Veterinary Microbiology 213 (2018) 28–34

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Development of an *in vivo* model of *Chlamydia abortus* chronic infection in mice overexpressing IL-10

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ARTICLE INFO

Keywords:
Chlamydia abortus
IL-10
Chronic infection
Macil10tg mice

ABSTRACT

Chlamydia abortus, like other members of the family Chlamydiaceae, have a unique intracellular developmental cycle that is characterized by its chronic nature. Infection of a flock can remain undetected for months, until abortion occurs the following reproductive season but, to date, neither the location nor the mechanisms that maintain this latent phase are fully understood. Studies have shown that IL-10 produced as a response to certain micro-organisms sustains the intracellular survival of pathogens and increases host susceptibility to chlamydial infections.

In order to induce a sustained infection *C. abortus*, transgenic mice that constitutively express IL-10 were infected and the immunological mechanisms that maintain infection in these mice were compared with the mechanisms of a resistant wild-type mouse strain. Viable bacteria could be detected in different tissues of transgenic mice up to 28 days after infection, as analysed by bacterial isolation and immunohistochemistry. Chronic infection in these mice was associated with an impaired recruitment of macrophages, decreased iNOS activity at the site of infection and a more diffuse distribution of inflammatory cells in the liver. This murine model can be of great help for understanding the immunological and bacterial mechanisms that lead to chronic chlamydial infections.

1. Introduction

Members of the family Chlamydiaceae are Gram-negative bacteria that follow a unique intracellular developmental cycle with the potential to cause both acute and chronic infections. There has been a sustained effort on the part of chlamydiologists to understand the features of chronic infection in both host cell and pathogen, as well as any possible association with aberrant ("persistent") chlamydial morphology, as observed *in vitro* (Goellner et al., 2006). Moreover, there is evidence that these bacteria are also able to persist within tissues of the host and to become reactivated, thus causing recurrent infections despite host defence mechanisms (Stephens, 2003). As an example, in human medicine, a connection between *Chlamydia* (*C. pneumoniae* and *C. trachomatis*) infections and chronic diseases, such as asthma (Black et al., 2000) and pelvic inflammatory disease (Darville and Hiltke, 2010), has been described.

In the same way, *Chlamydia abortus* is a major abortifacent pathogen in small ruminants world wide and the aetiological agent of Ovine Enzootic Abortion (OEA). Infection in the flock follows a sub-clinical course in non-pregnant sheep and can remain undetected for months until late-term abortions occur as the only sign of infection (Navarro et al., 2004). Even if animals are infected early in gestation, the pathogenic effects of infection start after 90 days of gestation, when high loads of bacteria multiply within placental membranes and foetal tissues, causing abortion and perinatal mortality. Interestingly, once they have aborted or delivered a weak lamb, affected animals will develop protective immunity and will not abort again as a result of *C. abortus* infection (Longbottom and Coulter, 2003). Although mice are not the natural host for *C. abortus*, there are several analogies in the pathogenesis of abortion and immune response which makes them a suitable model that has been extensively used for studying the pathogenesis of OEA and vaccine development (reviewed in Caro et al. (2009)).

In both the natural host and the mouse, *C. abortus* infection triggers the production of Th1 cytokines such as interferon (IFN-γ) and tumor necrosis factor TNF-α, which are essential mediators in mounting a protective cell response against the pathogen (reviewed in Kerr et al.

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https://doi.org/10.1016/j.vetmic.2017.11.009
Received 7 August 2017; Received in revised form 11 October 2017; Accepted 9 November 2017

(2005)). Nevertheless, the induction of pro-inflammatory cytokines in pregnant animals may have a deleterious effect in placental tissues. For instance, the local production of TNF-α in the placental tissues has been linked to inflammation of the chorionic epithelium in aborted placentas of sheep (Buxton et al., 2002), while in mice, this cytokine has been detected in the serum, but not locally in placental tissues during abortion (Kerr et al., 2010). In addition, cells of the innate immune response such as neutrophils and NK cells have been described as predominant in the inflammatory infiltrate of the placenta (Buxton et al., 2002; Navarro et al., 2004). In mouse models, not only neutrophils, but also cytotoxic CD8+ T cells have been shown to play an essential role in controlling *C. abortus* infection and the evolution of disease (Montes de Oca et al., 2000), (Martínez et al., 2006).

One of the main features of OEA is latency. In an experimental intranasal infection model of pregnant sheep, it has been shown that pregnancy outcome may depend on the infective dose and a protective response has been associated with a strong antibody response (Longbottom et al., 2013). However, the immunological mechanisms that determine the dormant stage of bacteria and their reactivation during pregnancy are still poorly understood. Due to the difficulties involved in the using of natural host models, an *in vivo* mouse model that mimics chlamydial latency could be of great use. Indeed, in chlamydial research, murine models have been extensively used to describe the immune mechanisms of host resistance and to develop new chlamydial vaccines (reviewed in Caro et al. (2009)), although in such *in vivo* models, chlamydial infection can only persist for a limited time in the tissues. More specifically, infection of resistant mouse strains triggers a strong inflammatory response that clears bacteria from tissues in a few days, whereas, susceptible mouse strains develop a systemic infection that results in immunopathogenesis and death. Therefore, in order to fully understand the immunological mechanisms that take place during chlamydial latency and bacterial reactivation, new approaches are needed to establish a valid *in vivo* model of sustained *C. abortus* infection.

It is known that certain pathogens can trigger cytokine production pathways that favour their persistence within the host, and IL-10 is strongly associated with such virulence strategies (Duell et al., 2012). Moreover, IL-10 is known to contribute to the chronic evolution of viral and bacterial infections (Brooks et al., 2006), and it is involved in long-lasting infections of intracellular pathogens, such as *C. burnetii* (Meghari et al., 2008) and *Mycobacterium tuberculosis* (Redford et al., 2011), and also parasites such as *Trichinella spiralis* (Huang et al., 2014). In addition, it has been shown to contribute to the chronic evolution of bacterial infectious diseases as part of immunosuppressive treatments (Mege et al., 2006).

Moreover, in several models of infectious diseases, IL-10 has been shown to moderate immunopathological events associated with the overproduction of IFN-γ or TNF-α, due to its anti-inflammatory role (Kulcsar and Griffin, 2016). Additionally, it has been described that IL-10 cannot only inhibit cytokine production by T cells, but can also prevent the maturation of DCs. It also plays a role in blocking pro-inflammatory cytokine production, co- stimulation, MHC class II expression and chemokine secretion (reviewed in Filippi and Von-Herrath (2008)). Particularly, studies using known chronic pathogens such as *Mycobacterium tuberculosis* (Redford et al., 2011) or *Leishmania major* (Belkaid et al., 2001) have shown that the absence of IL-10 leads to a better clearance of these micro-organisms preventing the development of chronic infections.

With regard to clamydial infections, some antigens such as major outer membrane protein (MOMP) are associated with IL-10 production in macrophages and T lymphocytes (Bermudez-Fajardo et al., 2011), and the inmunomodulatory role of IL-10 has also been widely described by several authors. In particular, IL-10 has been shown to control Th1 responses (Igietseme et al., 2000), acting on DCs maturation (Marks et al., 2010), and modulating antigen presentation through the regulation of the NLRP3 inflammasome assembly (Omosun et al., 2015). Nevertheless, due to the special features of chlamydial pathogenesis, IL-10 production has been described as exerting both negative and positive effects during infection (Thiel et al., 2000). More, detrimental effects of IL-10 production may be related with the inhibition of IFN-γ, consequently limiting bacterial clearance from tissues. In contrast, a positive effect of IL-10 linked to its anti-inflammatory effect, may reduce infection-associated immunopathogenesis.

The present model of experimental chlamydial infection using IL-10 overproducing mice (macIL10tg) was conceived to validate an *in vivo* model of a sustained infection, which may be of great use for monitoring the immunological and bacterial mechanisms present during chlamydial chronic infections and in vaccine development.

2. Material and methods

2.1. Ethics statement

This study protocol was approved by the Bioethical Committee of the University of Murcia, Spain (approval number C1310050301, date of approval 18 May 2010) and all experiments were carried out in accordance with the Spanish legislation on animal experimentation (RD 53/2013). Female control mice of FVB background, (WT mice) and transgenic mice overexpressing IL-10 (macIL10tg) were kept in a specific pathogen-free facility in the University of Murcia. During animal handling, all efforts were made to minimize suffering.

2.2. Microorganisms

C. abortus (AB7 strain) was propagated in the yolk sacs of developing chick embryos and titrated by counting inclusion-forming units (IFUs) in McCoy cells, as previously described (Buendía et al., 1999). Standardized aliquots were frozen at −80 °C until use.

2.3. Mice and experimental infection

Eight-week-old female control mice of FVB background (WT mice) and transgenic mice were challenged intraperitoneally with 4×10^5 IFUs of *C. abortus* in 0.2 ml of 0.1 M phosphate-buffered saline (PBS), pH 7.2 and their clinical status was recorded daily. Non-infected mice (5–7 per group) were injected with 0.2 ml of sterile PBS and killed at 4, 9, 17, 22 and 28 days post infection (pi.). Although macrophages of macIL10tg mice overexpress IL-10 but they have been described as being fully immunocompetent and as developing an adequate immune response after infection (Lang et al., 2002).

During necropsy, blood was collected by cardiac puncture, and tissue samples from liver were fixed in 10% formalin or Zinc fixative agent (BD Biosciences, Pharmingen, San Jose, CA) and embedded in paraffin for further histopathological or immunopathological analysis. All the experiments were repeated twice.

2.4. Morbidity and course of infection

Mice were monitored daily for possible signs of infection. In addition, weight loss was recorded as a sign of morbidity. The infectivity of *C. abortus* in WT and macIL10tg mice was evaluated in liver tissue. This organ has previously been described as the target organ after intraperitoneal infection, while showing no differences with the spleen (Del Río et al., 2001). The level of infection was measured by titration of IFUs on monolayers of McCoy cells, as described in (Buendía et al., 1999).

2.5. Histopathology and immunohistochemistry

Formalin-fixed liver sections (4 μ) were stained with Haematoxylin-Eosin for histopathological study and immunohistochemical labelling was carried out to demonstrate presence of chlamydial antigen (as