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MICROBIOLOGY

Ureaplasma diversum clearance in lung mice infection is mediated by neutrophils

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Abstract: Pneumonia in cattle is one of the causes of morbidity rates and economic loss. The host response to lung infections caused by *Ureaplasma diversum* in bovines is virtually unknown. Here in the immune response was evaluated in a murine model for an experimental pulmonary infection by *U. diversum*. Therefore, AJ, BALB/C and C57BL/6 mice received intratracheal inoculation of *U. diversum* and were evaluated after 1, 2, 3, 7 and 14 days and the clinical specimens were collected. In bronchoalveolar lavages (BAL) an increase of inflammatory cells was observed. Neutrophils were the main cells recruited to the site of infection and the infiltration was coincided with the production of pro-inflammatory cytokines. We found a large amount of neutrophil in this initial period, followed by a decrease 7 and 14 days post infection, accompanied by bacterial clearance. Our results evidenced the presence of *U. diversum* within the neutrophil that suggests a phagocytic role of this cell in the elimination of the infection. The immune response features reported here are the initial evidence that healthy immune systems may control these microorganisms. This may be the first step to design new strategies immune based to control the infections in naturally infected hosts.

Key words: infection pulmonary, neutrophil, mycoplasma, cattles.

INTRODUCTION

Bloodstream circulating neutrophils have a short-life and are important in initiating defenses against foreign microorganisms (Summers et al. 2010). After an infection or inflammation, these cells are attracted by mediators, such as complement fragment C5a (Dragomir et al. 2012), leukotriene B_4 (Afonso et al. 2012), CXCL-2 (Uchida et al. 2009), myeloperoxidase (Klinke et al. 2011) and antimicrobial peptides (Ma et al. 2012). These cells migrate and interact with the stimulated endothelial cell surface (Rigby & DeLeo 2012). Inside the tissues, granules and antimicrobial proteins of mobilized neutrophils kill the infectious agent, but also cause damage to the infected tissue site (Summers et al. 2010). Usually, the microorganism is destroyed in the phagocytic vacuole of activated neutrophil (Rigby & DeLeo 2012).

Neutrophils may also be involved in the response to *U. diversum* infections in bovines. This mollicute adheres to the mucosa of the reproductive tract of these animals and may cause important economic loss due to abortion, vulvovaginitis and other urogenital disturbances (ter Laak et al. 1993, Rottem 2003, Petit et al. 2008). Importantly, respiratory infections are common as well and pulmonary diseases might be associate with pneumonia and economic implications. As with all Mollicutes, *U. diversum* lacks a cell wall but may present an external glycocalix (Marques et al. 2016, Marques et al.

2010). Like other ureaplasmas, *U. diversum* hydrolyzes the urea and releases ammonia, intoxicating the host tissue (Glass et al. 2000).

Even after five decades of isolation (Taylor-Robinson et al. 1967), there is a little information about this organism and the immune response against *U. diversum* in cattle or other hosts. The mechanisms by which this organism exerts its virulence and pathogenicity are mostly unknown (Amorim et al. 2014, Kim et al. 1994, Marques et al. 2010, Silva et al. 2016).

Despite the description of causal associations, the relationship between *U. diversum* and disorders in its hosts remains controversial. Bovines may show different genetic backgrounds and distinct patterns of immune response to pathogens. This suggests that the interaction pathogen-host is one of the main factors involved in the development of pneumonia in calves.

In this context, a large number of species of mycoplasma have been isolated from the respiratory tract of calves. The three most important species, based on studies of occurrence and pathogenicity, are *Mycoplasma bovis, M. dispar*, and *Ureaplasma* sp. In the literature, authors report that ureaplasmal virulence factors include IgA protease, urease, phospholipases A and C, and production of hydrogen peroxide (Gourlay & Howard 1982, Viscardi & Hasday 2009, Howard 1983). These factors may allow the organism to evade mucosal immune defenses by degrading IgA, and injuring mucosal cells through the local generation of ammonia, membrane phospholipid degradation and prostaglandin synthesis, and membrane peroxidation, respectively (Viscardi & Hasday 2009, Xiao et al. 2014). However, murine models for the study of inflammation caused by *Ureaplasma diversum* are very rare in the literature.

We aimed to mimic the different types of immune responses that could occur in natural hosts using different inbred mice strains which ones have divergent immune response patterns as result of genetic mutations and polymorphisms. Although common inbred mice are considered "immune competent," many have variations in their immune system that may affect the phenotype.

Thus, inbred mice become an excellent model to investigate how hosts genetic variability influence immune response in different sort of infections. The knowledge about immune response may help clarify factors underlying *U. diversum* infection. However, there is no experimental model to study pulmonary infections by this mollicute. Here we investigate the initial immune response during an experimental lung infection by *U. diversum* in three different mice strains.

MATERIALS AND METHODS

Mice

BALB/c, A/J and C57BL/6 mice, age 6 to 8 weeks were obtained from the animal facility of Universidade Federal da Bahia – Instituto Multidisciplinar em Saúde - *Campus* Anísio Teixeira). Mice were housed in a controlled environment with free access to food and water. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Universidade Estadual de Feira de Santana (UEFS).

Bacterial strain and culture

U. diversum ATCC 49782 was obtained from the collection of Mycoplasma Laboratory, Institute of Biomedical Sciences at University of São Paulo/ Brazil. *U. diversum* was cultured in ureaplasma medium (UB) according to Ruhnke & Rosendal procedures. In a logarithmic growth phase,

the culture was centrifuged at 15,000 x g for 25 minutes at 4°C, pelleted and gently washed with Phosphate-buffered saline (PBS). The final pellet was homogenized in PBS aliquoted in 1 mL vials. This inoculum was quantified in 96-well microplates for Determination of Color Changing Units-methodology (CCU/mL) as described by Kim et al. (1994). For some experiments *U. diversum* was homogenized in PBS and incubated with carbocyanine dye solution (Vybrant™ Dil cell-labeling solution-Dil, V-22885, Molecular Probe, Eugene, Oregon, USA). The viable or inactivated by heat (100°C for 30 minutes) bacterial strain was analyzed by flow cytometry. The inactivation was confirmed by absence of a positive culture on UB medium. Negative controls without bacteria inoculation were also used.

Lung infection

Prior to intratracheal inoculation, mice (n=6) were anesthetized with an intraperitoneal injection of 65 mg/Kg ketamine and 10 mg/Kg xylazine. For experiments with unlabeled *Ureaplasma* each mouse was inoculated intratracheally with 10^4 CCU/mL of *U. diversum* by instilling the inoculum into the posterior pharynx of an anesthetized mouse while it was suspended in a vertical position and prevented from swallowing by gentle extension of the tongue. Control mice received saline alone. The mice were maintained in this position until aspiration was witnessed (disappearance of inoculum from the posterior pharynx and retraction of the chest wall) (Viscardi et al. 2002a). The animals were euthanized 1, 2, 3, 7 and 14 days postinoculation for bronchoalveolar lavage and analysis of lung tissue for *Ureaplasma* by culture and PCR. Additional groups of five mice (BALB/c strain) were infected with 10^4 CCU / mL of labeled viable and non-viable *U. diversum*, following the same procedures described above.

Bronchoalveolar lavage

After euthanasia, bronchoalveolar lavage (BAL) was performed *in situ*. The trachea was cannulated (21 GA Insyte, Becton Dickinson), and 1 mL of PBS was infused intratracheally and withdrawn, followed by instillation and recovery of PBS. This procedure was repeated three times. The BAL fluids were stored at 4°C until cells were collected by centrifugation at 300 x g for 10 minutes. Cell-free supernatants were stored at -80ºC for analysis of cytokine concentrations. Total-cell counts were performed manually with a hemacytometer. Differential cell counts were performed on cytoslides prepared using a cytocentrifuge stained with panoptic according to the manufacturer's instructions. The analysis was performed by two blinded observers using morphological criteria.

Cytokine ELISA

Concentrations of TNF-α, IL-1β, IFN-γ and IL-10 in BAL supernatants were measured by sandwich ELISA (Ready- SET-GO® kit – Bioscience) according to the manufacturer's recommendation.

Lung culture

To confirm the infection in our model, after euthanasia the mice lungs were removed and processed for *Ureaplasma* culture and PCR. Following lavage, the lungs were homogenized in 1mL of PBS and centrifuged at 300 x g for 10 minutes at 4 °C. The supernatant (200uL) was added in 1 mL of UB medium and e incubated at 37°C in 95% air–5% CO_2. The tubes were examined daily for two weeks for color change.

Polymerase chain reaction (PCR)

DNA was extracted from lung supernatants by boiling method (Fan et al. 1995) to detect *U. diversum*. The PCR methodology was performed as described by Cardoso & colleagues (2000). The DNA of the *U. divsersum* ATCC was the

positive control and the negative control was an aliquot of PCR mix. The PCR products were electrophoresed in 1.5% agarose gel, stained with ethidium bromide. A DNA product band of 216 bp visualized by UV illumination was considered positive.

Flow cytometry

The BAL fluids were collected 24 hours after the infection with labeled *U. diversum*. Cells were suspended in MACS buffer (PBS, 0.5% bovine serum albumin, 2 mM EDTA) and surface stained with anti-CD11b and anti-Gr-1 according to the manufacture's protocols. The neutrophil population was selected at the double-positive gateway for CD11b and GR-1. Other non-neutrophil cells were defined for the positive gate in CD11b and negative for GR-1. The buffers and antibodies were purchased from BD Biosciences (CA, USA). The cells were examined by flow cytometry using FACS Fortessa (BD Biosciences San Jose, CA/USA) and data were analyzed using the FlowJo software (TreeStar, OR, USA). Data are shown as median fluorescence intensity (MFI).

Statistical analysis

All data are represented as means ± standard errors (SE). Statistical analysis was performed by multiple comparisons in the different experiments using the test non-parametric (one-way ANOVA) Kruskal-Wallis. Statistical differences were considered significant when $p < 0.05$ using a confidence interval of 95%. Analyzes were performed at GraphPad Prism 4.0 program (GraphPad Software, San Diego, CA, USA). The obtained results were analyzed using Mann Whitney Test. Analyses were performed using GraphPad Prism® software (version 5.0, GraphPad Software, San Diego, CA, USA). Statistical differences were considered significant at p values <0.05.

RESULTS

U. diversum infection induces influx of neutrophils into the lungs

To assess the immune response after pulmonary infection by *U. diversum,* inbred mice strains BALB/c, A/J and C57BL/6 were infected and BAL were collected after 1, 2, 3, 7 and 14 days after infection. *U. diversum* induced a significant recruitment of cells to the site of infection; total BAL cell counts were higher at 1, 2 and 3 days postinoculation (Fig. 1). Peaks of BAL cell accumulation occurred at 24 hours in BALB/c and A/J lineage while C57BL/6 reached highest cell count at 48 hours in *Ureaplasma*-inoculated mice vs. in saline-inoculated controls. After 7 days, we observed a rescue in total BAL cells number to the control levels in all mouse lineages evaluated. It was maintained up to 14 days after infection (Fig. 1).

In order to investigate the cell types that were present in the BAL, we performed differential cell count. The BAL cellular profile of mice that remained healthy during the length of the experiment consisted mainly of macrophages. However, *Ureaplasma* inoculation stimulated significant changes in BAL cell composition characterized by an influx of neutrophils (Fig. 2). BALB/c and A /J mice showed an intense neutrophil infiltration during the first 72 hours (15 and 40-fold-higher numbers of neutrophils in *Ureaplasma*-inoculated mice than controls – Fig. 2a, b, c, d) with a peak at 24 hours post infection. While C57BL/6 mice showed an increase in the number of neutrophils into the lungs within 48 hours (Fig. 2e, f). There were a decrease in BAL neutrophils content of *Ureaplasma*-inoculated mice, 7 and 14 days postinoculation with levels similar to the control group (Fig. 2).

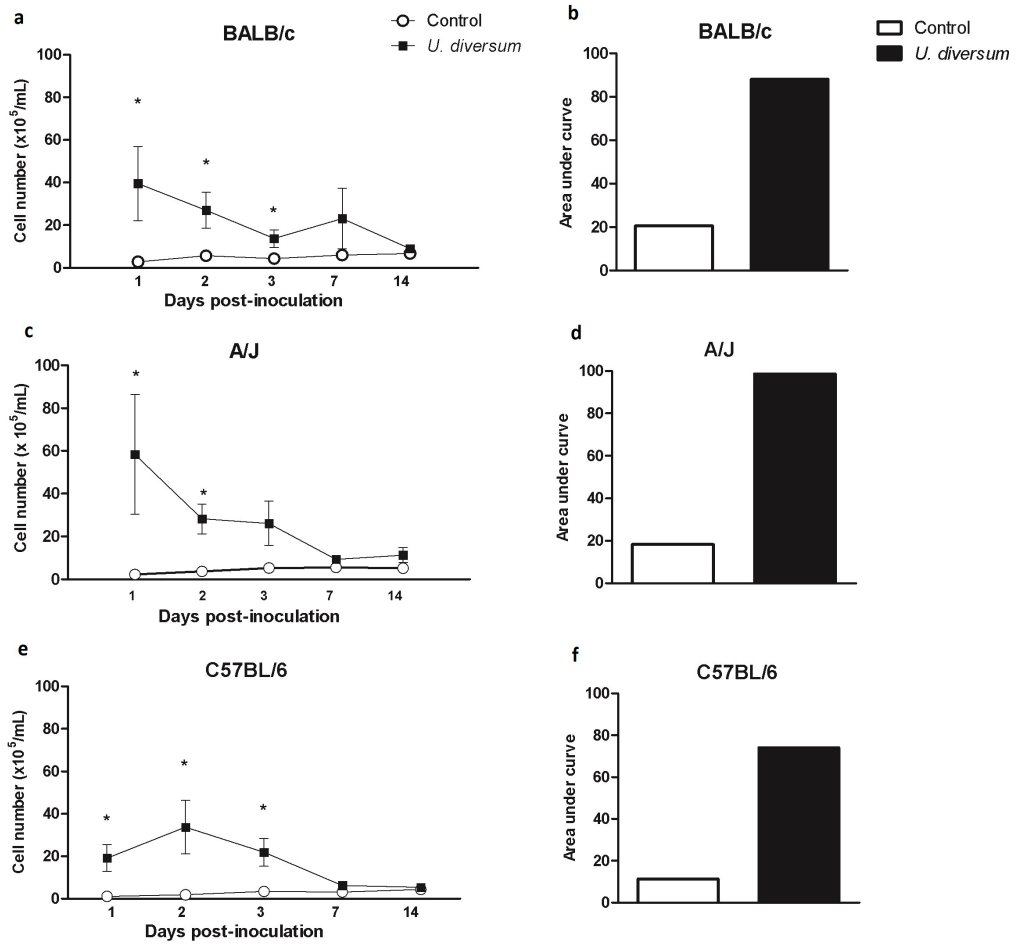

Figure 1. BAL fluid cell counts. Mice were infected intratracheally by 10^4 CCU of *U. diversum* and euthanized 1, 2, 3, 7 and 14 days after infection. The BALs were collected, centrifuged, the pellet resuspended in PBS and the total number of cells was determined manually with a hemacytometer. Data are expressed as the means ± SE of the numbers of cells per milliliter of BAL. (a) BALB/c total cell counts. (c) A/J total cell counts. (e) C57BL/6 total cell counts. (b), (d) and (f): area under the curve. n=6. *p<0.05 compared to control of the same day.

U. diversum-infected lungs stimulates production of pro-inflammatory cytokines

Since we observed the influx of inflammatory cells into the *Ureplasma*-inoculated mice lungs, we next investigated the cytokines profile present in the BAL fluids of these animals. Analysis of BAL cytokines composition showed an increase of pro-inflammatory profile after infection. BALB/c and A/J *Ureaplasma*-inoculated mice showed higher production of IL-1β and TNF-α one-day postinoculation in their BALs (Fig. 3a, b). In contrast, we did not observe any difference in concentration of these cytokines in C57BL/6 mice at the same time postinoculation. These ones showed an increase of IFN-γ at the first 48 hours after infection as well as A/J mice (Fig. 3c). There were no differences between *Ureaplasma*-inoculated mice and saline-inoculated mice after 24 hours in any lineages evaluated when we accessed the anti-inflammatory cytokine IL-10 (Fig. 3d).

U. diversum clearance is mediated by neutrophil

The infection by *U. diversum* was evaluated by culture of lung tissue and PCR. Culture from lungs tissues confirmed the presence of *U. diversum* in 100% of inoculated BALB/c all over the period analyzed. C57BL/6 inoculated mice showed positive culture in 100% one-day after inoculation, while A/J mice showed 66.6%. After 3 days, 66.6% of cultures were positive in

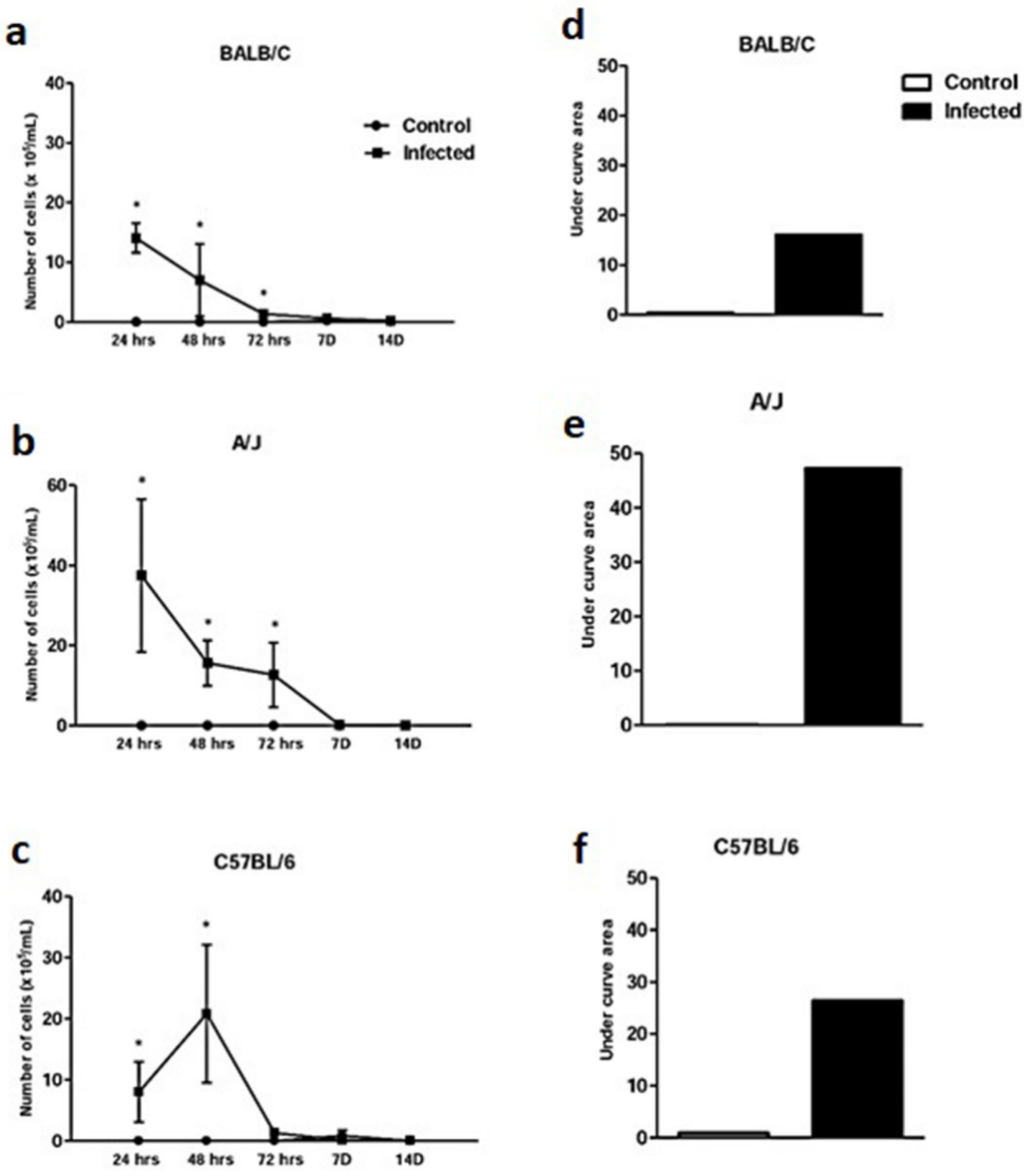

Figure 2. Neutrophil cell counts in BAL. Animals were infected intratracheally by 10^4 CCU of *U. diversum* and euthanized 24, 48, 72 hours, 7 and 14 days after infection. The BALs were centrifuged and the pellet resuspended in saline solution. The obtained cytospin slides were stained with Panotic and evaluated by light microscopy. Figures a, b and c, show the neutrophil numbers in the different times of infection. (a). Balb/C, (b). A/J, (c). C57BL/6 respectively. (d), (e), (f), show area under the curve. n=6. *p<0.05.

these both lineages. Samples in culture were considered positive by observation of color changed in UB medium due to alkalization, which is a feature exclusive to *Ureaplasmas* (not to other mycoplasmas species) since they have urease activity generating an electrochemical gradient through accumulation of ammonia/ammonium (Fig. 4a). All tissues from non-inoculated animals were culture negative.

In parallel, *U. diversum* was detected in lung tissue by PCR as well. We have shown the presence of *Ureaplasma* in BALB/c mice by positive PCR in all animals analyzed 1, 2, or 3 days postinoculation. In A/J and C57BL/6 lineages, all mice were positive one-day after inoculation, while the percentage of positive animals decreased to 66.6% 3 days post infection. These data suggest that C57BL/6 followed by A/J tend to control infection faster than BALB/c mice. Moreover, *Ureaplasma* DNA was not detected by PCR in all animals evaluated 7 and 14 days post infection (Fig. 4b). All lungs of

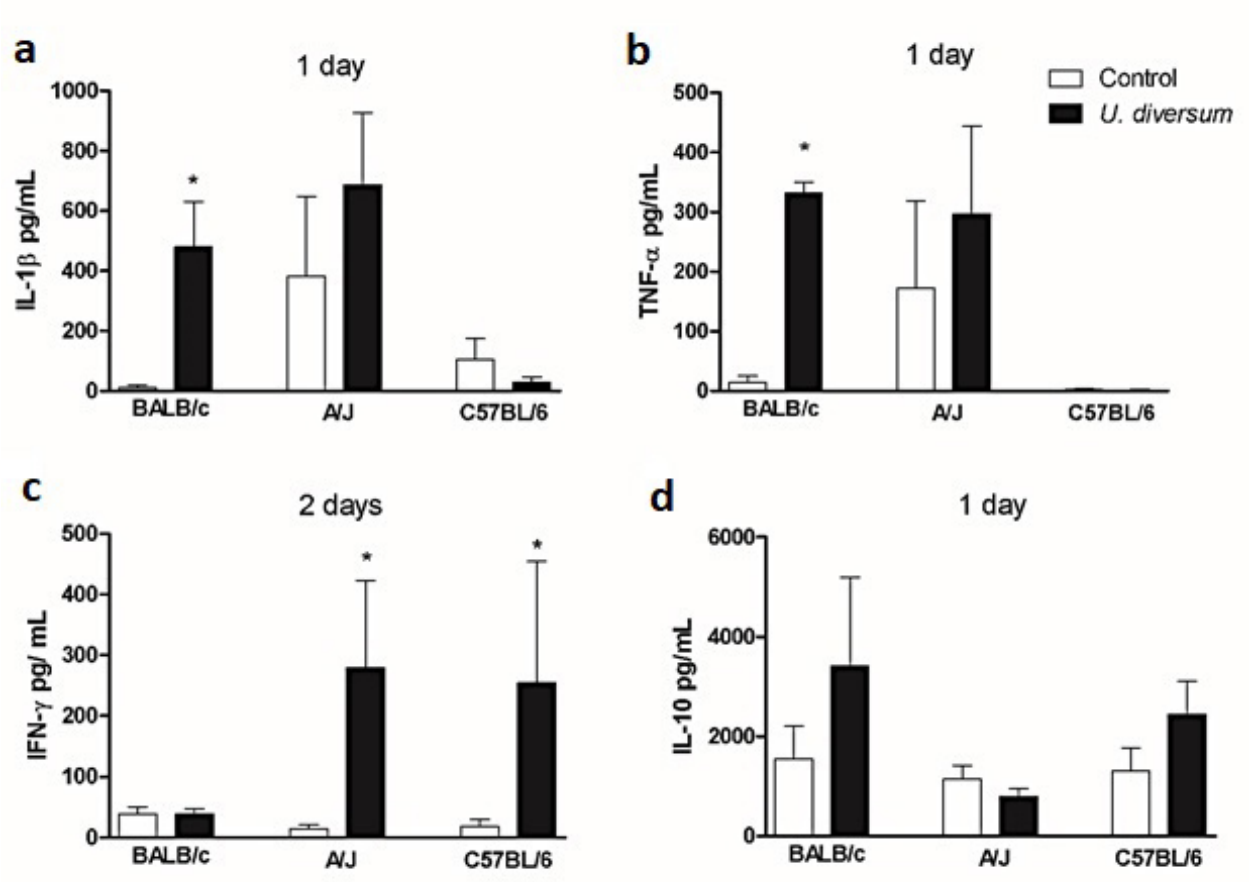

Figure 3. Pulmonary response cytokines. Mice were infected intratracheally by 10^4 CCU of *U. diversum* and euthanized 1 or 2 days after infection. The BALs were centrifuged and the supernatant of BALs were used for measurement of cytokines (IL-1β, TNF-α, IFN-γ and IL-10) by ELISA. (a) IL1- β; (b) TNF-α; (c) INF-γ; (d) IL-10.

saline-inoculated mice were PCR negative. The histology of lungs in infected mice showed an inflammatory infiltrate, unlike the non-infected mice. The infiltrate was more intense one-day postinoculation, but we did not observe severe pulmonary injury. However, in our model, none *Ureaplasma*-inoculated mice appeared ill and there was no mortality in any lineage evaluated.

Additionally, we assessed the correlation between neutrophils recruitment and the bacterial clearance into the lungs in *Ureaplasma*-inoculated mice. We hypothesize that the neutrophils influx into the lungs after infection promotes *U. diversum* phagocytosis and triggers bacterial clearance. To confirm this hypothesis, we used labeled *U. diversum* viable and inactivated by heat. Flow cytometry revealed that fluorescence emission of *U. diversum* strains (viable and inactivated) was present in neutrophils ($CD11b^+/Gr\text{-}1^+$ cells) postinoculation, on the other hand, there was no significant fluorescence for *U. diversum* in $CD11b^+/Gr\text{-}1^-$ (non-neutrophils population), indicating the presence of *Ureaplasma* inside neutrophils (Fig. 5a, b and c). As expected, cells $CD11b^+/Gr1^+$ were positive for either viable or inactivated *U. diversum* strain (Fig. 5). Together these data indicate that *U. diversum* is internalized by neutrophils and this process might not be mediated by bacterial invasion.

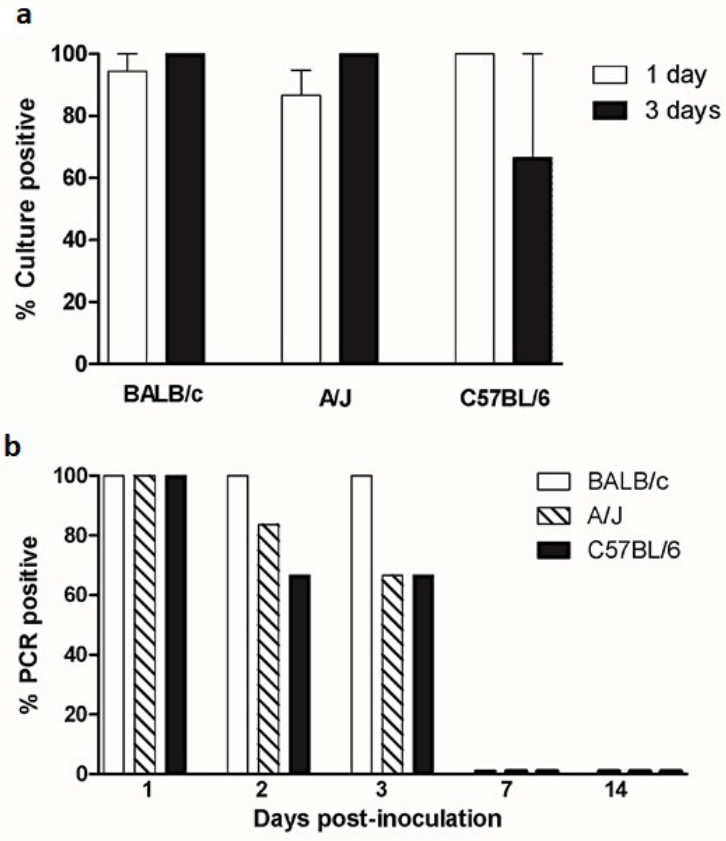

Figure 4. Lung detection of *U. diversum*. **(a)** The percentage of cultures in which there was a color change in the medium ureaplasma recovered from lungs of infected mice 1 and 2 hours post infection. It was considered positive culture, one that showed color change without turbidity, precipitation or any evidence of contamination. **(b)** Percent of ureaplasmal detecting DNA in infected lung 1, 2, 3, 7 and 14 days post infection by *U. diversum*. Mice of the control groups were negative for both culture and for PCR. n = 6.

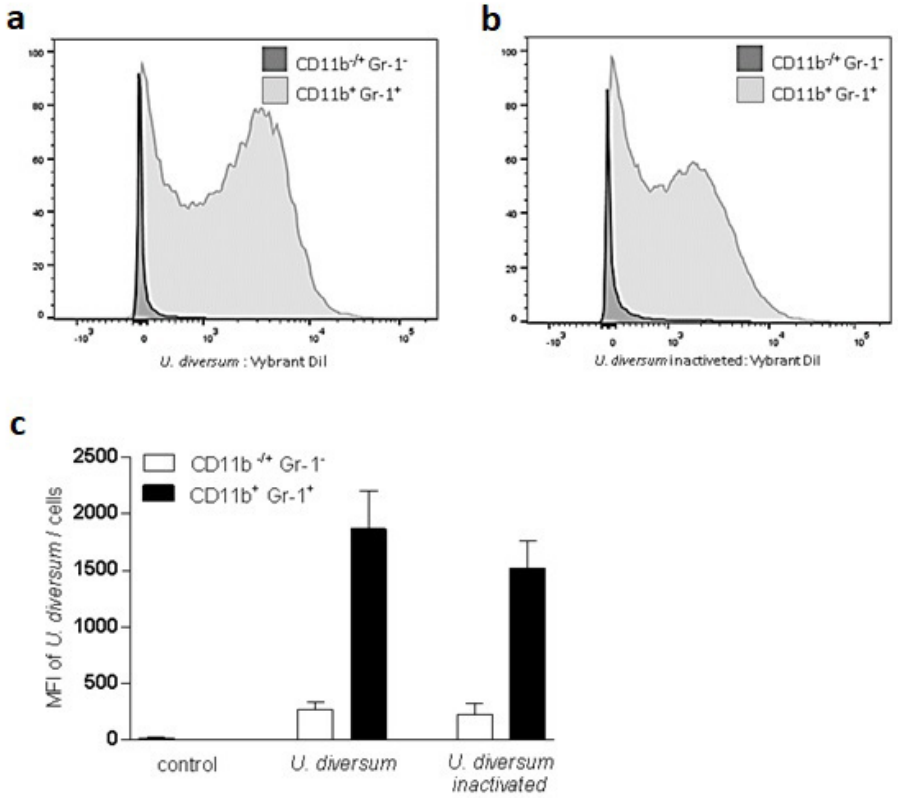

Figure 5. Neutrophil mediated *U. diversum* clearance into the lungs. BALB/c were infected intratracheally with 10^4 CCU *U. diversum* viable or inactivated, labed with vybrant. One day after infection, BAL cells were labeled for anti-CD11B-BV421 and anti-GR1-FITC. The mean vibrant dil fluorescence intensity (MFI) was measured on gate neutrophil (CD11b+/ GR-1+) and non-neutrophil cells (CD11b+/ Gr-1-). n = 5. *p<0.05.

DISCUSSION

We have shown in this study that pulmonary infection by *U. diversum* in three different mice lineages (BALB/c, A/J and C57BL/6) induced an early influx of cells into the airways and lung tissue, 1 to 3 days after infection. We found a large amount of neutrophil in this initial period, followed by a decrease 7 and 14 days post infection. It was correlated with the absence of *Ureaplasma* in lung tissue and an increase in pro inflammatory cytokines levels. Our findings suggest that there is a role of neutrophils in the clearance of *U. diversum*, which seems to have contributed to eliminate the infection in all tested lineages. These results provide evidence that the innate immunity may have an important role against of *U. diversum*. Here we demonstrated for the first time, the induction of a protective immune response in the host airways during *U. diversum* infection in a murine model.

U. diversum pulmonary infection induced an early influx of neutrophil into the airways and lung tissue after infection. Our experimental model the infection was characterized by an acute inflammation (days 1 to 3) and after that a resolution phase (days 7 to 14). Some studies have demonstrated that ureaplasmas can induce a systemic inflammatory response with elevated peripheral leukocyte counts, mainly neutrophils, in neonates with *Ureaplasma* isolated from the lower respiratory tract (Ohlsson et al. 1993, Ollikainen et al. 1998, Panero et al. 1995). In addition, acute inflammation in *Ureaplasma*-inoculated mice preserved histologic integrity of the lung parenchyma (data not show). However, in other species, for example, *Ureaplasma parvum*, pneumonic infection in non-human primate models showed that an established infection in the lung leads to inflammation and lung damage by a robust inflammatory infiltrate (Novy et al. 2009). These data suggest that the response generated in our model may be enough to control infection without signal of exacerbation that could compromise the infection site.

An important result of our study was the presence of neutrophils, representing the majority of cells, recruited to the lungs of infected mice. In fact, this data corroborates with others studies showing that infection with *Ureaplasma urealyticum* induces an early influx of neutrophils into the lung (Viscardi et al. 2002a). In addition, the clearance of infection by *Mycoplasma pneumoniae* in mice correlates with the presence of polymorphonuclear (Martin et al. 2001, Parker et al. 1987). Indeed, the resolution phase, characterized in that decreased number of neutrophils recruited at 7 and 14 days after infection coincided with the absence of *Ureaplasma* in lung tissue, suggesting a role of neutrophils in the clearance of infection by *U. diversum*. Furthermore, these data are in accordance with the fact that the rapid attraction of leukocytes to the lungs and subsequent production of pro-inflammatory mediators are necessary for effectively eliminate the infectious agent in mycoplasma infections (Wu et al. 2007).

Additionally, we observed an increase in the pro-inflammatory cytokines levels, such as IL-1β, IL-6 and TNF-α 24 hours after infection, as well as the production of IFN-γ at 48 hours. This same response profile was also observed *in vitro* when cultures of peritoneal macrophages stimulated by *U. diversum* were able to induce the production of pro-inflammatory cytokines (Chelmonska-Soyta et al. 1994). Our findings in murine models are in agreement with other studies, which have shown that ureaplasmal infection of the respiratory tract promotes a proinflammatory cytokine cascade in the lower respiratory tract. Neonatal *Ureaplasma*

infection is consistently associated with increases in TNF- α, IL-1 β, and IL-8 (De Dooy et al. 2001, Viscardi et al. 2002b). The dynamics of the inflammatory process is characterized by an innate immune response and involves the expression of inflammatory genes such as those that are responsible for coordinated cytokine secretion through activation receptors such as the family of Toll Like Receptor (TLR) (Xiang & Fan 2010). The activation mechanisms induced by *U. diversum* involving TLRs are not described in the literature. It is known that the infection of BALB/c and C57BL/6 mice by *U. parvum* involves the activation of TLR2 (Allam et al. 2014). Furthermore, the antigen lipoprotein exposed on the surface of the membrane and shared between the species of *U. urealyticum* and *U. parvum* interact with TLR2 and TLR4 activating NF-kB, which is involved in signaling to increase production of inflammatory cytokines (Peltier et al. 2007, Triantafilou et al. 2013). The production of IFN-γ suggests the involvement of TLRs receptors, which may be capable of inducing signaling pathways that culminate in the activation of expression of interferons (Bourgeois & Kuchler 2012). In the literature, authors have reported that they are capable of promoting a significant inflammatory response by activation of Toll Like Receptor 2 (TLR2), indicating that surface molecules are important for the activation of inflammatory response (Marques et al. 2016).

We assessed *U. diversum* infection in cultured lungs to confirm the infection within 24 to 72 hours and monitored by PCR. Among strains, C57BL/6 tends to control the infection faster than other strains. This is in agreement with the literature which demonstrates BALB/c mice more susceptible to *Ureaplasma* infections (von Chamier et al. 2012). Comparing the data, among the three strains recruitment of neutrophils was higher in A/J mice. The largest population of neutrophils present in this strain together with the production of cytokines may explain the differences obtained in PCRs from A/J and BALB/c. In this way, the genetic background of the animals and pathogen virulence factors may be responsible for modulating the pro-inflammatory response mediated by inflammatory cells involved in the control of infection by *U. diversum*. Here, all mouse lineages eliminate the infection after 7 days.

The results show that neutrophils are able to phagocyte *U. diversum*, performing the depuration of the microorganism. Exploration of the mechanisms involved in the interaction pathogen/host in different strains of mice may be useful in developing immune based strategies to control infections caused by mycoplasmas in bovines. Historically, these studies have been difficulties because of logistical and economic challenges of working with the natural host and the inability to successfully create a laboratory animal model that reproduce clinic signs observed in cattle.

Virulence factors of the *U. diversum* related to activation and stimulation of the host immune system remain unclear. Here we showed the first report of participation of neutrophils in the control of *U. diversum*. Neutrophils are the first cells to migrate to the sites of infection and play important roles in defense against *U. diversum*. The role of neutrophils involves phagocytosis, in addition to the production of cytokines, capable of activating other cells involved in the elimination of the pathogen (Fig. 6).

The immune response features reported here are the initial evidence that healthy immune systems may control these microorganisms. This may be the first step to design new strategies immune based to control the infections caused by *U. diversum* in naturally infected hosts.

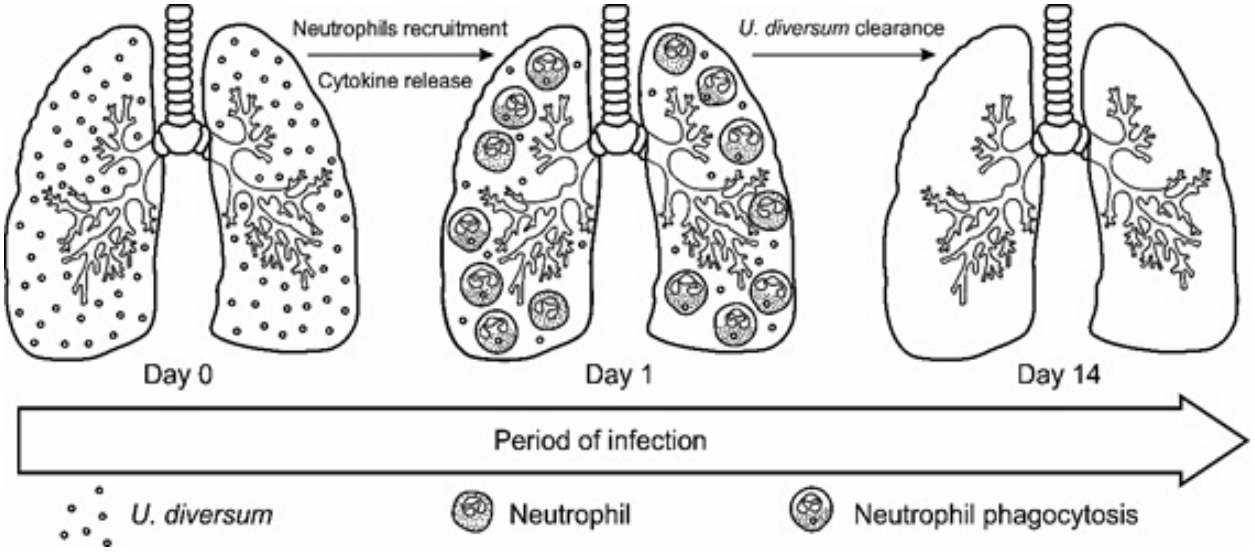

Figure 6. Schematic figure of *U. diversum* clearance. Mice were infected intratracheally by 10^4 CCU of *U. diversum* and euthanized 1, 2, 3, 7 and 14 days after infection. The results show that neutrophils are able to phagocyte *U. diversum*, performing the depuration of the microorganism and the role of neutrophils involves phagocytosis, in addition to the production of cytokines, capable of activating other cells involved in the elimination of the pathogen.

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