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Mini Review

Chlamydia psittaci: New insights into genomic diversity, clinical pathology, host–pathogen interaction and anti-bacterial immunity

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ABSTRACT

The distinctive and unique features of the avian and mammalian zoonotic pathogen *Chlamydia* (*C.*) *psittaci* include the fulminant course of clinical disease, the remarkably wide host range and the high proportion of latent infections that are not leading to overt disease. Current knowledge on associated diseases is rather poor, even in comparison to other chlamydial agents. In the present paper, we explain and summarize the major findings of a national research network that focused on the elucidation of host–pathogen interactions in vitro and in animal models of *C. psittaci* infection, with the objective of improving our understanding of genomics, pathology, pathophysiology, molecular pathogenesis and immunology, and conceiving new approaches to therapy. We discuss new findings on comparative genome analysis, the complexity of pathophysiological interactions and systemic consequences, local immune response, the role of the complement system and antigen presentation pathways in the general context of state-of-the-art knowledge on chlamydial infections in humans and animals and single out relevant research topics to fill remaining knowledge gaps on this important yet somewhat neglected pathogen.

Introduction

The first definition of *Chlamydia* (*C.*) *psittaci* was formulated in 1968 to distinguish it from *C. trachomatis*, the only other known chlamydial species at that time, and was based on the inability to accumulate glycogen in its inclusions and resistance to sulfadiazine (Page, 1968). More recently, the species was redefined to comprise mainly avian strains, but at the same time assigned to the separate genus *Chlamydophila* (Everett et al., 1999). The latter has been abolished in the meantime (Kuo et al., 2011; Stephens et al., 2009), so that *C. psittaci* is now one of nine accepted members of the recombined genus *Chlamydia*. Two more avian chlamydiae (Sachse et al., 2014) and one Candidatus taxon (Vorimore et al., 2013) were discovered recently and are about to be added to the same genus.

The groundwork for modern chlamydia research was laid in the 1960s and 1970s by the pioneering work of J.W. Moulder, A. Matsumoto, G.P. Manire, T.P. Hatch, P.B. Wyrick and several other scientists, who focused on *C. psittaci* in their studies. By analyzing the structure and chemical composition of isolated *C. psittaci* "particles", Moulder (1962) conducted one of the first molecular characterizations of chlamydiae. Based on experiments with *C. psittaci*-infected cells, Matsumoto and Manire (1970) were the first to provide high-resolution images of chlamydial bodies and to describe effects of antibiotics on their morphology. The groundbreaking studies of Hatch (1975) demonstrated that chlamydiae owe their obligate intracellular mode of reproduction to the requirement for energy intermediates produced by the host cell. Not least, Wyrick and colleagues (1978) were the first to describe structural properties of chlamydial compartments (Narita et al., 1976) and the ability of *C. psittaci* to infect immune cells (Wyrick and Brownridge, 1978).

C. psittaci is the causative agent of psittacosis (sometimes also called ornithosis or parrot fever), the most important animal

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chlamydiosis of zoonotic character. This systemic disease can take acute, protracted, chronic or subclinical courses and mainly affects psittacine birds and domestic poultry (Andersen and Vanrompay, 2000; Kaleta and Taday, 2003). Typical symptoms include lethargy, hyperthermia, abnormal excretions, respiratory distress, nasal and ocular discharges, weight loss and reduced egg production. *C. psittaci* infections and ensuing disease outbreaks are economically relevant to the poultry industry and significantly compromise animal welfare (Yin et al., 2013). Recent data also indicate that, in concert with *C. trachomatis*, *C. psittaci* may play a role in human trachoma (Dean et al., 2013).

The zoonotic dimension of avian chlamydiosis is another cause for concern, as cases of bird-to-man transmission are regularly reported in the literature (Gaede et al., 2008; Harkinezhad et al., 2009; Heddema et al., 2006; Laroucau et al., 2009). The fact that the overall number of reported cases remained generally low in the past decades is, to some extent, due to the absence of this pathogen in most routine diagnostic schemes. The symptoms in affected individuals are mainly non-specific and influenza-like, but severe pneumonia, endocarditis and encephalitis are not uncommon (Salisch et al., 1996). While it is generally perceived that the course and severity of disease depend on virulence properties of the *C. psittaci* strain involved, the immune response of the host, as well as transmission route and infectious dose, very little is known in concrete terms about any parameter of host–pathogen interactions. Furthermore, latent infection seems to be more widespread than clinical cases, both among birds and humans. It was shown in several studies in cattle that, in the absence of clinical signs, performance and health parameters of chlamydia carriers were clearly inferior to those of chlamydia-free animals (reviewed in Reinhold et al., 2011b). In this context, frequently observed recurrent infections by chlamydiae might pave the way for chronic disease.

The remarkable variety of clinical manifestations displayed by *C. psittaci* and chlamydial infections in general should, at least in part, be explicable through the distinctive features of the causative agent. *C. psittaci* is an obligately intracellular bacterium with a unique biphasic developmental cycle. It exists in basically two morphological stages, the infectious, but metabolically inactive elementary body (EB) and the non-infectious, but metabolically active reticulate body (RB). In the course of a replication cycle, EBs evolve into RBs in a vacuole-like inclusion of the host cell, where the latter undergo binary fission before transforming back into EBs to start a fresh cycle. The natural development can be halted at the intracellular stage through the action of adverse factors, such as interferon (IFN)-γ exposure, iron or amino acid depletion, and antibiotic treatment (Beatty et al., 1994; Wyrick, 2010). This persistent state is characterized by aberrant (enlarged) morphology of RBs, loss of cultivability and rescue of infectivity upon removal of inducers. In the case of *C. psittaci*, induction of persistence in vitro was accompanied by consistent downregulation of membrane proteins, chlamydial sigma factors, cell division protein and RB–EB differentiation proteins from 24 h post-infection onwards (Goellner et al., 2006). While it is not yet clear whether the host immune response can actually trigger transition of chlamydial bodies into the persistent state, although some evidence seems to suggest it (Borel et al., 2008; Pospischil et al., 2009), these observations provide an explanation for the failure of antibiotic treatment to eliminate chlamydial agents from an infected organism (Reinhold et al., 2011a).

Host preference of *C. psittaci* is an interesting yet largely unexplored topic. As the agent is generally referred to as an avian pathogen, there is still a widely held notion that it occurs only in birds. However, a number of surveys showed that *C. psittaci* can be found frequently in non-avian domestic animals, such as cattle (Kemmerling et al., 2009), sheep (Lenzko et al., 2011), swine (Kauffold et al., 2006; Vanrompay et al., 2004), horses (Szeredi et al., 2005; Theegarten et al., 2008), goats and cats (Pantchev et al., 2010), as well as in wildlife (Hotzel et al., 2004) and laboratory rodents (Henning et al., 2008). Although the role of the agent in these hosts remains to be clarified it seems certain that overt clinical manifestations are rare exceptions. In addition, the virulence to humans of non-avian *C. psittaci* strains appears to be negligible compared to avian isolates, since the reported cases of human psittacosis are usually traced back to contact with an avian source. Nevertheless, no genetic markers to distinguish between avian and non-avian strains have been found in comparative genomic studies (Voigt et al., 2012), nor have any virulence criteria been identified (Read et al., 2013). One can hypothesize from these findings that birds are the original and typical host for *C. psittaci*, while mammals merely act as alternate hosts and any passages of avian strains through non-avian hosts are accompanied by loss of virulence. Apart from these considerations, the factors determining host preference and adaptation of chlamydiae are largely unclear.

Comparative genomics: *C. psittaci* – a typical member of the family Chlamydiaceae with unique features

Second-generation sequencing efforts have led to the completion of more than 100 chlamydial genome sequences encompassing all recognized species, except *C. suis*. For *C. psittaci*, whole-genome sequences of 28 strains have become available so far (http://ftp.ncbi.nlm.nih.gov/genomes/ASSEMBLY_BACTERIA/Chlamydia_psittaci/), covering virtually all serotypes and avian and mammalian hosts (Grinblat-Huse et al., 2011; Schofl et al., 2011; Seth-Smith et al., 2011, 2013; Van Lent et al., 2012; Voigt et al., 2011). This wealth of data allows unprecedented insights in the genomic organization, evolutionary dynamics, and genetic diversity of the species.

Comparative analysis of chlamydial genomes showed a high level of sequence and gene order conservation (shared synteny) across members of the family and, as in other obligate intracellular bacteria, a considerably reduced genome, implying dependency on the host organism for many metabolic capabilities. All sequenced *C. psittaci* strains possess a single chromosome of approximately 1.1 Mb, and a conserved plasmid of ~8 kb containing 7–8 protein-coding sequences (CDS) was reported for 12 strains. A total of 911 core CDS are shared among all currently sequenced *C. psittaci* strains, which is equivalent to about 90% of the genes present in each of these genomes (Read et al., 2013). Among the species of *C. psittaci*, *C. abortus*, *C. pneumoniae*, and *C. trachomatis*, 736 genes are still shared (with a total CDS count ranging from 874 to 1097), which illustrates the degree of conservation across chlamydial genomes (Voigt et al., 2012). In contrast, one of the closest relative of *Chlamydiaceae* for which a genomic sequence is available, *Simkania negevensis*, features a genome of more than twice this size with a chromosome of 2.5 Mb and ~2500 predicted CDSs (Collingro et al., 2011).

Conserved synteny is extremely high among most chlamydial genomes. Major genomic rearrangements are present only between the closely related *C. trachomatis* and *C. muridarum* versus the remainder of the *Chlamydiaceae* (Fig. 1). Major deviations from synteny and/or sequence conservation, however, can be observed in genes encoding inclusion membrane proteins, in the hypervariable region near the predicted replication termination region known as the plasticity zone (PZ), as well as among the family of polymorphic membrane protein (*pmp*) genes (Fig. 1). These regions are assumed to harbor key genomic correlates to species-specific adaptation (see also Section "Host adaptation of *C. psittaci*: Escape from the avian innate immune response") to environmental niches, tissue tropism and differences in virulence and pathogenicity (Thomson et al., 2005).

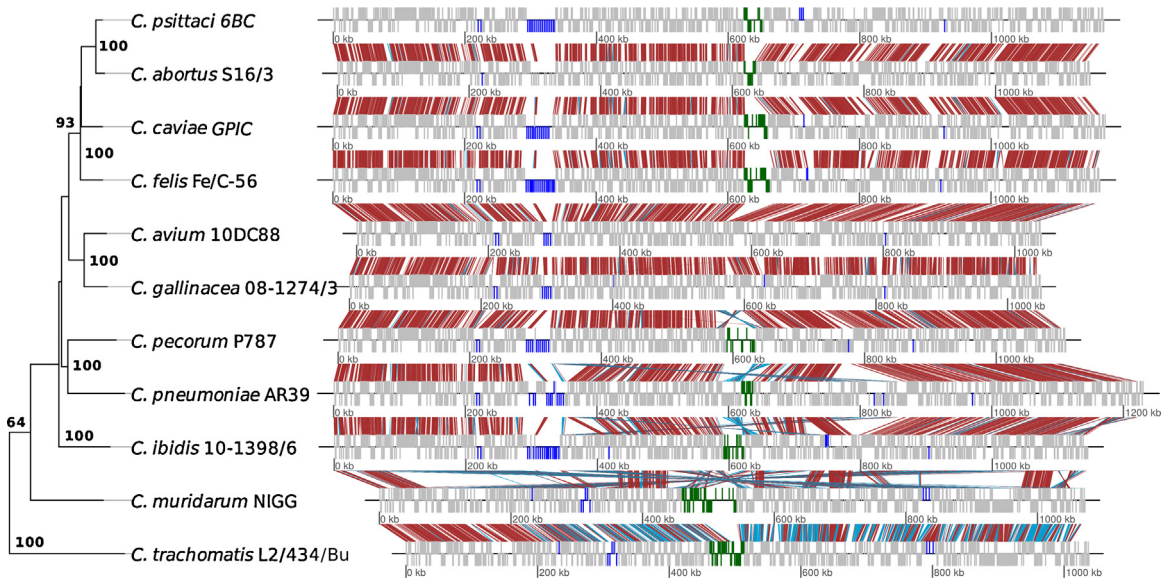

Fig. 1. Comparative genomics. Maximum-likelihood tree based on a random sample of 100 conserved orthologous genes and global comparison of synteny among the eleven fully sequenced species of the family *Chlamydiaceae* (C. ibidis is a taxon with *Candidatus* status). Gray tick marks above and below the sequence lines represent predicted CDSs on the plus and minus strands of the genomes, respectively. Color-marked are (blue) members of the polymorphic membrane protein (Pmp) family and (green) genes within the chlamydial plasticity zone (PZ). Red lines connecting genomes represent direct orthologous matches based on best reciprocal BLAST hits; blue lines represent reverse-complemented matches. Bootstrap values are based on 1000 resampled trees.

The size and organization of the PZ differs substantially among *Chlamydiaceae*, ranging from 45 genes in *C. trachomatis* to six genes in koala strain LPCoLN of *C. pneumonia* (Voigt et al., 2012). The PZ of *C. psittaci* is remarkable for its inclusion of a large intact toxin/adhesin gene similar to the EHEC adherence factor, as well as a *guaAB-add* cluster, which plays a role in salvaging biosynthesis of purine nucleotides necessary for bacterial growth. These genes are shared with *C. caviae* and *C. felis*, but are absent (or predicted to have loss-of-function frameshifts) in other chlamydial species, including the closely related *C. abortus* (Voigt et al., 2012). Interestingly, a recent comparative analysis of 20 *C. psittaci* strains revealed that only a subset of *C. psittaci* strains, the monophyletic"6BC clade", and its closest relatives may have acquired these genes through a single event of horizontal gene transfer, or they may have been lost from or degraded in more basal clades of *C. psittaci* on three independent occasions (Read et al., 2013). The 6BC clade comprises 10 strains including type strain 6BC/30 isolated from a parrot (Grinblat-Huse et al., 2011), and contains strains of both avian and mammalian origin corresponding to serotypes A and B (Read et al., 2013). In contrast, two further clades including serotypes C and D comprise only avian hosts. The 6BC clade shows extremely little internal variability (483 polymorphic nucleotide positions) coupled with a distinct lack of host specificity (Read et al., 2013). Interestingly, significant sequence differences between 6BC and non-6BC clades were observed in the large toxin locus. It is tempting to speculate that these remarkable differences among *C. psittaci* strains may have conferred adaptive advantages that facilitated host range expansion in the 6BC lineage.

In several chlamydial species, including *C. psittaci*, *pmp* genes have undergone a significant proliferation resulting in unusually high levels of mutational change within and across species. The Pmps are a large protein family, probably unique to the chlamydiae, with low overall amino acid similarity, but sharing multiple, often alternating motifs GGA (ILV) and FxxN. They are considered to be important in EB adhesion to host cells, molecular transport, and cell wall-associated functions (Longbottom et al., 1998), and have been identified as a critical factor in chlamydial pathogenesis (Tan et al., 2006). Pmps cluster phylogenetically into six subfamilies and are present in varying numbers ranging from a total of nine genes in *C. trachomatis* and *C. muridarum* to 21 in *C. pneumoniae* and *C. psittaci* (Voigt et al., 2012). Subfamily G/I is the largest and most rapidly evolving representative (Voigt et al., 2012). The tendency to proliferation of G/I family Pmps is especially pronounced in *C. psittaci* and its closest relatives, as well as in *C. pneumoniae*. While there are only two G/I *pmp* genes present in *C. trachomatis* and *C. muridarum*, there are 14 G/I family *pmp* genes present in the *C. psittaci* genome, several of which are unique to this species (Voigt et al., 2012). It has been suggested that the diversity in the *pmp* family of genes may have evolved in response to demands of the host environment, e.g. to escape systemic host response or adapt to multiple host environments (Tan et al., 2006). Thus, the extremely high variability of *pmp* genes in *C. psittaci* may also be related to its broad host spectrum.

While homologous recombination is a fundamental mechanism for genetic diversification in free-living bacteria, it is often thought to be limited in obligate intracellular bacteria, whose lifestyle should rarely allow encounters with sources of foreign DNA. With many genomes from the same species becoming available, it has become possible to apply traditional population genetics methods to whole genomes in order to test predictions as above with high precision. Thus, analysis of a large number of *C. trachomatis* genomes recently provided compelling evidence for relatively frequent homologous recombination in this species, despite the ecological barriers to contact posed by an intracellular lifestyle (Joseph et al., 2012). Similar evolutionary dynamics also play a surprisingly strong role in *C. psittaci*, where recombination events are spread evenly across the genome and were estimated to occur at roughly 1/20th the rate of mutation events. However, although recombination was less frequent than mutation, it accounted for three times more substitutions in a genome, because each recombination event affected several nucleotides at a time (Read et al., 2013). Strikingly, BLAST analysis of predicted recombinant segments indicated that roughly 50% of putative DNA imports derive from *C. abortus* or as-yet unknown/unsampled *C. psittaci* strains. This suggests that there is still substantial diversity in *C. psittaci* to be uncovered at the genome level.

Clinical pathology: The course of *C. psittaci* respiratory infection in a natural non-avian host

Animal models closely mimicking the relevant characteristics of human disease are still a key element for evaluating pathophysiological consequences and for understanding the underlying mechanisms of disease pathogenesis. Moreover, a valid animal model is a prerequisite for development and efficacy testing of therapeutics and vaccines.

With respect to research on respiratory *C. psittaci* infections, the bovine species offers a number of advantages stated in detail elsewhere (Reinhold et al., 2012). Morphological peculiarities of the bovine lung, including segmental anatomy and the lack of collateral airways, mirror pathophysiological mechanisms of pulmonary dysfunctions more clearly. The availability of a range of highly sensitive, non-invasive pulmonary function tests known from human medicine and previously validated for calves enables detailed evaluation of nature and scope of pulmonary disorders (Ostermann et al., 2014). Additionally, the body size of calves facilitates repeated sampling of biological specimens (e.g. blood and swabs), and thus follow-up monitoring of clinical and immunological parameters (see also Section "Local immune response: Development and resolution of pulmonary lesions after *C. psittaci* infection of calf and mouse"). A calf model using a bovine *C. psittaci* strain potentially allows detailed monitoring of transmission routes, molecular and physiological processes in the course of infection, as well as evaluation of a strain's virulence. The data obtained should be of interest for researchers in both veterinary and human medicine. In a recent series of infection trials, we were able to demonstrate and verify the essential characteristics of a *C. psittaci* infection in its various manifestations (Ostermann et al., 2013a,b, 2014; Reinhold et al., 2012).

First of all, the severity of disease during the acute phase of infection (Fig. 2A) proved clearly dose dependent. Respiratory and clinical signs, which peaked 2–3 days post-inoculation (dpi), were mild after inoculation of 10^6 inclusion-forming units (ifu) of *C. psittaci* strain DC15 per calf, moderate with doses of 10^7–10^8 ifu and severe for 10^9 ifu. This dose–response relation was highly reproducible in extent and quality of lung lesions, and also reflected in respiratory functions affecting pulmonary gas exchange (Ostermann et al., 2013b; Reinhold et al., 2012). Baseline effects due to chlamydial LPS or cell culture medium were ruled out by using controls that received UV-inactivated chlamydiae or medium, respectively.

Secondly, we were able to reproduce the whole variety of characteristic features that are observed in animals and humans affected by natural chlamydial infections. In the case of calves intra-bronchially inoculated with 10^8 ifu/calf, a typical course included initial acute clinical illness (2–3 dpi), which subsided considerably, but not completely, within 10 dpi (Ostermann et al., 2013a). Subsequently, a protracted clinically silent course was indicated by intermittent mild symptoms, fecal pathogen excretion, transient chlamydemia, and slightly elevated levels of monocytes and lipopolysaccharide-binding protein (LBP) in blood. Interestingly, these phenomena were also observed as a consequence of naturally acquired, initially mild infection, as induced in a group of naïve calves (sentinels) that socialized with the acutely diseased animals. Again, this underlines the validity of the present bovine infection model and its potential to emulate the natural circumstances. The fact that neither group of calves completely eliminated the chlamydiae within five weeks after exposure (Ostermann et al., 2013a) is also in line with observations in animal herds, where (permanent or intermittent) carriers of chlamydiae represent a permanent reservoir for recurrent outbreaks of disease (Reinhold et al., 2011b). Under field conditions, even asymptomatic chlamydial infections in cattle herds are known to be associated with numerous negative effects on health and performance including significantly reduced growth rate in calves (Jaeger et al., 2007; Poudel et al., 2012; Reinhold et al., 2008).

Thirdly, the humoral immune response was generally weak (see also Section "Local immune response: Development and resolution of pulmonary lesions after *C. psittaci* infection of calf and mouse"). Only two-thirds of calves experimentally challenged with 10^8 ifu developed specific antibodies against *C. psittaci* as detected by immunoblotting. The onset of the antibody response occurred between 7 and 14 dpi. In sentinels, no humoral immune response was observed (Ostermann et al., 2013a; Reinhold et al., 2012). Although innate and acquired humoral immunity are also critically involved in the clearance of chlamydiae, this supports the notion that the cellular immune response plays a central role in controlling anti-bacterial immunity in infected hosts (see also Section "The cellular immune response: New insights into MHC I-mediated presentation of chlamydial antigens") and also imposes basic constraints on disease control approaches based solely on classical immunoprophylaxis.

Fourthly, the pathological manifestations of acute respiratory *C. psittaci* infection included inflammatory cells (mainly neutrophil granulocytes) recruited onto the site of infection (Figs. 2B and 3B; see also Sections "Local immune response: Development and resolution of pulmonary lesions after *C. psittaci* infection of calf and mouse" and "The innate immune response and its interaction with the adaptive response: Chlamydial infection and the complement system"). Pulmonary inflammation resulted in damage of the alveolar-capillary barrier, as indicated by altered cytology, elevated concentrations of eicosanoids and total protein in broncho-alveolar lavage fluid (Reinhold et al., 2012). Inflammation and the accumulation of detritus and protein-rich fluid caused (i) ventilatory disorders due to both airway obstructions and pulmonary restrictions and (ii) inhibited pulmonary gas exchange. Among the resulting functional consequences were reduced alveolar ventilation (hypoventilation) and reduced oxygen supply to the blood (hypoxemia). Attempts of compensation included the elevation of both respiratory rate and minute ventilation, clinically visible as dyspnea (Ostermann et al., 2013b, 2014).

Pulmonary dysfunctions were accompanied by metabolic disorders in the acid–base equilibrium revealing additional systemic consequences of an acute *C. psittaci* infection on blood pH and blood homeostasis in the host (Ostermann et al., 2014).

Taken together, the bovine model of respiratory *C. psittaci* infection revealed unprecedented insights into the very complex interactions between pathological changes, pathophysiological consequences and clinical signs induced by this pathogen in the mammalian host (Fig. 2B). With respect to comparative and translational medicine, this model builds a useful bridge between basic molecular research and clinical work in patients, both humans and animals. The elucidated host–pathogen interactions provide a suitable basis for further research focusing on new strategies to control this infection through treatment or vaccination.

Local immune response: Development and resolution of pulmonary lesions after *C. psittaci* infection of calf and mouse

In recent studies, pulmonary lesions were investigated in models of experimental aerogenous infection with *C. psittaci* in calves (see Section "Clinical pathology: The course of *C. psittaci* respiratory infection in a natural non-avian host") and mice (see Sections "The innate immune response and its interaction with the adaptive response: Chlamydial infection and the complement system" and "The cellular immune response: New insights into MHC I-mediated presentation of chlamydial antigens") to analyze species-specific

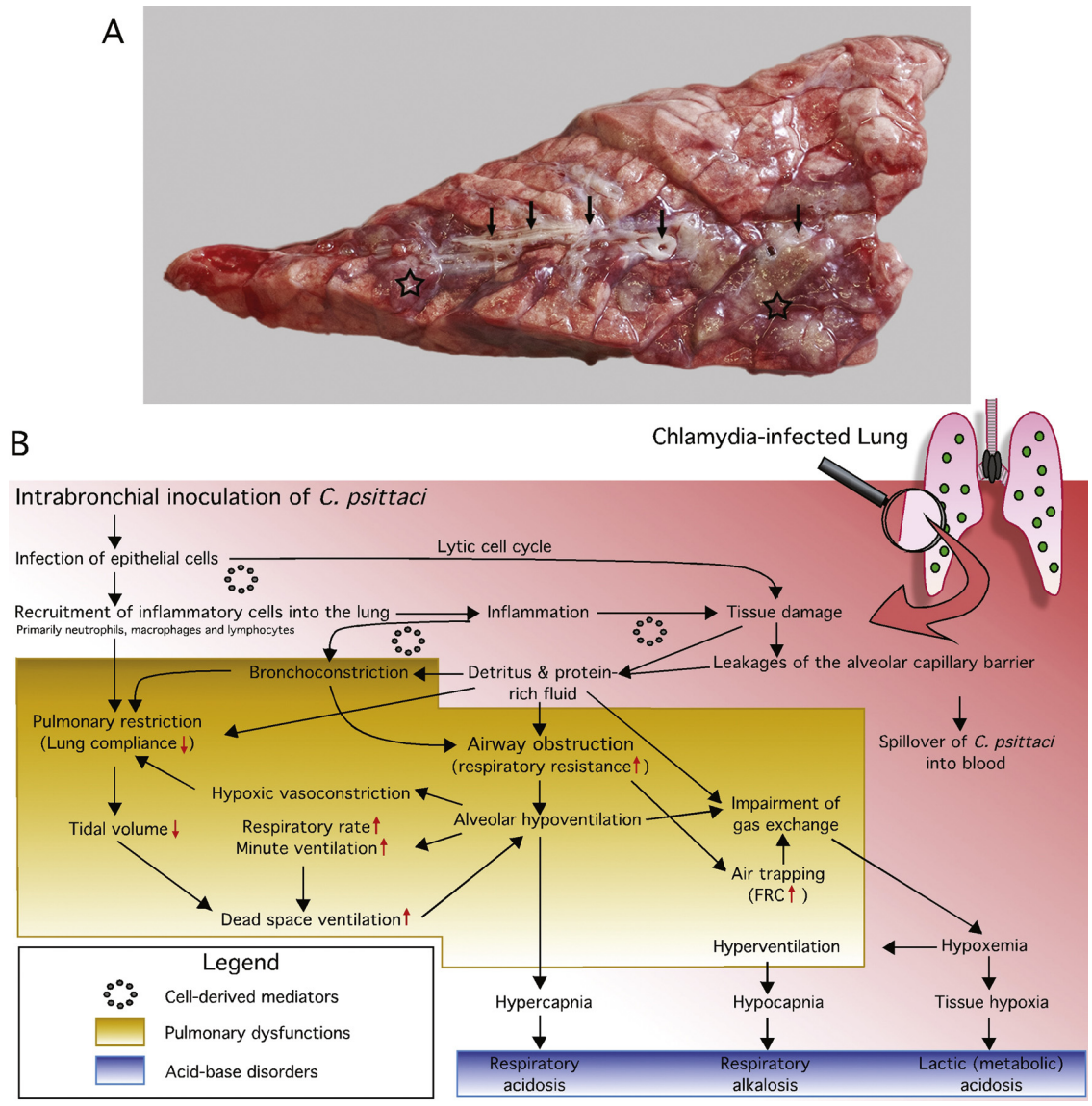

Fig. 2. Respiratory *Chlamydia psittaci* infection: complexity of pathogenetic mechanisms, pathophysiological interactions and systemic consequences in the mammalian host (bovine model). (A) Pulmonary lesions in the bovine lung. The section through a cranial lobe reveals several lobules with fibrinopurulent bronchopneumonia (asterisks) in close association with bronchi (arrows) in a calf 4 days after experimental inoculation with *C. psittaci*. (B) Inoculation of *C. psittaci* resulted in infection and replication of the pathogen in pulmonary epithelial cells and initiated an acute-phase reaction. Pro-inflammatory chemokines activated the inflammation cascade with recruitment and accumulation of phagocytic and reactive immune cells from the blood into the lung. Tissue damage and leakages of the alveolo-capillary membrane enabled a spillover of the pathogen into the bloodstream (chlamydemia). Within the lung, fluid, cells and debris in the alveoli and the interstitial tissue provided a barrier for oxygen transfer leading to oxygen deficiency in arterial blood (hypoxemia). Additional interacting factors induced by inflammatory mediators diminished multiple pulmonary functions. Analysis of respiratory mechanics revealed restriction of pulmonary tissue (i.e. limitations in lung compliance) and airway obstructions, both contributing to a reduction in alveolar ventilation (alveolar hypoventilation). Regarding the breathing pattern, expiration was more impaired than inspiration, which resulted in 'apped air', i.e. larger end-expiratory gas volume in the alveolar spaces due to incomplete deflation of the lung (i.e. increased functional residual capacity; FRC). The latter worsened the already existing alveolar hypoventilation and the already reduced oxygen delivery to the blood. In hypoventilated areas of the lung, the corresponding pulmonary arteries constricted (hypoxic pulmonary vasoconstriction; HPV), and those stiffer blood vessels with increased vascular resistance deteriorated the loss of compliant pulmonary properties. In the less compliant lung, the volume inhalable and exhalable per breath (tidal volume) was reduced. Due to the lack of oxygen in the blood, however, respiration was stimulated resulting in a compensatory increase of the respiratory rate and the volume of minute ventilation. Clinically, short, rapid breathing cycles appeared that were characterized by an increased percentage of dead space ventilation and a reduced percentage of alveolar ventilation. Tissue damage and pulmonary dysfunctions had a strong impact on acid–base equilibrium. While alveolar hypoventilation may result in accumulation of CO_2 in the body (hypercapnia) and a consecutive respiratory acidosis (blood pH ↓), the attempt to compensate hypoxemia by a dramatically intensified respired volume per time (hyperventilation) was associated with a loss of CO_2 (hypocapnia) driving blood pH in the direction of respiratory alkalosis (pH ↑). In addition, the demand of oxygen in peripheral tissues in relation to a reduced oxygen supply by the blood stimulated anaerobe metabolism (increased production of L-lactate and protons), which influenced blood pH in the direction of metabolic acidosis (pH ↓). Additional complex metabolic influences on blood pH are described in Ostermann et al. (2014). In general, numerous acidic effects were counterbalanced by a variety of alkalotic effects influencing the acid–base equilibrium in the host organism in multiple ways. In comparative and translational medicine, this integrated large animal model has fundamentally improved our understanding of pathogenetic mechanisms and complex pathophysiological interactions during respiratory *C. psittaci* infection.

reaction patterns to the pathogen (Fiegl et al., 2013; Lambertz, 2011; Möhle, 2011; Reinhold et al., 2012).

In calves, intra-tracheal application of viable *C. psittaci* induced pneumonia in all animals inoculated (Fig. 2), but in none of the controls (Reinhold et al., 2012). In addition to the dose-dependent clinical score (see Section "Clinical pathology: The course of *C. psittaci* respiratory infection in a natural non-avian host"), the type of lesions changed with increasing dose of inoculum from purulent

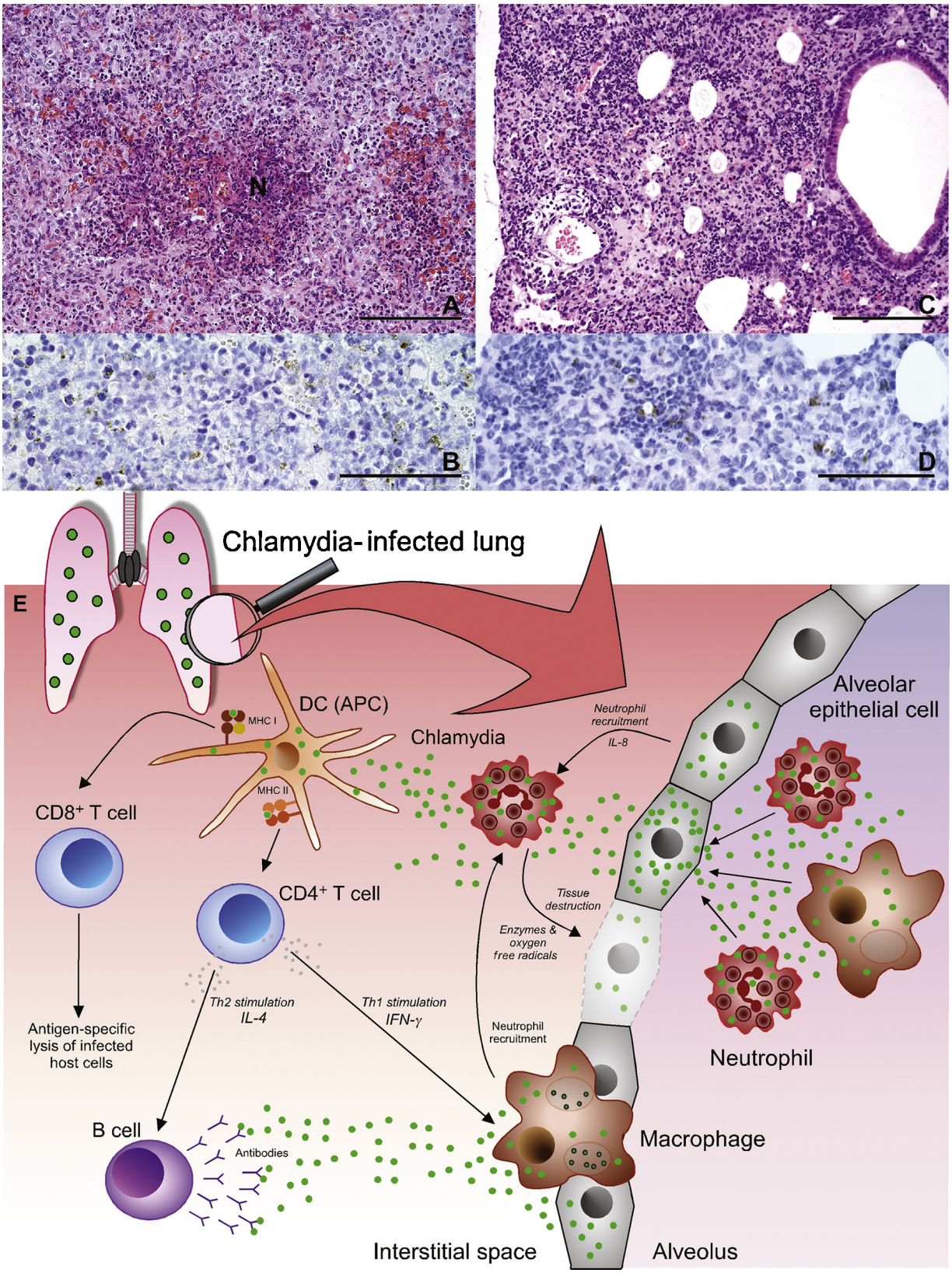

Fig. 3. Pulmonary lesions (A–D) and model of local immune response to Chlamydiae (E). Pulmonary lesions 4 days after experimental inoculation of a calf (A and B) and a mouse (C and D) with *C. psittaci*. Lesions in the calf (A) are characterized by fibrinopurulent exudate and foci of necrosis (N), whereas neutrophilic infiltrates predominate in the mouse (C). Numerous chlamydial inclusions (brown) are present in intra-lesional neutrophils and macrophages of calf (B) and mouse (D). A, C bars = 100 μm. B, D bars = 200 μm. In the model of local immune response to chlamydiae (E), infected alveolar epithelial cells secrete interleukin-8 (IL-8), which is a potent pro-inflammatory cytokine playing a key role in recruitment and activation of neutrophils. Neutrophils produce enzymes and reactive oxygen species to kill bacteria and provide the first line of defense against chlamydiae together with macrophages and dendritic cells (DCs). Binding of conserved pathogen-derived molecules by specific receptors on the cell

to fibrinous to necrotizing. Thus, the type of acute lesions differed distinctly from the interstitial and atypical pneumonia generally reported in calves and humans (Beeckman and Vanrompay, 2009; Jee et al., 2004).

The time course of infection was followed in inoculated calves from 2 days to 1 month after inoculation (Lambertz, 2011; Möhle, 2011). The pneumonic exudate predominantly consisted of neutrophil granulocytes (see also Sections "Clinical pathology: The course of *C. psittaci* respiratory infection in a natural non-avian host" and "The innate immune response and its interaction with the adaptive response: Chlamydial infection and the complement system") at 2 and 3 dpi. Lesions were most severe at days 3 and 4 pi with fibrinous exudate, areas of necrosis and increasing numbers of alveolar macrophages (Fig. 3A). Chlamydial inclusions were detected initially in alveolar epithelial cells and then increasingly in neutrophil granulocytes and alveolar macrophages, especially in areas of necrosis (Fig. 3B). Ultrastructural investigation revealed infection of alveolar epithelial cells, neutrophil granulocytes and alveolar macrophages with *C. psittaci*, but multiplication was seen in alveolar epithelial cells only. At day 7 pi, first signs of regeneration were seen: necrotic pulmonary tissue was demarcated by proliferating alveolar epithelial cells and dense infiltrates of macrophages. The zones of regeneration became confluent at day 10 pi and were infiltrated by dendritic cells (DCs) (see also Section "The cellular immune response: New insights into MHC I-mediated presentation of chlamydial antigens"), macrophages, T and B lymphocytes and plasma cells. At day 14 pi, the numerous lymphocytes, macrophages, DCs and ectopic lymphoid follicles in the lesions indicated a strong local immune response that caused a marked reduction of the number of chlamydiae. The pulmonary tissue had regenerated one month pi except for a few small foci of fibrous scar tissue. Number and distribution of unorganized immune cells were comparable to control calves, but bronchus-associated lymphoid tissue was more pronounced. The intrapulmonary infection of calves with *C. psittaci* allows a detailed study of the steps hypothesized to be involved in the development and organization of lung damage in animals and humans (Beers and Morrisey, 2011).

For the zoonotic agent *C. psittaci*, knowledge about shedding and potential routes of transmission is important. The detection of *C. psittaci* in nasal mucosa and trachea from 2 to 4 days pi was interpreted as excretion of the pathogen with infected exudate from the lung, since chlamydial inclusions were detected in luminal exudate in the trachea by immunohistochemistry. The excretion was limited to the short time period of severe lesions and to low numbers of chlamydial inclusions in the exudate (Lambertz, 2011). Dissemination of *C. psittaci* from the infected lung to sites that are considered to be preferential targets for chronic chlamydial diseases in humans, e.g. aorta and synovial membranes, was detected by PCR. The lack of tissue reaction in these target tissues argues against an active infection and confirms findings from infection experiments in rabbits with *C. pneumonia* (Gieffers et al., 2004).

In another experimental setting, mice were intranasally inoculated with the same strain of *C. psittaci* that was used in calves (see Sections "The innate immune response and its interaction with the adaptive response: Chlamydial infection and the complement system" and "The cellular immune response: New insights into MHC I-mediated presentation of chlamydial antigens"). The initial phase of infection was similar in both species. At day 4 pi, differences in reaction of mice versus calves became apparent, i.e. extensive purulent inflammation with increasing numbers of macrophages was found in mice (Fig. 3C), while calves developed severe fibrinous exudation with tissue destruction (Fig. 3A). Large numbers of chlamydiae were associated with neutrophil granulocytes and macrophages in the lesions of both hosts (Fig. 3B and D). The different types of inflammatory lesions resulted in distinct healing processes, i.e. mice developed lymphohistiocytic bronchointerstitial pneumonia, whereas regeneration with alveolar epithelial type 2 cell hyperplasia occurred in calves. Both mounted a massive local immune response that eliminated the chlamydiae (see also Sections "The innate immune response and its interaction with the adaptive response: Chlamydial infection and the complement system" and "The cellular immune response: New insights into MHC I-mediated presentation of chlamydial antigens").

Based on the morphological findings, the following hypothesis for the pathogenesis of pulmonary *C. psittaci* infection can be suggested (Fig. 3E). There is an initial infection of alveolar epithelial cells with *C. psittaci*. The multiplication of *C. psittaci* is followed by a rapid influx of neutrophil granulocytes most likely mediated by cytokines released from infected cells (see Sections "The innate immune response and its interaction with the adaptive response: Chlamydial infection and the complement system" and "The cellular immune response: New insights into MHC I-mediated presentation of chlamydial antigens"). These initial steps of the inflammatory reaction are consistent with the "cellular paradigm" in chlamydial infections (Stephens, 2003). Degranulation and decay of neutrophil granulocytes cause extensive species-specific damage of the pulmonary tissue. Progressive elimination of *C. psittaci* by adaptive immune response including DCs (see Section "The cellular immune response: New insights into MHC I-mediated presentation of chlamydial antigens"), T and B cells and regeneration of the pulmonary tissue follow. These findings go beyond the results from the large number of studies where only lesions at a single time point after their initiation or during their development were described. By focusing not only on the pathogen, but also the host's local immune response, our study allows insights into host–pathogen interactions at the commencement, progression and regression of the infection.

Host–pathogen interaction: Identifying the molecular partners on both sides of chlamydial cell infection

During the biphasic developmental cycle replicating bacteria acquire energy and biosynthetic precursors from the infected cell. Chlamydiae are capable of modulating cellular functions, such as apoptotic programs and immune response (see also Sections "The innate immune response and its interaction with the adaptive response: Chlamydial infection and the complement system" and "The cellular immune response: New insights into MHC I-mediated presentation of chlamydial antigens") (reviewed by Bastidas et al., 2013). Studies on inhibitors of bacterial protein synthesis suggest that modulation of host cell functions requires the activity of specific chlamydial proteins (see also Section "Host adaptation of *C. psittaci*: Escape from the avian innate immune response").

C. trachomatis dramatically rearranges the architecture of the eukaryotic host cell including the Golgi apparatus (GA), localization and appearance of the microtubule-organizing center (MTOC) and polarization of migrating cells (Heuer et al., 2009; Heymann et al., 2013; Johnson et al., 2009; Knowlton et al., 2011). These

surface of macrophages and DCs activates the production of pro-inflammatory cytokines and chemokines, which help to attract other effector cells to the site of infection. Chlamydia-activated DCs present bacteria-derived antigens to T cells and promote the differentiation of naive T cells to various subtypes of effector CD4+ and CD8+ T cells. CD4+ T helper 1 (Th1) cells secrete IFN-γ, which activates the anti-microbial activity of macrophages and helps B-cell production of chlamydia-specific antibodies, whereas Th2 cells provide help for B-cell production of antibodies.

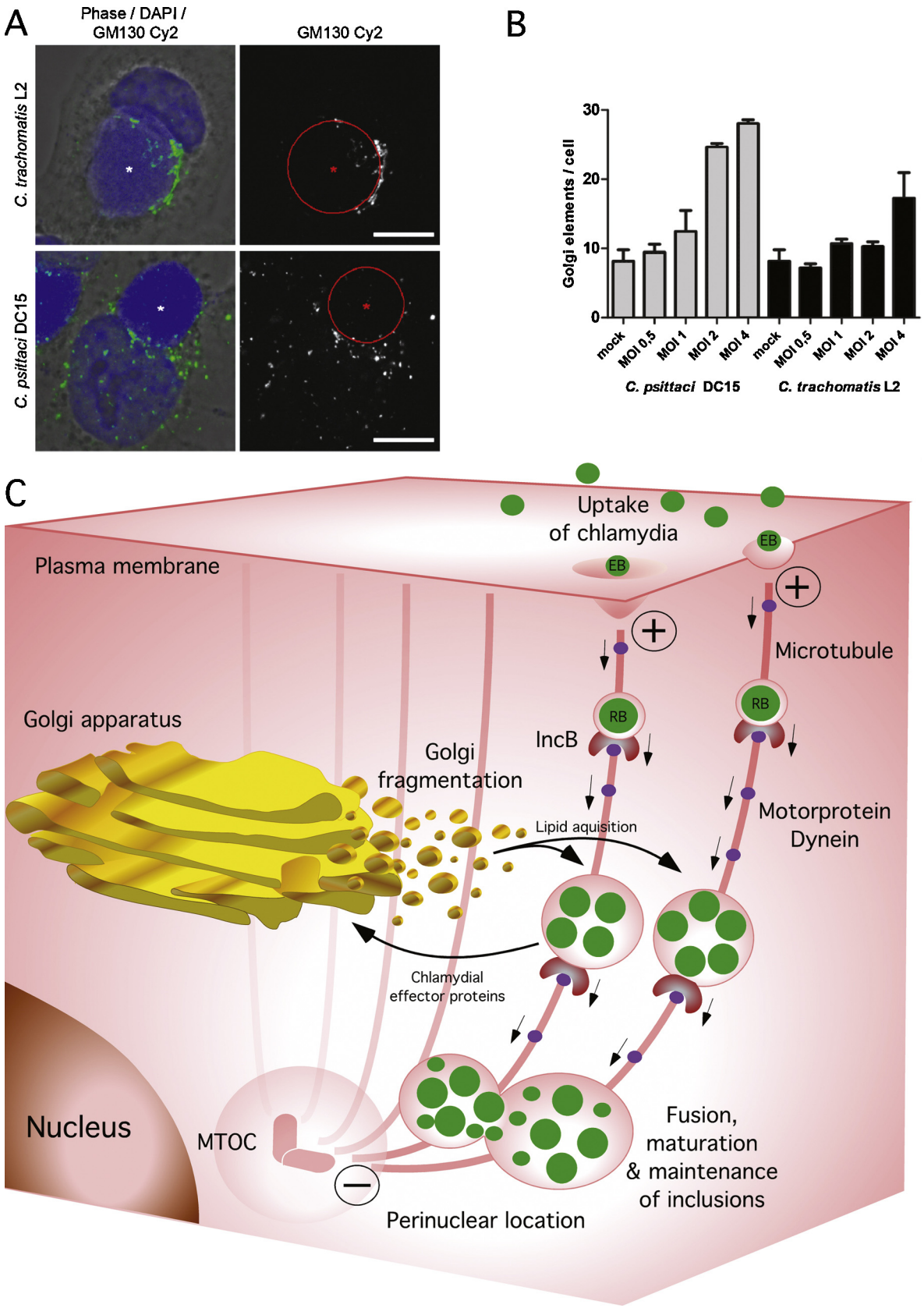

Fig. 4. Human and zoonotic chlamydial infections result in Golgi fragmentation in human cell lines. (A) HeLa cells were infected with either *C. trachomatis* L2 or *C. psittaci* at MOI 2. At 26 h p.i., cells were fixed and stained for the Golgi matrix protein, GM130 (in green). Cells were counter-stained using DAPI and analyzed at a Zeiss LSM 780. Overlay with a phase contrast is shown. Inclusions are indicated using a red line. Scale bar; 10 μm. (B) HeLa cells were infected with *C. trachomatis* L2 or *C. psittaci* using different MOIs. At 26 hpi, cells were fixed and the GA was visualized by GM130 staining. Golgi elements were then quantified using ImageJ. (C) Intracellular transport and lipid supply of chlamydial inclusion. Chlamydia exploits the host's intracellular trafficking machinery by recruiting the microtubule motor protein dynein to the outer surface of the vacuole (mediated by IncB), which drives the migration of inclusions toward the minus end of microtubules and the microtubule-organizing center (MTOC), where it

changes contribute either directly to bacterial growth or have implications on pathogenesis. Currently, it is unknown whether these observed changes are specific for *C. trachomatis* or if zoonotic *Chlamydia* strains including *C. psittaci* can induce similar changes. We have experimentally addressed this question by comparing the GA structure in a human cell culture model infected either with *C. psittaci* or *C. trachomatis* (Fig. 4). Infections resulted in fragmentation of the GA into smaller Golgi elements, and GM-130-positive Golgi elements were recruited to *C. trachomatis* inclusions. In contrast, *C. psittaci*-infected cells showed GM-130-positive Golgi elements scattered throughout the cytosol with no obvious recruitment to the inclusions (Fig. 4A). Quantification of the number of Golgi elements demonstrated Golgi fragmentation is MOI dependent but more pronounced in the case of *C. psittaci* infections (Fig. 4B). In summary, Golgi fragmentation is a hallmark of human and zoonotic chlamydial infections at least in a human cell culture model, but mode of fragmentation and/or trafficking of fragmented Golgi elements differ between species. These differences are likely reflected in divergence of chlamydial effector proteins.

All *Chlamydiaceae* spp. possess genes encoding core components of a Type III Secretion (TTS) apparatus, a protein transport system used by Gram-negative bacteria to translocate proteins into the cytoplasm of the host cell. Therefore, it is commonly accepted that chlamydial effector proteins are targeted by the TTS to the inclusion membrane and that their interaction with host proteins cause the modulation of host cell functions (Peters et al., 2007). Interactions of chlamydial effector proteins with host proteins seem to play a role at all stages of chlamydial development, i.e. from adhesion and internalization of EBs till their exit from the host cell.

An important group of chlamydial effector proteins are the Inc (inclusion) proteins, which share a common secondary structural feature of a bilobed hydrophobic domain. While this hydrophobic domain enables the anchoring of the proteins in the inclusion membrane, the cytoplasmic tail is responsible for interaction with host proteins (Dehoux et al., 2011). By direct interaction with host cell proteins, Inc proteins may affect different cell functions including signaling and trafficking. One of the first discovered protein–protein interactions was the association of IncG with mammalian 14-3-3β protein (Scidmore and Hackstadt, 2001), which contributes to circumvention of host cell apoptosis. The Inc protein CP0236 impairs IL-17 signaling via interaction with human Act1 (Wolf et al., 2009). Furthermore, IncD of *C. trachomatis* was shown to interact with the lipid transfer protein CERT (Derre et al., 2011), while another Inc protein, CT229, associated with the GTPase Rab4 (Rzomp et al., 2006). Both interactions may affect trafficking processes of the host cell. Finally, Inc protein CT228 was found to recruit elements of the myosin phosphatase pathway to regulate release mechanisms (Lutter et al., 2013).

In a recent study, we focused on the IncA and IncB proteins of the zoonotic agent *C. psittaci* using yeast two-hybrid screens to search for interacting host proteins that could indicate functions of these proteins. In a first attempt, the interaction between type III-secreted protein IncA of *C. psittaci* and host protein G3BP1 was identified. In GST-pull down and co-immunoprecipitation experiments both in vitro and in vivo interaction between full-length IncA and G3BP1 were shown. G3BP1 harbors a phosphorylation-dependent RNase activity that specifically cleaves the 3′-untranslated region of human *c-myc* mRNA. Using fluorescence microscopy, the localization of G3BP1 near the inclusion membrane of *C. psittaci*-infected Hep-2 cells was demonstrated. Notably, infection of Hep-2 cells with *C. psittaci* and overexpression of IncA in HEK293 cells led to a decrease in c-Myc protein concentration. This effect could be ascribed to the interaction between IncA and G3BP1 since overexpression of an IncA mutant construct disabled to interact with G3BP1 failed to reduce c-Myc concentration. We hypothesize that lowering the host cell c-Myc protein concentration may be part of a strategy employed by *C. psittaci* to avoid apoptosis and scale down host cell proliferation (Borth et al., 2011). Furthermore, it was shown that IncB in early inclusions of *C. psittaci* interacts and co-localizes with components of the dynein complex (data not shown). It is known that within the first few hours of infection, endocytosed *C. trachomatis* EBs are transferred to a perinuclear location corresponding to the MTOC of the host cell using the tubulin–dynein system (Grieshaber et al., 2003). Like *C. trachomatis*, *C. psittaci* also utilizes dynein motor proteins for optimal early development (Escalante-Ochoa et al., 2000). The interplay of IncB of *C. psittaci* with dynein components is the first indication of chlamydial proteins being involved in this transport process and possibly affecting the localization and structural appearance of the MTOC (Heuer et al., 2009) to support bacterial growth in the infected cell.

Host adaptation of *C. psittaci*: Escape from the avian innate immune response

Factors and mechanisms determining host specificity of *Chlamydia* spp. are still largely unknown. In a recent study (Braukmann et al., 2012), *C. psittaci* and *C. abortus* were compared for their invasiveness, virulence, and capability of eliciting an immune response in a chicken embryo model. This system has been used for decades, particularly to culture viruses and intracellular bacteria. As it involves inoculation onto the chorioallantoic membrane (CAM) the model resembles natural infection across epithelial layers. In fact it represents a valuable tool to investigate the innate immune response to chlamydial infection and to identify molecular processes on the pathogen's side in conjunction with the host's response that could explain differences in host adaptation between chlamydial species.

When compared with *C. abortus*, which is closely related but prefers non-avian hosts, *C. psittaci* displayed a significantly better capability of disseminating in host organs and elicited higher macrophage numbers and stronger expression of a sub-set of immune-related proteins. Nevertheless, the immune response patterns to both pathogens were largely similar, with expression of the pro-inflammatory mediators IL-1β, IL-6, IL-8, LITAF, IL-17 and iNOS, as well as IFN-γ, IL-12, IL-18, and TLR4, being significantly upregulated in CAM, liver and spleen of *C. psittaci*- and *C. abortus*-infected embryos. However, significant differences in favor of *C. psittaci* were seen in embryo mortality (Fig. 5) and mRNA expression rates of chlamydial genes, such as *incA*, *groEL* (in CAM, liver, spleen), *cpaf* and *ftsW* (in CAM). It is conceivable that elevated expression levels of the IncA protein would contribute to stabilization of the inclusion where the bacteria are residing (see also Section "Host–pathogen interaction: Identifying the molecular partners on both sides of chlamydial cell infection"). As expression of FtsW is used as an indicator of the pathogen's capability to replicate in the host, elevated mRNA expression rates of this gene could explain the better proliferation of *C. psittaci* throughout the infection. Furthermore, upregulation of the gene encoding the chlamydial heat shock protein GroEL (Hsp60) coincided with the elevated macrophage influx during *C. psittaci* infection. GroEL

resides in a central perinuclear position to establish its intracellular niche near the Golgi apparatus. Fusion, maturation and maintenance of chlamydial inclusions require clustering around the perinuclear MTOC. During the maturation of inclusions chlamydial effector proteins cause Golgi fragmentation and thereby ensure lipid acquisition and bacterial growth.

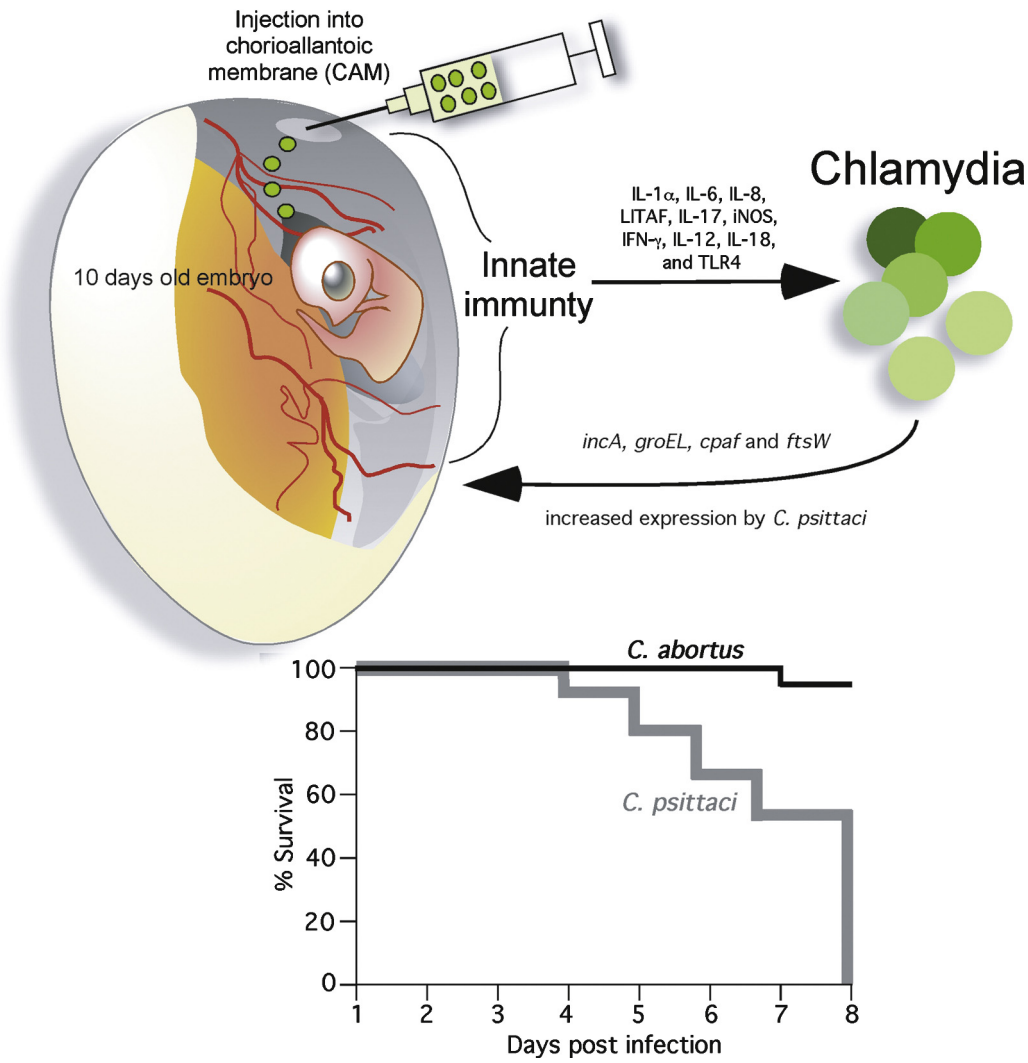

Fig. 5. Chlamydial infection in chicken embryos. Fertilized chicken eggs were incubated at 37.8 °C and a relative humidity of 60%. Inoculation of *C. psittaci* and *C. abortus* was conducted at embryonic developmental day (ED) 10. For infection, the bacterial suspension was transferred onto the chorioallantoic membrane (CAM (upper left panel)). The immune response patterns to both pathogens were largely similar, with expression of the pro-inflammatory mediators (upper right panel). Significant differences in favor of *C. psittaci* were seen mRNA expression rates of essential chlamydial genes (upper right panel), such as *incA*, *groEL*, *cpaf* and *ftsW* and in embryo mortality. Kaplan–Meier curves showing the survival of chicken embryos after infection with *C. psittaci* and, for comparison, *C. abortus* (lower panel). On ED 10, embryos ($n = 20$ per group) were inoculated with comparable amounts of chlamydiae (9×10^4–1×10^5 IFU/egg) and their vitality was determined by daily candling.

serves as a chaperone during intracellular development (Zugel and Kaufmann, 1999) and seems to be involved in the induction of immune response during human chlamydial infection, where it co-localizes with infiltrating macrophages (Kol et al., 1999).

Compared to *C. abortus*, *C. psittaci* appeared to be more efficient in actively suppressing the host immune response, which enabled better dissemination in the host. In this context, up-regulation of *cpaf* (see also Section "The cellular immune response: New insights into MHC I-mediated presentation of chlamydial antigens") probably enabled the pathogen to influence the host reaction in its favor. The results indicate a relevance of *incA* (see also Section "Host-pathogen interaction: Identifying the molecular partners on both sides of chlamydial cell infection"), *groEL*, *cpaf* and *ftsW* for the pathogenicity of *C. psittaci*. The data of the in vivo study provide a basis for a more detailed insight and better understanding of host–pathogen interactions in the course of infection. The fact that *C. psittaci* coped far better than *C. abortus* with the onset of the avian embryo's innate immune response by upregulating selected genes may be a key to understanding the mechanisms underlying host adaptation and etiopathology.

The innate immune response and its interaction with the adaptive response: Chlamydial infection and the complement system

The complement system consists of approximately 40 serum factors, mainly zymogens, their cleavage products and receptors. It is activated by LPS or lectins and other components of bacterial and viral surfaces. The activated complement system modulates inflammatory responses and protects against extracellular pathogens. Complement is often regarded as a key player of innate immunity (which can additionally be activated by immune complexes). However, there is increasing evidence that it can additionally trigger, enhance and orchestrate adaptive immune responses, at least in viral infections (reviewed by Klos et al., 2013). A complement system with homologous factors and identical function was found not only in mammals, but also in other vertebrate species including birds (Sharma, 1997).

Biologically active cleavage products of the circulating inert complement factors are locally released upon activation. Some of them become part of enzyme complexes, which permit successive steps in the cascade. Others remain soluble, bind to receptors on

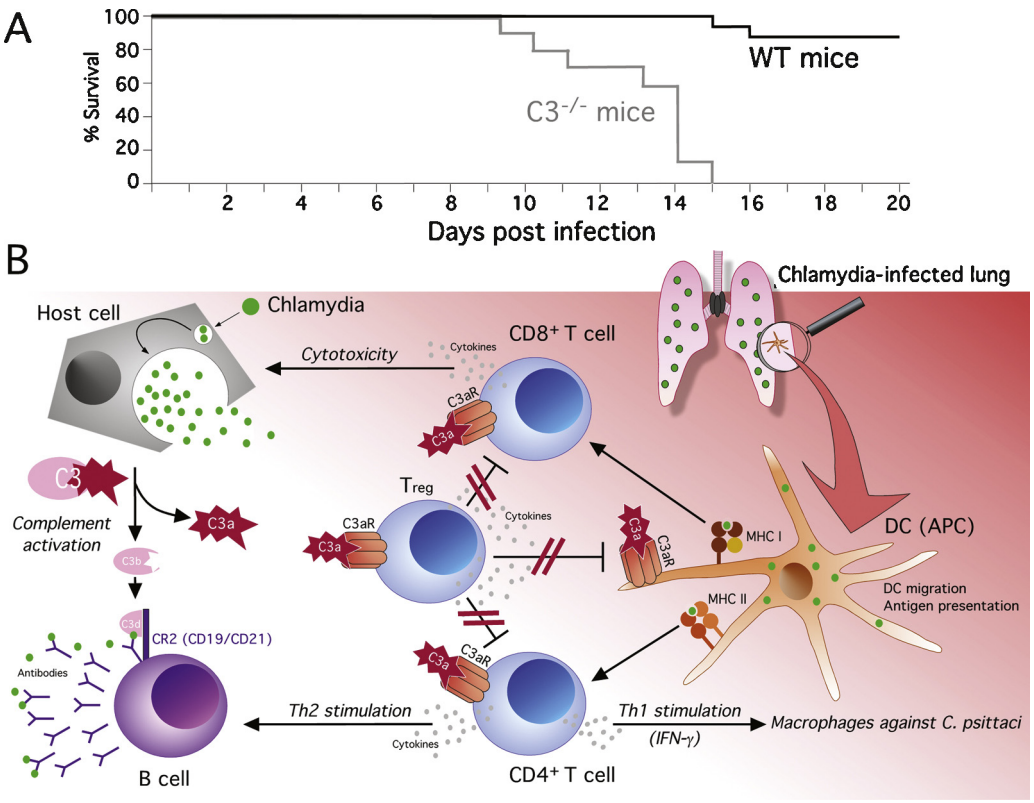

Fig. 6. (A) Complement factor C3 provides protection of mice against *C. psittaci*. $C3^{-/-}$ and the corresponding wild-type (WT) mice were intranasally infected with 10^4 ifu of *C. psittaci* strain DC15. Survival rates were determined for up to 21 days post-infection. (B) Assumed C3a/C3a-receptor (C3aR)-dependent regulation of the adaptive immune response against chlamydiae. Chlamydial elementary bodies (green) activate the complement cascade leading to the cleavage of inactive complement factor C3 into active C3a and C3b. Bound to its antigen, the C3b derivative C3d stimulates via complement receptor 2 (CR2) B cells and antibody production of plasma cells. The C3aR is expressed on mature dendritic cells (DCs) and, most likely, also on various subtypes of activated T cells including $CD4^+$ $CD25^+$ regulatory T cells (Treg cells). For the development of an effective adaptive protection against the bacteria, the immune-modulator C3a stimulates directly $CD4^+$ (Th1 and Th2) and cytotoxic $CD8^+$ T cells. Alternatively, C3aR-signaling down-regulates Treg cells thereby decreasing their inhibition of $CD4^+$ and $CD8^+$ T cells and DCs. Moreover, C3a/C3aR can activate DCs leading to their improved migration to the draining lymph nodes and thus, to an augmented presentation of chlamydial antigens to lymphocytes.

the host cell surface and act as extracellular mediators for intracellular signaling events. The three classical and broadly known effector functions of the complement cascade, i.e. opsonization via antigen-bound C3b, lysis by the membrane attack complex, pro-inflammation via the anaphylatoxins, are located downstream of complement factor C3. The anaphylatoxic peptides C3a and C5a, cleavage products of factor C3 and factor C5, respectively, activate their G-protein coupled receptors. C3a receptor (C3aR) and C5a receptor (C5aR, $C5a_1$ receptor, CD88) are expressed on cells of myeloic origin including granulocytes, monocytes/macrophages, DCs, mast cells, and even progenitor cells (see also Sections "Local immune response: Development and resolution of pulmonary lesions after *C. psittaci* infection of calf and mouse" and "The cellular immune response: New insights into MHC I-mediated presentation of chlamydial antigens"). Moreover, the anaphylatoxin receptors are also found on several non-myeloic cells and activated T cells. Albeit C3aR and C5aR are closely related, their functions overlap only partially.

EBs of *C. trachomatis* activate complement in vitro thereby reducing their infectivity in cell culture (Fedorko et al., 1987; Lin et al., 1992; Megran et al., 1985). Except for this fact, there was only limited knowledge about the role of complement during infections with *Chlamydia* or other intracellular bacteria at the beginning of the present research project. In a mouse model of intranasal *C. psittaci* lung infection, an early, high and long-lasting activation of the complement system was found (Bode et al., 2012). Intriguingly, only after the first week of infection complement became highly protective with an almost 100-fold increased susceptibility of $C3^{-/-}$ compared to wild type (WT) mice (Fig. 6A). In contrast, there were only minor differences after *C. psittaci* infection of complement factor C5-deficient and $C5aR^{-/-}$ in comparison to WT mice. This indicates that the main protective function of the cascade locates downstream of C3, but upstream of C5. It also points strongly toward C3a/C3aR as promising candidates of combatants for an effective defense against this intracellular microorganism.

Indeed, in our most recent study using $C3aR^{-/-}$ mice, the hypothesis that the C3aR is indispensable for effective protection and improved survival in *C. psittaci* lung infection has been confirmed (Dutow et al., 2014). During the first week, the course of disease was almost identical in infected WT and $C3aR^{-/-}$ mice. However, from then on, the ability to respond via C3aR to C3a significantly shortened severe acute pneumonia, led to drastically higher survival rates, more weight gain and lower clinical score. The bacterial clearance from lung and spleen was impaired in the absence of C3aR. Inflammatory parameters in lung homogenate including several cytokines and neutrophil granulocytes (and myeloperoxidase as granulocyte marker) (see also Section "Local immune response: Development and resolution of pulmonary lesions after *C. psittaci* infection of calf and mouse") remained elevated in $C3aR^{-/-}$ mice, as determined 14 days p.i. The most likely explanation for the sustained inflammatory response is the ongoing *C. psittaci* infection and the higher bacterial burden in the absence of C3aR.

Furthermore, the most likely reason for the impaired clearance of chlamydiae and the high lethality of the $C3aR^{-/-}$ mice during the second and third weeks p.i. has been addressed The time course suggested C3a/C3aR-dependent effects on the adaptive immune

response. Indeed, the size of the lung-draining lymph nodes of C3aR$^{-/-}$ mice increased less during chlamydial infection until day 9, i.e. the last time point before C3$^{-/-}$ and C3aR$^{-/-}$ animals can no longer control chlamydial infection and start to die. Intriguingly, cellular and adaptive immune responses were impaired in the absence of C3aR. The numbers of CD4$^+$ T cells and of re-stimulated chlamydia-specific IFN-γ$^+$ CD4$^+$ and CD8$^+$ cells in lung-draining lymph nodes were smaller; and there was no significant increase in B cells during infection. Following the same line, C3aR$^{-/-}$ mice were largely incapable of chlamydia-specific IgM or IgG production. To address causality and mechanism of C3aR-dependent protection in regard to the diminished antibody response, a serum transfer experiment was performed. Transfer of hyperimmune serum before infection could partially reverse the lethal outcome in C3aR$^{-/-}$ mice, indicating that antibody responses participate in protection against *C. psittaci* re-infection. This is in agreement with earlier reports, which indicated antibody-mediated protection in *C. trachomatis* and *C. muridarum* reinfections, suggesting a general function of antibodies in protection against secondary chlamydial infections (Morrison and Morrison, 2005; Su et al., 1997).

In contrast, transfer of *C. psittaci*-specific IgG to C3aR$^{-/-}$ mice on day 7, i.e. during ongoing acute infection, had only a very limited effect on disease progression and did not reduce lethality at all. This indicates that, in addition to the humoral response, other C3a-dependent functions must be critical for recovery and the much better survival rate of WT mice in our model of primary infection. CD4$^+$ and CD8$^+$ T cells each are sufficient for effective defense against *C. pneumoniae*, with IFN-γ as key player (Rothfuchs et al., 2004), suggesting a similar role of these T cell subsets in the defense against *C. psittaci* (see also Section "The cellular immune response: New insights into MHC I-mediated presentation of chlamydial antigens"). Thus, impaired *C. psittaci*-specific CD4$^+$ and cytotoxic CD8$^+$ T cell responses in infected C3aR$^{-/-}$ mice are presently the most likely causal link and explanation for the C3a-dependent protection in primary infection. Regulatory T cells, which suppress functions of both T cells and DCs and which can be downregulated by C3a (and C5a), might also participate in the immune dysfunction observed in the absence of C3aR in our model (for an illustration of the assumed C3aR-dependent regulation of the adaptive immune response against *C. psittaci* and protection, see Fig. 6B).

The studies described in this section showed that complement, and C3a and its receptor in particular, are critical for defense against *C. psittaci* in murine lung infection. However, it appears that the classical effector functions of the complement system are not important in this defense. Intriguingly, it seems as though C3a acts less as a weak pro-inflammatory mediator, but more as a powerful modulator of the adaptive cellular and humoral reactions against *C. psittaci*. Enhancement of specific B and T cell responses upon infection with an intracellular bacterium were identified as hitherto unknown features of complement and C3aR. These new functions might be of general immunological importance beyond chlamydial infections.

The cellular immune response: New insights into MHC I-mediated presentation of chlamydial antigens

It has been speculated that CD8$^+$T cells primed during chlamydia infection may lyse infected cells and deprive the pathogen of its intracellular niche (Balsara and Starnbach, 2007). If lysis of infected cells occurs early enough in the chlamydial developmental cycle, when the majority of organisms are non-infectious RBs, this mechanism could dramatically slow down the spread of infection within the host. In addition to this protective role of CD8$^+$ T cells during infection, there is evidence for the importance of IFN-γ release from activated CD8$^+$ T cells in controlling chlamydia infection (Starnbach et al., 1994). The initiation of the adaptive immune response (see also Section "Local immune response: Development and resolution of pulmonary lesions after *C. psittaci* infection of calf and mouse") requires MHC-mediated antigen presentation (Raghavan et al., 2008). In particular, DCs (see also Sections "Local immune response: Development and resolution of pulmonary lesions after *C. psittaci* infection of calf and mouse" and "The innate immune response and its interaction with the adaptive response: Chlamydial infection and the complement system") are equipped with a specialized machinery that promotes effective display of MHC/peptide complexes, rendering them the most potent stimulators of T lymphocytes (Thacker and Janssen, 2012). DCs perform a kind of sentinel function due to their ability to process internalized antigens before migration to secondary lymphoid organs, where they stimulate different sets of T cells (Thacker and Janssen, 2012). In the classical MHC I-pathway of antigen presentation, cytosolic antigens are degraded into peptide fragments by the proteasome and transported from the cytosol into the ER lumen by peptide transporter TAP (Raghavan et al., 2008). Peptides are then loaded on newly synthesized MHC I, and these complexes are released from the ER and transported to the cell surface via the Golgi (Peaper and Cresswell, 2008). DCs, however, have developed the ability to efficiently present peptides derived from endo- as well as exogenous antigens on MHC I via a process called cross-presentation (Neefjes and Sadaka, 2012). DCs are among the first professional antigen presenting cells (APCs) that are encountered by chlamydiae during infection (Gervassi et al., 2004) (see also Section "Local immune response: Development and resolution of pulmonary lesions after *C. psittaci* infection of calf and mouse"). It is thought that T cells primed by infected DCs play an important role in the effective immune response against chlamydia infection (Ojcius et al., 1998). The strategies used by the mammalian adaptive immune system (e.g. human and murine) are also mirrored in other jawed vertebrates, such as birds, so that, generally speaking, the avian innate and adaptive immune systems strongly resemble those of mammals (Foster and Berndt, 2013; Rolstad and Fossum, 1990; Sharma, 1997). Notably, pathogen-specific T and B cell responses combating chlamydial (Vanrompay et al., 1999) or other bacterial infections (Carvajal et al., 2008) and providing protection were also observed in birds. Moreover, since the avian immune system is also comprised of B, T, and NK cells, DCs, macrophages, as well as cyto/chemokines, it seems straightforward to speculate that mammals and birds use comparable immune mechanisms to elicit anti-chlamydial immunity.

In recent studies, a mouse model (see also Sections "Local immune response: Development and resolution of pulmonary lesions after *C. psittaci* infection of calf and mouse" and "The innate immune response and its interaction with the adaptive response: Chlamydial infection and the complement system") was used to analyze cellular and molecular requirements of MHC I-cross presentation of *C. psittaci*-infected myeloid (m)DCs (Fiegl et al., 2013). These DCs display morphological as well as functional maturation, which depend mainly on TLR2 signaling and are characterized by formation of dendritic veils, decreased phagocytic activity, elevated expression of distinct activation/maturation markers, as well as secretion of mDC-specific chemo/cytokines that are associated with optimal antigen presentation to T cells and the clearance of infection (Fiegl et al., 2013). Most interestingly, infected DCs permit only a restricted developmental cycle of chlamydiae. Thus, multiple small inclusions containing predominantly RBs, also at late stages of cell infection, are a typical feature (Fiegl et al., 2013). A controlled survival of chlamydiae in infected DCs might be critical to ensure a broad antigen reservoir allowing sustained and efficient chlamydial antigen presentation. The restricted growth of chlamydiae in DCs is induced by TNF-α that interferes with the developmental cycle. The limited growth of *C. psittaci* in infected DCs is accompanied by

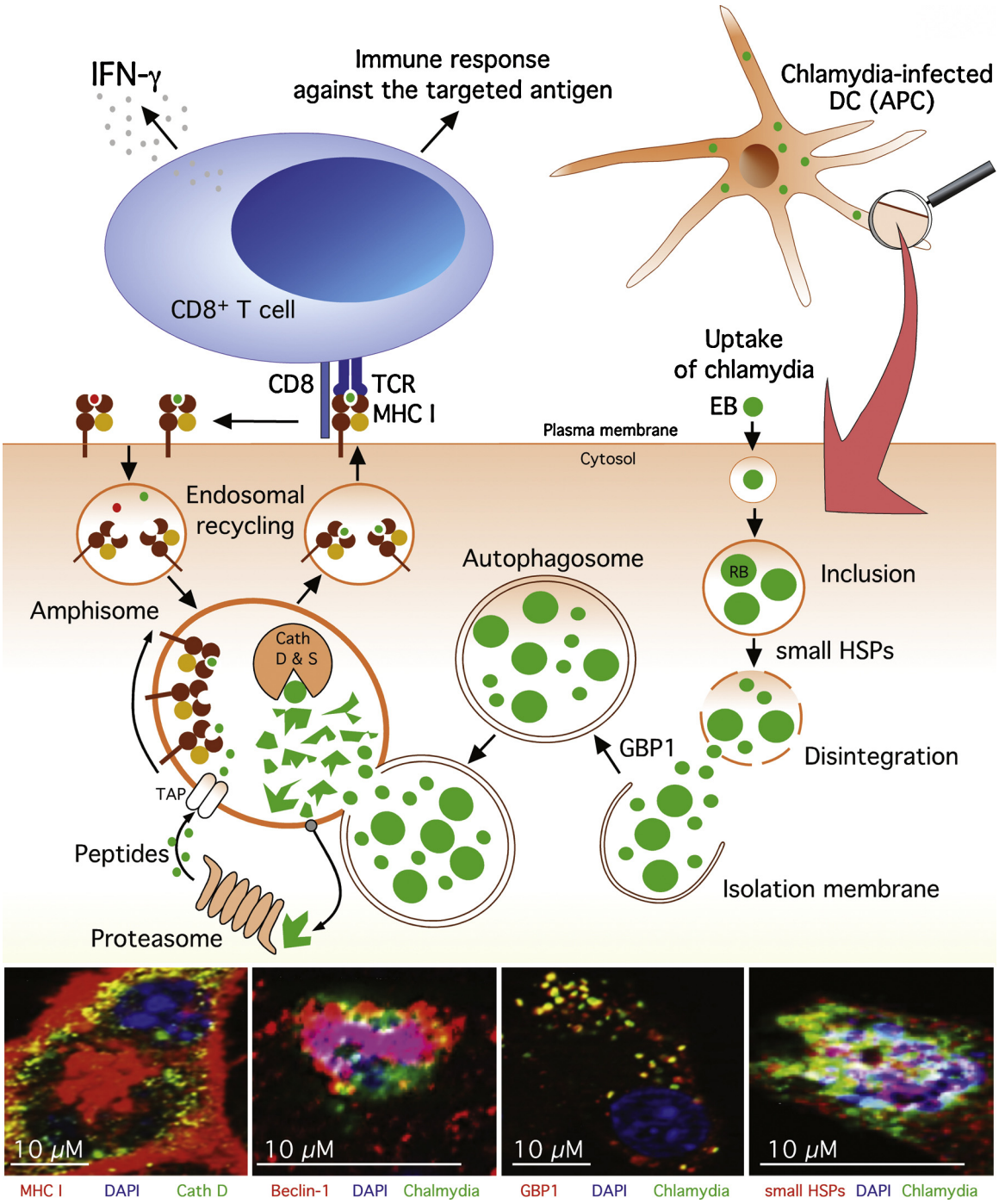

Fig. 7. Amphisomal cross-presentation of chlamydial antigens. Chlamydial inclusions formed in infected DCs are characterized by structural disintegration. Cytosolic released bacteria are engulfed by autophagosomes, which are able to fuse with endosomal compartments. Chlamydial antigens are preprocessed in these amphisomes and are then transferred across endo-vacuolar membranes for final processing by the proteasome. Antigenic peptides are reimported by TAP into amphisomal compartments and bound on MHC I derived from endosomal recycling of surface molecules. MHC I successfully loaded with chlamydial antigens is presented on the cell surface to $CD8^+$ T cells from which they recycle back to the "loading compartment". Depicted immunofluorescence images show colocalization of chlamydia with different effectors of the host cell defense machinery including small HSPs, GBP1 as well as autophagy markers and the presence of MHC I in cathepsin D-positive compartments.

retention of the chlamydial protease CPAF (see also Section "Host adaptation of *C. psittaci*: Escape from the avian innate immune response") within bacterial compartments, structural disintegration of inclusions and autophagosomal degradation of released free cytosolic chlamydiae (Fiegl et al., 2013). CPAF had been postulated to be the main virulence factor of chlamydiae (Shaw et al., 2002). It was proposed that the bacterial protease degraded various host factors relevant for chlamydial intracellular survival and that the cytosolic presence of CPAF played an essential role in maintaining the structural integrity of bacterial inclusions (Jorgensen et al., 2011). However, the findings of a recent study by Chen et al. (2012) have raised significant doubt that the identified host factors represent real substrates for CPAF in infected host cells. These new data exact a re-interpretation of the role of this bacterial enzyme during infection and fresh investigations on any CPAF functions.

By analyzing the stress response of *Chlamydia*-infected DCs, we observed an infection-dependent up-regulation of cytoprotective small HSPs (heat-shock proteins), which are known to interact

with the cellular cytoskeleton. This process is accompanied by a pronounced co-localization between the HSPs and chlamydial structures. Further experiments showed that knockdown of small HSPs in infected cells resulted in an increase in the bacterial load of infected cells with intact parasitophorous vacuoles encapsulated by actin ring structures. We suggest that the observed effects of infection-induced HSPs are likely due to direct interventions on chlamydial cytoskeleton subversion and in turn structural disintegration of chlamydial compartments. Moreover, our studies revealed up-regulation of the host defense factor GBP1 in chlamydia-infected DCs, which targets cytosolic released chlamydiae to autophagosomes to confer cell-autonomous protection and defense against the pathogen (Fiegl et al., 2013). Autophagy is a fundamental cellular mechanism essentially involved in the degradation of intracellular structures (Huang and Brumell, 2009) and has a protective function against cell-invading microbes. Our results suggest that chlamydiae from disintegrated inclusions are efficiently guided to autophagosomal compartments termed amphisomes, which are in close proximity to the collapsed chlamydial structures (Fiegl et al., 2013).

It was also found that chlamydiae do apparently not interfere with the ability of DCs to present MHC I-bound antigens (Fiegl et al., 2013). Thus, *C. psittaci*-infected mature DCs showed increased expression, surface recycling and presentation of MHC I (Fiegl et al., 2013). The induction of MHC I depends on the presence of TNF-α (Fiegl et al., 2013), which is released by chlamydia-infected DCs in response to TLR2 signaling (Prebeck et al., 2001). Chlamydia-infected DCs are also characterized by increased presence of MHC I and TAP in endosomal compartments containing cathepsin D and S (Fiegl et al., 2013). Both hydrolases are known to function as important downstream proteases in autolysosomal/amphisomal degradation (Boland et al., 2008; Pan et al., 2012). Inhibition of the two cathepsins in infected DCs has a dramatic influence on the MHC I-mediated stimulation of chlamydia-specific $CD8^+$ T cells suggesting important functions for the MHC I-processing of chlamydial antigens (Fiegl et al., 2013). Cathepsin-generated precursor peptides are thought to require further downstream trimming for MHC I loading (Fonteneau et al., 2003). In accordance with this idea, we found that the proteasome inhibition affects functional MHC I-presentation to chlamydia-specific $CD8^+$ T cells, indicating a significant contribution of the proteasome in the generation of chlamydial antigens. It appears that chlamydial antigens are first processed in amphisomal compartments, where cathepsins cleave bacterial proteins into large peptides. After cytosolic re-translocation, predigested peptides are further degraded by the proteasome and are then handled by TAP, which is also critically required for functional processing of chlamydial antigens. On the basis of the work described in this section, we propose a new cross-presentation model in which autophagy constitutes an important pathway promoting proteasome/TAP-dependent MHC I processing of chlamydial antigens (Fig. 7).

Clearance of chlamydiae depends on the ability of $CD8^+$ T cells to recognize epithelial cells, in which the bacteria predominantly replicate (Balsara and Starnbach, 2007) (see also Sections 3 and 4). Our recent studies demonstrate that chlamydia-infected epithelial cells retain their full ability to perform MHC I-mediated antigen presentation in the presence and absence of IFN-γ (Kägebein et al., 2014). One could imagine that, in chlamydia-infected epithelial cells, IFN-γ from activated T cells creates a situation reflecting the above-described scenario for infected DCs. Indeed, our own studies and those of other groups showed that, in infected epithelial cells, IFN-γ restricts chlamydial growth (Kägebein et al., 2014; Morrison, 2003) affects cytoplasmic CPAF translocation (Heuer et al., 2003), and induces autophagic degradation of chlamydiae (Al-Zeer et al., 2009).

Our recent findings on MHC I antigen presentation in chlamydia-infected DCs, epithelial cells and fibroblasts provide important novel insights for the development of such future strategies designed to support and maintain T cell-mediated immune responses against chlamydial infections.

Conclusions and outlook

Morbidity and mortality resulting from chlamydial diseases are of major global significance. The zoonotic potential of *C. psittaci* is an important and ongoing challenge for research and practice in human and veterinary medicine. The German national network on "Zoonotic chlamydiae – Models for chronic and persistent infections in man and animals" addressed distinct interconnected questions relating to chlamydial genomics and pathology, as well as host–pathogen interaction and anti-bacterial immunity. The individual studies of this coordinated research effort revealed

- the plasticity zone of the *C. psittaci* genome harboring key genomic correlates to species-specific adaptation to environmental niches, tissue tropism, virulence and pathogenicity,
- a broadened understanding of the complexity between pathological changes, pathophysiological interactions and the clinical outcome of an acute *C. psittaci* infection in the mammalian lung including the resulting systemic consequences,
- new insights into the early events of anti-chlamydial immune response and tissue regeneration in different hosts,
- significant differences and similarities between molecular factors and mechanisms of individual chlamydial pathogens, which might help to develop future strategies for disease control,
- the experimental basis to unravel the mechanisms underlying immune escape and host adaptation in the course of *C. psittaci* infection,
- a previously unknown important protective function of the complement system, particularly of C3a/C3aR, in *C. psittaci* infection of the lung,
- new insights into the role of C3a/C3aR on the development of an effective adaptive immune response against these (and most likely also other) intracellular bacteria, and
- new intracellular pathways of cell-intrinsic immunity and MHC I-mediated antigen presentation.

Although efficacious antimicrobial therapies exist, vaccination is still considered to be the best approach to reduce the prevalence of chlamydial infections. However, currently, there are no vaccines available against chlamydial infection despite the many efforts that have been made throughout the years. Our findings will not only help to develop innovative intervention and vaccination strategies against chlamydial infections, but also provide comparative data for other infectious agents. Clearly, further intensive research on chlamydial infections is required, especially with regard to treatment of persistent varieties, host adaptation, co-infection with other intracellular pathogens and bacterial mechanisms of immune evasion.

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References

Al-Zeer, M.A., Al-Younes, H.M., Braun, P.R., Zerrahn, J., Meyer, T.F., 2009. IFN-gamma-inducible Irga6 mediates host resistance against *Chlamydia trachomatis* via autophagy. PLoS ONE 4, e4588.

Andersen, A.A., Vanrompay, D., 2000. Avian chlamydiosis. Rev. Sci. Tech. 19, 396–404.

Balsara, Z.R., Starnbach, M.N., 2007. CD8+ T cell recognition of cells infected with *Chlamydia*. In: Bavoil, P.M., Wyrick, P.B. (Eds.), *Chlamydia*: Genomics, Pathogenesis and Implications for Control, Horizon Scientific Press, Norfolk, UK, pp. 381–412.

Bastidas, R.J., Elwell, C.A., Engel, J.N., Valdivia, R.H., 2013. Chlamydial intracellular survival strategies. Cold Spring Harb. Perspect. Med. 3, a010256.

Beatty, W.L., Morrison, R.P., Byrne, G.I., 1994. Persistent chlamydiae: from cell culture to a paradigm for chlamydial pathogenesis. Microbiol. Rev. 58, 686–699.

Beeckman, D.S., Vanrompay, D.C., 2009. Zoonotic *Chlamydophila psittaci* infections from a clinical perspective. Clin. Microbiol. Infect. 15, 11–17.

Beers, M.F., Morrisey, E.E., 2011. The three R's of lung health and disease: repair, remodeling, and regeneration. J. Clin. Invest. 121, 2065–2073.

Bode, J., Dutow, P., Sommer, K., Janik, K., Glage, S., Tummler, B., Munder, A., Laudeley, R., Sachse, K.W., Klos, A., 2012. A new role of the complement system: C3 provides protection in a mouse model of lung infection with intracellular *Chlamydia psittaci*. PLoS ONE 7, e50327.

Boland, B., Kumar, A., Lee, S., Platt, F.M., Wegiel, J., Yu, W.H., Nixon, R.A., 2008. Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease. J. Neurosci. 28, 6926–6937.

Borel, N., Summersgill, J.T., Mukhopadhyay, S., Miller, R.D., Ramirez, J.A., Pospischil, A., 2008. Evidence for persistent *Chlamydia pneumoniae* infection of human coronary atheromas. Atherosclerosis 199, 154–161.

Borth, N., Litsche, K., Franke, C., Sachse, K., Saluz, H.P., Hanel, F., 2011. Functional interaction between type III-secreted protein IncA of *Chlamydophila psittaci* and human G3BP1. PLoS ONE 6, e16692.

Braukmann, M., Sachse, K., Jacobsen, I.D., Westermann, M., Menge, C., Saluz, H.P., Berndt, A., 2012. Distinct intensity of host–pathogen interactions in *Chlamydia psittaci*- and *Chlamydia abortus*-infected chicken embryos. Infect. Immun. 80, 2976–2988.

Carvajal, B.G., Methner, U., Pieper, J., Berndt, A., 2008. Effects of *Salmonella enterica* serovar Enteritidis on cellular recruitment and cytokine gene expression in caecum of vaccinated chickens. Vaccine 26, 5423–5433.

Chen, A.L., Johnson, K.A., Lee, J.K., Sutterlin, C., Tan, M., 2012. CPAF: a chlamydial protease in search of an authentic substrate. PLoS Pathog. 8, e1002842.

Collingro, A., Tischler, P., Weinmaier, T., Penz, T., Heinz, E., Brunham, R.C., Read, T.D., Bavoil, P.M., Sachse, K., Kahane, S., Friedman, M.G., Rattei, T., Myers, G.S., Horn, M., 2011. Unity in variety – the pan-genome of the Chlamydiae. Mol. Biol. Evol. 28, 3253–3270.

Dean, D., Rothschild, J., Ruettger, A., Kandel, R.P., Sachse, K., 2013. Zoonotic Chlamydiaceae species associated with trachoma, Nepal. Emerg. Infect. Dis. 19, 1948–1955.

Dehoux, P., Flores, R., Dauga, C., Zhong, G., Subtil, A., 2011. Multi-genome identification and characterization of chlamydiae-specific type III secretion substrates: the Inc proteins. BMC Genomics 12, 109.

Derre, I., Swiss, R., Agaisse, H., 2011. The lipid transfer protein CERT interacts with the *Chlamydia* inclusion protein IncD and participates to ER-*Chlamydia* inclusion membrane contact sites. PLoS Pathog. 7, e1002092.

Dutow, P., Fehlhaber, B., Bode, J., Laudeley, R., Rheinheimer, C., Glage, S., Wetsel, R.A., Pabst, O., Klos, A., 2014. The complement C3a receptor is critical in defense against *Chlamydia psittaci* in mouse lung infection and required for antibody and optimal T cell response. J. Infect. Dis. 209, 1269–1278.

Escalante-Ochoa, C., Ducatelle, R., Haesebrouck, F., 2000. Optimal development of *Chlamydophila psittaci* in L929 fibroblast and BGM epithelial cells requires the participation of microfilaments and microtubule-motor proteins. Microb. Pathog. 28, 321–333.

Everett, K.D., Bush, R.M., Andersen, A.A., 1999. Emended description of the order Chlamydiales, proposal of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and standards for the identification of organisms. Int. J. Syst. Bacteriol. 49 (Pt 2), 415–440.

Fedorko, D.P., Clark, R.B., Nachamkin, I., Dalton, H.P., 1987. Complement dependent in vitro neutralization of *Chlamydia trachomatis* by a subspecies-specific monoclonal antibody. Med. Microbiol. Immunol. 176, 225–228.

Fiegl, D., Kagebein, D., Liebler-Tenorio, E.M., Weisser, T., Sens, M., Gutjahr, M., Knittler, M.R., 2013. Amphisomal route of MHC class I cross-presentation in bacteria-infected dendritic cells. J. Immunol. 190, 2791–2806.

Fonteneau, J.F., Kavanagh, D.G., Lirvall, M., Sanders, C., Cover, T.L., Bhardwaj, N., Larsson, M., 2003. Characterization of the MHC class I cross-presentation pathway for cell-associated antigens by human dendritic cells. Blood 102, 4448–4455.

Foster, N., Berndt, A., 2013. Immunity to *Salmonella* in farm animals and murine models of disease. In: Barrow, P.A., Methner, U. (Eds.), Salmonella in Domestic Animals. , 2nd ed, pp. 136–161.

Gaede, W., Reckling, K.F., Dresenkamp, B., Kenklies, S., Schubert, E., Noack, U., Irmscher, H.M., Ludwig, C., Hotzel, H., Sachse, K., 2008. *Chlamydophila psittaci* infections in humans during an outbreak of psittacosis from poultry in Germany. Zoonoses Public Health 55, 184–188.

Gervassi, A., Alderson, M.R., Suchland, R., Maisonneuve, J.F., Grabstein, K.H., Probst, P., 2004. Differential regulation of inflammatory cytokine secretion by human dendritic cells upon *Chlamydia trachomatis* infection. Infect. Immun. 72, 7231–7239.

Gieffers, J., van Zandbergen, G., Rupp, J., Sayk, F., Kruger, S., Ehlers, S., Solbach, W., Maass, M., 2004. Phagocytes transmit *Chlamydia pneumoniae* from the lungs to the vasculature. Eur. Respir. J. 23, 506–510.

Goellner, S., Schubert, E., Liebler-Tenorio, E., Hotzel, H., Saluz, H.P., Sachse, K., 2006. Transcriptional response patterns of *Chlamydophila psittaci* in different in vitro models of persistent infection. Infect. Immun. 74, 4801–4808.

Grieshaber, S.S., Grieshaber, N.A., Hackstadt, T., 2003. *Chlamydia trachomatis* uses host cell dynein to traffic to the microtubule-organizing center in a p50 dynamitin-independent process. J. Cell Sci. 116, 3793–3802.

Grinblat-Huse, V., Drabek, E.F., Creasy, H.H., Daugherty, S.C., Jones, K.M., Santana-Cruz, I., Tallon, L.J., Read, T.D., Hatch, T.P., Bavoil, P., Myers, G.S., 2011. Genome sequences of the zoonotic pathogens *Chlamydia psittaci* 6BC and Cal10. J. Bacteriol. 193, 4039–4040.

Harkinezhad, T., Verminnen, K., De Buyzere, M., Rietzschel, E., Bekaert, S., Vanrompay, D., 2009. Prevalence of *Chlamydophila psittaci* infections in a human population in contact with domestic and companion birds. J. Med. Microbiol. 58, 1207–1212.

Hatch, T.P., 1975. Utilization of L-cell nucleoside triphosphates by *Chlamydia psittaci* for ribonucleic acid synthesis. J. Bacteriol. 122, 393–400.

Heddema, E.R., van Hannen, E.J., Duim, B., de Jongh, B.M., Kaan, J.A., van Kessel, R., Lumeij, J.T., Visser, C.E., Vandenbroucke-Grauls, C.M., 2006. An outbreak of psittacosis due to *Chlamydophila psittaci* genotype A in a veterinary teaching hospital. J. Med. Microbiol. 55, 1571–1575.

Henning, K., Reinhold, P., Hotzel, H., Moser, I., 2008. Outbreak of a *Chlamydophila psittaci* infection in laboratory rats. Bull. Vet. Inst. Pulawy 52, 347–349.

Heuer, D., Brinkmann, V., Meyer, T.F., Szczepek, A.J., 2003. Expression and translocation of chlamydial protease during acute and persistent infection of the epithelial HEp-2 cells with *Chlamydophila* (*Chlamydia*) *pneumoniae*. Cell. Microbiol. 5, 315–322.

Heuer, D., Rejman Lipinski, A., Machuy, N., Karlas, A., Wehrens, A., Siedler, F., Brinkmann, V., Meyer, T.F., 2009. *Chlamydia* causes fragmentation of the Golgi compartment to ensure reproduction. Nature 457, 731–735.

Heymann, J., Rejman Lipinski, A., Bauer, B., Meyer, T.F., Heuer, D., 2013. *Chlamydia trachomatis* infection prevents front-rear polarity of migrating HeLa cells. Cell. Microbiol. 15, 1059–1069.

Hotzel, H., Berndt, A., Melzer, F., Sachse, K., 2004. Occurrence of Chlamydiaceae spp. in a wild boar (*Sus scrofa* L.) population in Thuringia (Germany). Vet. Microbiol. 103, 121–126.

Huang, J., Brumell, J.H., 2009. Autophagy in immunity against intracellular bacteria. Curr. Top. Microbiol. Immunol. 335, 189–215.

Jaeger, J., Liebler-Tenorio, E., Kirschvink, N., Sachse, K., Reinhold, P., 2007. A clinically silent respiratory infection with *Chlamydophila* spp. in calves is associated with airway obstruction and pulmonary inflammation. Vet. Res. 38, 711–728.

Jee, J., Degraves, F.J., Kim, T., Kaltenboeck, B., 2004. High prevalence of natural *Chlamydophila* species infection in calves. J. Clin. Microbiol. 42, 5664–5672.

Johnson, K.A., Tan, M., Sutterlin, C., 2009. Centrosome abnormalities during a *Chlamydia trachomatis* infection are caused by dysregulation of the normal duplication pathway. Cell. Microbiol. 11, 1064–1073.

Jorgensen, I., Bednar, M.M., Amin, V., Davis, B.K., Ting, J.P., McCafferty, D.G., Valdivia, R.H., 2011. The Chlamydia protease CPAF regulates host and bacterial proteins to maintain pathogen vacuole integrity and promote virulence. Cell Host Microbe 10, 21–32.

Joseph, S.J., Didelot, X., Rothschild, J., de Vries, H.J., Morre, S.A., Read, T.D., Dean, D., 2012. Population genomics of *Chlamydia trachomatis*: insights on drift, selection, recombination, and population structure. Mol. Biol. Evol. 29, 3933–3946.

Kägebein, D., Gutjahr, M., Grosse, C., Vogel, A.B., Rodel, J., Knittler, M.R., 2014. *Chlamydia* trachomatis-infected epithelial cells and fibroblasts retain the ability to express surface-presented MHC class I molecules. Infect. Immun. 82, 993–1006 (Epub 18 Dec 2013).

Kaleta, E.F., Taday, E.M., 2003. Avian host range of *Chlamydophila* spp. based on isolation, antigen detection and serology. Avian Pathol. 32, 435–461.

Kauffold, J., Melzer, F., Berndt, A., Hoffmann, G., Hotzel, H., Sachse, K., 2006. Chlamydiae in oviducts and uteri of repeat breeder pigs. Theriogenology 66, 1816–1823.

Kemmerling, K., Muller, U., Mielenz, M., Sauerwein, H., 2009. *Chlamydophila* species in dairy farms: polymerase chain reaction prevalence, disease association, and risk factors identified in a cross-sectional study in western Germany. J. Dairy Sci. 92, 4347–4354.

Klos, A., Wende, E., Wareham, K.J., Monk, P.N., International Union of Pharmacology, 2013. LXXXVII. Complement peptide C5a, C4a, and C3a receptors. Pharmacol. Rev. 65, 500–543.

Knowlton, A.E., Brown, H.M., Richards, T.S., Andreolas, L.A., Patel, R.K., Grieshaber, S.S., 2011. *Chlamydia trachomatis* infection causes mitotic spindle pole defects independently from its effects on centrosome amplification. Traffic 12, 854–866.

Kol, A., Bourcier, T., Lichtman, A.H., Libby, P., 1999. Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages. J. Clin. Invest. 103, 571–577.

Kuo, C.-C., Stephens, R.S., Bavoil, P.M., Kaltenboeck, B., 2011. Genus *Chlamydia* Jones, Rake and Stearns 1945, 55. In: Krieg, N.R., Staley, J.T., Brown, D.R., Hedlund, B.P., Paster, B.J., Ward, N.L., Ludwig, W., Whitman, W.B. (Eds.), Bergey's Manual of Systematic Bacteriology, vol. 4, 2nd ed. Springer, Heidelberg, pp. 846–865.

Lambertz, J., (Doctoral thesis) 2011. Pathology and Pathogenesis of an Experimental Aerogenous Infection of Calves With *Chlamydophila psittaci* of Non-Avian Origin. University of Veterinary Medicine Hanover, TiHo, Hannover (in German) http://elib.tiho-hannover.de/dissertations/lambertzj_ss11.pdf

Laroucau, K., de Barbeyrac, B., Vorimore, F., Clerc, M., Bertin, C., Harkinezhad, T., Verminnen, K., Obeniche, F., Capek, I., Bebear, C., Durand, B., Zanella, G., Vanrompay, D., Garin-Bastuji, B., Sachse, K., 2009. Chlamydial infections in duck farms associated with human cases of psittacosis in France. Vet. Microbiol. 135, 82–89.

Lenzko, H., Moog, U., Henning, K., Lederbach, R., Diller, R., Menge, C., Sachse, K., Sprague, L.D., 2011. High frequency of chlamydial co-infections in clinically healthy sheep flocks. BMC Vet. Res. 7, 29.

Lin, J.S., Yan, L.L., Ho, Y., Rice, P.A., 1992. Early complement components enhance neutralization of *Chlamydia trachomatis* infectivity by human sera. Infect. Immun. 60, 2547–2550.

Longbottom, D., Russell, M., Dunbar, S.M., Jones, G.E., Herring, A.J., 1998. Molecular cloning and characterization of the genes coding for the highly immunogenic cluster of 90-kilodalton envelope proteins from the *Chlamydia psittaci* subtype that causes abortion in sheep. Infect. Immun. 66, 1317–1324.

Lutter, E.I., Barger, A.C., Nair, V., Hackstadt, T., 2013. *Chlamydia trachomatis* inclusion membrane protein CT228 recruits elements of the myosin phosphatase pathway to regulate release mechanisms. Cell Rep. 3, 1921–1931.

Matsumoto, A., Manire, G.P., 1970. Electron microscopic observations on the effects of penicillin on the morphology of *Chlamydia psittaci*. J. Bacteriol. 101, 278–285.

Megran, D.W., Stiver, H.G., Bowie, W.R., 1985. Complement activation and stimulation of chemotaxis by *Chlamydia trachomatis*. Infect. Immun. 49, 670–673.

Möhle, K., (Doctoral thesis) 2011. Host–Pathogen Interactions in the Respiratory Tract of Calves After Intrapulmonary Infection With *Chlamydia psittaci*. University of Veterinary Medicine Hanover, TiHo, Hannover (in German) http://elib.tiho-hannover.de/dissertations/moehlek_ws11.pdf

Morrison, R.P., 2003. New insights into a persistent problem – chlamydial infections. J. Clin. Invest. 111, 1647–1649.

Morrison, S.G., Morrison, R.P., 2005. A predominant role for antibody in acquired immunity to chlamydial genital tract reinfection. J. Immunol. 175, 7536–7542.

Moulder, J.W., 1962. Some basic properties of the psittacosis-lymphogranuloma venereum group of agents. Structure and chemical composition of isolated particles. Ann. N. Y. Acad. Sci. 98, 92–99.

Narita, T., Wyrick, P.B., Manire, G.P., 1976. Effect of alkali on the structure of cell envelopes of *Chlamydia psittaci* elementary bodies. J. Bacteriol. 125, 300–307.

Neefjes, J., Sadaka, C., 2012. Into the intracellular logistics of cross-presentation. Front. Immunol. 3, 31.

Ojcius, D.M., Bravo de Alba, Y., Kanellopoulos, J.M., Hawkins, R.A., Kelly, K.A., Rank, R.G., Dautry-Varsat, A., 1998. Internalization of *Chlamydia* by dendritic cells and stimulation of *Chlamydia*-specific T cells. J. Immunol. 160, 1297–1303.

Ostermann, C., Linde, S., Siegling-Vlitakis, C., Reinhold, P., 2014. Evaluation of pulmonary dysfunctions and acid base imbalances induced by *Chlamydia psittaci* in a bovine model of respiratory infection. Multidiscip. Respir. Med. 9, 10, http://dx.doi.org/10.1186/2049-6958-9-10.

Ostermann, C., Ruttger, A., Schubert, E., Schrodl, W., Sachse, K., Reinhold, P., 2013a. Infection, disease, and transmission dynamics in calves after experimental and natural challenge with a bovine *Chlamydia psittaci* isolate. PLoS ONE 8, e64066.

Ostermann, C., Schroedl, W., Schubert, E., Sachse, K., Reinhold, P., 2013b. Dose-dependent effects of *Chlamydia psittaci* infection on pulmonary gas exchange, innate immunity and acute-phase reaction in a bovine respiratory model. Vet. J. 196, 351–359.

Page, L.A., 1968. Proposal for the recognition of two species in the genus *Chlamydia* Jones, Rake and Stearns 1945. Int. J. Syst. Bacteriol. 18, 51–66.

Pan, L., Li, Y., Jia, L., Qin, Y., Qi, G., Cheng, J., Qi, Y., Li, H., Du, J., 2012. Cathepsin S deficiency results in abnormal accumulation of autophagosomes in macrophages and enhances Ang II-induced cardiac inflammation. PLoS ONE 7, e35315.

Pantchev, A., Sting, R., Bauerfeind, R., Tyczka, J., Sachse, K., 2010. Detection of all *Chlamydophila* and *Chlamydia* spp. of veterinary interest using species-specific real-time PCR assays. Comp. Immunol. Microbiol. Infect. Dis. 33, 473–484.

Peaper, D.R., Cresswell, P., 2008. Regulation of MHC class I assembly and peptide binding. Annu. Rev. Cell Dev. Biol. 24, 343–368.

Peters, J., Wilson, D.P., Myers, G., Timms, P., Bavoil, P.M., 2007. Type III secretion a la *Chlamydia*. Trends Microbiol. 15, 241–251.

Pospischil, A., Borel, N., Chowdhury, E.H., Guscetti, F., 2009. Aberrant chlamydial developmental forms in the gastrointestinal tract of pigs spontaneously and experimentally infected with *Chlamydia suis*. Vet. Microbiol. 135, 147–156.

Poudel, A., Elsasser, T.H., Rahman, Kh.S., Chowdhury, E.U., Kaltenboeck, B., 2012. Asymptomatic endemic *Chlamydia pecorum* infections reduce growth rates in calves by up to 48 percent. PLoS ONE 7, e44961.

Prebeck, S., Kirschning, C., Durr, S., da Costa, C., Donath, B., Brand, K., Redecke, V., Wagner, H., Miethke, T., 2001. Predominant role of toll-like receptor 2 versus 4 in *Chlamydia pneumoniae*-induced activation of dendritic cells. J. Immunol. 167, 3316–3323.

Raghavan, M., Del Cid, N., Rizvi, S.M., Peters, L.R., 2008. MHC class I assembly: out and about. Trends Immunol. 29, 436–443.

Read, T.D., Joseph, S.J., Didelot, X., Liang, B., Patel, L., Dean, D., 2013. Comparative analysis of *Chlamydia psittaci* genomes reveals the recent emergence of a pathogenic lineage with a broad host range. MBio 4, e00604–e00612.

Reinhold, P., Jaeger, J., Liebler-Tenorio, E., Berndt, A., Bachmann, R., Schubert, E., Melzer, F., Elschner, M., Sachse, K., 2008. Impact of latent infections with *Chlamydophila* species in young cattle. Vet. J. 175, 202–211.

Reinhold, P., Liebler-Tenorio, E., Sattler, S., Sachse, K., 2011a. Recurrence of *Chlamydia suis* infection in pigs after short-term antimicrobial treatment. Vet. J. 187, 405–407.

Reinhold, P., Ostermann, C., Liebler-Tenorio, E., Berndt, A., Vogel, A., Lambertz, J., Rothe, M., Ruttger, A., Schubert, E., Sachse, K., 2012. A bovine model of respiratory *Chlamydia psittaci* infection: challenge dose titration. PLoS ONE 7, e30125.

Reinhold, P., Sachse, K., Kaltenboeck, B., 2011b. Chlamydiaceae in cattle: commensals, trigger organisms, or pathogens? Vet. J. 189, 257–267.

Rolstad, B., Fossum, S., 1990. Non-adaptive cellular immune responses as studied in euthymic and athymic nude rats. Spontaneous rejection of allogeneic lymphoid cell grafts by natural killer (NK) cells. Anat. Embryol. (Berl.) 181, 215–226.

Rothfuchs, A.G., Kreuger, M.R., Wigzell, H., Rottenberg, M.E., 2004. Macrophages, CD4+ or CD8+ cells are each sufficient for protection against *Chlamydia pneumoniae* infection through their ability to secrete IFN-gamma. J. Immunol. 172, 2407–2415.

Rzomp, K.A., Moorhead, A.R., Scidmore, M.A., 2006. The GTPase Rab4 interacts with *Chlamydia trachomatis* inclusion membrane protein CT229. Infect. Immun. 74, 5362–5373.

Sachse, K., Laroucau, K., Riege, K., Wehner, S., Dilcher, M., Myers, G., Weidmann, M., Vorimore, F., Vicari, N., Magnino, S., Liebler-Tenorio, E., Ruettger, A., Hufert, F.T., Bavoil, P.M., Rosselló-Móra, R., Marz, M., 2014. Evidence for the existence of two new members of the family *Chlamydiaceae* and proposal of *Chlamydia avium* sp. nov. and *Chlamydia gallinacea* sp. nov. Syst. Appl. Microbiol. 37, 79–88, http://dx.doi.org/10.1016/j.syapm.2013.1012.1004.

Salisch, H., Malottki, K., Ryll, M., Hinz, K.-M., 1996. Chlamydial infections of poultry and human health. World's Poult. Sci. J. 52, 279–308.

Schofl, G., Voigt, A., Litsche, K., Sachse, K., Saluz, H.P., 2011. Complete genome sequences of four mammalian isolates of *Chlamydophila psittaci*. J. Bacteriol. 193, 4258.

Scidmore, M.A., Hackstadt, T., 2001. Mammalian 14-3-3beta associates with the *Chlamydia trachomatis* inclusion membrane via its interaction with IncG. Mol. Microbiol. 39, 1638–1650.

Seth-Smith, H.M., Harris, S.R., Rance, R., West, A.P., Severin, J.A., Ossewaarde, J.M., Cutcliffe, L.T., Skilton, R.J., Marsh, P., Parkhill, J., Clarke, I.N., Thomson, N.R., 2011. Genome sequence of the zoonotic pathogen *Chlamydophila psittaci*. J. Bacteriol. 193, 1282–1283.

Seth-Smith, H.M., Sait, M., Sachse, K., Gaede, W., Longbottom, D., Thomson, N.R., 2013. Genome sequence of *Chlamydia psittaci* strain 01DC12 originating from swine. Genome Announc., 1, e00078-12.

Sharma, J.M., 1997. The structure and function of the avian immune system. Acta Vet. Hung. 45, 229–238.

Shaw, A.C., Vandahl, B.B., Larsen, M.R., Roepstorff, P., Gevaert, K., Vandekerckhove, J., Christiansen, G., Birkelund, S., 2002. Characterization of a secreted *Chlamydia* protease. Cell. Microbiol. 4, 411–424.

Starnbach, M.N., Bevan, M.J., Lampe, M.F., 1994. Protective cytotoxic T lymphocytes are induced during murine infection with *Chlamydia trachomatis*. J. Immunol. 153, 5183–5189.

Stephens, R.S., 2003. The cellular paradigm of chlamydial pathogenesis. Trends Microbiol. 11, 44–51.

Stephens, R.S., Myers, G., Eppinger, M., Bavoil, P.M., 2009. Divergence without difference: phylogenetics and taxonomy of *Chlamydia* resolved. FEMS Immunol. Med. Microbiol. 55, 115–119.

Su, H., Feilzer, K., Caldwell, H.D., Morrison, R.P., 1997. *Chlamydia trachomatis* genital tract infection of antibody-deficient gene knockout mice. Infect. Immun. 65, 1993–1999.

Szeredi, L., Hotzel, H., Sachse, K., 2005. High prevalence of chlamydial (*Chlamydophila psittaci*) infection in fetal membranes of aborted equine fetuses. Vet. Res. Commun. 29 (Suppl. 1), 37–49.

Tan, C., Spitznagel, J.K., Shou, H., Hsia, R.C., Bavoil, P.M., 2006. The polymorphic membrane protein gene family of the Chlamydiaceae. In: Bavoil, P.M., Wyrick, P.B. (Eds.), *Chlamydia*: Genomics and Pathogenesis, Horizon Bioscience, Norfolk, UK, pp. 195–218.

Thacker, R.I., Janssen, E.M., 2012. Cross-presentation of cell-associated antigens by mouse splenic dendritic cell populations. Front. Immunol. 3, 41.

Theegarten, D., Sachse, K., Mentrup, B., Fey, K., Hotzel, H., Anhenn, O., 2008. *Chlamydophila* spp. infection in horses with recurrent airway obstruction: similarities to human chronic obstructive disease. Respir. Res. 9, 14.

Thomson, N.R., Yeats, C., Bell, K., Holden, M.T., Bentley, S.D., Livingstone, M., Cerdeno-Tarraga, A.M., Harris, B., Doggett, J., Ormond, D., Mungall, K., Clarke, K., Feltwell, T., Hance, Z., Sanders, M., Quail, M.A., Price, C., Barrell, B.G., Parkhill, J., Longbottom, D., 2005. The *Chlamydophila abortus* genome sequence reveals an array of variable proteins that contribute to interspecies variation. Genome Res. 15, 629–640.

Van Lent, S., Piet, J.R., Beeckman, D., van der Ende, A., Van Nieuwerburgh, F., Bavoil, P., Myers, G., Vanrompay, D., Pannekoek, Y., 2012. Full genome sequences of all nine *Chlamydia psittaci* genotype reference strains. J. Bacteriol. 194, 6930–6931.

Vanrompay, D., Cox, E., Volckaert, G., Goddeeris, B., 1999. Turkeys are protected from infection with *Chlamydia psittaci* by plasmid DNA vaccination against the major outer membrane protein. Clin. Exp. Immunol. 118, 49–55.

Vanrompay, D., Geens, T., Desplanques, A., Hoang, T.Q., De Vos, L., Van Loock, M., Huyck, E., Mirry, C., Cox, E., 2004. Immunoblotting, ELISA and culture evidence for Chlamydiaceae in sows on 258 Belgian farms. Vet. Microbiol. 99, 59–66.

Voigt, A., Schofl, G., Heidrich, A., Sachse, K., Saluz, H.P., 2011. Full-length de novo sequence of the *Chlamydophila psittaci* type strain, 6BC. J. Bacteriol. 193, 2662–2663.

Voigt, A., Schofl, G., Saluz, H.P., 2012. The *Chlamydia psittaci* genome: a comparative analysis of intracellular pathogens. PLoS ONE 7, e35097.

Vorimore, F., Hsia, R.C., Huot-Creasy, H., Bastian, S., Deruyter, L., Passet, A., Sachse, K., Bavoil, P., Myers, G., Laroucau, K., 2013. Isolation of a new *Chlamydia* species from the Feral Sacred Ibis (*Threskiornis aethiopicus*): *Chlamydia ibidis*. PLoS ONE 8, e74823.

Wolf, K., Plano, G.V., Fields, K.A., 2009. A protein secreted by the respiratory pathogen *Chlamydia pneumoniae* impairs IL-17 signalling via interaction with human Act1. Cell. Microbiol. 11, 769–779.

Wyrick, P.B., 2010. *Chlamydia trachomatis* persistence in vitro: an overview. J. Infect. Dis. 201 (Suppl. 2), S88–S95.

Wyrick, P.B., Brownridge, E.A., 1978. Growth of *Chlamydia psittaci* in macrophages. Infect. Immun. 19, 1054–1060.

Yin, L., Kalmar, I.D., Lagae, S., Vandendriessche, S., Vanderhaeghen, W., Butaye, P., Cox, E., Vanrompay, D., 2013. Emerging *Chlamydia psittaci* infections in the chicken industry and pathology of *Chlamydia psittaci* genotype B and D strains in specific pathogen free chickens. Vet. Microbiol. 162, 740–749.

Zugel, U., Kaufmann, S.H., 1999. Role of heat shock proteins in protection from and pathogenesis of infectious diseases. Clin. Microbiol. Rev. 12, 19–39.