Innate immune response in avian macrophages elicited by *Chlamydia psittaci*

Aangeboren immuniteit van aviaire macrofagen geïnduceerd door Chlamydia psittaci

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ABSTRACT

***Chlamydia psittaci* is a gram-negative, obligate, intracellular bacterium, which mainly infects birds and mammals. Not much is known about innate immunity initiated by *C. psittaci*. The focus of the present study is on chicken macrophage activation and expression of cytokine, chemokine, caspase-1, iNOS and TLR genes during the early phase and mid-cycle period of the developmental cycle of the highly virulent *C. psittaci* strain 92/1293. *C. psittaci* significantly augmented the transcript levels for all genes investigated, especially during the mid-cycle period. These results demonstrate a robust innate immune response of chicken macrophages initiated by a *C. psittaci* infection.**

SAMENVATTING

Chlamydia psittaci is een obligate, gramnegatieve bacterie. Deze bacterie infecteert voornamelijk vogels en zoogdieren. Er is weinig bekend over hoe *C. psittaci* de aangeboren immuniteit initieert van zijn gastheercel. In deze studie worden de activering van macrofagen en de expressie beschreven van cytokinen, chemokinen, caspase-1, iNOS en TLR genen gedurende de vroege en middelste fase van de ontwikkelingscyclus van de hoog virulente *C. psittaci* stam 92/1293. Een significante opregulatie van alle genen werd geobserveerd na infectie, vooral tijdens de middelste fase van de ontwikkelingscyclus. De resultaten geven een beter beeld van hoe het aangeboren immuunsysteem van aviaire macrofagen beïnvloed wordt door een *C. psittaci*-infectie.

INTRODUCTION

Chlamydiaceae are gram-negative, obligate, intracellular bacteria, which mainly infect birds and mammals. In birds, *C. psittaci* replicates in epithelial cells and macrophages of the avian respiratory tract, which may result in a systemic infection (Vanrompay et al., 1995). They possess a unique biphasic developmental cycle, thereby switching between a metabolically inactive, infectious state, the elementary body (EB) and a metabolically active, non-infectious state, the reticulate body (RB). Following attachment of EBs to the host cell membrane and the subsequent internalization, EBs start to differentiate into RBs within an inclusion, which is derived from the host cell membrane during internalization (Vanrompay et al., 1995). Reticulate bodies start to migrate to the periphery of the inclusion, whereby the replication starts by binary fission. Afterwards, RBs may re-differentiate into new infectious EBs followed by the release of those EBs by host cell lysis or reverse endocytosis. In some cases, the developmental cycle can be altered in favor of persistence. Persistent *Chlamydiaceae* or so-called aberrant bodies fail to complete their development from RBs into infectious EBs, but retain their metabolic activity.

Not much is known about how the innate immune system of the host is influenced by a *C. psittaci* infection. *C. psittaci* replicates in epithelial cells and macrophages of the avian respiratory tract. Subsequently, *C. psittaci* can be demonstrated in plasma and blood monocytes, resulting in a systemic infection (Vanrompay et al., 1995). Monocytes/macrophages are part of the innate immune system and capable of engulfing and killing pathogens; but probably, their most important function is to recruit other myeloid cells, in particular polymorphonuclear phagocytes, to the site of infection by the release of chemotactic cytokines. Macrophages can also activate the adaptive immune response by presenting antigens

to $CD4^+$ T cells via class II MHC antigen (Beuttler, 2004). Although monocytes/macrophages play an important role in clearing pathogens, *C. psittaci* as well as other *Chlamydiaceae* are able to survive and even replicate within those cells. Moreover, *C. psittaci* uses blood monocytes as vehicles to establish a systemic infection in birds. Although not much is known about how the host innate immune system is influenced by *C. psittaci*, Beeckman et al. (2010) demonstrated an increased expression of IL-1β and IL-6, CXCLi2, CXCLi1 and CCLi2 following inoculation with the highly virulent *C. psittaci* strain 92/1293 (*ompA* genotype D) at 4 hours post infection (p. i.). Interestingly, exceptionally high IL-10 and no TGF-β4 responses were observed at 4 hours post inoculation. This could induce macrophage deactivation and NF-κB suppression (Beeckman et al., 2010) and thereby, dampen innate immunity and promote *C. psittaci* survival in macrophages.

Toll-like receptor (TLR)-mediated recognition of components derived from a wide range of pathogens and their role in the subsequent initiation of innate immune responses are widely accepted (Kawai and Akira, 2011).

The goal of the present study was to examine the expression of cytokines (IL-1β, IL-6, MIF, LITAF (lipopolysaccharide-induced TNF facor) , IL-12p35, IL-10), caspase-1, GM-CSF, iNOS, chemokines (CXCLi1, CXCLi2, CCLi3, IL-16) and TLRs (TLR2, TLR3, TLR4, TLR5, TLR7, TLR21) during an infection of chicken macrophages (HD11 cells) with the virulent *C. psittaci* strain 92/1293 at different time points.

MATERIALS AND METHODS

Chlamydia and cell lines

The well-characterized, virulent *Chlamydia psittaci* strain 92/1293 used in this study was isolated from the lung, spleen and cloaca of a diseased turkey (Vanrompay et al., 1993). The bacterium was grown in buffalo green monkey (BGM) cells as described previously (Vanrompay et al., 1993), and the median tissue culture infective dose ($TCID_{50}$) was determined using the method of Spearman and Kaerber (Mayr et al., 1974).

HD11 chicken monocytes/macrophages (Beug et al., 1979) were cultured in Dulbecco's modified eagle's minimal essential medium (DMEM) supplemented with 1% L-glutamine, 0.5% gentamicin, 5% fetal calf serum (FCS) and 1% sodium pyruvate (Invitrogen, Merelbeke, Belgium), and were incubated in a humidified atmosphere at 37°C and 5% CO_2.

C. psittaci infection of HD11 cells

HD11 cells were seeded in a 25 cm^2 tissue culture flask at a concentration of 300.000 cells/ml and grown for 24 hours at 37°C and 5% CO_2. The medium was aspirated and 1.5 x 10^6 HD11 cells were infected with *C. psittaci* at a multiplicity of infection (MOI) of 1. Irreversible attachment and cell entry were accomplished by incubating the HD11 cells for 3 hours on a rocking platform at 37°C. The unbound organisms were washed away with DMEM (37°C) and culture medium enriched with 5.5 mg/l glucose (Sigma-Aldrich, United Kingdom) was added to each tissue culture flask. Infected cells were incubated until RNA extraction at 2, 4, 8, 12 and 18 hours p.i.

Transcription analysis of cytokine, chemokine, caspase-1, iNOS and TLR genes

The innate immune response following *C. psittaci* infection of HD11 cells was determined by examining gene transcript levels of IL-1β, IL-6, MIF, LITAF, IL-12p35, IL-10, caspase-1, GM-CSF, iNOS, CXCLi1, CXCLi2, CCLi3, IL-16, TLR2, TLR3, TLR4, TLR5, TLR7, TLR21 in infected and control HD11 cultures. Specific primers were designed using primer 3 (http://frodo.wi.mit.edu/primer3/) and DINA melt (http://www.bioinfo.rpi.edu/ applications/hybrid) software programs (Table 1). The specificity of all RT-PCR primers was initially checked by conventional PCR followed by cloning (pGEM-T Easy Vector System, Promega, Leiden, the Netherlands) and DNA sequencing of the inserts (LGC Genomics, Berlin, Germany). As it was not possible to design primers, which were 100% specific for IL10, IL12p35 and GM-CSF, probes, kindly provided by P. Kaiser and L. Rothwell (Institute for Animal Health, Compton, Berkshire, UK), were needed to verify the specificity of the amplified targets (Table 1).

The total RNA from 1,5 x 10^6 infected HD11 cells (MOI 1) was prepared using the total RNA isolation reagent (TRIR, ABgene, Westburg, Leusden, the Netherlands) according to the manufacturer's protocol. RNA from uninfected cells served as negative controls. After RNA extraction, samples were treated with RNase-free amplification grade DNase I (Promega) following the manufacturer's instructions and were confirmed to be DNA-free by performing a PCR for the *C. psittaci* 16S rRNA gene. One microgram of total RNA was reverse transcribed (reverse-IT™ 1st Strand Synthesis, Thermo Scientific, Waltham, USA) into host cell cDNA using the anchored oligo-dT molecule. Each RNA sample was spiked with 5ng coliphage MS2 control RNA (RNA Control Kit, Thermo Scientific, Waltham, USA). All experiments were performed in duplicate, with replicates performed at different days.

Following cDNA synthesis, cDNA amplification was performed for 6 cytokine genes, 4 chemokine genes, the caspase-1 gene, the GM-CSF gene, the iNOS gene, 6 TLR genes, the HD11 28S rRNA normalization gene and the MS2 spike. cDNA amplification was performed using the Absolute™ QPCR SYBR® Green

Table 1. Real-time quantitative RT-PCR primers and probes.

Target	Accession No.	Primer and probe sequence (5'-3')	T_a
HD11 28S rRNA	X59733	F: TTTGGGTTTTAAGCAGGAGGT R: TTGCGACAACACATCATCAGT	58°C
C. psittaci 16S rRNA	CPU68447	F: GTCAAGTCAGCATGGCCCTT R: CCCAGTCATCAGCCTCACCT	58°C
MS2 spike	Unpublished	F: Unpublished R: Unpublished	60°C
IL-1β	Y15006.1	F: CACAGAGATGGCGTTCGTT R: GTGACGGGCTCAAAAACCT	58°C
IL-6	NM_204628	F: AGAAATGCCTGACGAAGCTCT R: CACGGTCTTCTCCATAAACGA	58°C
Caspase-1	AF031351	F: TGCCATGAAGACAAAACTTCC R: TCTACACATCTCCAGCCATCC	58°C
MIF	M95776	F: CAGAACAAGACCTACACCAAGC R: CTAACAAGGAGCCATCCATC	58°C
IL-10	AJ621254	F: CATGCTGCTGGGCCTGAA R: CGTCTCCTTGATCTGCTTGATG P: CGACGATTCGGCGCTGTCACC	55°C
LITAF	AY765397	F: TCCTCACCCCTACCCTGTC R: TCAGAGCATCAACGCAAAAG	58°C
IL-12p35	AJ262751	F: TGGCCGCTGCAAACG R: ACCTCTTCAAGGGTGCACTCA P: CCAGCGTCCTCTGCTTCTGCACCTT	55°C
GM-CSF	AJ621253	F: CCTGGAAGAAATAACGAGTCACTTG R: ACAGGTTTATCCCTGATGTCCAT P: AGCGGCCACAGCAGGTCTGTCC	55°C
CXCLi1	Y14971	F: CCACTGCTTACTGGCTTATCG R: CTTGGGATGGATGAACTTGG	55°C
CXCLi2	NM_205498	F: CTCGCTCTTCTCATCGCATC R: GGCAGCAGTGTCCCATCC	58°C
CCLi3	Y18692	F: AGCCTGCCATCATCTTCATC R: AAACAGCACCTGCCATGAG	58°C
IL-16	NM_204352	F: CTCAGCCCAAAACCATCAGT R: GGTGGCAGTAAGTGGAAAGC	58°C
TLR2	AB046533	F: CCTGGTGTTCCTGTTCATCC R: AGCGTCTTGTGGCTCTTCTC	58°C
TLR3	NM_001011691	F: GGCTAAACGACACTCAAGCA R: GGCGTCATAATCAAACACTCC	58°C
TLR4	NM_001030693	F: TGGCACCTACCCTGTCTTTC R: GGCTTGGAGTGGCTTGTATG	58°C
TLR5	NM_001024586	F: AACTCCCTTCCTTCCCACAT R: AACCTCTCTCCCACACAAGC	58°C
TLR7	NM_001011688	F: ATCAGCACAGGGATGGAAAG R: GGGGAACGGTAGTCAGAAGG	58°C
TLR21	NM_001030558	F: AGAAGCAACCACAGGGAGAA R: AAGCACTTTTGGGGTCCTTT	58°C
iNOS	D85422	F: ACCCACCCAACAACTGCTAA R: GCCCTTGTCCATCTCTTGTC	58°C

F: forward primer; R: reverse primer; P: 5'-FAM (5-carboxyfluorescein) + 3'-TAMRA (6- carboxytetramethylrhodamine) probe.

Mix (Thermo Scientific, Waltham, USA). The DNA polymerase was initially activated for 15 minutes at 94°C. Then 40 cycles of amplification were carried out using the Rotor Gene RG-3000 cycler (Westburg, the Netherlands) according to the following cycle profile: DNA was denatured at 95°C for 10 minutes and during 40 cycles of 95°C for 30 seconds, primers annealed at 55-60°C for 30 seconds and extended at 72°C for 30 seconds (Table 1). Program settings included the acquisition on the FAM/Sybr channel in the extension step and a gain of six. The quantification was done as described by Beeckman et al. (2008), using standard graphs of the cycle treshold (Ct) values obtained by testing 10-fold serial dilutions (10^9 to 10^1 molecules/µl) of the purified PCR products. All samples and standards were tested in duplicate. Ct-values of the samples were automatically converted into initial template quantities (N_0) by the use of the RotorGene software 6.0 (Westburg, the Netherlands) using imported standard curves from previous runs. The quantification results of the coliphage MS2 RNA were used to correct for intersample variability, while the quantification results of the HD11 28S rRNA were used to correct during the developmental cycle and cell growth. No difference in mRNA level is therefore shown as a fold change of 0.

HD11 activation assay

The activation of HD11 cells was determined by measurement of the accumulation of nitrite (NO_2) in the culture medium at 2, 4, 8, 12 and 18 hours post infection with *C. psittaci*. One hundred microliter of the collected cell free supernatant was added to equal amount of Griess reagent (Sigma-Aldrich, United Kingdom) and incubated for 15 minutes at room temperature. The amount of NO_2^- (NO) was determined by measuring the absorbance of the reaction product with a spectrophotometer (Tecan Genios Plus) at a wavelength of 585 nm. A 10-fold $NaNO_2$ dilution series ranging from 320 µM to 0.3125 µM (in triplicate) was created to generate a standard curve. This standard curve was used to determine the amount of NO_2^- (NO) in the samples.

Statistical analysis

The experiment was performed twice, each time testing all samples in duplicate. Data were pooled for statistical analysis. The mean and standard error mean (SEM) for cytokine-, chemokine-, caspase-1-, GM-CSF-, iNOS-, and TLR-gene transcript levels were calculated. Statistically significant differences ($p<0.05$; $p<0.005$) between the results obtained to investigate the innate immune response elicited by *C. psittaci* was determined using an unpaired Student's *t*-test (SPSS Inc., Chicago Illinois, USA). Secondly, an analysis of variance (ANOVA, SPSS Inc.) with post-hoc analysis (both Tukey HSD and Tukey-b) was performed along the time axis to determine significant upregulation time points for cytokine-, chemokine-, caspase-1-, iNOS- and TLR-genes and NO.

RESULTS

Transcription analysis of the caspase-1 gene and cytokine genes

Statistical differences were observed when comparing gene transcript levels of infected cells versus mock-infected controls (Table 2). The influence of an infection on gene expression by comparing gene transcript levels in infected cells versus mock infected controls was examined. The mRNA levels in mock-infected controls were presented as an mRNA-fold change of 0. For the pro-inflammatory cytokines IL-1β and IL-6, gene expression upregulation was already noticed at 2 hours p.i., as compared to the mock-infected controls. The upregulation continued, resulting in the maximal upregulation of the IL-1β (1671-fold) and IL-6 (650-fold) gene expression at 18 and 12 hours p.i., respectively. IL-1β and IL-6 gene upregulation was most significant from 8 to 12 hours p.i. The genes for caspase-1, LITAF, MIF, IL-12p35, IL-10 and GM-CSF were all downregulated (mRNA-fold change < 0) during the first 4 hours p.i, as compared to the results of the mock-infected controls. However, at 8 hours p.i., mRNA-levels for all these genes were comparable to the ones for the mock-infected controls, as they were close to a mRNA-fold change of 0. A significant upregulation of the expression of the caspase-1, LITAF , MIF, IL-12p35, IL-10 and GM-CSF genes was observed towards 12 hours p.i., the beginning of the mid-cycle period. Regarding the caspase-1, LITAF , MIF, IL-12p35, IL-10 and GM-CSF genes, the upregulation of gene expression was most pronounced for LITAF (23-fold) and IL-12p35 (106-fold). Interestingly, during mid-cycle (from 12 to 18 hours p.i.), mRNA levels for all genes significantly declined towards the base line level of 0, except for IL-1β, GM-CSF and IL-10. The expression of those genes was significantly upregulated during mid-cycle, resulting in a 1671-, 27- and 9.8-fold change in mRNA level, as compared to the mock-infected controls (Figure 1).

Transcription analysis of chemokine genes

The expression of the pro-inflammatory chemokine genes CXCLi1 (K60), CXCLi2 (IL-8), CCLi3 (K203) and IL-16 in *C. psittaci* infected avian macrophages were compared with mock-infected controls. Statistical differences were observed when comparing gene transcript levels for infected cells versus mock-infected controls (Table 2). All chemokine genes, except for the IL-16 gene, were significantly upregulated during the early phase of the bacterial reproduction cycle (85-fold for CXCLi1, 66-fold for CXCLi2 and 89-fold for CCLi3). During mid-cycle, the gene expression upregulation continued (1493-fold for CXCLi1, 471-fold for CXCLi2 and 767-fold for CCLi3). For the IL-16 gene, a significant, but rather moderate expression upregulation was noticed no earlier than mid-cycle (10-fold rise and 2.2-fold rise at 12 and 18 hours p.i., respectively) (Figure 2).

Transcription analysis of TLR genes

The expression of six known avian TLR genes were compared; TLR2, TLR3, TLR4, TLR5, TLR7 and TLR21 in *C. psittaci* infected avian macrophages versus mock-infected controls. During the early phase of the developmental cycle, TLR gene expression was significantly downregulated, as compared to the mock-infected controls. TLR21 was significantly upregulated (3.6-fold rise) at 8 hours p.i. In contrast, the expression of all TLR genes, with the exception of the TLR2 gene, was significantly upregulated during mid-cycle (especially, at 12 hours p.i.). Gene expression upregulation was rather moderate for TLR3 (5.9-fold rise), TLR4 (6.2-fold rise), TLR5 (5.2-

Table 2. X-fold changes of the mRNA expression levels of cytokines, chemokines, caspase-1, iNOS and TLR of infected versus non-infected HD11cells.

Accession No	Gene	Hour post infection				
		2h p.i	4h p.i	8h p.i	12h p.i.	18h p.i.
Y15006.1	IL-1β	46 ± 6.1	478 ± 68	123 ± 14	1599 ± 79	1701 ± 75
NM_204628	IL-6	15.4 ± 2.3	1195 ± 130	141 ± 17	482 ± 192	0.8 ± 0.8
AF031351	Caspase-1	-5.2 ± 0.5	1.5 ± 0.2	1.8 ± 0.3	6.8 ± 0.2	3.9 ± 0.4
M95776	MIF	-5.1 ± 0.04	1.3 ± 0.1	0.6 ± 0.6	4.2 ± 0.2	2.5 ± 0.1
AJ621254	IL-10	-1.3 ± 0.1	-2.1 ± 0.3	-1.3 ± 0.1	3.2 ± 0.8	9.9 ± 0.9
AY765397	LITAF	-13 ± 4.1	0.9 ± 0.9	-1.8 ± 0.2	25 ± 0.7	4.2 ± 0.6
AJ262751	IL-12p35	6.5 ± 3.2	-7.3 ± 1.5	0.9 ± 1.1	119 ± 36	10 ± 1.9
AJ621253	GM-CSF	-5.1 ± 1.6	-2.8 ± 0.1	-1.5 ± 0.2	3.1 ± 0.3	28 ± 11
Y14971	CXCLi1	4.1 ± 0.2	34 ± 3.2	118 ± 20	1601 ± 12	1367 ± 132
NM_205498	CXCLi2	32 ± 2.8	414 ± 43	94 ± 11	530 ± 33	78 ± 3.3
Y18692	CCLi3	21 ± 1.6	330 ± 50	123 ± 20	865 ± 61	1441 ± 27
NM_204352	IL-16	-9.8 ± 0.6	2.5 ± 0.2	1.7 ± 0.1	11 ± 2.8	2.2 ± 0.2
AB046533	TLR-2	-12.6 ± 1.8	-1.0 ± 0.06	-3.2 ± 0.3	1.2 ± 0.08	-17 ± 1.8
NM_001011691	TLR-3	-2.6 ± 0.2	2.6 ± 0.8	-1.6 ± 0.9	6.5 ± 0.6	0.3 ± 0.9
NM_001030693	TLR-4	-13 ± 4.3	0.5 ± 0.7	-1.6 ± 0.09	6.9 ± 0.4	3.6 ± 0.2
NM_001024586	TLR-5	-5.2 ± 0.1	0.4 ± 1.0	-1.8 ± 0.4	5.8 ± 0.4	-1.6 ± 0.2
NM_001011688	TLR-7	-2.7 ± 0.3	3.6 ± 0.3	0.9 ± 0.7	4.8 ± 0.5	0.02 ± 0.6
NM_001030558	TLR-21	-3.4 ± 0.2	3.4 ± 0.2	5.1 ± 0.7	38 ± 0.3	6.1 ± 0.4
D85422	iNOS	-3.7 ± 0.9	-2.4 ± 0.7	1.8 ± 0.3	9.8 ± 0.9	350 ± 10
	NO	1.4 ± 0.09	1.4 ± 0.06	1.7 ± 0.03	3.3 ± 0.1	68 ± 3.0

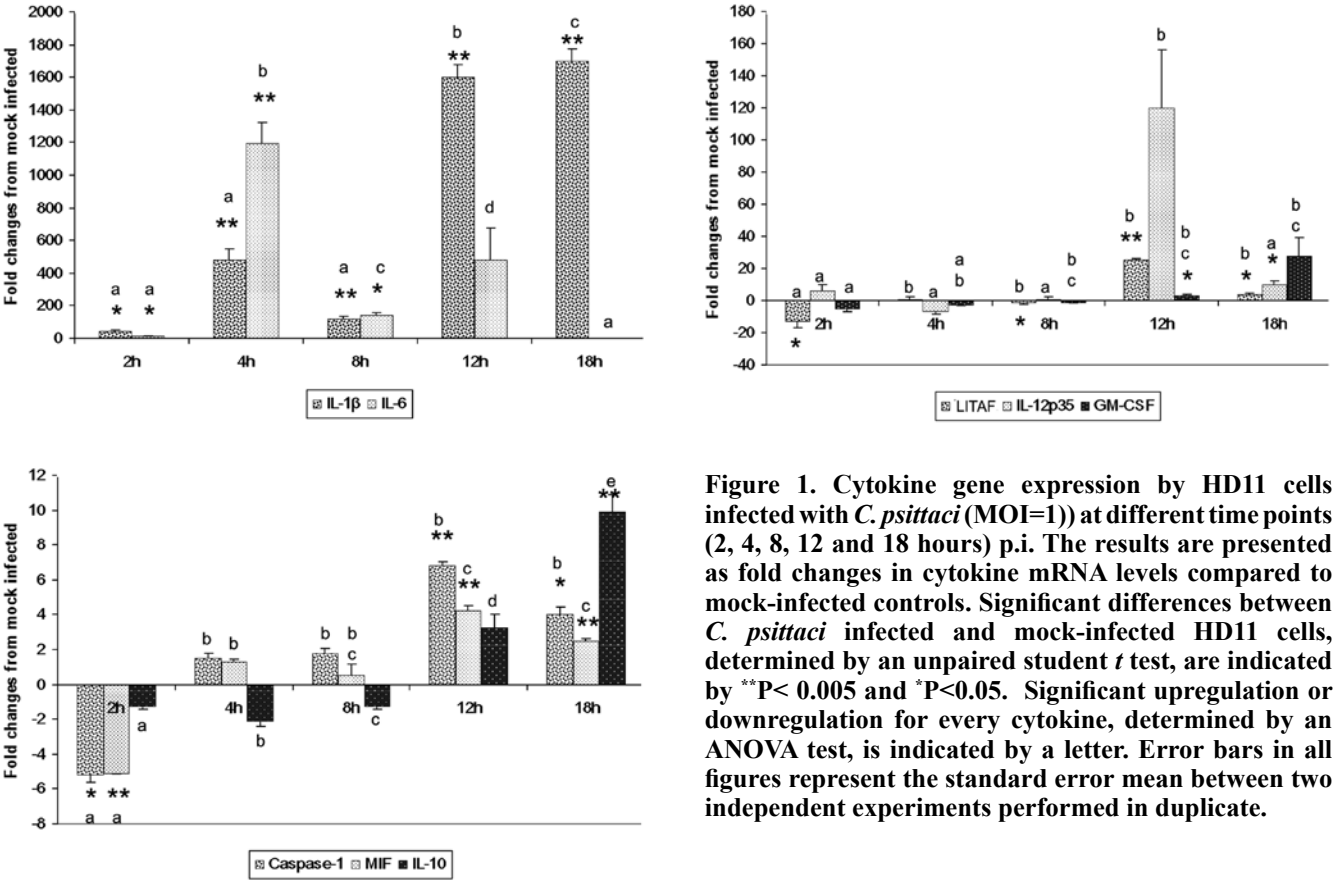

Figure 1. Cytokine gene expression by HD11 cells infected with *C. psittaci* (MOI=1)) at different time points (2, 4, 8, 12 and 18 hours) p.i. The results are presented as fold changes in cytokine mRNA levels compared to mock-infected controls. Significant differences between *C. psittaci* infected and mock-infected HD11 cells, determined by an unpaired student *t* test, are indicated by $^{**}P< 0.005$ and $^{*}P<0.05$. Significant upregulation or downregulation for every cytokine, determined by an ANOVA test, is indicated by a letter. Error bars in all figures represent the standard error mean between two independent experiments performed in duplicate.

fold rise) and TLR7 (4.5-fold rise) genes, whereas gene expression upregulation was more pronounced for the TLR21 gene (34-fold rise) (Figure 3).

Transcription analysis of the iNOS gene and HD11 activation assay

The activation of HD11 cells by *C. psittaci* was evaluated by comparing iNOS gene transcription and NO (NO_2^-) production in infected versus mock-infected cells. The expression of the iNOS gene was significantly upregulated during mid-cycle, resulting in a 344.6-fold rise of the mRNA level at 18 hours p.i. The same was observed for the NO production resulting in 91.02 µM ± 4.06 at 18 hours p.i. versus 1.32 µM ± 0.17 for the mock-infected controls (Figure 4).

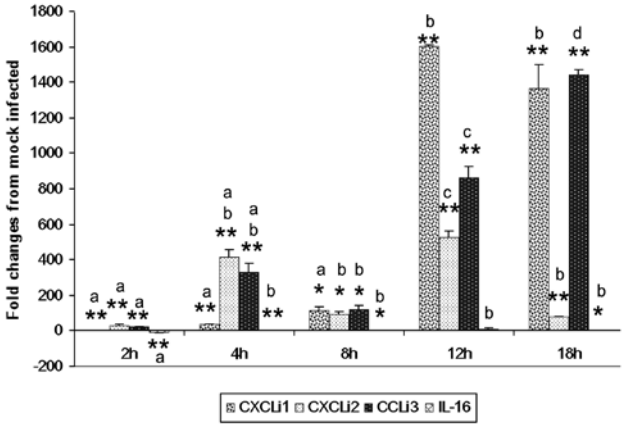

Figure 2. Chemokine gene expression by HD11 cells infected with *C. psittaci* (MOI=1)) at different time points (2, 4, 8, 12 and 18 hours) p.i. The results are presented as fold changes in cytokine mRNA levels compared to mock-infected controls. Significant differences between *C. psittaci* infected and mock-infected HD11 cells, determined by an unpaired student *t* test, are indicated by $^{**}P< 0.005$ and $^{*}P<0.05$. Significant upregulation or downregulation for every cytokine, determined by an ANOVA test, is indicated by a letter. Error bars in all figures represent the standard error mean between two independent experiments performed in duplicate.

DISCUSSION

As a member of the obligate intracellular *Chlamydiaceae* family, *Chlamydia psittaci* engages in an intimate relation with respiratory epithelial cells and macrophages. Not much is known about the innate immunity during a primary *Chlamydia* infection. Former studies on chlamydial immunology mainly focused on adaptive immunity against *C. trachomatis*, *C. muridarum*, *C. pneumoniae*, and *C. caviae*, whereas it is becoming increasingly clear that the innate immune response influences the migration and activation of immune cells and thereby directing the adapative immune response (Germain, 2004). Very few studies have investigated the innate immune system of the avian respiratory tract (Ariaans et al., 2008; Sarmento et al., 2008 and Wang et al., 2006) and only one study has examined innate immunity to *C. psittaci* in its natural host cell, the respiratory epithelial cell or avian macrophage (Beeckman et al., 2010), although knowledge of the innate immune mechanisms to *C. psittaci* infections and chlamydial antigens is crucial to understand the pathogenesis of and immunity to this zoonotic pathogen.

The objective of this study was to examine the innate immune response generated after an avian *C. psittaci* infection in a matched avian host cell line. The use of natural host cells in in vitro experiments is important, as previously demonstrated by Roshick et al. (2006).

The current study focused on chicken macrophage activation and expression of cytokine and TLR genes

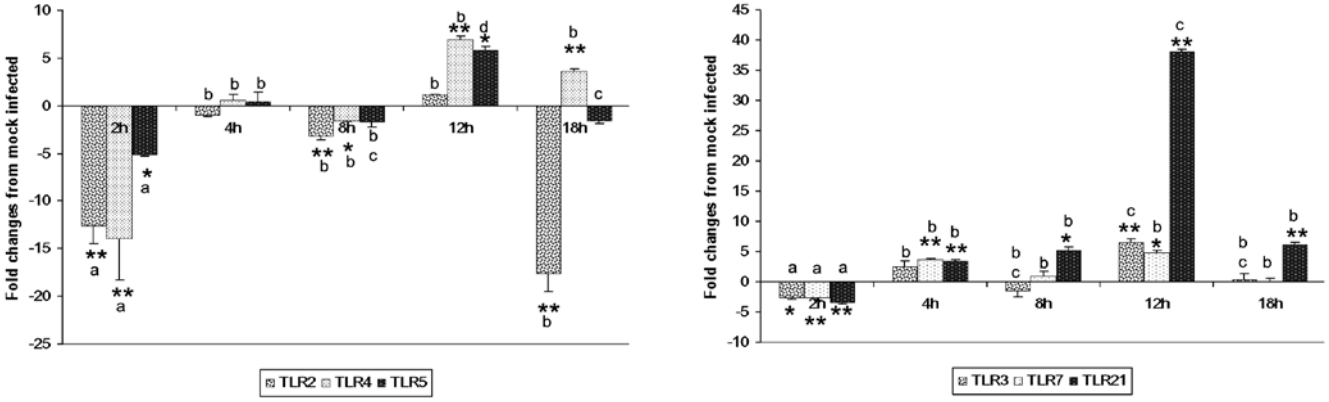

Figure 3. TLR gene expression by HD11 cells infected with *C. psittaci* (MOI=1)) at different time points (2, 4, 8, 12 and 18 hours) p.i. The results are presented as fold changes in cytokine mRNA levels compared to mock-infected controls. Significant differences between *C. psittaci* infected and mock-infected HD11 cells, determined by an unpaired student *t* test, are indicated by $^{**}P< 0.005$ and $^{*}P<0.05$. Significant upregulation or downregulation for every cytokine, determined by an ANOVA test, is indicated by a letter. Error bars in all figures represent the standard error mean between two independent experiments performed in duplicate.

by chicken macrophages during the early phase (2-8 hours p.i.) and mid-cycle (12-18 hours p.i.) period of the developmental cycle of the highly virulent *C. psittaci* strain 92/1293 (*ompA* genotype D) was examined. This study on avian macrophages was performed as it is well known that macrophages play a key role in directing the innate immune response during infection.

First, the expression of inflammatory cytokine genes in *C. psittaci* infected macrophages was investigated. Genes encoding the NF-kB-regulated pro-inflammatory cytokines IL-1b and IL-6 were highly expressed during *C. psittaci* infections of avian macrophages. mRNA Levels for both genes showed a significant upregulation at 4 hours post infection. The same mRNA-fold changes were obtained by Beeckman et al. (2010), using the same model, but inoculating *C. psittaci* at a multiplicity of infection (MOI) of 100 instead of 1 and monitoring cytokine production at 4 hours p.i. Thus, the expression of the IL-1b and IL-6 genes early on during the *C. psittaci* developmental cycle seemed to be MOI-independent. A continuously augmenting IL-1b and IL-6 gene expression upregulation was observed leading to the highest IL-1b and IL-6 gene expression levels during mid-cycle (12-18 hours p.i.). Continuously augmenting IL-1b and IL-6 gene expression upregulation was also observed using other in vitro models, like for instance *C. muridarum* in primary mouse macrophages (Prantner and Nagarajan, 2009) and *C. trachomatis* in human monocytes/macrophages and THP-1 cells (Bas et al., 2008). In the present study, the IL-1b mRNA levels continuously augmented in infected cells, but they were only accompanied by caspase-1 gene upregulation from mid-cycle onwards. IL-1b protein expression is controlled at the posttranslational level, since it requires cleavage of pro-IL-1b by the host protease caspase-1. The effector protein CopB (Fields et al., 2005) may play a role in caspase-1 activation because the homologous T3S translocator proteins in *Shigella* (IpaB) and *Salmonella* (SipB) (Hersh et al., 1999; Hilbi et al., 1998; Thirumalai et al., 1997) have been shown to co-localize with caspase-1 and are necessary and sufficient for its activation. Beeckman et al. (2008), examined the expression of *C. psittaci* T3S effector genes including *copB1* (but not *copB2*). *copB1* was expressed late (>24 hours p.i.) during the developmental cycle.

The expression of the LITAF gene, another NF-kB-regulated pro-inflammatory cytokine, was not upregulated till the beginning of the mid-cycle period and this 23-fold upregulation corresponded with a 6.2-fold increase in TLR4 expression. This is in contrast with the study of Prantner and Nagarajan (2009) in murine macrophages; they observed the highest induction of LITAF mRNA early during infection (8 hours p.i.) and ascribed this to dominant TLR2-MyD88 signaling.

The importance of the contribution of TLR2 or TLR4 signaling in chlamydial inflammation is still a

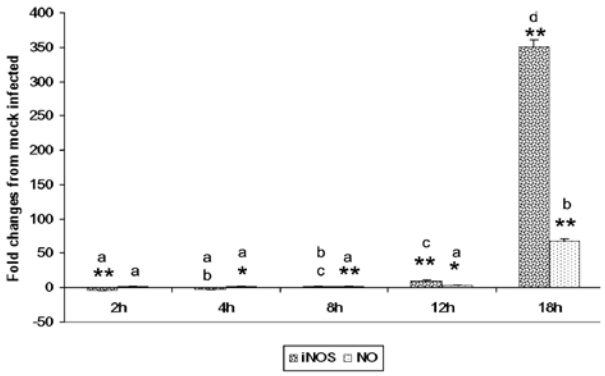

Figure 4. iNOS gene expression and NO production by HD11 cells infected with *C. psittaci* (MOI=1)) at different time points (2, 4, 8, 12 and 18 hours) p.i. The results are presented as fold changes in cytokine mRNA levels compared to mock-infected controls. Significant differences between *C. psittaci* infected and mock-infected HD11 cells, determined by an unpaired student *t* test, are indicated by **P< 0.005 and *P<0.05. Significant upregulation or downregulation for every cytokine, determined by an ANOVA test, is indicated by a letter. Error bars in all figures represent the standard error mean between two independent experiments performed in duplicate.

matter of debate. Joyee and Yang (2008) stated in their review on the role of TLRs in immune responses to chlamydial infections that, although chlamydial LPS and hsp are recognized by TLR4, intact organisms stimulate innate immune cells mainly through TLR2. Interestingly, the TLR2 gene is the only TLR gene in the present study, which is not upregulated after internalization of *C. psittaci*, when comparing infected and mock-infected cells. The question rises whether this might present an immune evasion strategy, reducing early secretion of pro-inflammatory cytokines as they may aid in eradicating a chlamydial infection (Darville et al., 2003), or whether this is a crucial mechanism that exists to switch this pathway off to prevent over-amplification of the TLR-2-mediated signal. The gene encoding the chicken macrophage migration inhibitory factor (MIF) was the least upregulated cytokine gene during a *C. psittaci* infection of HD11 cells. This might be beneficial for the pathogenesis of the infection, as high MIF levels could negatively influence the spreading of *C. psittaci* from the lungs to various tissues throughout the body. Unlike mammalian MIF, avian MIF alone does not promote the expression of IL-1b, IL-6, IL-12 and IL-8 or NO production in monocytes/macrophages. This only occurs in previously stimulated (primed) cells (Bernhagen et al., 1994; Kim et al., 2010). Thus, in the present study, MIF probably played no role in enhancing cytokine and/or chemokine expression by chicken macrophages.

IL-12 gene expression was actually downregulated in the early phase of the infection and it became highly (106.7-fold) upregulated during mid-cycle. A study of

Agrawal et al. (2009) showed that IL-12 is involved in the protection against *C. trachomatis*.

C. psittaci infection downregulated the expression of the anti-inflammatory IL-10 gene during the early phase of the chlamydial developmental cycle. This is in contrast with a former study of the authors, examining IL-10 expression only at 4 hours p.i. (Beeckman et al., 2010). Previously, a 581-fold upregulated IL-10 mRNA level was found at 4 hours p.i. This may most likely be attributed to the MOI, which was 100 times higher in the former study.

Similar to the downregulation of the IL-10 gene early in the infection, other genes like IL-12, GM-CSF, LITAF , MIF, caspase-1, TLR-2, TLR-3, TLR-4, TLR-5, TLR-7 and TLR21 were also significantly downregulated, suggesting an immune evasion strategy induced by *C. psittaci*.

The pro-inflammatory chemokines CXCLi1, CXCLi2 and CCLi3 were highly expressed, especially during mid-cycle. CXCLi1, CXCLi2 and CCLi3 mediate neutrophil, heterophil and monocyte attraction to the place of infection. Buchholz and Stephens (2008) revealed that the endogenous CXCLi2 response induced by *C. trachomatis* was dependent upon NOD-1 PRR signaling. As TLR-21 gene expression was significantly upregulated by *C. psittaci*, the gene expression analysis for additional intracellular PRR, like NOD-1 will be performed in future experiments. Expression of IL-16 was upregulated by *C. psittaci* during mid-cycle. According to Ghigo et al. (2010), IL-16 promotes replication of *Tropheryma whipplei* by inhibiting phagolysosomal fusion. It is possible that IL-16 also plays a role in the inhibition of the fagolysosomal fusion of *C. psittaci* by the activation of T3SS.

The expression of all examined TLR genes was downregulated during the first 4 hours p.i. Gene upregulation was first observed for TLR21 (3.6-fold at 8h p.i.). The avian TLR21 is an intracellular, endosomal, nucleotide signaling receptor that senses and responds to bacterial genomic DNA (Keestra et al., 2010). Thus, *C. psittaci* is certainly recognized by intracellular signaling receptors. This might influence the expression of pathogen recognition receptors (PRRs) directly or their downstream signaling. Unfortunately, other intracellular receptors like RIG-I-like receptors, NOD-like receptors and inflammasomes have not been investigated yet.

In conclusion, the results of the present study show a clearer view on how *C. psittaci* is recognized by avian macrophages and its influence on the host innate immune response. High expression of cytokines, chemokines, iNOS, caspase-1 and GM-CSF genes with a peak during the mid-cycle of the developmental infection were observed. Further research on other pattern recognition receptors and their pathways is necessary to map the innate immune responses elicited by *C. psittaci* in avian macrophages.

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