover, little is known about the uptake and phagocytosis of *Legionella* spp. by macrophages. *L. pneumpohila* as well as *L. longbeachae* multiply rapidly inside macrophages, but when phagocytic activity is inhibited the bacteria failed to growth (Elliott and Winn 1986). Taken together, there are notable similarities in the processes by which *L. pneumophila* and *L. longbeachae* infect protozoa and mammalian phagocytic cells (Horwitz 1984, Bozue and Johnson 1996, Faulkner and Garduno 2002). Furthermore, there are also several differences in the genome analysis and the virulence mechanisms, which are an interesting aspect of research in order to understand these particular features of the two *Legionella* species.

1.1.6 *Legionella* life cycle

The virulence mechanisms of *L. pneumophila* and *L. longbeachae* provide the ability to infect and multiply within the cells. In contrast to *L. pneumophila*, the

intracellular life cycle and the complexity of the virulence mechanisms of the *L. longbeachae* are still poor understood. Using the flagella, *L. pneumophila* reaches the host cell surface where the adhesion is triggered through self- produced adhesins and the Icm/Dot secretion system-dependent uptake takes place (Hilbi, Segal et al. 2001, Watarai, Derre et al. 2001, Hoffmann, Harrison et al. 2014). In alveolar macrophages *L. pneumophila* replicates inside a unique phagosome, which excludes early and late endosomal markers, resist fusion with lysosomes, but recruits endoplasmatic reticulum (ER) and mitochondria. Inside of this protective or *Legionella* containing vacuole (LCV), *L. pneumophila* is able to replicate and down-regulate the expression of virulence factors. It is believed that the nutrient limitation inside the protective vacuole induces the bacteria to express many virulence-associated factors. This transition to the transmissive phase allows the release of bacteria out of the host cell and infecting new host cells (Molofsky and Swanson 2004, Cazalet, Gomez-Valero et al. 2010). Therefore, *L. pneumophila* undergoes a biphasic life cycle in which it switches from a transmissive and virulent, named post-exponential phase, to a replicative and non-virulent, named exponential phase. Similarly, *L. longbeachae* also exhibits two developmental stages characterized as exponential and post exponential growth phase. In order to characterize the biphasic life cycle of *L. longbeachae*, the gene expression program was investigated in the exponential and post exponential growth phase. *L. longbeachae* up regulates more than 300 genes in the post-exponential phase, of which 208 have no orthologs in *L. pneumophila*. For 70% of these genes no function was still discovered. Consequently, the post-exponential phase of *L. longbeachae* is distinct from the one of *L. pneumophila*. Unfortunately, the invasion strategies of *L. longbeachae* are not well known. The chemotactic system enables to *L. longbeachae* to find favorable conditions by migrating towards higher concentrations of attractants (Cazalet, Gomez-Valero et al. 2010).

1.1.7 Secretion system in *Legionella* spp.

Secretion system is the transport or translocation of effector molecules such as proteins, enzymes or toxins from the interior of a bacterial cell to its exterior. It is a very important adaption and survival mechanism for bacterial functioning and operation in the surrounding environment trough efficient and rapid delivery of

effector molecules into the phagocytic host cell. Two different types of secretion system are encoded by *L. longbeachae*; type II secretion system (T2SS) and type IV secretion system (T4SS). The Lsp type II secretion system (T2SS) and the Dot/Icm type IVB secretion system (T4BSS) are both found in *L. pneumophila* and *L. longbeachae*. However, *L. longbeachae* is missing 45% of the type II secretion system substrates, which are described for *L. pneumophila*.

L. longbeachae encodes four T4SS; the T4BSS, the T4ASS present on the plasmid and two more, which are embedded on putative mobile genomic islands (GI) in the chromosome (Cazalet, Gomez-Valero et al. 2010). The Dot/Icm T4SS of *L. longbeachae* and *L. pneumophila* makes part of T4BSS and is T4ASS independent. The Dot/Icm T4SS is necessary for the efficient formation of replicative vacuole and for the transport of effector proteins into the host cell (Berger and Isberg 1993, Roy, Berger et al. 1998, Vogel, Andrews et al. 1998, Vincent, Friedman et al. 2006). The T4BSS present in *L. longbeachae* displays 47% to 92% conserved protein identities and gene organization when compared with the T4BSS of *L. pneumophila* (Morozova, Qu et al. 2004, Gomez-Valero, Rusniok et al. 2011). Interestingly, in 1998 two different laboratories discovered around 20 genes required for establishment of the replicative niche, intracellular replication, or macrophage killing. These genes have two independent names; defect in organelle trafficking (dot) and/or intracellular multiplication (icm). This happens because of the simultaneous discovery of the genes from two different research groups (Purcell and Shuman 1998, Roy, Berger et al. 1998, Segal and Shuman 1998, 2007). In 2002 it was demonstrated the ability of the Dot/Icm system to translocate effector proteins into the host cell cytosol (Nagai, Kagan et al. 2002). Moreover, over hundred proteins, which are translocate via the Dot/Icm system into the host cytosol have been discovered (Hubber and Roy 2010). Twenty-seven proteins build up the Dot/Icm T4BSS system of *Legionella*; 5 of them are present in the cytoplasm, 16 are attached to the inner and 4 on the outer membrane of the replicative vacuole and one is a periplasmic protein (Vincent, Friedman et al. 2006). Today is well established that Dot/Icm T4BSS in *L. pneumophila* posses genes called *dot* or *icm,* which code for a membrane transport complex necessary for substrate translocation and intracellular replication (Berger and Isberg 1993, Brand, Sadosky et al. 1994, Swanson and Isberg 1996, Segal and Shuman 1997, Vogel,

Andrews et al. 1998, Qiu and Luo 2013). The *L. longbeachae dotA* mutant is strongly attenuated for intracellular growth in *A. castellanii* as well as for pulmonary mouse infection confirming that the *Dot/Icm* type IV secretion system is essential for *L. longbeachae* virulence (Cazalet, Gomez-Valero et al. 2010). According with this finding, data from our group demonstrate that the virulence of *L. longbeachae* in C57Bl/6 mice model of *Legionella* infections is mediated by lethal lung damage in Dot/Icm-dependent manner. Moreover, the lung damage induced by infection or lung injury is often correlated with complement system activation. Furthermore in the last years the importance of the complement system in the context with pneumonia and acute lung injury has become a focus of several researches.

1.2 COMPLEMENT SYSTEM

The complement system is an innate immune surveillance and effector mechanisms of humoral immunity, important not just in the defense against pathogens, but also in host homeostasis and inflammation. The complement system is composed of more than 40 fluid phase or plasma proteins and membrane proteins expressed on cell surface (Walport 2001). The plasma proteins are mostly synthetized in the liver and found in the blood, lymph, and interstitial fluids; while the membrane proteins are expressed on cell surface, and include receptors and regulators of complement activation fragments(Ehrlich 1900, Muller 1911, Nesargikar, Spiller et al. 2012). Microorganism which trespass the host barriers and invade deeper tissues are detected by fluid phase complement proteins, which, in turn, leads to the activation of the complement proteolytic cascade and to the elimination of the microbial target. The elimination of the microbial targets is the consequence of the complement proteolytic cascade activation and formation of the membrane attached complex (MAC) (Nilsson and Mueller-Eberhard 1965, Nilsson 1967, Hadding and Muller-Eberhard 1969, Manni and Muller-Eberhard 1969, Morgan 1999). The MAC build pores on the cell or microbial surface, thus inducing the cell lysis and consequently leads to death (H 1891, Skarnes and Watson 1957). Three different pathways are responsible for the activation of complement; classical, alternative and the lectin pathway, each leading to a common terminal pathway (Figure 1) (Pillemer, Blum et al. 1954, Pillemer 1955, Kawasaki, Etoh et al. 1978, Kozutsumi, Kawasaki et al. 1980). Independent of which pathway was activated, the generations of the enzimatic complexes of C3 and C5 convertase occur. C3 convertase cleaves the C3 into the functional fragments C3a and C3b. Subsequently the strong activation of C3 convertase and the accumulation of C3b induce the formation of C5 converatse which cleave the C5 into functional fragments C5a and C5b. The formation and deposition of C5b on the target membrane surface induces the formation of the MAC and the target lysis. The opsonin C3b can bind covalently, tag any surface in the immediate proximity to the site of its generation, and promote phagocytosis by neutrophils, monocytes and mast cells (Walport 2001, Walport 2001, Nesargikar, Spiller et al. 2012). The C3a and C5a are inflammation mediators and promote cell migration to

the site of infection (Ricklin, Hajishengallis et al. 2010). Nevertheless, complement system does not have just the function of innate and systemic defense against pathogens. Complement system plays a central role in regulation of innate and adaptive immunity as well as generative, degenerative, and regenerative processes (Carroll 1998, Carroll 2004). Moreover, complement is activated within cells, engages intracellular complement receptors, and interacts with several other cell effectors such as growth factor receptors, inflammasomes and metabolic pathways(Hawlisch, Belkaid et al. 2005, Bosmann, Patel et al. 2011, Arbore and Kemper 2016, Cumpelik, Ankli et al. 2016). All of these findings confirm the importance of the complement system and the necessity to discover more about.

1.2.1 Pathways of complement activation

As mentioned before, there are three main pathways of complement activation, which differs from each other primarily in their way of activation (Figure 1). The classical pathway was discovered first. However, the alternative pathway is phylogenetically older. The classical pathway is activated by specific types of antibodies, which are bound to antigens. On the other hand, the alternative pathway is activated in the absence of antibody on the microbial cell surface. The lectin pathway is activated by binding of microbial polysaccharides to circulating lectins. The classical pathway is an important component of the humoral immunity, while the alternative and lectin pathways are effector mechanism of innate immunity.

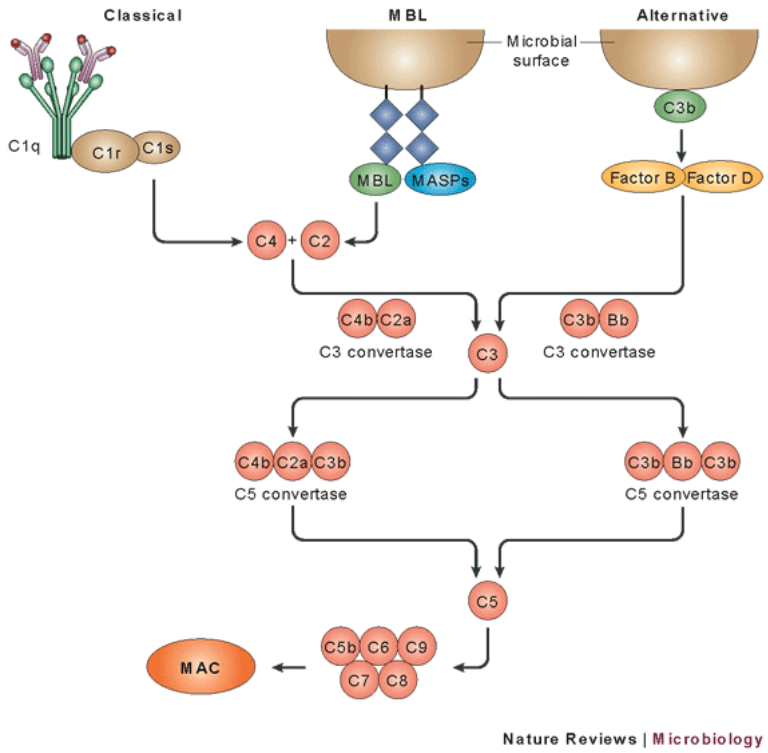


Figure 1. Pathways of complement activation and the late steps of complement activation (Connolly and Benach 2005).

1.2.1.1 The alternative pathway

The alternative pathway of complement activation begins with the proteolysis of C3 in to C3a and C3b and the stable attachment of the C3b to microbial surfaces, without the antibody involvement (Pillemer, Blum et al. 1954, Pillemer 1955, Pillemer, Schoenberg et al. 1955, Ehrnthaller, Ignatius et al. 2011). The C3 protein is constitutively expressed and found in the plasma and other body fluids as well as other plasma proteins that build up the complement system. Moreover, the plasma C3 protein is being continuously cleaved to its breakdown products; the C3a and C3b. This continuously cleavage of C3 is called C3 tickover (Muller-Eberhard 1988, Thurman and Holers 2006). The C3 protein contains reactive thioester bond, which is protected inside of the thioester domain. When the C3 is cleaved, the C3b fragment undergoes a conformational change and the thioester domain became exposed (Muller-Eberhard 1988, Dodds, Ren et al. 1996, Law and Dodds 1996). The thioester group reacts with proteins or polysaccharides found on the cell or microbial surface and forms amide or ester bonds (Dalmasso and Muller-Eberhard 1967, Muller-

Eberhard, Polley et al. 1967, Johnston, Klemperer et al. 1969, Nicholson, Brade et al. 1974). When the C3b fragment do not attach to the membrane surface of some cells or microbes, it remains in the fluid phase and the thioester domain becomes rapidly hydrolyzed inducing the C3b inactivation (Merle, Church et al. 2015). In contrast, when the C3b is covalently bound to the cell or microbial surface it can interact and bind through covalent interactions to the B factor. Subsequently, the B factor is in turn cleaved by factor D releasing a small fragment Ba. The major Bb fragment remains covalently attached to the C3b. The cell surface protein complex C3bBb represents the C3 convertase of the alternative pathway, which function is to cleave more C3 molecules into C3a and C3b (Ehrnthaller, Ignatius et al. 2011, Nesargikar, Spiller et al. 2012). Interestingly, when C3b is generated by the classical pathway, it can still bind the factor B and induce the formation of the C3bBb that is the C3 convertase (Law and Dodds 1996). Therefore, the function of the C3 convertase is to amplify the complement activation when initiated by the alternative, classical or even the lectine pathway. The amplification loop induces the production of many C3b proteins, which in turn can bind to the C3 convertase and build the C3bBbC3b protein complex (Merle, Church et al. 2015). This protein complex, attached to the membrane, represents the alternative pathway C5 convertase. The C5 convertase can cleave the C5 in to C5a and C5b and in this way induce the late steps of complement formation.

1.2.1.2 The classical pathway

The classical pathway is activated by IgG and/or IgM molecules, which could be present as antigen-antibody complex or as membrane bound antibodies. The classical pathway of complement activation begins when the C1q protein binds to the Fc region of IgG or IgM or another ligand. The C1q is a hexamer made up of six chains that recognizes and specifically binds to the Fc CH2 domains of the γ heavy chain and CH3 domain of the μ heavy chain (Reid 1989). In order to be activated C1q must bind to at least two heavy chains, thus the free circulating antibodies are not capable to activate the C1q protein. Since each antibody has only one Fc region, in order to activate the classical pathway, multiple IgG molecules must be brought together on a multivalent antigen. In the case of the pentameric IgM, the C1q

activation is prevented because of the planar configuration of the FC regions. When an IgM molecule binds to an antigen, the Fc conformation of the pentameric IgM changes and allows the access of the C1q to the Fc regions. Binding of the C1q to the Fc regions of IgG or IgM activates the serine protease C1r that in turn cleaves and activates the serine protease C1s (Ravetch and Bolland 2001, Walport 2001, Walport 2001). The next protein of the cascade is C4, which is homologus to C3. C4 is cleaved, by the activated C1s and generates the C4a and C4b. The C4b protein is similar to the C3b, because of the thioester bond, which binds to the antigen-antibody complex or to the cell surface trough covalent amide or ester bonds(Muller-Eberhard 1988). Once, the C4b protein is attached to the cell surface or immune complex, the C2 protein can bind to the C4b and subsequently be cleaved by the CIs molecule. The formed proteins are the soluble C2a and the larger C2b, with the latter remaining bound to the C4b. In this way, the C4b2b complex is formed on the cell surface, which corresponds to the classical pathway C3 convertase (Walport 2001, Merle, Church et al. 2015). The C3 convertase (C4b2b) has the ability to bind and proteolitically cleave the C3 proteins into C3a and C3b. In this process, the C3 convertase, which is build up of C4b2b cleaves the C3, where the C4b is important for the binding of C3 and the C2b catalyzes the C3 proteolysis. Once the C3b is formed, it can covalently bind to the cell surface or with the antibody where the classical pathway was initiated. Subsequently, C3b can bind the factor B and generate more C3 convertase (Bottomley and Muller-Eberhard 1988). As described before, the alternative pathway is responsible for the generation and amplification of the C3 convertase. These repetitive enzymatic steps can lead to the deposition of many C3b on the cell surface and subsequently activate the complement system. The deposition of C3b to the cell surface, and the generation of C3 convertase represent the early steps of the complement activation. In the alternative and classical pathway the early steps are analogous (Merle, Church et al. 2015). The C3 of the alternative pathway is homologous to the C4 in the classical pathway and the B factor of the alternative pathway is homologous to the C2 of the classical pathway (Merle, Church et al. 2015, Merle, Noe et al. 2015). The late steps of the classical pathway initiate with the formation of the C5 convertase. This occurs, when the C3b molecule binds to the C3 convertase, and build on the cell surfaces the C4b2b3b (or the alternative pathway C3bBbC3b) protein complex. This C4b2b3b protein complex represents the C5 convertase and it cleaves the C5 into the C5a and C5b fragment. The proteolytical

cleavage of C5 represents the initiation of the late steps of the complement activation (Morgan 1999).

1.2.1.3 The lectin pathway

The lectin pathway of the complement system is induced when the polysaccharides, found on the cell membrane of microorganisms, bind to circulating lectins (Kawasaki, Etoh et al. 1978, Kozutsumi, Kawasaki et al. 1980). Lectins are carbohydrate-binding proteins such as C-type lectins, mannose binding lectin (MBL), and N-acetylglucosamine recognizing lectins. The most well-characterized target recognition molecule of the lectin pathway is the MBL, which recognizes carbohydrates (Diepenhorst, van Gulik et al. 2009). The MBL as well as other soluble lectins are members of the collectin family and posses a structure similar to the C1q protein (Petersen, Thiel et al. 2000, Thiel, Petersen et al. 2000, Vorup-Jensen, Petersen et al. 2000). Mannose residues present on the polysaccharides of microorganisms are bound by the MBL. The MBL simultaneously bind to the MBL-associated serine protease (MASPs) such as MASP-l, MASP-2, and MASP-3 and small MBL-associated protein (sMAP) (Matsushita, Thiel et al. 2000, Hajela, Kojima et al. 2002). Oligomers of MBL associate with MASP-2 and MASP-3 and sMAP. In this conformation MAPS-2 initiate the lectin pathway by cleavage of C4 and C2 and the C4b2b complex (which correspond to C3 convertase) is formed (Matsushita, Thiel et al. 2000, Hajela, Kojima et al. 2002). As described in the classical pathway, the C3 convertase cleaves the C3 protein into the C3a and C3b fragments what leads to the formation of C5 convertase and the induction of the late steps of the complement activation.

1.2.2 The late steps of complement activation

The late steps of complement activation initiate by the generation of C5 convertase through the alternative, classical or lectin pathway. Subsequently, the C5 convertase cleaves C5 into C5a and C5b fragments. The C5b fragment remains bound to the complement proteins, which are deposited on the membrane surface (Morgan 1999, Morgan 2000, Song, Sarrias et al. 2000). The C5b fragment has the ability to

bind the next proteins of the cascade; C6 and C7. The hydrophobic C7 component has the ability to inserts into lipid bilayer of cell membranes. In this way, the C7 can bind the C8 protein with high affinity. The C8 is build up of 3 chains of which one binds to the C5b,6,7 complex and simultaneously to the second chain of the C8 and the third is inserted into the lipid bilayer of the cell membrane. The C5b-8 complex is a stable protein complex situated on the membrane surface with two tails, the C7 and C8, which anchored the protein complex into the lipid bilayer of the cell membrane (Morgan 1999). In order to fully form the active MAC, the serum proteins C9 are required (Tegla, Cudrici et al. 2011, Tegla, Cudrici et al. 2011). C9 posses the ability to polymerize and form pores in the plasma membranes. However, up to 15 C9 serum proteins are required to polymerize around the C5b-8 complex in order to form the MAC. Consequently, the MAC induces pores in the membrane and allows the free movement of water and ions into the cell and induces cell lysis (Koski, Ramm et al. 1983, Ramm, Whitlow et al. 1983).

Besides the MAC formation and lysis of the target cell, the complement system posses other side effects induced by the broad spectrum of fragments arose during the early steps of complement activation. The complement system and its derivatives are involved in the development of many inflammatory and immunological diseases, such as sepsis, acute respiratory distress syndrome, rheumatoid arthritis, glomerulonephritis, multiple sclerosis, ischemia-reperfusion injury, and asthma (Linton and Morgan 1999, Welch 2002, Welch, Frenzke et al. 2002, Albrecht and Ward 2004, Arumugam, Woodruff et al. 2004, Glovsky, Ward et al. 2004, Hawlisch, Wills-Karp et al. 2004). Complement protein split products, especially C3a and C5a, known also as anaphylatoxines, promote phagocytosis, induce cell migration, cell activation, and appear to be responsible for promoting and perpetuating inflammatory reactions.

1.2.3 The anaphylatoxins and its receptors

The C3a and C5a complement protein fragments are also known as anaphylatoxins, because of their ability to activate the mast cells to induce anaphylaxis (Drouin, Kildsgaard et al. 2001). However, it is known that C3a and C5a

have a broad spectrum of functions and its pro-inflammatory abilities are already well established (Sacks 2010). The C3a and C5a complement protein fragments are obtained by the proteolytic cleavage of C3 and C5, respectively, during the complement activation (Song, Sarrias et al. 2000, Amara, Rittirsch et al. 2008). With just 77 amino acids (C5a) and 74 amino acids (C3a), these small polypeptides have important physiological functions. Moreover, they share 36% of aminoacid identity; both are cationic with an almost identical conformational structure, but just the C3a peptide exercise antimicrobial function (Nordahl, Rydengard et al. 2004, Sonesson, Ringstad et al. 2007, Pasupuleti, Walse et al. 2008). C3a and C5a exercise their function by binding the respective receptors. The C3a interacts with the C3a receptor (C3aR) (Nettesheim, Edalji et al. 1988, Daffern, Pfeifer et al. 1995), while the C5a can interact with two different receptors; the C5a receptor (C5aR/CD88) and the C5a receptor-like 2 (C5aL2) (Gerard, Hodges et al. 1989, Bamberg, Mackay et al. 2010, Bosmann, Haggadone et al. 2013). The C3aR is expressed on neutrophils, monocytes/macrophages, mast cells, hepatocytes, bronchial and alveolar epithelial cells, vascular endothelium, and among other cells (Klos, Bank et al. 1992, Daffern, Pfeifer et al. 1995, Zwirner, Werfel et al. 1998, Gutzmer, Lisewski et al. 2004).

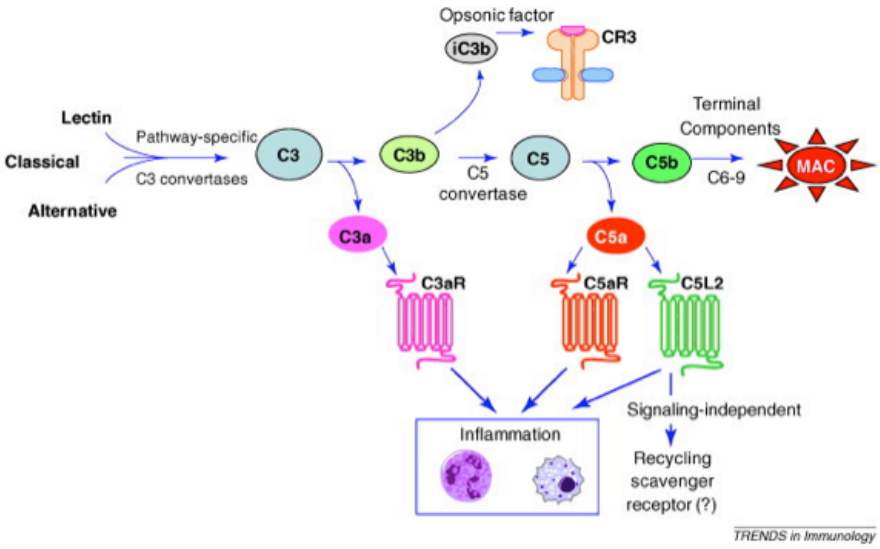


Figure 2. Anaphylatoxin C3a and C5a are complement protein fragments obtained by the proteolytic cleavage of C3 and C5 during the complement activation (Hajishengallis and Lambris 2010).

Interestingly, once released, the C5a and C3a undergoes the cleavage of Arg in the C-terminal end by serum carboxypeptidases. The desArg form of C5a and C3a posses a two to three orders of magnitude lower cell-stimulating activity. Furthermore, C5a-desArg maintains 1–10% of the inflammatory activity of C5a, while C3a-desArg lacks the pro-inflammatory activity (Sayah, Jauneau et al. 2003). Moreover, if there is some impairment, by the process of cleavage from C5a into C5a desArg form, it leads to a substantial increase in inflammation in the joints (Matthews, Mueller-Ortiz et al. 2004, Song, Hwang et al. 2011).

The anaphylatoxins are potent inflammatory mediators and act on immune and non-immune cells. These small proteins can increase the permeability of small blood vessels, induce contraction of smooth muscles as well as regulate vasodilatation. C5a and C3a can trigger the oxidative burst in macrophages, neutrophils, and eosinophils as well as the histamine release in mast cells and basophils (Murakami, Imamichi et al. 1993, Elsner, Oppermann et al. 1994, Elsner, Oppermann et al. 1994) (Kretzschmar, Jeromin et al. 1993). Mast cell migration is also mediated trough C5a and C3a gradient. Furthermore, unlike C3a, C5a induces macrophage and neutrophils migration as well as B and T cell activation (Lett-Brown and Leonard 1977, Ehrengruber, Geiser et al. 1994, Hartmann, Henz et al. 1997, Nataf, Davoust et al. 1999, Ottonello, Corcione et al. 1999). As already described the C5a protein is released during the C5 activation, and exerts its effects through the high-affinity C5a receptors; the C5aR/CD88 and the C5L2. The C5aR is a G-protein coupled receptor, built of seven transmembrane segments, which belongs to the rhodopsin family; in contrast, the C5L2 is not a G-protein-coupled receptor. Immune and non-immune cells express both of the C5a receptors, but the C5aR is expressed in much higher levels compared to C5L2.

It is already known that the C5a interacts and binds to the C5L2 with high affinity, but its exact role remains unclear (Gerard, Hodges et al. 1989, Gerard and Gerard 1991, Ohno, Hirata et al. 2000, Okinaga, Slattery et al. 2003, Holers 2014). Recently, it was described that the C5L2 receptor posses pro-inflammatory activity in sepsis and allergic asthma (Rittirsch, Flierl et al. 2008, Zhang, Schmudde et al. 2010). In contrast the C5L2 seems to be important in the development of Th17 cells, which are involved in asthma and autoimmune arthritis (Lajoie, Lewkowich et al. 2010). The

C5a-C5aR interactions induce an increase in the intracellular calcium that leads to the cell activation and triggers the intracellular signaling cascades. Depending of the cell type, these events lead to induction of several functional responses such as recruitment and activation of inflammatory cells, enhancement of cell adhesion and molecule expression, delayed or enhanced apoptosis, phagocytosis, oxidative burst, release of granule enzymes, histamine secretion and release, cytokine and chemokine production, vasodilatation and chemotaxis (Guo and Ward 2005). It is already shown that C5L2 can also be important in the regulation of the extra-cellular concentration and bioavailability of C5a in order to limit the C5a induced pro-inflammatory response. Furthermore, the C5L2 can negatively modulate the C5aR signalling (Bamberg, Mackay et al. 2010). Taken together, the role of C5L2 receptor and its signaling pathway is controversial and still not well understood (Woodruff, Nandakumar et al. 2011).

The expression of the C5aR was first discovered on myeloid cells such as neutrophils, eoosinophils, basophils, and monocytes. However, recent studies demonstrate the expression of C5aR on various non-myeloid cells of many organs, especially lung and liver (Chenoweth and Hugli 1978, Gerard, Hodges et al. 1989, Kurimoto, de Weck et al. 1989, Werfel, Oppermann et al. 1992). The C5aR signaling plays an important role in pathogenesis of sepsis and development of multiple organ failure and many inflammatory diseases are attributable to the C5a activity. The activation of C5aR in sepsis inhibits the neutrophil functions, promote cardiomyophaty, and induce the apoptosis of thymocytes (Huber-Lang, Sarma et al. 2001, Niederbichler, Hoesel et al. 2006). The C5aR is also significantly up regulated in several organs such as the lung, liver, kidney, heart and thymus and the absence of C5aR and C5L2 in sepsis improves survival (Strunk, Eidlen et al. 1988, Gasque, Chan et al. 1995, Haviland, McCoy et al. 1995, Lacy, Jones et al. 1995, Drouin, Kildsgaard et al. 2001, Riedemann, Guo et al. 2002, Riedemann, Guo et al. 2002, Rittirsch, Flierl et al. 2008).

The C5aR plays also a key role in the development of the innate immune response. As already described, the stimulation of C5aR on neutrophils and monocytes induce the oxidative burst. In a whole blood model of *Escherichia coli (E. coli)* infection, the C5aR is important for the induction of the oxidative burst and

indirectly for the induction of phagocytosis of *E. coli*. Therefore, the opsonisation and phagocytosis of *E. coli* are dependent of the activation of C5aR. Similarly, by blocking the C5aR pathway, the oxidative burst induced by *Neisseria meningitides* is prevented and subsequently the phagocytosis of *N. meningitides* is disabled. Mice deficient for the C5aR exhibits an increase of inflammatory cells, reduced survival as well as reduced bacterial clearance in the lung after *Pseudomonas aeruginosa* infection (Hopken, Lu et al. 1996, Rhein, Perkins et al. 2008). Thus, the host defenses in the lung, as well as other organs, are influenced by C5a-C5aR signalling pathway.

1.2.4 Legionella and the complement system

Legionella has evolved mechanisms able to infect both mammalian cells and aquatic protozoa and consequently use them as a replication niche. Nevertheless, the ability to multiply within alveolar macrophages and monocytes as well as the ability to evades the antimicrobial defences of mononuclear phagocytes, because phagosomes that contain legionellae fail to fuse with lysosomes, makes legionella a high pathogenic bacterium (Horwitz M. A. 1983, Horwitz M. A. and S. C. Silverstein 1980). The uptake of *L. pneumophila* by mononuclear phagocytes is independent of opsonizing antibodies and other serum components. *Legionellae* incubated in human serum are phagocytosed at a much greater rat (Horwitz M. A. and S. C. Silverstein 1981, Horwitz M. A. and S. C. Silverstein 1981, King C. H. 1991). The ability to directly bind C1q independently of antibody indicates that *Legionellae* can activate the classical pathway in an antibody-independent manner (Clifford S. Mintz, 1995). Phagocyte complement receptor CR1 and CR3 as well as complement component promote the uptake of *L. pneumophila* by alveolar macrophages and monocytes (Payne N. and Horwitz M. A 1987). In addition to immune antibody, the classical pathway of complement plays an important role in the opsonization and phagocytosis of *L. pneumophila*. (H. A. Verbrugh, 1984). Furthermore, the ability of lipopolysaccharide (LPS), present on the surface of *L. pneumophila*, to interact and activate the complemen tsystem suggests a role for LPS in the uptake of *L. pneumophila* by mononuclear phagocytes (Clifford S. Mintz, 1992).

Little is known about the *L. longbeachae* uptake and pathogenesis. Studies in mouse model of Legionnaire's disease have demonstrated a high virulence of *L. longbeachae* (Pereira, Marques et al. 2011). A comparison in the susceptibility of different mice strains demonstrated that C57Bl/6 mice, that are resistant to *L. pneumophila* infection, are highly susceptible to *L. longbeachae* infection (Asare, Santic et al. 2007, Pereira, Marques et al. 2011). In a recent evaluation of a mouse model of *Legionella* infections, it was demonstrated that *L. longbeachae* is significantly more virulent then *L. pneumophila*. This virulence is mediated by fatal lung damage in a Dot/Icm-dependent manner (Liliana M Massis in preparation). Complement system activation has been demonstrated in many form

s in lung damage induced by infection or acute lung injury. The concentration of the anaphylatoxin C5a in the bronchoalveolar fluid increases during acute lung injury. C5a can directly attract neutrophils into lung, and it can also activate neutrophils, macrophages, and endothelial cells and is essential for the full development of acute lung injury. However, in a mouse model of intrapulmonary *Pseudomonas aerugionosa* infection, the presence of the C5aR was crucial for mouse recovery and for mucosal host defence in the lung (Uta E. Höpken, nature, 1996). Because of the importance of the C5aR during damage or infection induced lung injury, we decide to evaluate the role of the C5a receptor in a lethal model of mouse Legionnaires' disease.

Objectives

2. OBJECTIVE

2.1 General objective

To evaluate the role of the C5a receptor in a lethal model of mouse Legionnaires' disease.

2.2 Specific objectives

→ To evaluate the effect of C5aR in mouse survival in a lethal model of *L. longbeachae* infection.

→ To evaluate replication of *L. longbeachae* in the lungs of wild-type and $C5aR^{-/-}$ mice.

→ To evaluate the production of cytokines and recruitment of inflammatory cells to the lungs of wild-type and $C5aR^{-/-}$ mice in response *L. longbeachae* infection.

→ To evaluate the mouse resistance to *L. longbeachae* infection using littermate control mice $C5aR^{-/-}$ and $C5aR^{+/-}$.

Material and methods

3. MATERIAL AND METHODS

3.1 Mice

The experiments were performed on BALB/c mice housed in the animal care facility of the Ribeirao Preto Medical School. C5a-receptor-deficient mice ($C5aR^{-/-}$) on a BALB/c background were maintained in Center of Special Animal Care of Ribeirao Preto Medical School. The heterozygote $C5aR^{+/-}$ (F_1) mice were generated by mating BALB/c with $C5aR^{-/-}$ mice. The F_2 littermate control mice ($C5aR^{-/-}$ (F_2-) and $C5aR^{+/-}$ (F_2+)) were generated by backcrossing the F_1 heterozygote with $C5aR^{-/-}$ mice. To identify F_2+ heterozygote ($C5aR^{+/-}$) and F_2- homozygote ($C5aR^{-/-}$) animals, ear DNA genotyping with a following sets of primer was performed:

5`GGT CTC TCC CCA GCA TCA TA 3' wild type forward

5`GGC AAC GTA GCC AAG AAA AA 3' common

5`GCC AGA GGC CAC TTG TGT AG 3' mutant forward.

PCR assays were performed in a final volume of 10 µl buffer consisting of 10X PCR buffer (Fermentas), 2 mM MgCl2 (Thermo), 0.2 mM dNTPs (Fermentas), 1 µM each primer (Sigma), 0.06 µl Taq polymerase (5 U/µl, Thermo) and 2 µl genomic DNA. PCR conditions were: 3 minutes (min) at 94°C; followed by 35 cycles of 30 seconds at 94°C, 1 min at 63°C, and 1 min at 72°C, following by 2 min at 72°C. The reaction products were analyzed on a 1 % agarose gel.

Six to 12 weeks old male or female mice were used for the experiments. The care and mouse protocol were in compliance with the institutional guidelines on ethics in animal experiments approved by CETEA (Comissão de Ética em Experimentação Animal da Faculdade de Medicina de Ribeirão Preto; approved protocol number 126/2015).

3.2 Bacterial culture

Legionella longbeachae, strain NSW150 streptomycin resistant (DZ420), was cultivated in charcoal yeast extract agar (BCYE: 1% yeast extract, 1% N-(2 acetoamido)-2-aminoethaneosulfonic (ACES) pH 6.9, 3.3 mM L-cysteine, 0.33 mM $Fe(NO)_3$, 1.5% bacto agar and 0.2% activated charcoal). For the *in vivo* and *in vitro* infections, bacteria were grown at 37℃ in BCYE agar plates. After 48 hours, the

bacteria were diluted in sterile RPMI 1460 (Sigma-Aldrich) and the OD at 600 nm was measured in a spectrophotometer (Bio - Photometer; Eppendorf, Hamburg, Germany) to determine the bacterial concentration in the solution, that was previously determined that the OD 600nm = 1 correspond to 10^9 bacteria.

3.3 Bone marrow derived macrophages and bacterial multiplication *in vitro*

Bone marrow cells were collected from femurs and differentiated with RPMI 1640 (Gibco), 10% FBS (Invitrogen), 30% L-929 cell-conditioned media (LCCM), 2 mM L-glutamine (Sigma-Aldrich) and 100 U/ml penicillin–streptomycin (Sigma- Aldrich) cultivated at 36 °C, 5% CO2. BMDMs were collected and seeded on tissue culture plates and kept in RPMI 1640 media 10% FBS, 5% LCCM and 2 mM L-glutamine (Marim, Silveira et al. 2010). To measure intracellular multiplication, BMDMs were seeded at a density of 2 x 10^5 cells/well into 24-well tissue culture plates well in 1 ml RPMI 1640, 10% FBS, 5% L-929 cell-conditioned media (LCCM), 2 mM L-glutamine. Each macrophage sample was plated in triplicate. These cells were then allowed to adhere overnight before infection. Cultures were infected at an MOI of 0.015, followed by centrifugation for 5 min at 1200 rpm at room temperature and incubation at 37℃ in a 5% CO2 atmosphere. For CFU determination, the cultures were lysed in sterile water after 24, 48, and 72 hours of infection; then, the cell lysates were combined with the cell culture supernatants from the respective wells. Lysates plus supernatants from each well were diluted in RPMI 1460, plated on BCYE agar plates, and incubated for 4 days at 37℃ for CFU determination.

3.4 In vivo assays

3.4.1 In vivo infection

For in vivo infections, the mice were anesthetized with ketamine and xylazine (90 mg/kg and 5 mg/kg, respectively) by intraperitoneal administration (i.p.), followed by intranasal administration of 40 µL of RPMI containing 5x10^3, 10^4, 5x10^4 or 10^5 bacteria per mouse. Control groups received 40 µL of RPMI by nasal route.

3.4.2 Mice survival

After infection, performed as described above, the survival rates and the body weight were determined by daily examination of the infected animals for a period of 15 days. After this interval the surviving mice were euthanized. The mice that died during the period were recorded for the animal's survival curve.

3.4.3 Colony Forming Units determination

After 4 48, 72 and 96 hours of infection the animals were euthanized and lungs were removed. The harvested lungs were homogenized in 5 mL of sterile RPMI for 30 seconds, using a tissue homogenizer (Power Gen 125; Thermo Scientific, Waltham, MA). Lung homogenates were diluted in sterile RPMI, plated on CYE agar plates and incubated for 4 days at 37 °C for Colony Forming Units (CFU) determination.

3.4.4 Neutrophil recruitment and myeloperoxidase determination

Infected animals were euthanized after 24 and 48 hours. One ml of PBS-EDTA (5mM EDTA) was injected in the trachea of each mice and the bronco alveolar lavage (BAL) was used for the determination of the total number of cells by flow cytometry Acuri 6 (Beckton Dickson, BD). The percentages of neutrophils were estimated by microscopic enumeration of BAL cells, which were previously stained by hematoxylin and eosin (HE). Neutrophils accumulation in the lung tissue was evaluated by assaying myeloperoxidase activity (MPO). Lungs of mice infected for 48 and 96 hours were perfused with 10 ml of PBS and the left superior lobules were homogenized with a tissue homogenizer in 0.2 ml of pH 4.7 buffer (NaCl 0.1 M, $NaPO_4$ 0.02 M, Na-EDTA 0.015 M, pH 4.7). The suspensions were centrifuged at 3000g for 15 min, the pellet was resuspended in lysis buffer (NaCl 0.2%) and further centrifuged for 15 min. The pellet cells were resuspended and homogenized in 0.5 mL of H-TAB buffer (0.05 M $NaPO_4$ buffer (pH 5.4) containing 0.5% dexadecyltrimethylammonium bromide (Sigma)), centrifuged at 10,000g for 15 min and the supernatants were used for determination of MPO activities. Three μL of the supernatant were diluted in mixed with 25 mL of tetramethylbenzidine (1.6 mM) plus

0.1 mL of H_2O_2 (0.5 mM) for 5 min in the dark. The reaction was stoped with 75 ml of H_2SO_4 (4 M) and measured at OD 450 nm. A standard curve was used to determinate the MPO concentration.

3.4.5 Cytokine and chemokine measurements

Animals were infected and euthanized after 48 and 72 hours. The Bronchoalveolar lavage (BAL) was collected by the cannulation of the trachea followed by three washes with 1 ml of PBS-EDTA. The cells were centrifuged and the supernatant was used to measure the cytokine levels by ELISA using BD OptEIA kits following the manufactures instructions.

3.4.6 Total protein measurement

As described before, BAL of mice infected for 48 and 96 hours was collected and the concentration of total protein was determinate using Bradford protein assay (SIGMA).

3.4.7 Histopathology

Infected or control mice were humanely sacrificed 48 hours after inoculation. Before lung removal, the pulmonary vasculature was perfused with 10 ml of PBS, via the right ventricle. The excised lungs were then inflated and fixed in 10% formaldehyde for at least 48 hours, dehydrated, and then embedded into paraffin blocks, sectioned at 5μm. The sections were stained with hematoxylin and eosin. Pathological changes were qualitatively assessed by imaging with microscopy Olympus Virtual Scanscope Image (VSI).

3.4.8 Flow cytometric analysis

The animals were sacrificed after 48 hours of infection, lungs were removed and digested in 1 mL of HANKS with collagenase (Gibco) 0.005g/ml for 1h at 37℃. The cells were passed through a 100μm cell strainer (Becton Dickinson, BD) by gentle rubbing and washing with HANKS (Gibco). After red blood cell lysis, the total lung cell counts were determined and the cell suspension was adjusted to 10^7 cells/200μL in RPMI 1640, 10% FBS.The frequency of specific cells was determined by staining

with appropriate concentrations of fluorochrome-conjugated mAb specific for murine Ly6G (BioScience), MHC-II (BioScience), Ly6G (ByoScience), F4/80 (BioScience), CD11b (BioScience) and CD11c (BioScience). Flow cytometry acquisitions were performed in FACs CantoII (BD Bioscience) and analysed using Flow Jo (Tree Star).

3.4.9 Statistical analysis

Statistical analyses were performed using Prism 5.0 software (GraphPad, San Diego, CA). Unpaired Student t test was used to compare two groups. One-way ANOVA followed by multiple comparisons according to Tukey's procedure was used to compare three or more groups. A log-rank test was used for mice survival statistical analyses. Unless otherwise stated, data are expressed as the mean ± SEM. Differences were considered statistically significant at a p value <0.05.

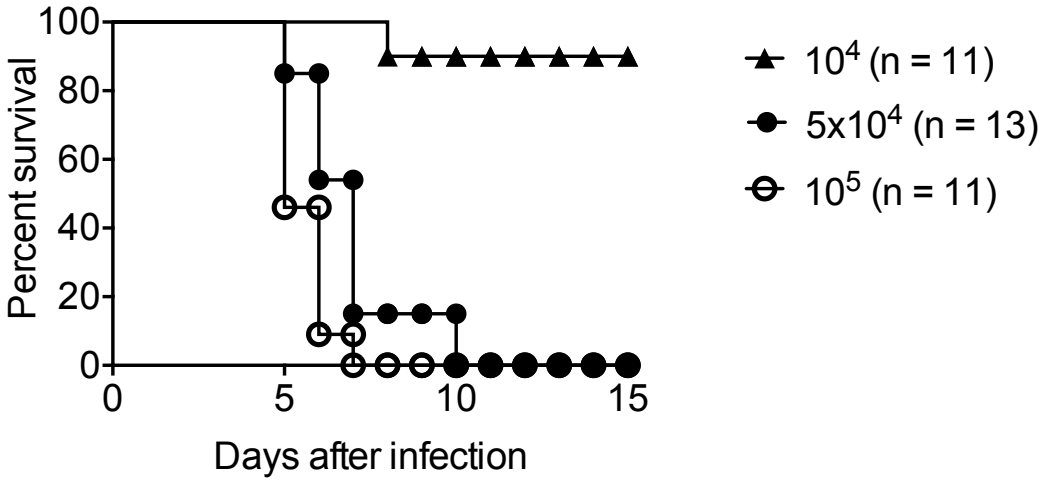
Results

4. RESULTS

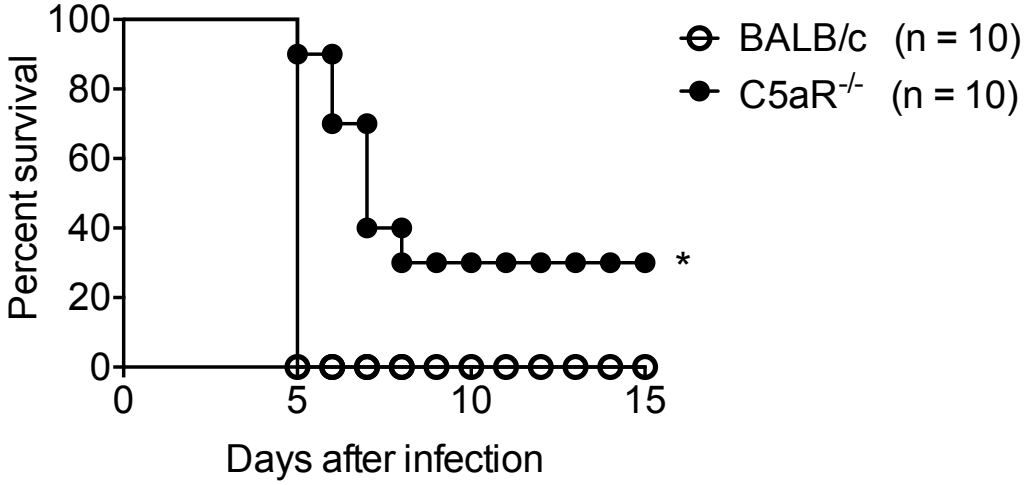
4.1 The C5aR is important for mice susceptibility in *L. longbeachae* pulmonary infection.

In mouse model, *L. longbeachae* induces an acute lung infection and injury, which can cause mice death (Asare, Santic et al. 2007, Gobin, Susa et al. 2009). It is already well described that *L. longbeachae* is lethal for C57BL/6 mice strain, while nothing is known about *L. longbeachae* infection in BALB/c mice. As shown in figure 3A the infected BALB/c mice die after 7-10 days infection with *L. Longbeachae* in a dose dependent manner. The BALB/c mice, infected with 10^5 bacteria die 7 day after infection and mice infected with 5 x 10^4 bacteria, die in a period of 9 days. In contrast, mice infected with 10^4 shows a survival rate of 90% (Figure 3A). Therefore the dose of $5x10^4$ was set as the optimal dose in our model. Complement activation, a key component of the innate immune system, is triggered in response to multiple types of tissue injury. As mentioned before, the complement system builds an important part of the innate immunity, is able to recognize the bacterial surface, as well as the capsule and therefore is one of the first and fastest defense mechanism after an infection. In order to test if the complement system could be involved in the defense against *L. longbeachae* (DZ420) infection, we test the survival rate of mice that lack the receptor for the C5a protein ($C5aR^{-/-}$). Interestingly, the absence of the C5a receptor (C5aR), protect the mice infected with the DZ420 compared with the BALB/c. As shown in figure 3B and 3C, which represent two independent experiments, the $C5aR^{-/-}$ mice have a survival rate of 30%, while 100% of BALB/c mice die after an infection with $5x10^4$ bacteria (Figure 3B, C). Taken together, these results show that the absence of the C5aR contributes to mice survival in response to lethal dose of *L. longbeachae.*

A


Percent survival
100
80
60
40
20
0
0
5
10
15
Days after infection
10^4 (n = 11)
5x10^4 (n = 13)
10^5 (n = 11)

B


Percent survival
100
80
60
40
20
0
0
5
10
15
Days after infection
BALB/c (n = 10)
C5aR-/- (n = 10)
*

C

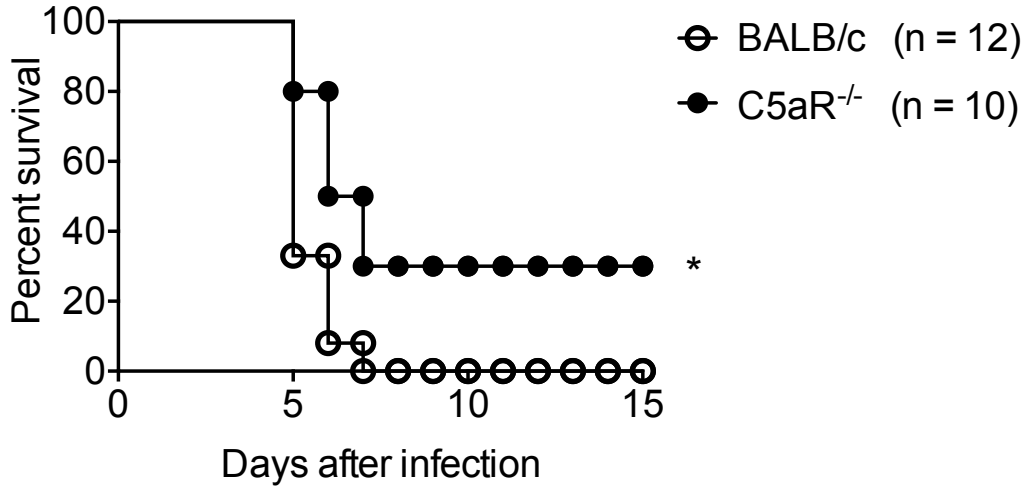

Percent survival
100
80
60
40
20
0
0
5
10
15
Days after infection
BALB/c (n = 12)
C5aR-/- (n = 10)
*

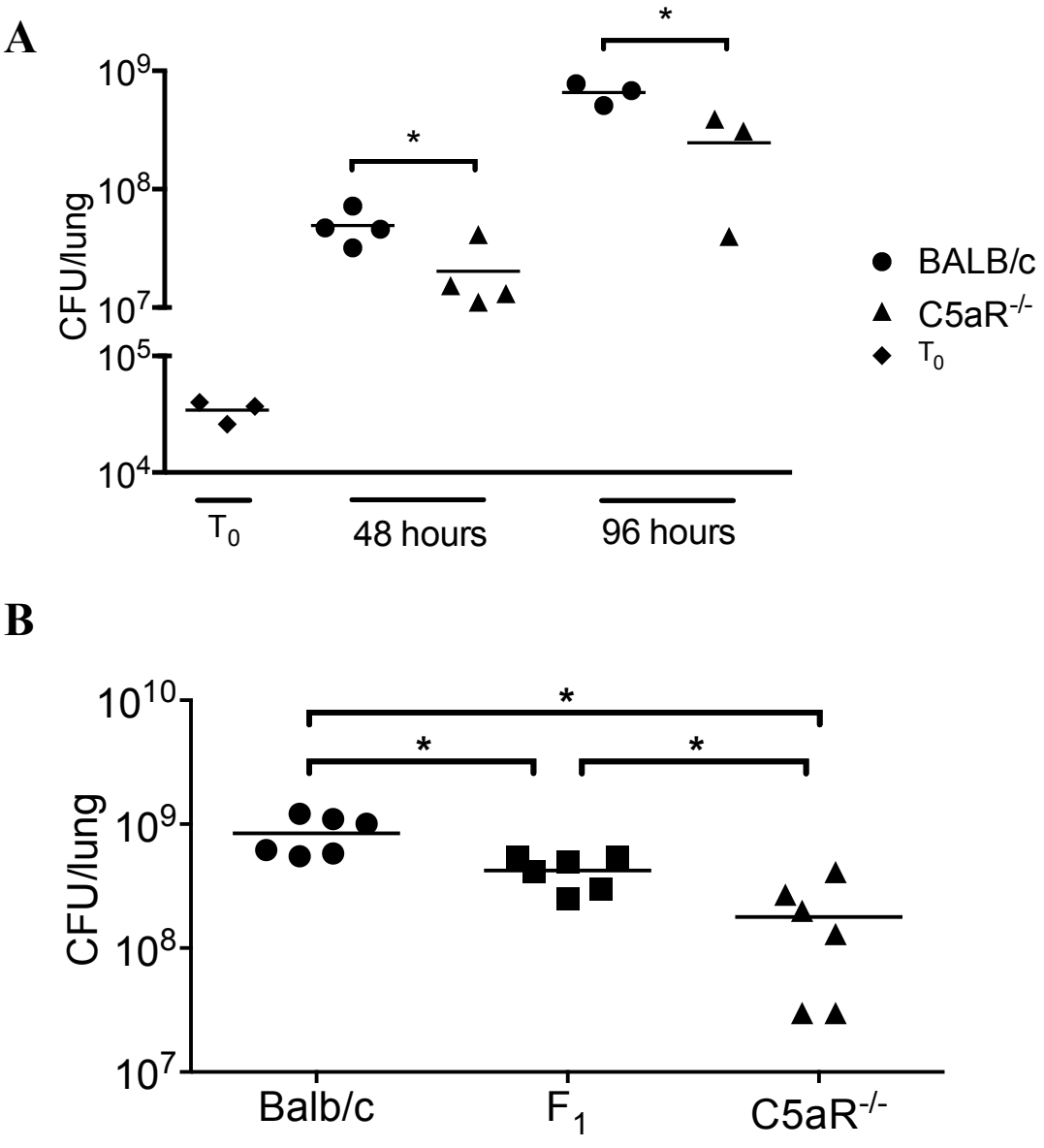
Figure 3. Evaluation of the mice susceptibility in *L. longbeachae* pulmonary infection. (A) BALB/c mice were infected with 10^5, $5x10^4$ or 10^4 DZ420 bacteria per mice. (B, C) BALB/c and C5aR$^{-/-}$ mice were infected with $5x10^4$ bacteria per mice. "B" and "C" represent two independent experiments. The survival was followed during 15 days. The number of mice used in each group (n) is indicated in the figure legend. *p<0.05.

4.2 C5aR contributes for clearance of pulmonary *L. longbeachae* infection.

It is already known that *L. longbeachae* is able to multiply in mouse lungs leading to mice death (Cazalet, Gomez-Valero et al. 2010). However, the previous results demonstrate a contribution of the C5aR for mice susceptibility in *L. longbeachae* pulmonary infection. In order to evaluate if the C5aR also affect bacterial replication inside the lungs, intranasal infection of BALB/c, C5aR$^{-/-}$ and F_1 (BALB/c x C5aR$^{-/-}$) mice were performed. The mice were infected by nasal rote with $5x10^4$ DZ420, and the bacterial multiplication in the lungs was estimated at 48 and 96 hours. The numbers of CFU 48 hours after infection increase dramatically in BALB/c as well as in C5aR$^{-/-}$ mice and enhance after 96 hours (Figure 4A). Moreover, comparing the BALB/c and C5aR$^{-/-}$ mice, there is a significant diminished CFU of a C5aR$^{-/-}$ mouse at the time point of 48 as well as 96 hours. These data indicate that in the absence of C5aR influences the DZ420 replication inside the lung, respectively the CFU of C5aR$^{-/-}$ is impaired when compared with the BALB/c mice. To evaluate if the F_1 C5aR heterozygote mice (C5aR$^{+/-}$) display differences in bacterial clearance inside the lung, C5aR$^{+/-}$ was compared with the C5aR homozygote (C5aR$^{-/-}$) and wild-type (BALB/c) mice. The CFU was estimated 48 hours after infection. As shown in figure 4B, the CFU of heterozygote mice displays an intermediate phenotype; the F_1 mice display significantly less CFU compared with the BALB/c mice and significantly more CFU compared with the C5aR$^{-/-}$mice (Figure 4B). Taken together, these dates indicate that the absence of the C5aR impair the ability of bacterial replication and/or improve the bacterial clearance inside the lung. Next, we investigated whether the impaired bacterial clearance in the lungs of mice deficient in C5aR was caused by an intrinsic macrophage defect in the restriction of bacterial

replication. To test this hypothesis, bone marrow derived macrophages (BMDMs) from BALB/c and C5aR$^{-/-}$ mice were generated and infected with a *L. longbeachae* (MOI 0.015). The bacterial replication was followed for 24, 48 ad 72 hours after infection. The result shows that *L. longbeachae* replicate in BALB/c as well as C5aR$^{-/-}$ BMDMs and there are no differences between the bacterial replication inside the BMDMs from these two mice strains (Figure 4C).

Taken together, these results demonstrate that the C5aR is important for the DZ420 replication and the absence of the receptor increase the clearance of pulmonary *L. longbeachae* infection. However, the DZ420 effectively replicate in the BMDMs regardless of the presence of C5aR.



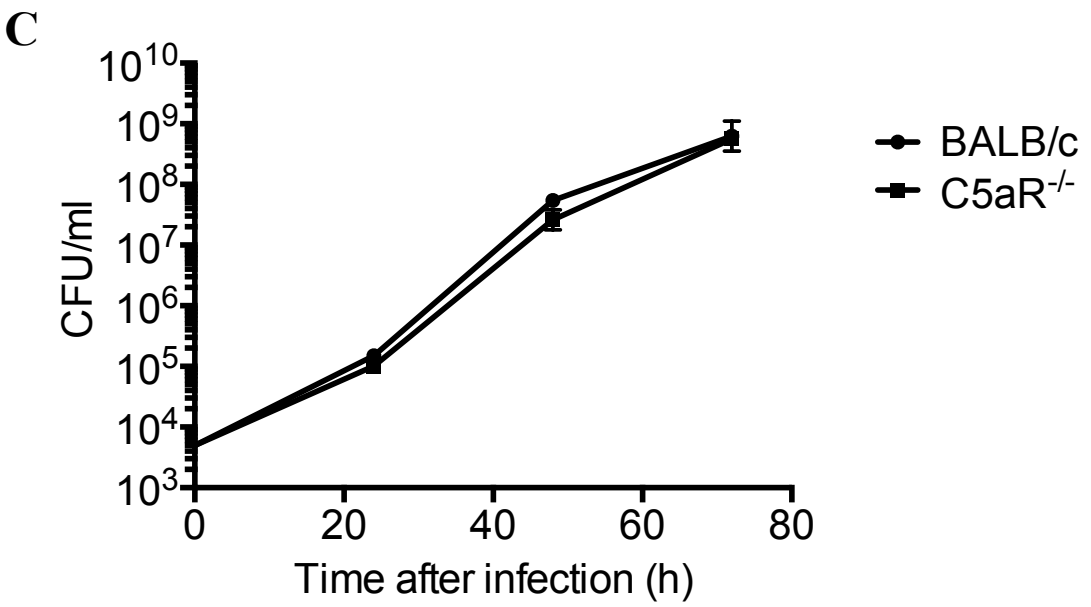
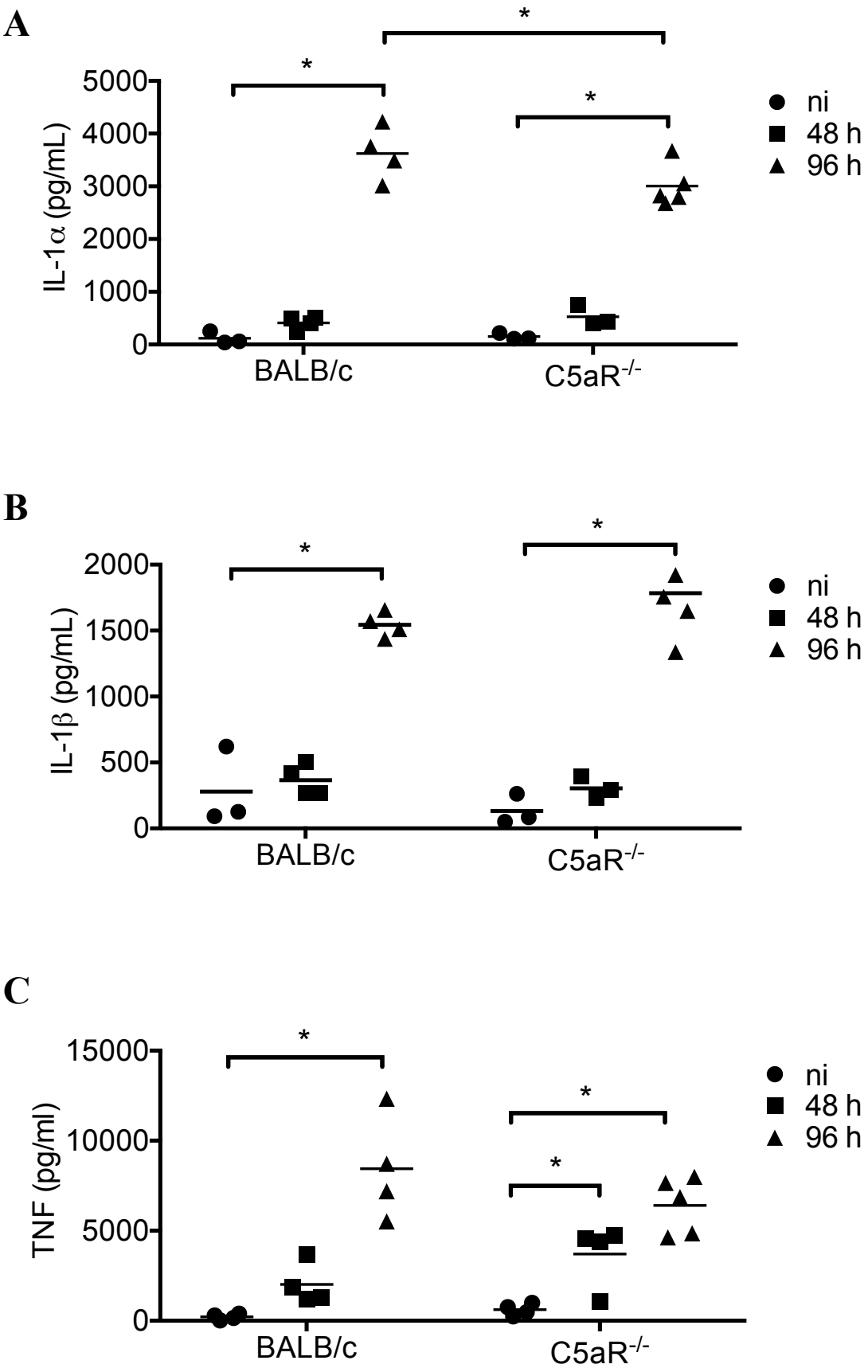


Figure 4. The importance of the C5aR in the clearance of *L. longbeachae* lung infection. BALB/c, C5aR$^{-/-}$ and/or F_1 (C5aR$^{+/-}$) mice were infected intranasally with 5x10^4 DZ420 per mice. BALB/c and C5aR$^{-/-}$ mice were infected with 5x10^4 and euthanized after (A) 48 or 96 hours and (B) 72 hours. T_0 indicates the Colony Forming Units (CFU) recovered after 4 hours of infection. (A, B) Dilutions of the lung homogenates were added to CYE agar plates for CFU determination. Each dot represents a single animal and the horizontal lines represent the average *p<0.05. Results are expresses individually and the bars represent the means. (C) Bone marrow derived macrophages (BMDMs) from BALB/c and C5aR$^{-/-}$ mice were infected with DZ420 (MOI 0.015) and after 24, 48 and 72 hours of infection the cells were homogenized to count the numbers of CFU. n = 3, *p<0,05. Results are expressed as means ± SEM.

4.3 Pulmonary *L. longbeachae* infection induces cytokines and chemokines production independently of the C5aR.

The C5a protein has immunomodulatory properties such as modulation of cytokines and chemokine expression (Guo and Ward 2005). Tumor necrosis factor (TNF) – α is a cytokine that can stimulate release of chemotactic factors from alveolar macrophages and lung epithelial cells and subsequently amplify pulmonary

inflammatory response (Nelson, Bagby et al. 1989). Other pro-inflammatory cytokines such IL-1α, IL-1β, IFN-γ and IL-12 are also involved in pulmonary infection, inflammation and bacterial clearance (Yang, Hooper et al. 2002, Thanawongnuwech, Amonsin et al. 2004). In order to verify the importance of these pro-inflammatory cytokines, and their eventual involvement in the C5aR signalling in *L. longbeachae* pulmonary infection, these cytokines from BAL were measured by ELISA. Thus, BALB/c and C5aR$^{-/-}$ mice were infected for 48 and 96 hours and the concentration of cytokine present in the BAL was measured. As shown in figure 5A, the production of IL-1α, 48 hours after infection, remains almost unchanged, but increases significantly 96 hours after infection in both BALB/c and C5aR$^{-/-}$ mice. Nevertheless, there is a significant decrease of IL-α production in C5aR$^{-/-}$ mice 96 hours after infection, compared with the BALB/c mice. As shown in figure 5B, there is a significant increase of IL-1β concentration 96 hours after infection, but there are no differences in BALB/c and C5aR$^{-/-}$ mice, respectively. Next, the production of TNF and IL-12 was determinate, as shown in figure 5C and 5D. The levels of these cytokines, recovered from the BAL of infected mice, are significantly higher compared with the non-infected mice. The cytokines levels 48 hours after infection the cytokines levels are elevated, and increased after of 96 hours of infection. However, there is no statistic difference between the cytokines levels produced in BALB/c when compared with C5aR$^{-/-}$ mice. The pro-inflammatory cytokine IFN-γ increases slightly in infected mice compared to the uninfected (Figure 5E). Moreover, the IFN-γ levels remain unchanged in BALB/c and C5aR$^{-/-}$ mice, respectively. Taken together, these results indicate that the absence of the C5aR does not influences, or very little, the inflammatory cytokine production in non-infected as well as infected mice.


A
*
*
*
5000
4000
3000
2000
1000
0
IL-1α (pg/mL)
BALB/c
C5aR-/-
ni
48 h
96 h
B
*
*
2000
1500
1000
500
0
IL-1β (pg/mL)
BALB/c
C5aR-/-
ni
48 h
96 h
C
*
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15000
10000
5000
0
TNF (pg/ml)
BALB/c
C5aR-/-
ni
48 h
96 h

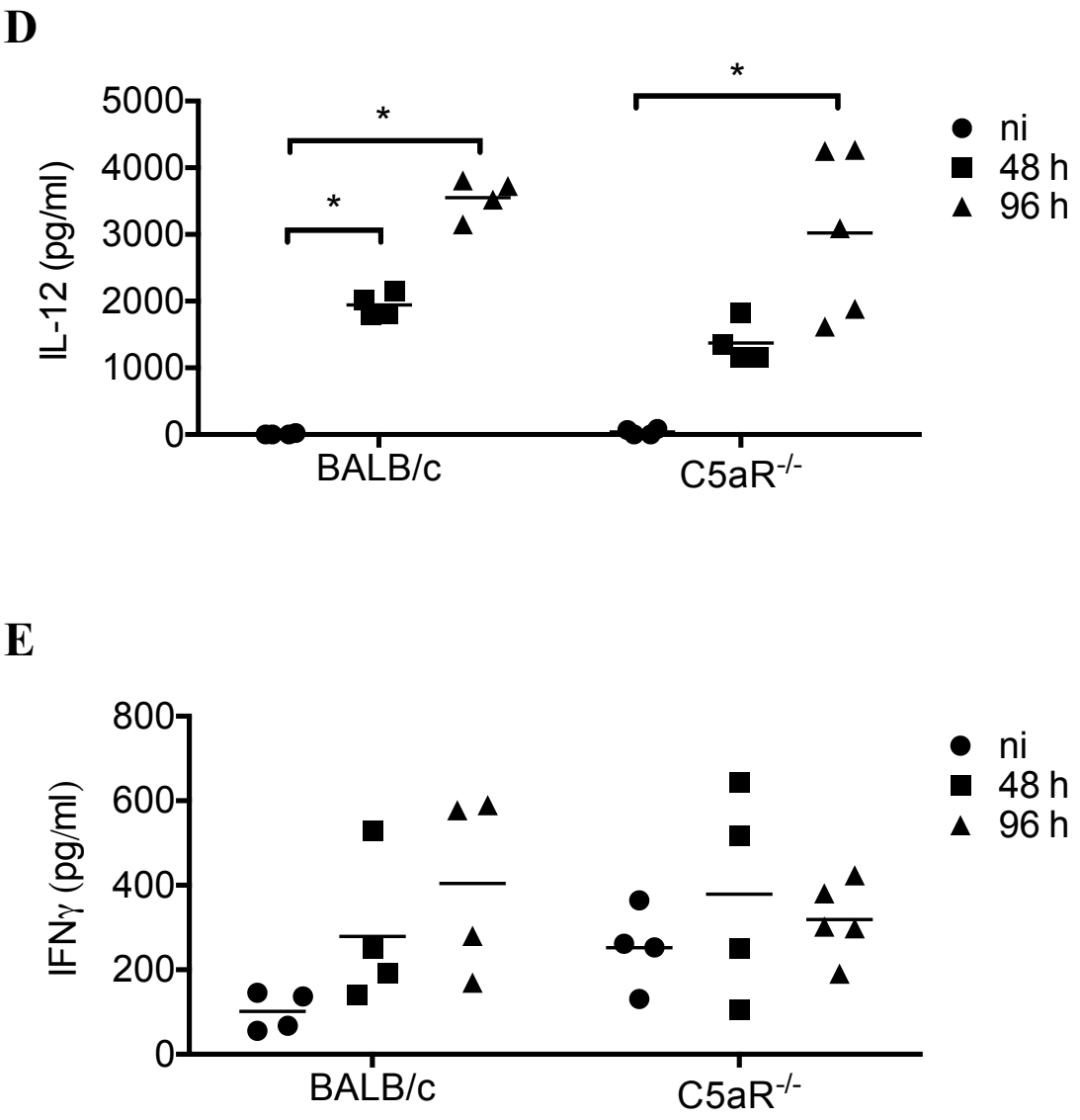


Figure 5. Evaluation of cytokines and chemokines production in response to pulmonary infection with *L. longbeachae*. BALB/c and C5aR$^{-/-}$ mice were infected intranasally with 5x10^4 DZ420 per mice. After 48 and 96 hours, the broncoalveolar fluid (BAL) was collected and used to define the concentration of (A) IL-1α (B) IL-1β, (C) TNF-α, (D) IL-12 and (E) IFN-γ. ni – non infected mice. n = 3-5 mice in each group. *p<0.05. Results are expresses individually and the bars represent the means.

4.4 The total bronchoalveolar lavage proteins and myeloperoxidase activity are increased after *L. longbeachae* infection in C5aR independent manner.

It is already well established that the C5a is a potent inflammatory peptide and strong chemoattractant for neutrophils (Marder, Chenoweth et al. 1985). Moreover, increases in C5a concentration and neutrophil accumulation constitute the foundation of many forms of lung injury (Ishii, Kobayashi et al. 1989, Winter, Paradis et al. 1989, Guo and Ward 2002, Guo and Ward 2005). The myeloperoxidase (MPO) assay indicates the presence of neutrophils in lung tissue. In order to test if the absence of the C5aR influences the neutrophil migration to the lung and possibly explain the protected phenotype of $C5aR^{-/-}$ mice, the MPO assay of BALB/c and $C5aR^{-/-}$ infected mice was performed. In comparison to uninfected mice, a high activity of MPO in the lungs of mice infected for 48 hours was detected (Figure 6A). However, there is no significant difference in MPO activity after 48 as well as 96 hours of infection comparing the BALB/c and $C5aR^{-/-}$ mice. Moreover, an increase of the total protein concentration of the BAL fluid may reflect an increase in the permeability of the alveolar capillary membrane and consequently indicate to tissue damage or lung injury. To test this hypothesis, Bradford protein assay was performed in order to measure the amount of proteins present in the BAL after 48 and 96 hours of infection. As shown in figure 6B, 48 hours after infection the amount of total protein detected in the BAL remains almost unchanged. Nevertheless, 96 hours after infection there is a dramatic increase of total protein detected in the BAL in BALB/c and $C5aR^{-/-}$ mice, respectively. However, there are no differences in the amount of proteins present in the BAL of BALB/c compared with $C5aR^{-/-}$ mice. Taken together, these results indicate that the C5aR does not influences neither the MPO nor total BAL protein present in the lung.

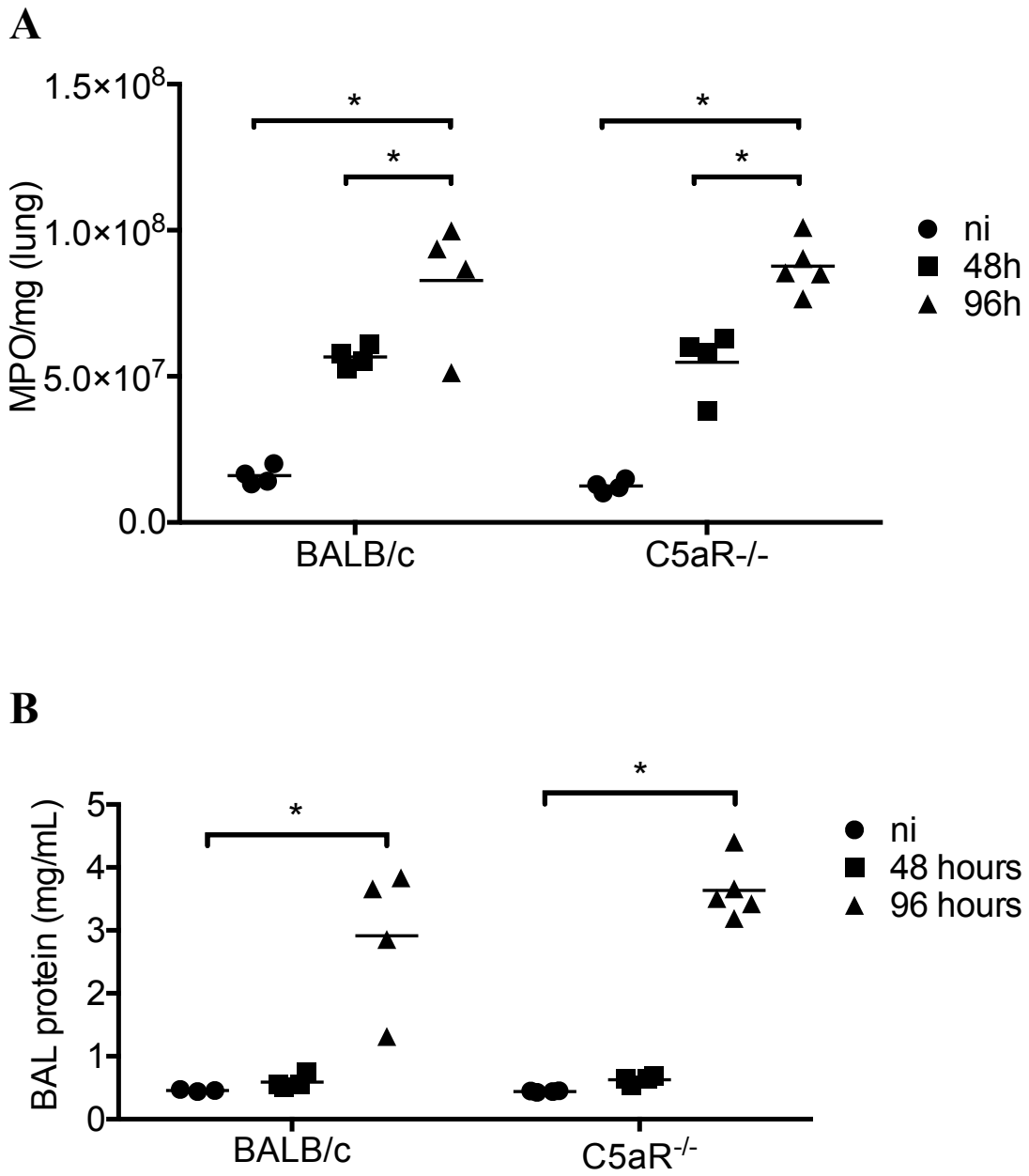
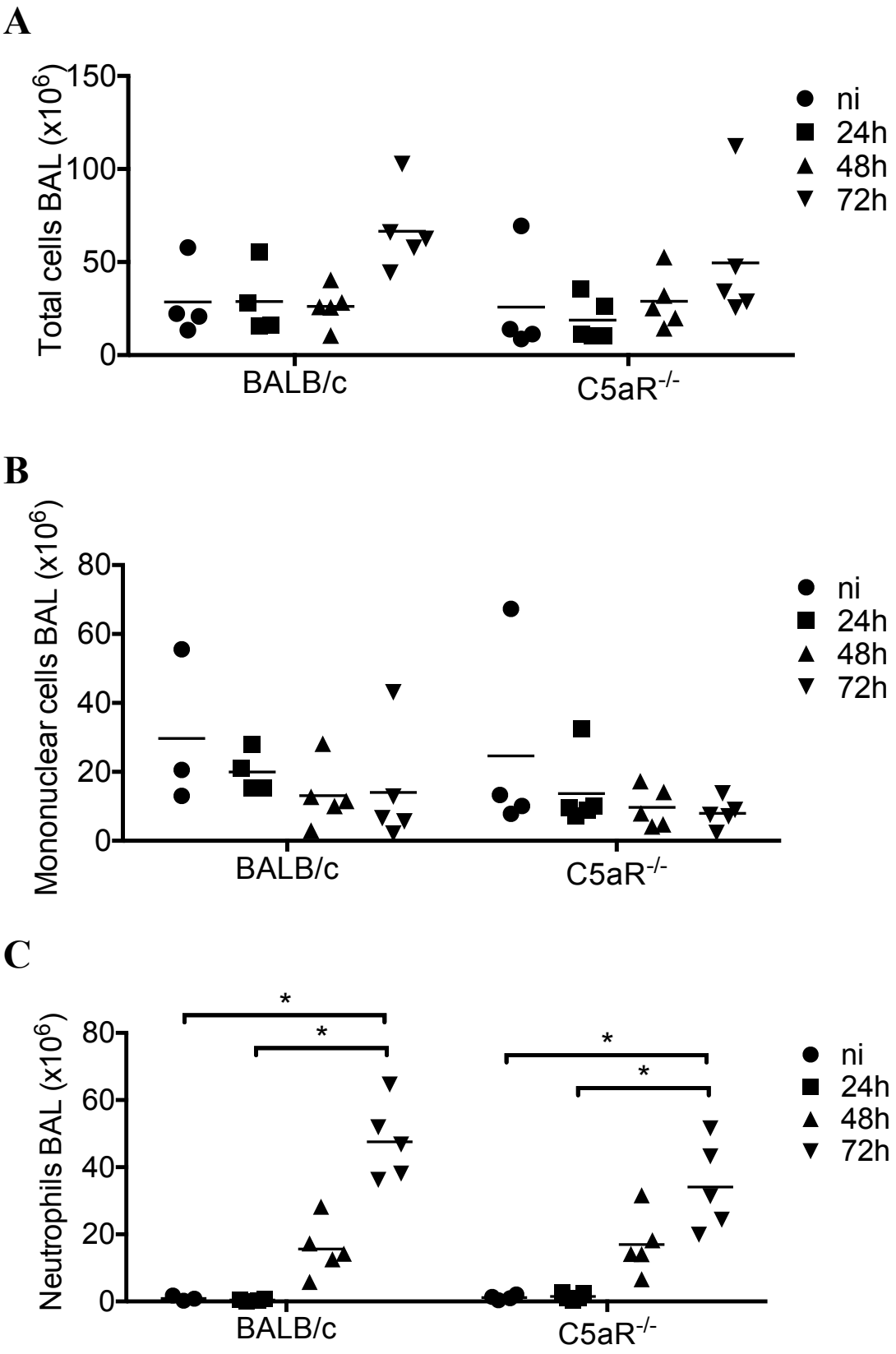


Figure 6. Evaluation of the total BAL proteins and MPO activity in the lung after *L. longbeachae* infection. The bronco alveolar lavage (BAL) and lung tissue were collected 48 and 96 hours after nasal infection with DZ420 ($5x10^4$) of BALB/c and $C5aR^{-/-}$ mice. (A) Measurement of myeloperoxidase (MPO) activity in the lungs and (B) represents total protein present in BAL. n = 4-5 mice in each group. ni – not infected; *p<0.05. Results are expresses individually and the bars represent the means.

4.5 Infection by *L. longbeachae* increases significantly the cell migration to the pulmonary site in a C5aR independent manner.

As mentioned before, the C5a peptide posses inflammatory properties and it can recruit neutrophils as well as other proinflamatory cells at the site of infection (Riedemann, Guo et al. 2003, Sarma and Ward 2012). In order to analyze the role of C5aR in the cell migration into the lung compartment in our model of *L. longbeachae* lung infection, BALB/c and $C5aR^{-/-}$ mice were infected intranasally with DZ420 ($5x10^4$). The BAL was collected 24, 48 and 72 hours after infection and total cell number was estimated. As shown in figure 7A, the total cell number in the lung cavity increases 72 hours after infection, in both, BALB/c and $C5aR^{-/-}$. However, the absence of the C5aR seems to not affect the cell migration to the lung compartment. In order to verify if there are some differences between the cell populations, which are migrating into the lung compartment, differential cell count was performed. As shown in figure 7C, there is a significant increase in neutrophil migration, especially 48 and 72 hours after infection. In contrast, the number of monocyte present in the BAL does not change and seems to not be influenced by the infection (Figure 7B). Comparing the BALB/c mice with the $C5aR^{-/-}$ mice, which display a protected phenotype, there are no significant differences in cell migration either in total cells, neutrophils or monocytes (Figure 7A- C). Histological analysis of the lungs of BALB/c and $C5aR^{-/-}$ mice infected for 48 and 96 hours shown a higher number of cells in the lungs when compared to non-infected mice (Figure 7D). Additionally, Image J software was used to confirm that more cells are present in samples from infected mice, but in a C5aR independent manner (Figure 7E). According to these results, the absence of C5aR does not influence the total cell migration in lung compartment, amount of neutrophils and monocytes as well as the amount of cells present in the lung tissue. All together, these results demonstrate that infection by *L. longbeachae* induces a strong cell migration in the lung cavity as well as a cell infiltration and high inflammation of the lung tissue and this process is C5aR independent.


A
Total cells BAL (x10^6)
150
100
50
0
BALB/c
C5aR-/-
ni
24h
48h
72h
B
Mononuclear cells BAL (x10^6)
80
60
40
20
0
BALB/c
C5aR-/-
ni
24h
48h
72h
C
Neutrophils BAL (x10^6)
80
60
40
20
0
*
*
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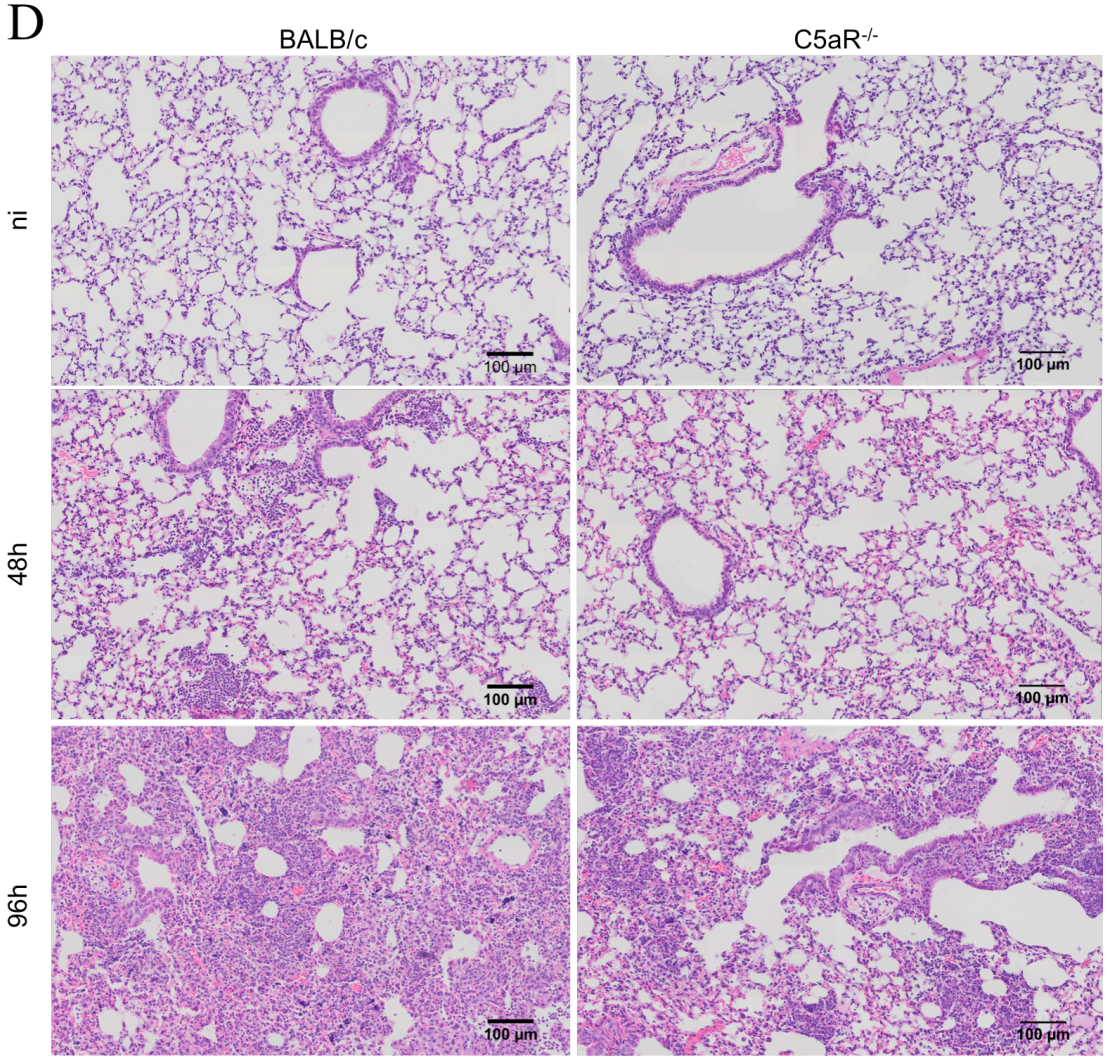
*
BALB/c
C5aR-/-
ni
24h
48h
72h

D


BALB/c
C5aR-/-
ni
48h
96h
100 µm

E

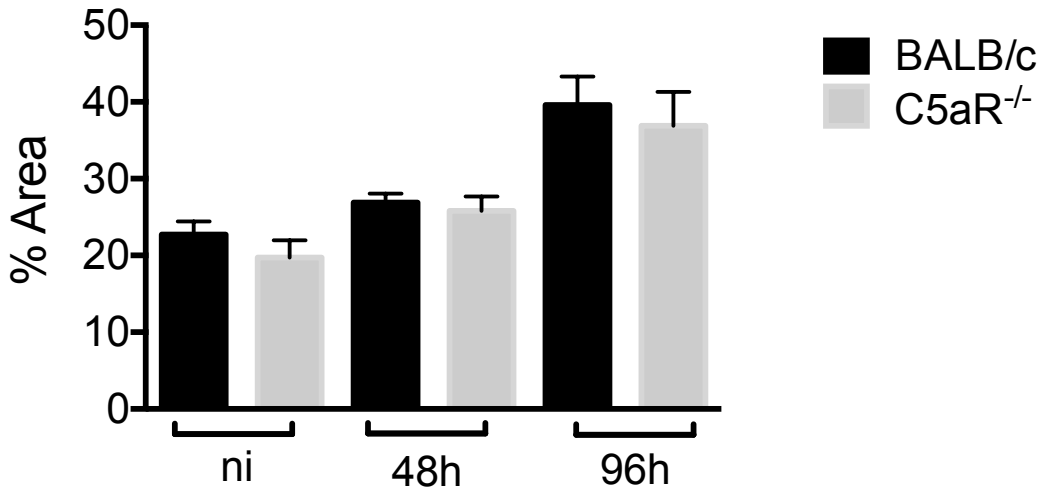
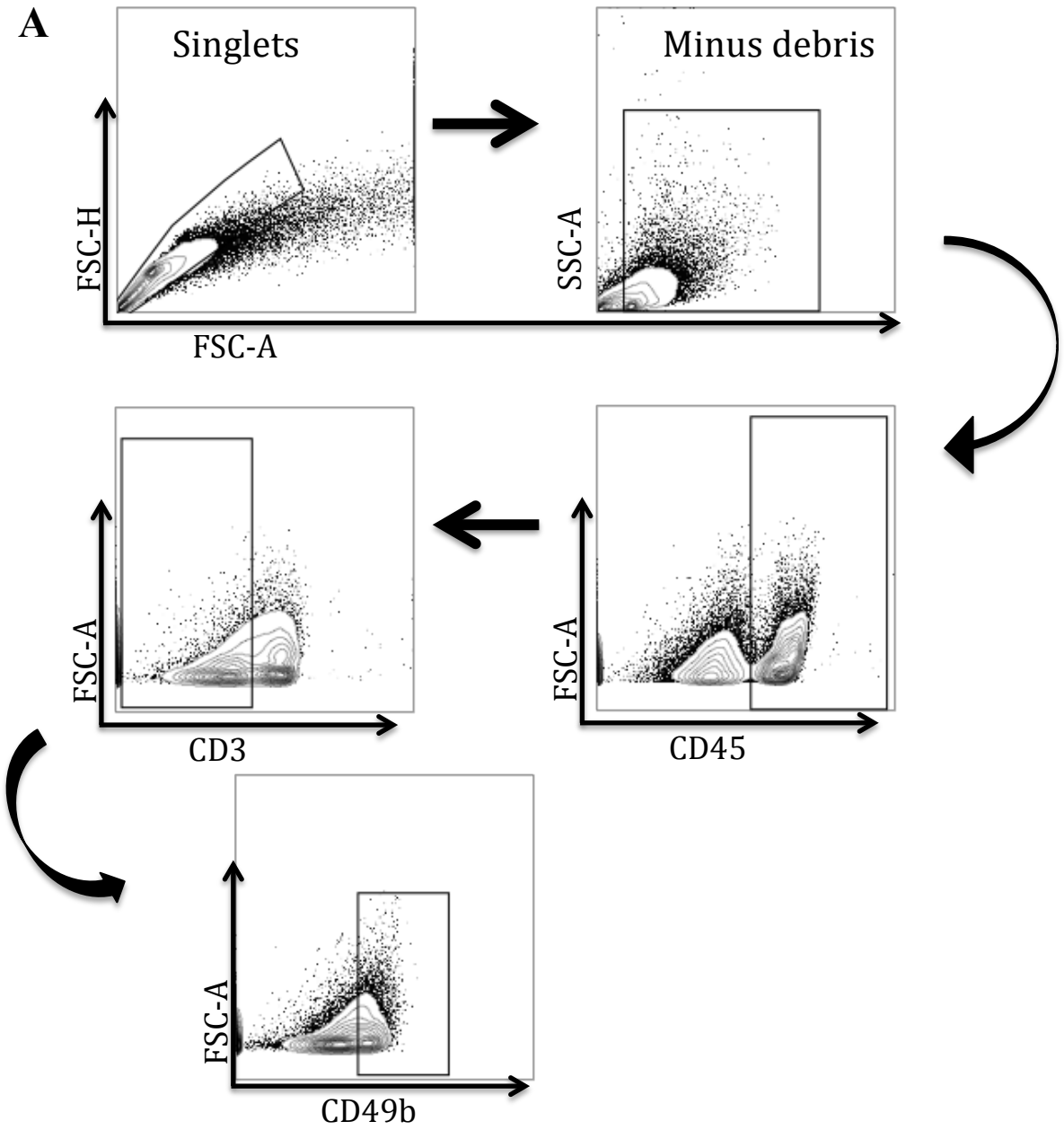

% Area
50
40
30
20
10
0
ni
48h
96h
BALB/c
C5aR-/-

Figure 7. Investigation of *L. Longbeachae* induced cell migration to the pulmonary site. The bronco alveolar lavage (BAL) was performed 24, 48 and 72 hours after nasal infection of BALB/c and C5aR$^{-/-}$ mice with DZ420 (5X10^4). Cytospin with H & E staining of slides were prepared from BAL and the number of (A) total cells, (B) mononuclear cells and (C) neutrophils were differentially counted. Results are expresses individually and the bars represent the means. (D) Histology of the lungs with H&E staining, 48 and 96 h after infection. Original magnification: 10X. The image represents the findings for one mouse of 4 or 5 mice in each group and all mice showed similar results. (E) Indirect quantification of inflammatory infiltrate using ImageJ's area measurement. Original magnification: 40X. Results are expressed as means ± SEM. n = 4-5 mice in each group. ni – not infected. *p<0.05.

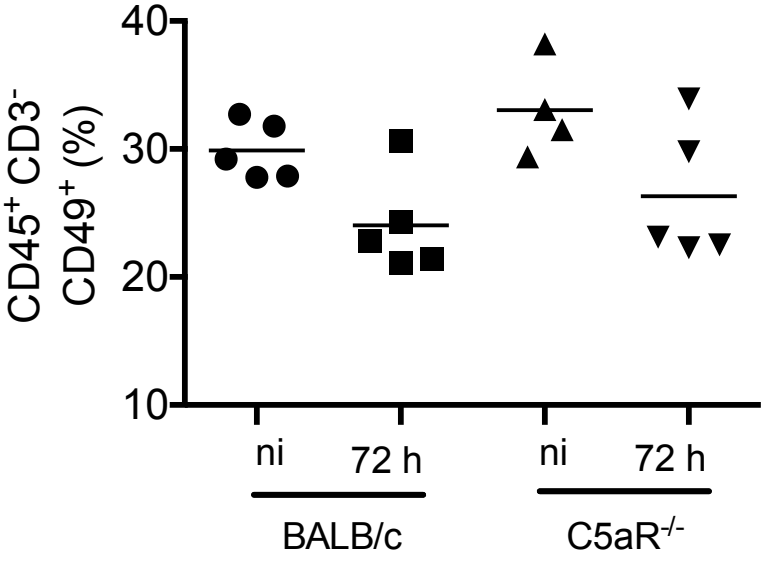
4.6 Flow cytometry analysis of lung tissue of BALB/c and C5aR$^{-/-}$ mice infected with *L. longbeachae.*

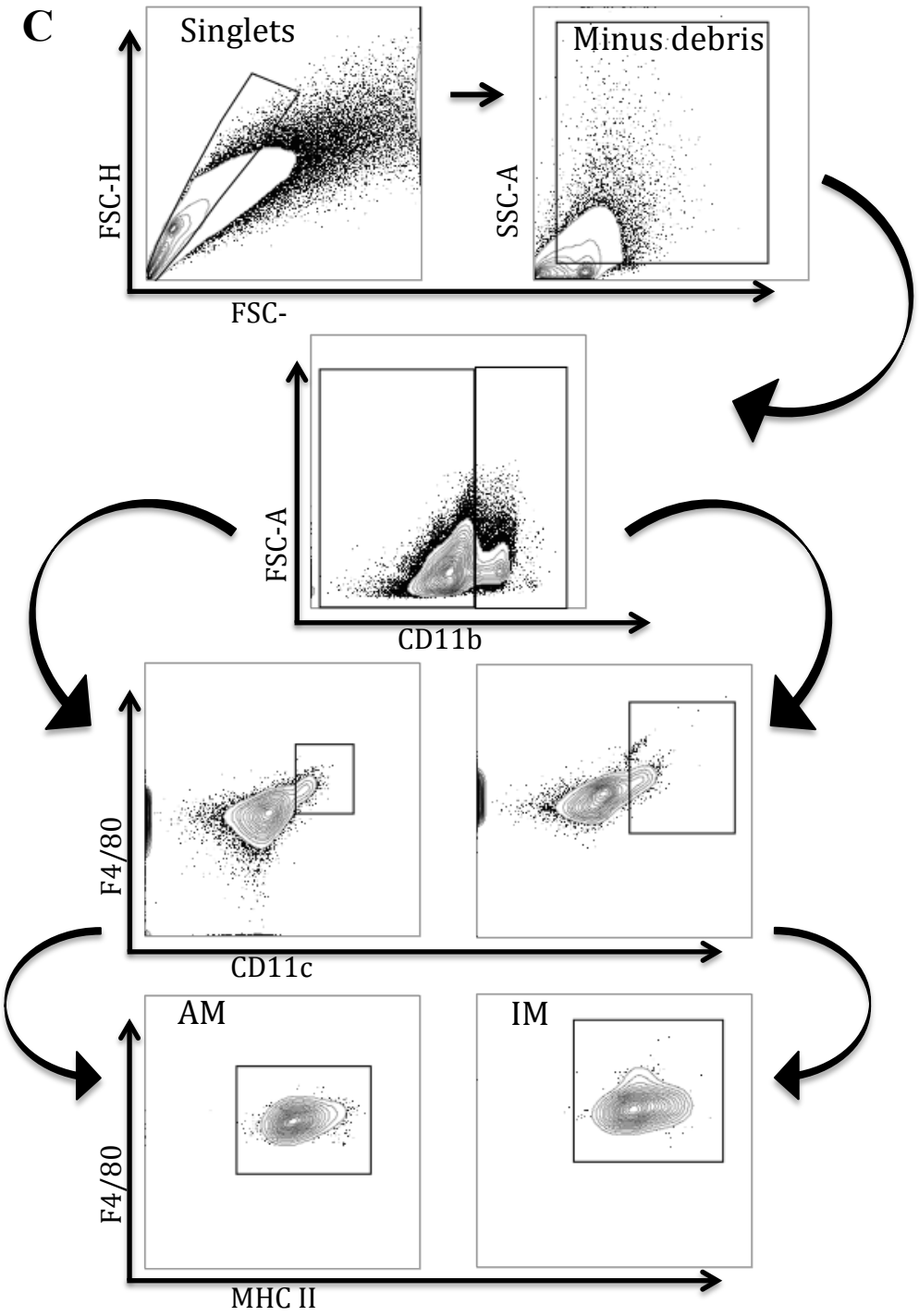
As shown in figure 7A, the total cell number in the lung cavity increases 72 hours after infection, in both, BALB/c and C5aR$^{-/-}$ mice. The increase in total cells corresponded to an increase in the neutrophil infiltrate to the lung cavity (Figure 7C), but the monocyte migration remains almost unchanged in infected and non-infected mice, respectively. However, there were no significant differences found between the BALB/c and C5aR$^{-/-}$ mice. Therefore, to further evaluate the cell infiltrate into the pulmonary tissue, the lungs of BALB/c and C5aR$^{-/-}$ mice infected for 72 hours were digested with collagenase and the released cells were stained with specific antibodies to analyze by flow cytometry. The figure 8A represents the gating strategy used to analyze the natural killer (NK) cell population present in the lung. The percentage of NK cells decrease slightly 72 hours after infection, but there are no significant differences between the BALB/c and C5aR$^{-/-}$ mice. Next the alveolar macrophages (AM) and the interstitial macrophages (IM) populations were analyzed (Figure 8C-E). The AM population decreases significantly 72 hours after infection in bought BALB/c and C5aR$^{-/-}$ mice, when compared with the non-infected (Figure 7D). However, there are no significant changes in the IM populations, as shown in figure 8E. Furthermore, the inflammatory monocytes population was evaluated in both infected and non-

infected BALB/c and C5aR$^{-/-}$ mice. As shown in figure 8F, the inflammatory monocytes population is present just in infected mice, while in non-infected mice the population could not be detected. Regardless the significant increase in the inflammatory monocyte population 72 hours after infection, there are no significant differences between the BALB/c and C5aR$^{-/-}$ mice (Figure 8G). The present data indicate that there are no differences in macrophage, NK or inflammatory monocyte cell populations of the BALB/c and C5aR$^{-/-}$ mice lung neither before the infection nor 72 hours after infection with *L. Longbeachae*.

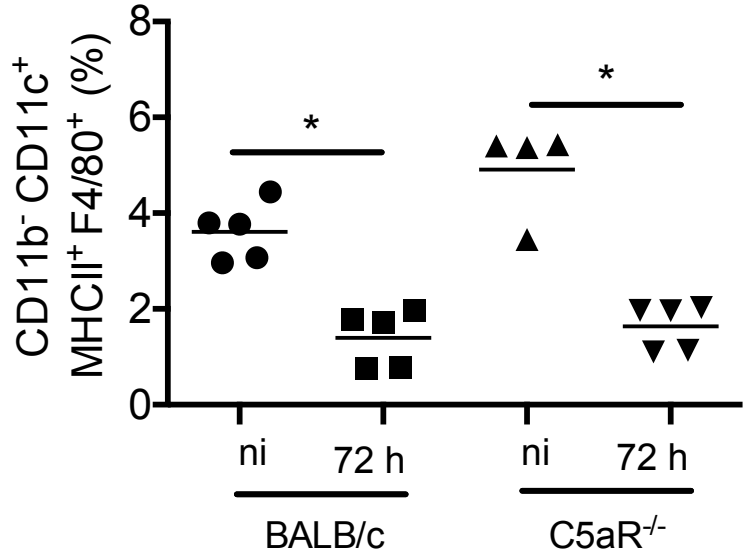

A
Singlets
FSC-H
FSC-A
Minus debris
SSC-A
FSC-A
CD45
FSC-A
CD3
FSC-A
CD49b

B

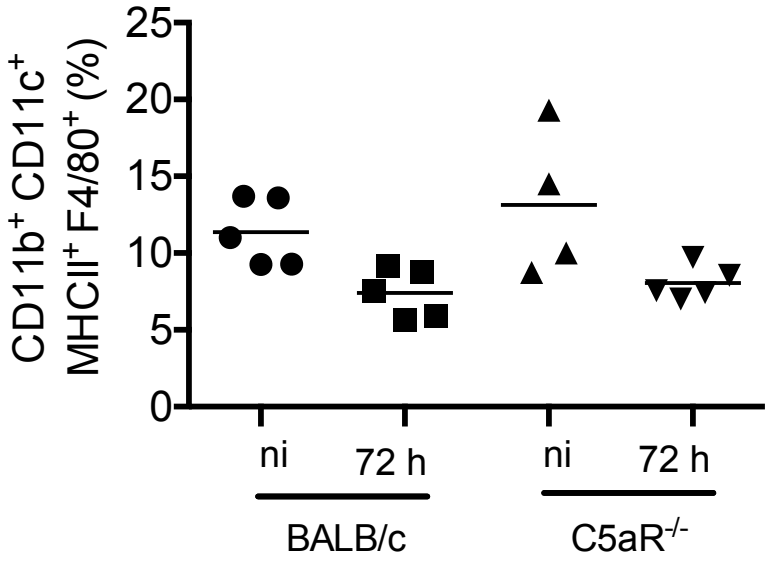



C
Singlets
FSC-H
FSC-
Minus debris
SSC-A
FSC-A
CD11b
F4/80
CD11c
AM
IM
F4/80
MHC II

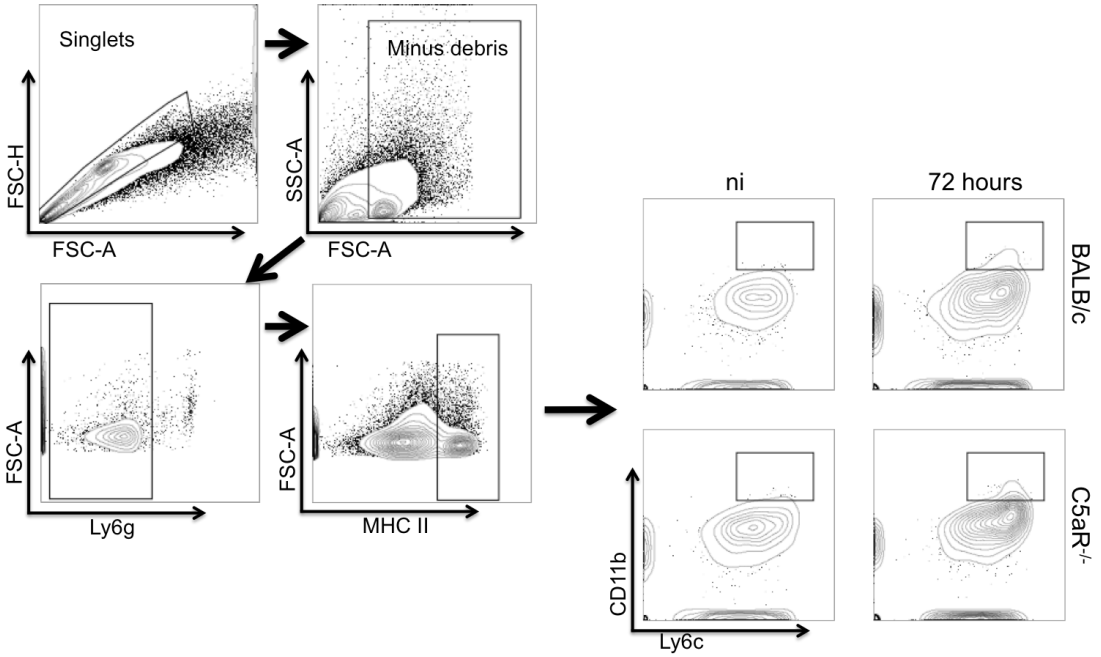
D


CD11b- CD11c+
MHCII+ F4/80+ (%)
8
6
4
2
0
*
*
ni
72 h
ni
72 h
BALB/c
C5aR-/-

E


CD11b+ CD11c+
MHCII+ F4/80+ (%)
25
20
15
10
5
0
ni
72 h
ni
72 h
BALB/c
C5aR-/-

F


Singlets
FSC-H
FSC-A
Minus debris
SSC-A
FSC-A
FSC-A
Ly6g
FSC-A
MHC II
ni
72 hours
BALB/c
C5aR-/-
CD11b
Ly6c

G

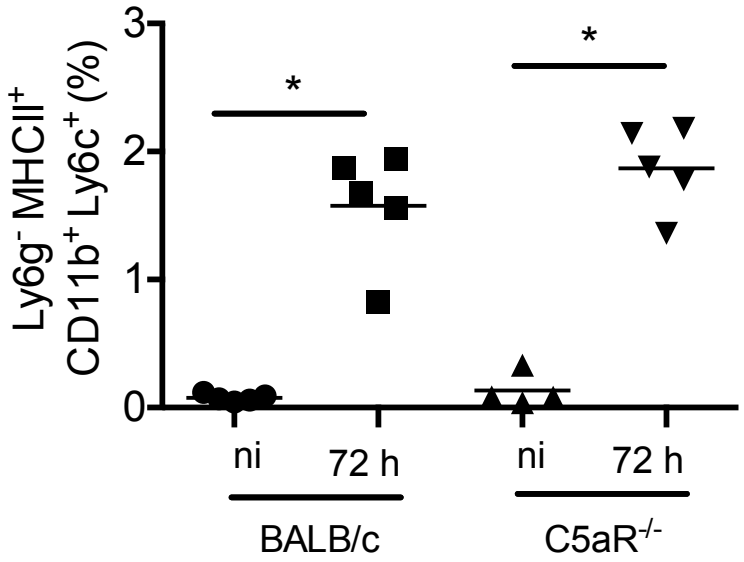
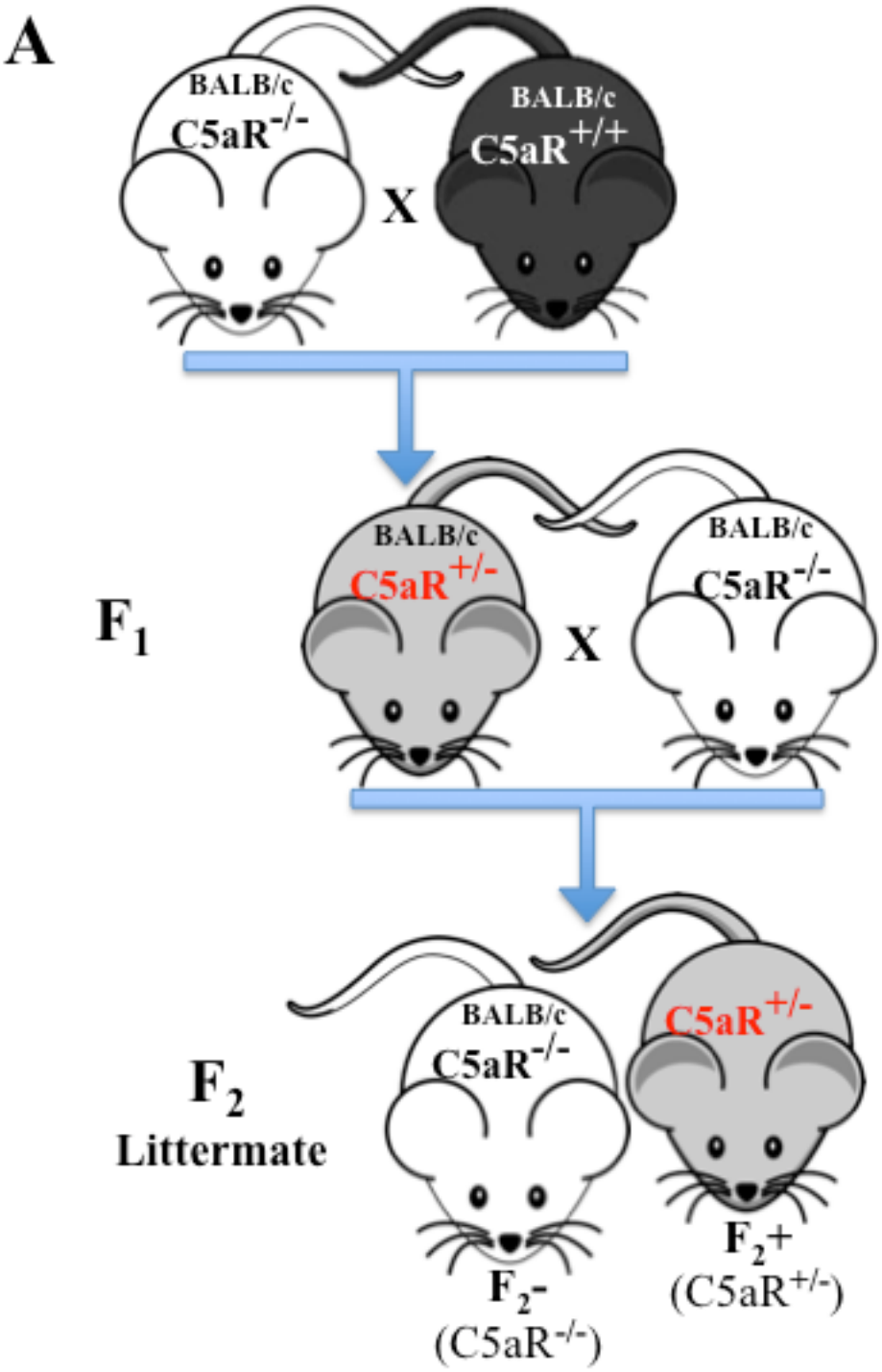

Ly6g- MHCII+
CD11b+ Ly6c+ (%)
3
2
1
0
*
*
ni
72 h
ni
72 h
BALB/c
C5aR-/-

Figure 8. Flow cytometric analysis of cells in the lung tissue of BALB/c and C5aR$^{-/-}$ mice. Lung tissues were explanted from non-infected mice (ni) or after 72 hours of infection (5x10^4 DZ420), digested with collagenase and processed for specific antibodies labelling and flow cytometry analyses. Gating strategy and percentage of cells stained for natural killer cells (NK) (CD45$^+$, CD3$^-$, CD49b$^+$) (A and B), alveolar macrophages (AM) (CD11b$^-$, MHCII$^+$, CD11c$^+$ and F4/80$^+$) and interstitial macrophage (IM) (CD11b$^+$, MHCII$^+$, CD11c$^+$ and F4/80$^+$) (C, D and E) and inflammatory monocytes (CD11b$^+$, MHC II$^+$, Ly6G$^-$ and Ly6C$^+$) (F and G). ni - non infected mice. Each symbol represents data from a single mouse. *p<0.05. Results are expressed individually and the bars represent the means.

4.7 Littermate control mice confirm the detrimental role of C5aR in mice fected with *L. longbeachae*.

The use of littermate controls for mouse experiments have increased over the years in because of the important influence of microbiota in the immunity. Here we performed crossings to generate a F_2 of littermate control mice (Figure 9A). The F_2 progeny of mice used in the experimental procedures, as well as the parental generation were genotyped in order to confirm the genotypes (Figure 9B).


A
BALB/c
C5aR-/-
X
BALB/c
C5aR+/+
F1
BALB/c
C5aR+/-
X
BALB/c
C5aR-/-
F2
Littermate
BALB/c
C5aR-/-
C5aR+/-
F2-
(C5aR-/-)
F2+
(C5aR+/-)

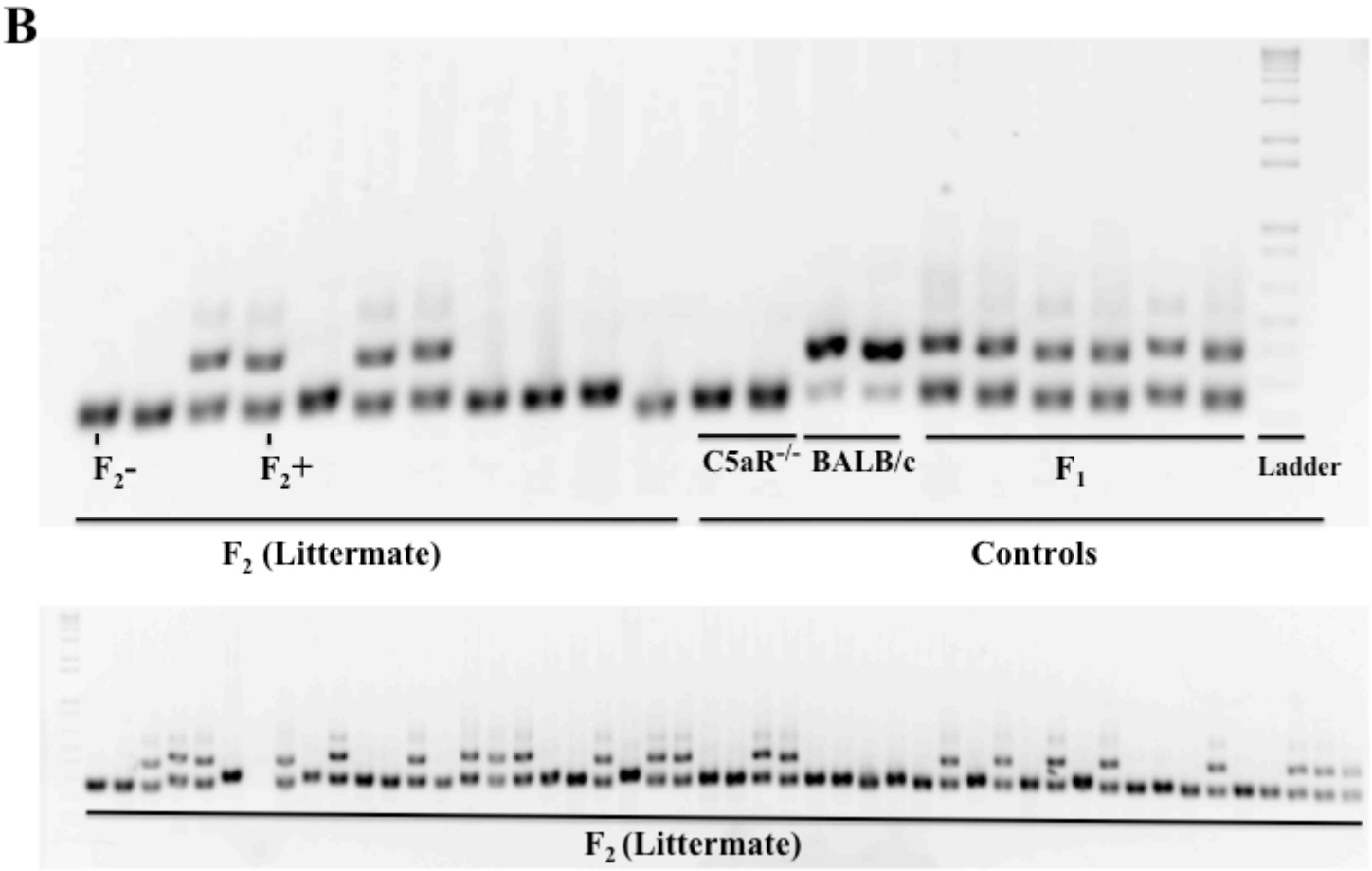
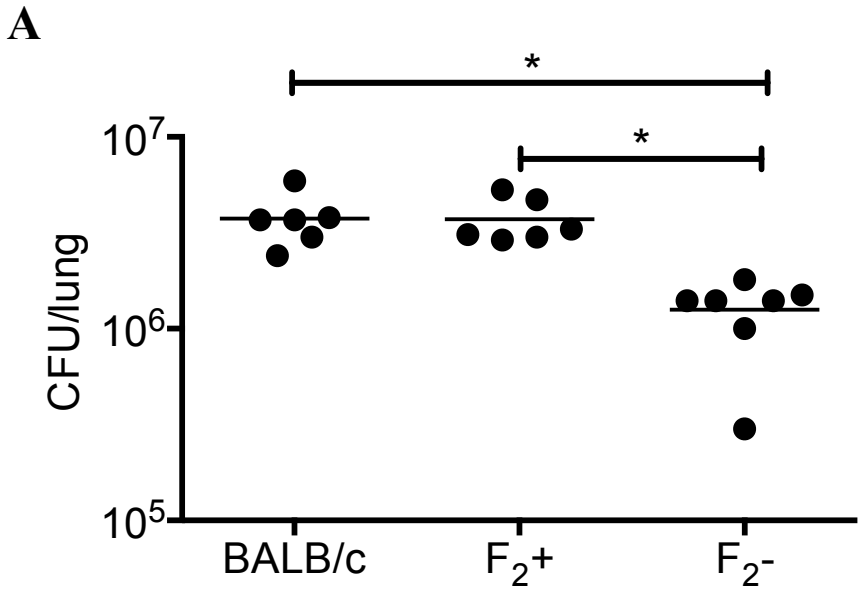
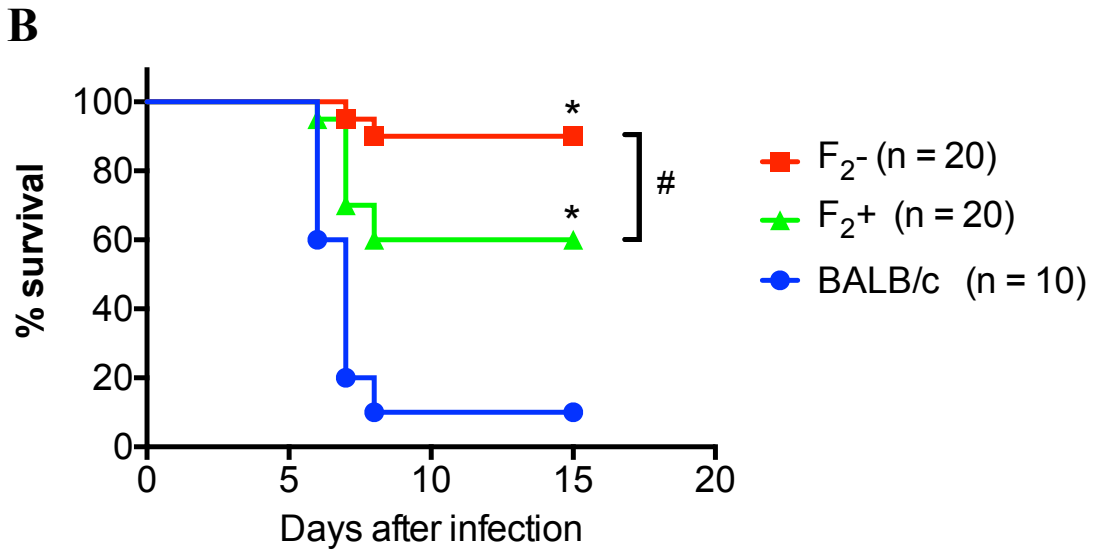


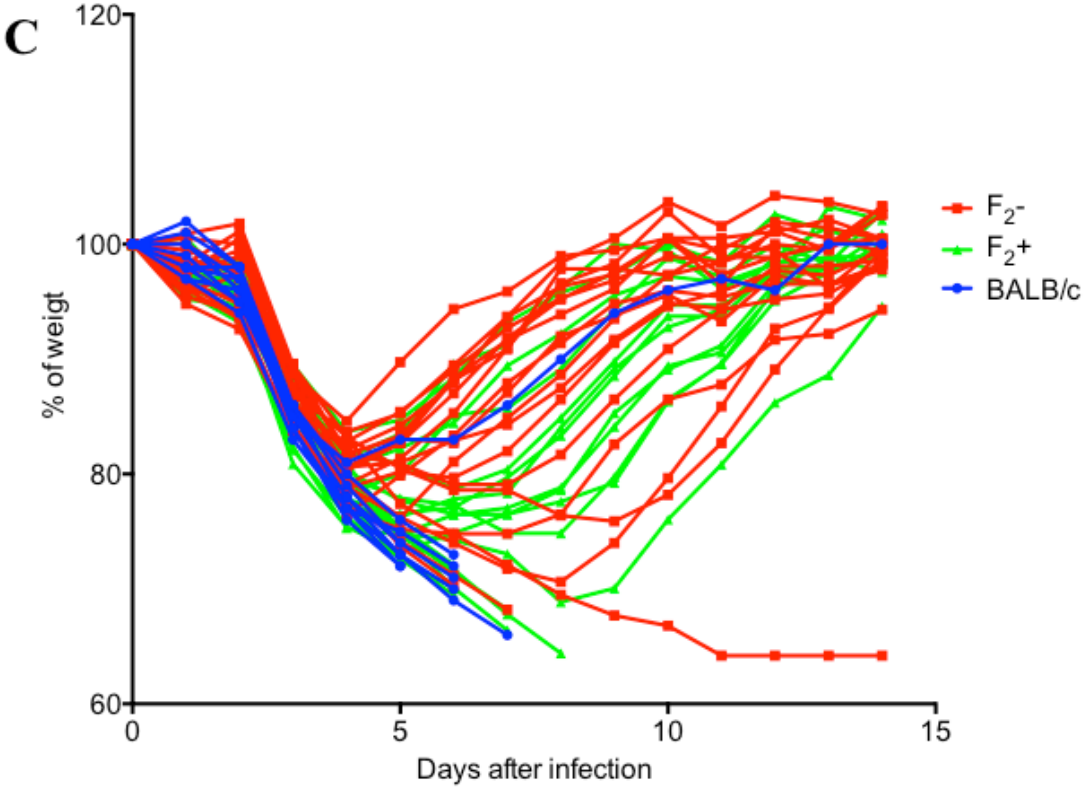
Figure 9. Generation of littermate control mice C5aR$^{+/-}$ and C5aR$^{-/-}$.
(A) Schematic representation of the generation of C5aR$^{+/-}$ heterozygote controls F_1 asd F_2, obtained from the mating of BALB/c with C5aR$^{-/-}$. "F_2-" indicates the homozygotec (C5aR$^{-/-}$) and the "F_2+" represent the heterozygote (C5aR$^{+/-}$). (B) Ear DNA genotyping of BALB/c, C5aR$^{-/-}$, F_1 and F_2 littermate control mice. Mice with F_2 genotypes were used for experiments.

In order to confirm the detrimental effect of C5aR in a *L. longbeachae* lung infection, the F_2 littermate controls mice were used to examine the bacterial replication inside the lung as well as the mice survival rate. First the BALB/c, C5aR$^{+/-}$ and C5aR$^{-/-}$ littermate control mice were infected by a nasal rote with DZ420 5x10^4, and the bacterial replication inside the lung 48 hours after infection was analysed. As shown in figure 10A, the CFU of C5aR$^{-/-}$ mice is significantly decreased when compared with the C5aR$^{+/-}$ and BALB/c mice. Furthermore, confirming the previous

result, the absence of the C5aR induces an increase in survival. The $C5aR^{-/-}$ littermate control mice have a survival rate of 90%, while the BALB/c shows a survival of just 10%. Moreover, the homozygote $C5aR^{+/-}$ mice show a significantly higher survival rate when compared with the BALB/c mice, while when compared with the $C5aR^{-/-}$ littermate control mice the survival is decreased (Figure 10B). Next, the change in percentage of body weight was followed together with the mice survival during 14 days after infection (Figure 10C and 10D). As shown in figure 10D, the average of the percentage of the body weight is very different between the BALB/c, $C5aR^{+/-}$ and $C5aR^{-/-}$ mice. On the third and fourth day after infection, the bodyweight decrease abnormally in all the three mice groups. However, while the BALB/c mice bodyweight continue to decrease, the F_2- mice start to recover and restore almost 100% of the bodyweight 14 days after infection. The $C5aR^{+/-}$ mice show a more slowly recover, however they were able to restore their bodyweight among 14 days after infection. Collectively, our results confirm the importance of the C5aR for mice susceptibility in *L. longbeachae* pulmonary infection.




B
100
80
60
40
20
0
% survival
0
5
10
15
20
Days after infection
*
*
#
F2- (n = 20)
F2+ (n = 20)
BALB/c (n = 10)


C
120
100
80
60
% of weigt
0
5
10
15
Days after infection
F2-
F2+
BALB/c

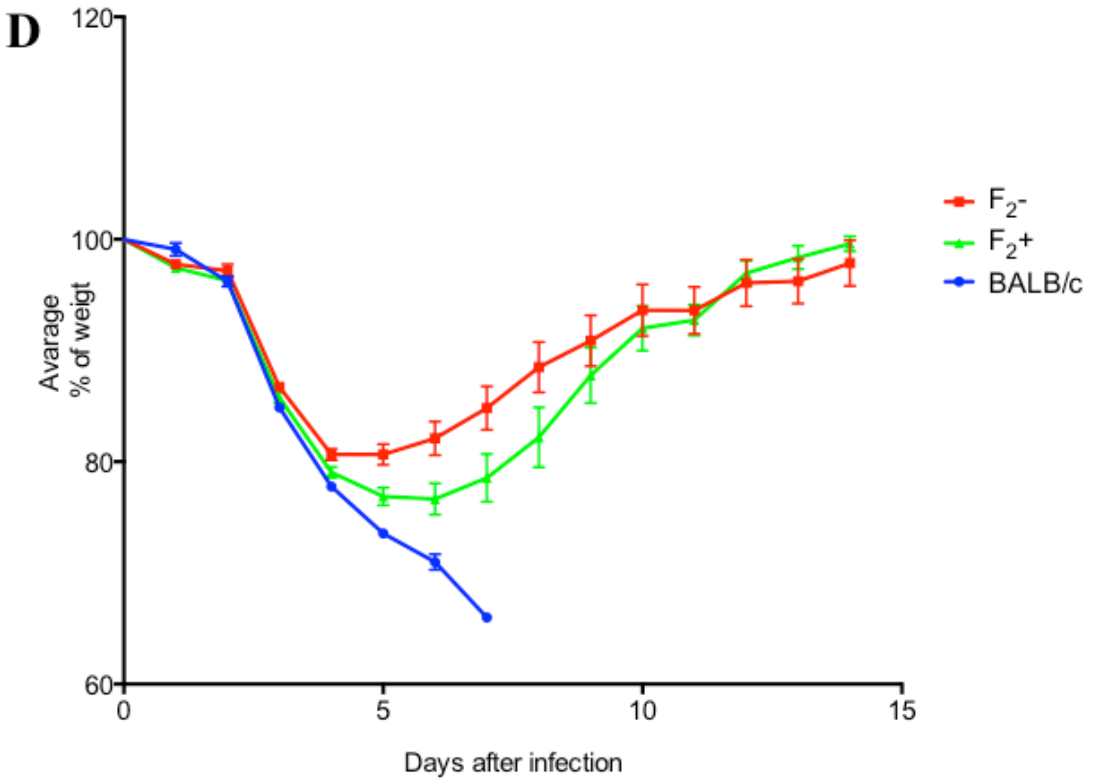


Figure 10. Evaluation of the susceptibility to *L. longbeachae* pulmonary infection using littermate control mice.

BALB/c, C5aR$^{+/-}$ and C5aR$^{-/-}$ mice were infected by a nasal rote with 5x10^4 DZ420 per mice. (A) Mice were sacrificed 72 hors after infection and dilutions of the lung homogenates were added to CYE agar plates for Colony Forming Units (CFU) determination. Each dot represents a single animal and the horizontal lines represent the means. Unpaired Student t test, *p<0,05. (B) The survival and (C) change in percentage of body weight was followed during 15 days. The number of mice used in each group (n) is indicated in the figure legend. *p<0.05 when compared with BALB/c. #p<0.05 when compared F_2+ with F_2-. (D) Change in the percentage of body weight represented as average. One outlier from the BALB/c group was excluded. Data are expressed as mean ± SEM.

Discussion

5. DISCUSSION

L. longbeachae is a predominant cause of Legionnaires' disease in some parts of the world, particularly in Australia (O'Connor, Carman et al. 2007). In the last decade, the number of *L. longbeachae* cases of Legionnaires' disease has increased markedly across Europe and Asia (Joseph and European Working Group for Legionella 2004, Ricketts, Joseph et al. 2005, Kubota, Tomii et al. 2007, Casati, Gioria-Martinoni et al. 2009). Infection by *L. longbeachae* in immunocompromised individuals or elderly people causes a severe pneumonia often leading to hospitalization and death (Diederen, van Zwet et al. 2005). The prevalence of these bacteria as cause of pneumonia is not well defined and certainly underscored since the usual diagnostic methods detect only the *L. pneumophila* species. However, recently, the prevalence of infections by *L. longbeachae* has increased and some atypical *L. longbeachae* infections have emerged. Data generated in our lab demonstrate that mice infection by *L. longbeachae* is significantly more virulent then the well-known and studied *L. pneumophila*, and this virulence is mediated by fatal lung damage in a Dot/Icm dependent manner (Liliana M Massis in preparation).

The function of C5a and its respective C5a receptor has been investigated in several lung diseases. Inhibition or deletion of the C5aR has been shown to alleviate ALI (acute lung injury) induced by paraquat and highly pathogenic viruses as well as hiperoxic induced lung injury. In contrast, lipopolysaccharide induced lung injury is C5a independent, while mice infected by *Pseudomonas aeruginosa* exhibited impaired lung clearance, control of bacterial infection as well as mice survival in the absence of C5a or C5aR (Larsen, Mitchell et al. 1982, Hopken, Lu et al. 1996, Flierl, Perl et al. 2008, Rittirsch, Flierl et al. 2008, Sun, Wang et al. 2011, Wang, Xiao et al. 2015). Moreover, the role of the C5aR during *L. longbeachae* infection in a mouse model of Legionnaires' disease has not yet been investigated. Therefore, in the present study, it was investigated the importance of the C5aR in mouse model of Legionnaires' disease induced by *L. longbeachae*.

C57BL/6 mice infected by *L. longbeache* develop a strong lung damage and die by pulmonary dysfunction (Liliana M Massis in preparation). *L. longbeachae* infection rapidly causes a dose dependent death in BALB/c mice. Furthermore, by the same infection conditions mice that lack the C5aR have a protected phenotype (Figure 3). Similar observations were made in several models of ALI. In the case of influenza

A virus infection, reducing the C5 activation decrease the inflammation and pathology (Garcia, Weston-Davies et al. 2013) and in hyperoxia-induced lung injury the C5aR determinates the severity of the disease (Russkamp, Ruemmler et al. 2015). In several studies it was reported that mice deficient in C5aR demonstrate enhanced bacterial clearance in the lung (Hopken, Lu et al. 1996, Tong, Lambert et al. 2014). In agreement to this finding, a significant impact of the C5aR deficiency on the restriction of bacterial multiplication in the lungs *in vivo,* but not *in vitro,* was found (Figure 4C). Furthermore, *in vitro*, *L. longbeachae* is immunologically "silent" in murine and human macrophages, because of the low cytokine production even with high infection dose (Liliana M Massis in preparation). *L. Longbeachae* replication inside the mice lung is rapid and already 48 hours after infection the CFU is a thousand times higher than the initial infecting dose (Figure 4). Beside the capacity to multiply in the lungs, *L. longbeachae* disseminate to the circulation and spleen but does not cause evident damage to others organs (Liliana M Massis in preparation). This could be driven because of the rapid multiplication and high amount of bacteria leading to a lung damage and therefore ending up randomly in the circulation and consequently in other organs such spleen, where the bacteria does not show its virulence activity.

Data generate in our lab demonstrate that infection by L. longbeachae induces the secretion of several proinflamatory cytokines in C57BL/6 mice *in vivo* (Liliana M Massis in preparation). The binding of C5a to its receptor exerts a wide range of biological functions including the chemoattraction of inflammatory cells and induction of the cytokine production during the inflammatory response (Sarma and Ward 2012). In order to test if the C5aR is involved in the secretion of some pro-inflammatory cytokines, the secretion of TNFα, IL-12, IFNγ, IL-1α and IL-1β was evaluated. Infection by *L. longbeache* induces a strong cytokine secretion in the BAL, thus this secretion is C5aR independent (Figure 5). *L. longbeachae* causes significant cell migration to the pulmonary site and induces the secretion of pro-inflammatory cytokines (Liliana M Massis in preparation). During ARDS (acute respiratory distress syndrome) and ALI the number of neutrophils in BAL increases significantly and it plays a key role in the development and control of the disease (Zimmerman, Renzetti et al. 1983, Zimmerman, Renzetti et al. 1983) The chemotactic and inflammatory functions of the anaphylatoxin C5a induces the recruitment and activation of neutrophils and monocytes/macrophages by binding to the C5aR. The

C5aR is expressed on many immune and non-immune cells; therefore the effects elicited by C5a/C5aR interaction appear to be organ and cell-type-specific. The expression of the C5a receptors on bronchial epithelial and smooth muscle cells was already noticed (Drouin, Kildsgaard et al. 2001, Riedemann, Guo et al. 2003, Sarma and Ward 2012). Furthermore, the complement component C5a effector functions are extensive, such increase in intracellular Ca+, promote inflammation, and mast cell, macrophage and neutrophils migration (Ehrengruber, Geiser et al. 1994, Hartmann, Henz et al. 1997, Nataf, Davoust et al. 1999, Ottonello, Corcione et al. 1999). Several publications connect the severity of lung injury with the presence of C5a and its interaction with the C5aR inducing strong cell migration, in particularly neutrophils. (Larsen, Mitchell et al. 1982, Sun, Wang et al. 2011, Wang, Xiao et al. 2015). *L. longbeachae* infection in BALB/c mice induces a significant cell migration to the pulmonary site (Figure 7) and most of these cells are neutrophils (Figure 7C). However, these data are unchanged in $C5aR^{-/-}$ mice, indicating that the C5aR does not influence neither the amount of cells nor the number of neutrophil found in the BAL in the present model of *L. longbeachae* pulmonary infection. Furthermore, the morphological changes in the lung tissue induced by *L. longbeachae* infection are not alternated in the absence of the C5aR (Figure 7 D and E).

Myeloperoxidase (MPO) is a peroxidase enzyme most abundantly expressed in neutrophil and present antimicrobial activity (Klebanoff 2005). *L. longbeachae* infection induces neutrophil migration into lung tissue, leading to an increase in MPO activity (Liliana M Massis in preparation). Corroborating that finding, the results obtained in the present study indicate an increase in MPO activity after *L. longbeachae* infection, as a consequence of recruitment of neutrophils into lung tissue. However the neutrophil recruitment, as well as MPO activity in the lung tissue, does not appears to be influenced by the absence of the C5aR in *L. longbeachae* pulmonary infection. These finding is conflicting with the well established function of the C5a-C5aR interaction as a strong chemoattractant for neutrophils (Marder, Chenoweth et al. 1985). In order to deeper investigate the cell populations present in the lung as a consequence of *L. longbeachae* pulmonary infection we phenotyped the cells present on the mice lung. Several models of lung infection indicate that NK cells are one of the first cells migrating and activated in the lung. NK cells are the early responders responsible for IFN-γ secretion leading to, among other functions, such as macrophages activation. (Hussell and Openshaw 1998, Junqueira-Kipnis, Kipnis et al.

2003, Lopez, Duckett et al. 2004, Schroder, Hertzog et al. 2004). However, in the present model of *L. longbeachae* pulmonary infection, there was no difference in IFN-γ levels in the BAL, nor in lung NK cells count (Figure 8B).

Based on their localization, different origins and life spans, macrophages in the lung include the alveolar macrophages and the interstitial macrophages, which are identified as important regulators of pathological and reparative processes. Alveolar macrophages populate lung tissue in early embryogenesis and are considered lung resident macrophages. These macrophages are placed within the alveolar surfactant film in the interphase between air and lung tissue, therefore they are the only macrophages exposed to air. Because of this, the alveolar macrophages represent the first line of defence against inhaled pathogens and display a high phagocytic and microbicidal potential (Zwilling, Campolito et al. 1982, Jonsson, Musher et al. 1986, Franke, Freihorst et al. 1994, Lohmann-Matthes, Steinmuller et al. 1994). Resident alveolar macrophages persist in LPS or influenza A-induced acute lung injury and simultaneously participate in the resolution of inflammation by phagocytosing apoptotic cells and recruiting monocyte-derived macrophages (Janssen, Barthel et al. 2011). Alveolar macrophage exerts protective anti-inflammatory effect in murine model of pneumococcal pneumonia, while depletion of alveolar macrophages have protective role against *Mycobacterium tuberculosis* mice infection (Leemans, Juffermans et al. 2001, Eichfeld, Einax et al. 2002). As shown in Figure 8C and 8D, the percentage of alveolar macrophages present in the lung is not affected by the absence of the C5aR; and infection by *L. longbeachae* induce a dramatically decrease in alveolar macrophage population in both BALB/c and $C5aR^{-/-}$ mice. The reason for the decreased alveolar macrophage population after the infection could be because *L. longbeachae* replicates and subsequently destroy the alveolar macrophage and the consequent lung damage leads to mice death. However, there is no consistent explanation for the protected phenotype of mice deficient in the C5aR. It is published that C5a, by interaction with alveolar macrophages, induces the secretion of acid hydrolases and proteolytic enzymes that can cause tissue damage and therefore may play a role in lung injury (McCarthy and Henson 1979). Unfortunately, there are no further correlations with bacterial infections or infection induced lung injury.

While alveolar macrophages are classified as long-lived tissue resident cells, which maintain lung homeostasis by removing pathogens without inducing inflammation, the interstitial macrophages have a short half-life and originate from

bone marrow-derived monocytes. They are recruited to the lung from the circulation in response to acute lung injury, and play a key role in the inflammatory response (Balhara and Gounni 2012, Gwyer Findlay and Hussell 2012, Lee 2012). In the present study, there was no change in the interstitial macrophage population in infected or uninfected BALB/c and C5aR$^{-/-}$ mice (Figure 8C).

Recently, a novel subpopulation of monocytes, the Ly6c^{hi} inflammatory monocytes, has emerged as essential to host defense against *Toxoplasma gondii, Listeria monocytogenes* and other infections (Serbina and Pamer 2006, Dunay, Fuchs et al. 2010, Neal and Knoll 2014). The inflammatory monocytes critically contributes also to lung protective immunity against *Streptococcus pneumonia (Winter, Herbold et al. 2009)*. Data generated in our lab, confirm the importance of the inflammatory monocytes in mice model of Legionnaires' disease. In the present study, the Ly6C^{hi} inflammatory monocytes population was evaluated in both BALB/c and C5aR$^{-/-}$ mice. The Ly6C^{hi} inflammatory monocyte population was not detected in the non-infected mice, neither BALB/c nor C5aR$^{-/-}$, different from what is observed in C57BL/6 mice. After 72 hours of infection with *L. longbeachae* there is a significantly increase in the Ly6C^{hi} inflammatory monocyte migration to the site of infection (Figure 8F and 8G). However, the *L. longbeachae* lung infection induced Ly6C^{hi} inflammatory monocyte migration do not appears to be influenced by the C5aR, since the percentage of Ly6C^{hi} inflammatory monocytes present in the lung of infected C5aR$^{-/-}$ mice do not show significant differences when compared with the infected BALB/c mice. Therefore the increased survival as well as impaired CFU of C5aR$^{-/-}$ mice infected by *L. longbeachae* cannot be attributed to the amount of Ly6C^{hi} inflammatory monocytes present in the lung during the infection.

Taken together, the present results indicate that the absence of the C5aR has a protective role to the lethal *L. longbeachae* pulmonary infection. Unfortunately, the mechanism by which the absence of C5aR partially protects mice from lethal infection could not be identified.

In order to testify the credibility of our result and exclude some microbiota implication, which could eventually generate false positive results, littermate control mice were generated and mice survival, bodyweight measurements as well as bacterial replication inside the lung were evaluated. The results obtained with the F_2 littermate control mice confirm the previous result obtained using C5aR$^{-/-}$ mice. Furthermore, the survival rate of C5aR$^{-/-}$ littermate control mice increases to 90%,

while the C5aR$^{-/-}$ mice have a survival of 30%. The C5aR$^{+/-}$ littermate control mice displayed an intermediate phenotype with a survival rate of 60%. By comparing their bodyweight, the C5aR$^{-/-}$ littermate control mice show reduced loss of weight as well as fastest recovery of the bodyweight when compared to the C5aR$^{+/-}$ littermate control and BALB/c mice. Corroborating the survival rate, bodyweight lost in the C5aR$^{+/-}$ littermate controls mice show an intermediate phenotype. The BALB/c mice present a survival rate of 10%, and excluding the one outlier, 100% of the BALB/c mice die within 7 days after infection. By observing their bodyweight change, there is an abnormally fast decline with a lost of 30% of the bodyweight at the day 5 after infection (Figure 10). The CFU of the C5aR$^{-/-}$ littermate control is significantly decreased compared to the BALB/c mice, confirming the previously detected phenotype with the C5aR$^{-/-}$ mice. In contrast, by comparing the CFU of the C5aR$^{-/-}$ and C5aR$^{+/-}$ littermate control with the BALB/c, the intermediate phenotype of the C5aR$^{+/-}$ was not detected (Figure 10A). However, the experiment performed with the F_1 mice shows clearly that there is a significant difference and intermediate phenotype in the CFU (Figure 2B). The reason for this disagreement between the two experiments could be the different infection time. The CFU with the F_1 mice was determinate within an infection time of 72 hours, while the CFU with the C5aR$^{+/-}$ littermate control mice was determinate within an infection time of 48 hours. This result could indicate a delayed effect of the C5aR and exclude some difference in phagocytic ability related to the C5aR. Furthermore, by observing the percentage of the bodyweight change within the first 96 hours, all the mice show a similar behavior, while after 96 hours of infection the difference between the C5aR$^{-/-}$, C5aR$^{+/-}$ and BALB/c mice became visible.

Taken together, there is an important implication of the C5aR in the bacterial clearance and mice survival in a mouse model of *L. longbeachae* infection. However, further investigation is needed in order to understand the mechanism by which the C5aR is involved in the pathogenesis of the *L. longbeachae* pulmonary infection.

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6. REFERENCES

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