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**Citation for published version:**

Wu, Z, Rothwell, L, Hu, T & Kaiser, P 2009, 'Chicken CD14, unlike mammalian CD14, is trans-membrane rather than GPI-anchored', *Developmental and Comparative Immunology*, vol. 33, no. 1, pp. 97-104.  
<https://doi.org/10.1016/j.dci.2008.07.008>

**Digital Object Identifier (DOI):**

[10.1016/j.dci.2008.07.008](https://doi.org/10.1016/j.dci.2008.07.008)

**Link:**

[Link to publication record in Edinburgh Research Explorer](#)

**Document Version:**

Publisher's PDF, also known as Version of record

**Published In:**

*Developmental and Comparative Immunology*

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# Chicken CD14, unlike mammalian CD14, is trans-membrane rather than GPI-anchored

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## ARTICLE INFO

### Article history:

Received 14 May 2008

Received in revised form 21 July 2008

Accepted 21 July 2008

Available online 30 August 2008

### Keywords:

Avian

CD14

Myelomonocytic differentiation antigen

## ABSTRACT

A cDNA encoding the chicken homologue of the human myelomonocytic differentiation antigen, CD14, was cloned by RT-PCR from chicken bone marrow cell RNA, using oligonucleotide primers based on the predicted cDNA sequence. The cloned chicken CD14 (chCD14) cDNA encodes an open reading frame of 465 amino acids (aa), with 31–34% aa identity to mouse, bovine and human (hu) CD14. As in mouse and man, chCD14 is a leucine-rich protein. In mammals, CD14 is a GPI-anchored protein. Protein structure analysis suggested that chCD14, by contrast, was potentially a trans-membrane protein. The predicted aa sequence comprises an extracellular domain of 417 aa, followed by a 23-aa trans-membrane segment, and a 25-aa intracytoplasmic region, the latter containing no obvious signalling motifs. COS-7 cells were transfected with p3XFLAG-CMV<sup>TM</sup>-8::chCD14 or pCDM8::huCD14, incubated with or without PI-PLC and stained with anti-FLAG or anti-huCD14 antibody respectively. PI-PLC cleaved huCD14 but not chCD14, suggesting that chCD14 is not GPI-anchored. Real-time quantitative RT-PCR analysis revealed that chCD14 mRNA was expressed in most lymphoid and non-lymphoid tissues, except muscle. ChCD14 mRNA was also expressed in most cells examined but strongly expressed in chicken peripheral blood monocyte/macrophages and KUL01<sup>+</sup> splenocytes.

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## 1. Introduction

CD14 is a myelomonocytic differentiation antigen in mammals [1]. Human CD14 is a single-copy gene encoding two protein forms: a 50–55-kDa glycosylphosphatidylinositol (GPI)-anchored membrane protein (mCD14) and a monocyte or liver-derived soluble serum protein (sCD14) that lacks the anchor [2–5]. CD14 is the lipopolysaccharide (LPS)-LPS binding protein (LBP) receptor, forming a multi-protein complex containing at least CD14, MD-2 and TLR4 [6–8]. The crucial role of CD14 in LPS signalling has been confirmed with knock-out mice; CD14-deficient mice are highly resistant to septic shock initiated by injection of either LPS or live bacteria [9–11]. Both mCD14 and sCD14 are critical for LPS-dependent signal transduction, and sCD14 confers LPS sensitivity to cells lacking mCD14. In addition to the LPS of Gram-negative bacteria, CD14 can bind other microbial products such as peptidoglycan (PGN) and lipoproteins [12].

CD14 was first identified on the surface of monocytes and macrophages [13] and ever since CD14 has been widely used as a monocyte/macrophage marker. Recently it has become clear that

CD14 is also expressed by other cells and not even restricted to the myeloid cell lineage [14]. In mammals, CD14 has been well characterized [2,15–18] but this molecule has yet to be described in a non-mammalian species. In this study we report for the first time the identification and characterization of chicken CD14 (chCD14).

## 2. Materials and methods

### 2.1. Bioinformatic identification and cloning of chicken CD14

BLAST was used to search Ensembl and predict the cDNA sequence of the chicken homologue of the human myelomonocytic differentiation antigen, CD14.

Primers were designed according to the predicted sequence (Table 1). Primer CD14-1 corresponds to nt positions –29 to –4 of the coding sequence. Primer CD14-2 is reverse complementary to nt positions 1391–1413. Nested PCR primer CD14-3 corresponds to nt positions 1–18 and primer CD14-4 is reverse complementary to nt positions 1380–1398.

Femurs were dissected from 4-week-old inbred line 7<sub>2</sub> chickens, the bone marrow was flushed out with PBS using a syringe and bone marrow cells were isolated by centrifugation over Histopaque 1.119 (Sigma–Aldrich, Poole, UK). Cells were

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**Table 1**  
Primer/probe sequences

Primer/probe <sup>a</sup>	Sequence (5'–3')
CD14-1	ACCGCGTCTCCTCCAGGCTGCTC
CD14-2	GGACGCCAGGAGCCCTTACAAGT
CD14-3	ATGCGTGGGCGCCGCTCTG
CD14-4	TTACAAGTCTCTGCACCG
CD14-5	ACAAAGCTTCGGTGTCTTCAACCGCAC
CD14-6	TCCTCTAGATTACAAGTCTGACCCGC
CD14 F	GGAGGACTCCACCATTGACAT
CD14 R	GGAGGACTCAGGAACAGAA
CD14 probe	(FAM)-AATGATCTTCTGATTGACAGCTGCCAA-(TAMRA)
28S F	GGCGAAGCCAGAGAAACT
28S R	GACGACCGATTGACGTC
28S probe	(FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA)

<sup>a</sup> F, Taqman forward primer; R, Taqman reverse primer.

harvested and total RNA isolated using an RNeasy Mini kit (Qiagen, Crawley, UK), with on-column DNaseI treatment. Separate RNA preparations were isolated from bone marrow from three different birds and RT-PCRs were carried out separately using the RNAs from these three preparations. First strand synthesis was for 50 min at 42 °C in a 20- $\mu$ l volume containing 4 pmol of oligo-dT, 200 U Superscript II (Invitrogen, Paisley, UK) and 500 ng bone marrow RNA. After denaturation of the reverse transcriptase at 94 °C for 3 min, 2  $\mu$ l of this reaction mix was added as template to a 20- $\mu$ l PCR reaction, containing 20 pmol of each primer (CD14-1 and CD14-2), 0.4 mM dNTPs, 1  $\mu$ l Taq polymerase and 4  $\mu$ l Q solution (Qiagen). Cycling conditions were 30 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min. The PCR products were purified and primers CD14-3 and CD14-4 were used to reamplify and clone the entire coding sequence of chCD14 from the initiation to the stop codons inclusive. The resulting PCR product was ligated into TOPO pCR2.1 (Invitrogen) and the complete sequence of three clones determined on each strand. Based on the protein structure prediction, primers (CD14-5 and CD14-6) were designed to subclone the cDNA encoding the predicted mature protein of chCD14 into p3XFLAG-CMV<sup>TM</sup>-8 for eukaryotic expression. Several clones of this construct (p3XFLAG-CMV<sup>TM</sup>-8::chCD14) were sequenced to confirm the integrity of the inserts. The cDNA sequence has been submitted to Ensembl with the accession number AM933591.

## 2.2. Tissues and cells

Lymphoid (thymus, spleen, bursa, Harderian gland, caecal tonsil, Meckel's diverticulum and bone marrow) and non-lymphoid (brain, muscle, heart, liver, kidney, lung and skin) tissues were obtained from three 6-week-old inbred line 7<sub>2</sub> chickens. Different cell populations (splenocytes, splenocytes stimulated with Concanavalin A [ConA] (1  $\mu$ g/ml), bursal cells, bursal cells stimulated with phorbol myristate acetate [PMA] (500 ng/ml), thymocytes, thymocytes stimulated with phytohaemagglutinin [PHA] (25  $\mu$ g/ml), peripheral blood monocytes and peripheral blood monocyte-derived macrophages) were obtained as described before [19]. In order to isolate different lymphocyte subsets, splenocytes were isolated as described [20]. Briefly, spleens were digested in Hank's buffered salt solution (HBSS) containing 556  $\mu$ g/ml DNase I (Roche Diagnostics Ltd., Burgess Hill, West Sussex, UK) and 2.2 mg/ml collagenase D (Roche Diagnostics Ltd.) for 1 h. Cells were then collected into HBSS containing 10 mM EDTA and passed through a cell strainer. The cell suspension was centrifuged at 250  $\times$  g for 10 min to pellet the cells. Cells were then resuspended in PBS and layered over 5–7 ml Histopaque 1.077 (Sigma–Aldrich) followed by centrifugation at 1000  $\times$  g for 20 min. Cells at the interface were collected, washed

and counted. Cells ( $1 \times 10^8$ ) were labelled with mouse anti-chicken CD4, CD8, Bu-1, TCR1, TCR2, TCR3, or KUL01 monoclonal antibodies (SouthernBiotech, Birmingham, UK). Chicken Bu-1 is a marker for chicken B cells and is also expressed on subsets of macrophages and monocytes [21,22]. The monoclonal antibody KUL01 identifies chicken monocytes and macrophages as well as interdigitating cells and activated microglia cells [23,24]. Cell subsets were isolated using polyclonal goat anti-mouse IgG-coated microbeads (Miltenyi Biotec, Bisley, Surrey, UK) and an autoMAC-Spro separator (Miltenyi Biotec). The cell subsets were then cultured with or without LPS (400 ng/ml) for 24 h. Heterophils were isolated from 4-week-old chickens as described [25]. Briefly, heterophils were isolated from peripheral blood of eight chickens of a Salmonella enteritidis-susceptible commercial line (line B) at 4 weeks of age, on three separate occasions; in total, therefore, 24 birds were used as cell donors.

## 2.3. Total RNA isolation

RNA from the tissues and cells described above was extracted using an RNeasy Mini kit following the manufacturer's instructions. To avoid contamination with genomic DNA, as CD14 is a single exon gene, the samples were exposed to an on-column treatment with RNase-free DNase I (Qiagen) for 60 min at room temperature (RT). The RNA was eluted in RNase-free water and stored at –80 °C until required.

## 2.4. Real-time quantitative RT-PCR analysis of chCD14 mRNA expression

ChCD14 mRNA levels in different tissues and lymphoid cells were quantified using a well-described method (e.g. [19,26–28]).

Primers and probe were designed using the Primer Express software program (PE Applied Biosystems, Warrington, UK). Details are given in Table 1. All probes were labelled with the fluorescent reporter dye 5-carboxyfluorescein (FAM) at the 5' end and the quencher *N,N,N,N'*-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3' end.

Real-time quantitative RT-PCR was performed using the Reverse Transcriptase qPCR Master Mix RT-PCR kit (Eurogentec, Seraing, Belgium). Amplification and detection of specific products were performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with the following cycle profile: one cycle of 50 °C for 2 min, 60 °C for 30 min, and 95 °C for 5 min, and 40 cycles of 94 °C for 20 s, 59 °C for 1 min. Quantification was based on the increased fluorescence detected due to hydrolysis of the target-specific probes by the 5'-exonuclease activity of the *rTth* DNA polymerase during PCR amplification. The passive reference dye 6-carboxy-c-rhodamine, which is not involved in amplification, was used for normalization of the reporter signal. Results are expressed in terms of the threshold cycle value (Ct), the cycle at which the change in the reporter dye passes a significance threshold ( $\Delta R_n$ ).

To account for variation in sampling and RNA preparation, the Ct values for CD14-specific product for each sample were standardised using the Ct value of 28S rRNA product for the same sample. To normalise RNA levels between samples within an experiment, the mean Ct value for 28S rRNA-specific product was calculated by pooling values from all samples in that experiment. Tube-to-tube variations in 28S rRNA Ct values about the experimental mean were calculated. The slope of the 28S rRNA log<sub>10</sub> dilution series regression line was used to calculate differences in input total RNA. Using the slopes of the respective CD14 or 28S rRNA log<sub>10</sub> dilution series regression lines, the difference in input total RNA, as represented by the 28S rRNA, was then used to adjust CD14-specific Ct values, as follows: corrected

Ct value =  $Ct + (Nt - Ct') \times S/S'$  where Ct = mean sample Ct, Nt = experimental 28S mean, Ct' = mean 28S of sample, S = CD14 slope, S' = 28S slope. Results were then expressed as 40-Ct values.

2.5. Transient expression of chCD14 in COS-7 cells

COS-7 cells were routinely grown in DMEM (Invitrogen) containing 10% FCS (PAA Laboratories, Linz, Austria), 1% nonessential amino acids, 1% L-glutamine, 1 U/ml penicillin, and 1 μg/ml streptomycin at 37 °C in 5% CO<sub>2</sub> and passaged using standard conditions [29].

Pre-warmed medium was added to each chamber of chamber slides (Lab-Tek™ Chamber Slide™ System, Nunc, VWR, Lutterworth, UK), 500 μl per well for 4-chamber slides. Trypsinised COS-7 cells were added to each well (1 × 10<sup>5</sup> cells/well), left at RT for

10 min to allow cells to settle and then returned to 37 °C for 18–24 h. Growth medium was aspirated and Hepes-buffered DMEM (HBD, 700 μl) containing 3 μg DNA (p3XFLAG-CMV<sup>TM</sup>-8::chCD14 or pCDM8::huCD14 [30]), 258 μg chloroquine, and 600 μg/ml DEAE-dextran was added to the cells, which were then incubated for 4 h at 37 °C in 5% CO<sub>2</sub>. The transfection medium was then removed and cells washed once with PBS. HBD containing 10% DMSO was added for 2 min, removed, replaced with 700 μl of growth medium and incubated for 40–60 h. One chamber on each slide was washed twice with cold PBS and incubated with phosphatidylinositol-specific phospholipase C (PI-PLC) (1.0 U/ml, Invitrogen) at 4 °C for 30 min and the other three chambers were incubated with PBS only. Afterwards the whole slides were washed twice with cold PBS, fixed in acetone and incubated with PBS/1.0% BSA/0.01% NaN<sub>3</sub> for 1 h at RT in a humidified chamber. Mouse anti-

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1   ATGCGTGGGGCCGCTCTGCTGCTCCTGGCGCTGGGGCTGCAGCAGGCCGAGGGCCGGTGTGTCTTCAACCCGACGCAGGAG
1   M R G A A L L L L A L G L Q Q A E G R C V F N R T Q E

82  TACTGCCTGTGTACAGGCTGACCCAGGAGAGCGCCGGAGCGTCATCCAGTGCCTCGCCGCTCCGCGTGGAGTTCACG
28  Y C L C Y R L T Q E S A G S V I Q C L A A S A V E F Q

163 GGGGGGACCTGGAGAAATACGCTGACTTCCCCATCGAGGACCTGGACGACTCCACCATTGACATGCTTGGCAGTCTGC
55  G G D L E K Y A D F P I E D L D D S T I D M L G S L Q

244 ATCAGGAAGATCATTTCGACCTTCTGGTTCCTGAGGTCCTCCTGGCCCGCTCCTGCGGTTCTTCTCCTACACTCAG
82  I R K I I F S D L L V P E V L L A R V L R F F S Y T Q

325 GTGCAGGAGATGGTGTTCAGAGCTGCAGCTTGTGGGGAGGAGCAGCTGGGCGGACATGGCGGGCCGGCCCTTGCCCAT
109 V Q E M V F K S C S F V G R S S W A D M A G R A L P I

406 GTGTCACTGCGCTCCACAACGTGACGGCCGCGCTGGCAGGCCGCGAGCAGGACCTGTCCCCTCAGCCGCTGGCTG
136 V S L R F H N V T A A A L A G R E Q D L S P L S R W L

487 GGAGCCCTGCAGGAGCTGTGCTCACCCTCACGCCCTGGCCGTGCTGCCCTGTGCCGTGGGCCAGGCGCTGCGGGCGCTG
163 G A L Q E L S V T A S R L A V L P C A V G Q A L R A L

568 CGCTCGCTGGACCTGGCGGTAACAGCCTGGGGGATGACAGCCTGGCGCCCGCTTCTGCCAGGGGGCCTTACACAGCTG
190 R S L D L A R N S L G D D S L A P A F C Q G A F T Q L

649 CGGGTGTGAGCTGCGGCACAACAACCTGACGTGCTGACAGCGGTGTGTGTGGCAGCGTGCAGTGTGCTGGGCGAGCTGC
217 R V L S L R H N N L T S Y S G V C G S V R L L G E L Q

730 CACCTGGATCTCAGCCACAACGCGCTCACTGCCGGCACGCCTTCGTCCCCCTGCCAGTGGCCGGCAACCTCCGCATCTTT
244 H L D L S H N A L T A G T P S S P C Q W P A T L R I F

811 AACTTGTCCAGCGCCGGCTTGGACCAGTGCTCGCACCTCTGCCCCCGCTCTGGAGTTCTGGACCTGAGCTCCAACCGC
271 N L S S A G L D H V L A P L P P A L E V L D L S S N R

892 CTCCACGCTGTGGACATCTCCCTGCGCTCCCTGCAGGCGCTCTTCCCTCAGCCGCAACGCGCTGCCGGCCGCGCCGTCCATC
298 L H A V D I S L R S L Q A L F L S R N A L P A A P S I

973 CGGGGCTGCCCGCGCTGCACACCTGCACCTGGACAACAACCTGATCGCGGAGCTGCCGCGGGACGAGGCGCTGCTGCTG
325 R G C P A L H T L H L D N N L I A E L P R D E A L L L

1054 GAGCACCTGCAGGATGTGGCCGTGGCCGGGAACCCCTTCAACTGCACCTGTGGCGGGCCGGGCGGTGCAGGCGCTGGCG
352 E H L Q D V A V A G N P F N C T C G G A G A V Q A L A

1135 GCCGTGGGGTGCCTGAGGCGAGGCTGGCCGCGAGGCTACGTGTGCCACGCGCCTGCCCGCTACCAGGGGGTGTGCTGCTGCGG
379 A V G C L R Q G W P Q G Y V C H A P A R Y Q G V L L R

1216 GACGTGCCACCTCCGTGCTGCAGTGAACCCCGCGCTGTGCTGGCTCCCGTCTGCACGGGCTGGCGCTGCTGCTGCTG
406 D V P T S V L Q C N P A A V L A P V C T G L A L L C V

1297 GCCATGGCGGGCGGGCTGCTGTGGGCGGGGGGACGGCCCCGGTGGTTGTGCGCCTGGGGCAGCGGGAGGATGCCTGC
433 A M A G G L L W A R G A R P R W L C G L G Q R E D A C

1378 AGCGGCGCAGAGCACTTGTA
460 S G A E H L *
    
```

Fig. 1. Nucleotide (nt) and deduced amino acid (aa) sequences of chCD14. Numbers to the left of each row refer to nt and aa position alternately. The putative signal peptide (aa 1–18) is shown in bold and the predicted trans-membrane region is underlined. The potential N-linked glycosylation sites (Asn-X-Thr/Ser) are in bold and underlined.



FLAG M2 monoclonal antibody (mAb) (Sigma–Aldrich) was applied to the chamber containing COS-7 cells transfected with p3XFLAG-CMV<sup>TM</sup>-8::chCD14. Appropriately diluted mouse anti-human CD14 mAb (UCMH1) was added to the chambers containing COS-7 cells transfected with pCDM8::huCD14 and one chamber transfected with p3XFLAG-CMV<sup>TM</sup>-8::chCD14. The slides were incubated at RT for 1 h and then washed with PBS/0.05% Tween 20. Alexa Fluor<sup>®</sup> 568 goat anti-mouse IgG (H + L) (Invitrogen) was then added for 1 h at RT. 4',6-Diamidino-2-phenylindole (DAPI, Sigma–Aldrich) was used to detect the nuclei. A microscope with UV light (Leica DM IRB) was used for imaging. Positively staining cells were easily identifiable as red-coloured cells and cell nuclei were blue.

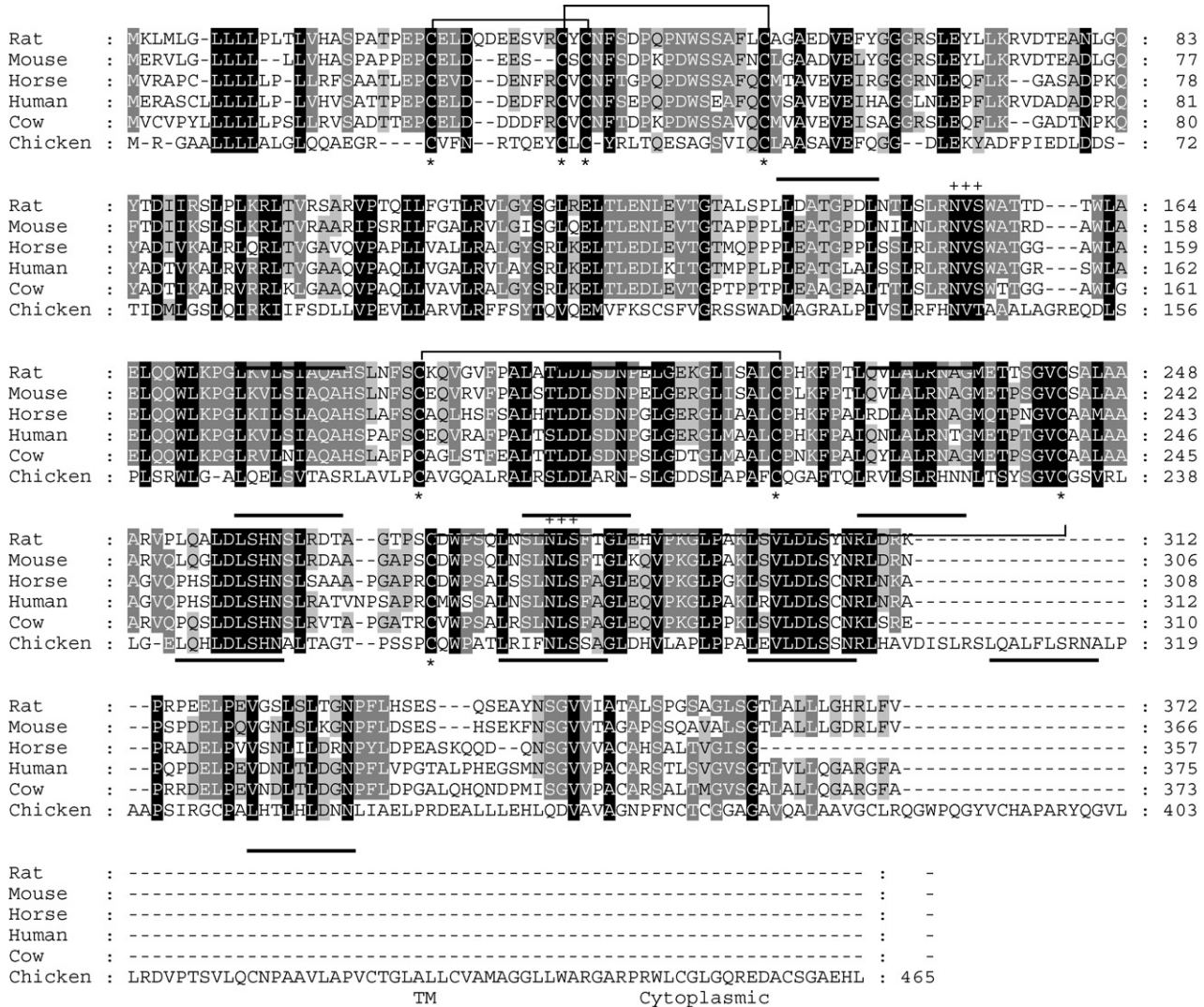
Flow cytometry analysis was used to detect the expression of chCD14. Briefly, COS-7 cells were cultured in T75 flasks and transfected with p3XFLAG-CMV<sup>TM</sup>-8::chCD14 or pCDM8::huCD14. 48 h post-transfection, cells were trypsinised and divided into two aliquots. One aliquot of cells was incubated with PI-DIVC (1.0 U/ml, Invitrogen) at 4 °C for 30 min and the other with PBS only. Cells were then incubated with mouse anti-FLAG M2 mAb or anti-

human CD14 mAb. FITC-labelled F(ab')<sub>2</sub> fragments of polyclonal goat anti-mouse immunoglobulins (DAKO, Ely, Cambridgeshire, UK) were used as secondary antibodies (Ab). For all the labelling steps, cells (0.5–1.0 × 10<sup>6</sup> cells/ml) were incubated for 10 min at RT with appropriate dilutions of the primary or secondary Ab in U-bottomed 96-well microtiter plates with two washes between each step. PBS containing 1.0% BSA and 0.1% NaN<sub>3</sub> was used as dilution and washing buffer. After the final wash, cells analysed on a FACSCalibur (BD Biosciences, Oxford, UK).

### 3. Results and discussion

#### 3.1. Cloning and sequence analysis of chCD14 cDNA

In man, the CD14 gene is on chromosome 5 in a region which contains a cluster of genes that encode several myeloid-specific growth factor and receptor genes, including platelet-derived growth factor receptor (PDGFR), beta-2-adrenergic receptor (ADRB2R), endothelial cell growth factor (ECGF) [16] and heparin-binding



**Fig. 2.** Alignment of the predicted aa sequence of chCD14 with its counterparts from different species: *Rattus norvegicus* (rat) NM\_021744, *Mus musculus* (mouse) NM\_009841, *Equus caballus* (horse) AY731081, *Homo sapiens* (human) NM\_000591, *Bos taurus* (cow) NM\_174008. Shaded areas represent conservation of aa similarity. Gaps introduced for optimal alignment are indicated by dashes. Conserved cysteines are indicated by asterisks, with the mammalian disulphide bridges drawn above the sequences, and conserved N-linked glycosylation sites indicated by '+++'. The 12 potential leucine-rich repeats in chCD14 are indicated under the sequences with thick bars. The potential trans-membrane (TM) and cytoplasmic domains of chCD14 are underlined.

growth factor (HBGF). In mouse, CD14 is encoded on mouse chromosome 18 which also contains at least five genes encoding receptors (PDGFR, ADRB2R, Ia-associated invariant chain (Ii), glucocorticoid receptor (Grl-1) and colony stimulating factor 1 receptor (CSF-1R or Fms)) [15]. Thus CD14 forms a conserved syntenic group with these growth factor and growth factor receptor genes [15]. We used the huCD14 sequence to search the chicken genome in Ensembl (by BLAST) and predicted the cDNA sequence of the chicken homologue of huCD14. It is encoded on chicken chromosome 13, Contig158.29, alongside several receptor genes, such as Cadherin-related neuronal receptor (CNR, ENSGALG00000000760), ADRB2R (ENSGALG00000002808) and heparin-binding growth factor 1 precursor (HBGF, ENSGALG00000007343). This suggests that, as in mammals, CD14 and the growth factor and growth factor receptor genes show conservation of synteny in the chicken.

The Ensembl novel protein coding prediction ENSGALT00000034888 identifies part of chCD14, but incorrectly as part of a three-exon gene spanning contigs 158.26–29, and overlapping another novel protein coding prediction ENSGALT00000001235. However, the *ab initio* Genscan translation prediction GENSCAN00000074788 actually correctly identifies chCD14, although of course it is not annotated as such.

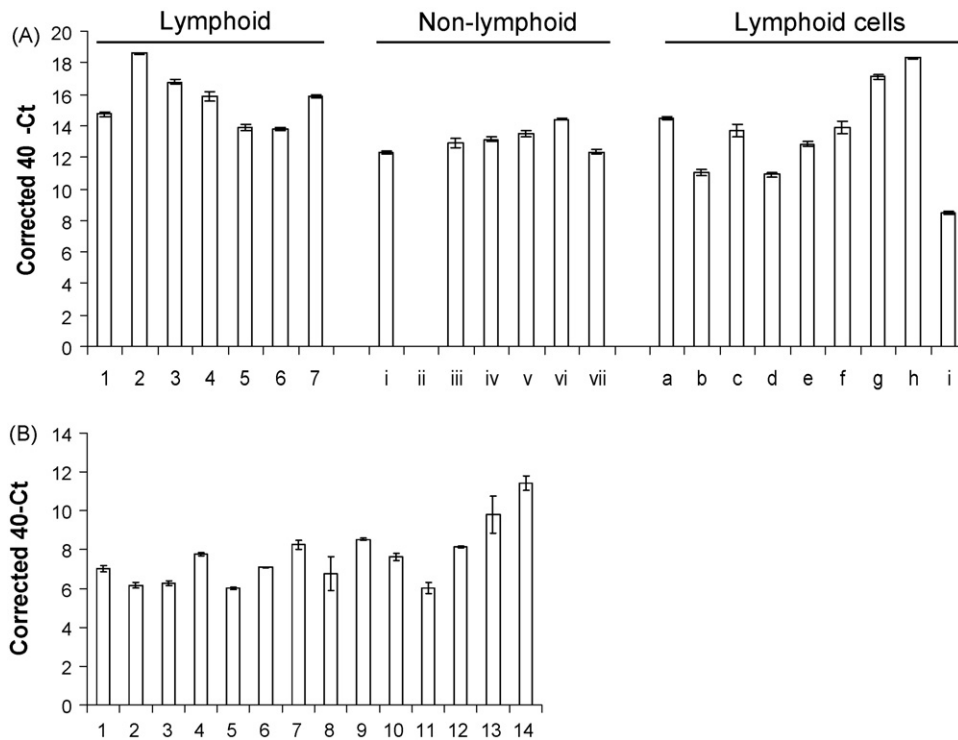
RNA isolated from chicken bone marrow cells was used as a template to amplify chCD14 cDNA with primers CD14-1 and CD14-2 and nested primers CD14-3 and CD14-4. The PCR product was 1398 bp in length with a high-GC content (Fig. 1). The primary structure of the chCD14 protein was deduced from the cDNA and consists of 465 aa (Fig. 1). A potential signal peptide cleavage site was predicted in the aa sequence (SignalP 3.0 server) between residues 18 and 19. ChCD14 contains nineteen cysteine residues,

eight of which are conserved between chicken and mammals. In mammalian CD14, there are four disulphide bridges between these eight conserved cysteines (as shown in Fig. 2), suggesting that chCD14 has a similar secondary structure. There are five potential N-linked glycosylation sites (Asn-X-Thr/Ser), two of which are conserved with mammalian CD14. Alignment of the aa sequences of chCD14 and mammalian CD14 (Fig. 2) reveals 31–34% aa identity. As shown in Fig. 2, chCD14 contains 12 leucine-rich repeats (LRR, LxxLxLxx) [31], as opposed to the 11 found in mammalian CD14 [15]. LRRs provide a structural framework for the formation of protein–protein interactions [31].

In mammals, CD14 is a glycosylphosphatidylinositol (GPI)-anchored protein [2]. These proteins lack a trans-membrane domain, have no cytoplasmic tail, and are, therefore, located exclusively on the extracellular side of the plasma membrane. GPI proteins have been found in a wide variety of eukaryotes, including mammals, chickens and fish. On average, ~0.5% of cellular proteins in eukaryotes are GPI-anchored [32]. Unlike in mammals, protein structure analysis suggests that chCD14 is not a GPI-anchored protein but a type I trans-membrane protein. The predicted aa sequence comprises an extracellular domain of 417 aa, followed by a 23-aa trans-membrane segment, and a 25-aa intracytoplasmic region. There are no obvious signalling motifs in the potential cytoplasmic tail, as assessed directly by eye and also by using the SCANSITE program (<http://scansite.mit.edu/>) [33].

### 3.2. Expression of chicken CD14 mRNA in different tissues and cell populations

Expression of chCD14 mRNA in lymphoid and non-lymphoid tissues and lymphoid cells was assessed by real-time quantitative



**Fig. 3.** (A) Expression patterns of chCD14 in chicken lymphoid tissues (1–7), non-lymphoid tissues (i–vii), stimulated/non-stimulated chicken lymphocytes (a–f), blood-derived monocytes (g), blood-derived macrophages (h) and blood-derived heterophils (i), as measured by real-time quantitative RT-PCR, with results expressed as 40-Ct values  $\pm$  S.E.: 1, thymus; 2, spleen; 3, bursa of Fabricius; 4, Harderian gland; 5, caecal tonsil; 6, Meckel's diverticulum; 7, bone marrow; i, brain; ii, muscle; iii, heart; iv, liver; v, kidney; vi, lung; vii, skin; a, unstimulated splenocytes; b, ConA-stimulated splenocytes; c, unstimulated bursal cells; d, PMA-stimulated bursal cells; e, unstimulated thymocytes; f, PHA-stimulated thymocytes. (B) Expression patterns of chCD14 in chicken splenocyte subsets: 1, CD4<sup>+</sup> cells; 2, LPS-stimulated CD4<sup>+</sup> cells; 3, CD8<sup>+</sup> cells; 4, LPS-stimulated CD8<sup>+</sup> cells; 5, TCR1<sup>+</sup> cells; 6, LPS-stimulated TCR1<sup>+</sup> cells; 7, TCR2<sup>+</sup> cells; 8, LPS-stimulated TCR2<sup>+</sup> cells; 9, TCR3<sup>+</sup> cells; 10, LPS-stimulated TCR3<sup>+</sup> cells; 11, Bu-1<sup>+</sup> cells; 12, LPS-stimulated Bu-1<sup>+</sup> cells; 13, KUL01<sup>+</sup> cells; 14, LPS-stimulated KUL01<sup>+</sup> cells.

RT-PCR (Fig. 3). ChCD14 mRNA was constitutively expressed in all the lymphoid and non-lymphoid tissues tested except for muscle. ChCD14 was also expressed in splenocytes, bursal cells and, thymocytes. Expression was down-regulated in mitogen-stimulated splenocytes and bursal cells ( $P < 0.05$ ) but expression was not significantly altered ( $P > 0.05$ ) in mitogen-stimulated thymocytes. ChCD14 was highly expressed in monocyte-derived macrophages and blood monocytes. ChCD14 was also expressed in blood-derived heterophils.

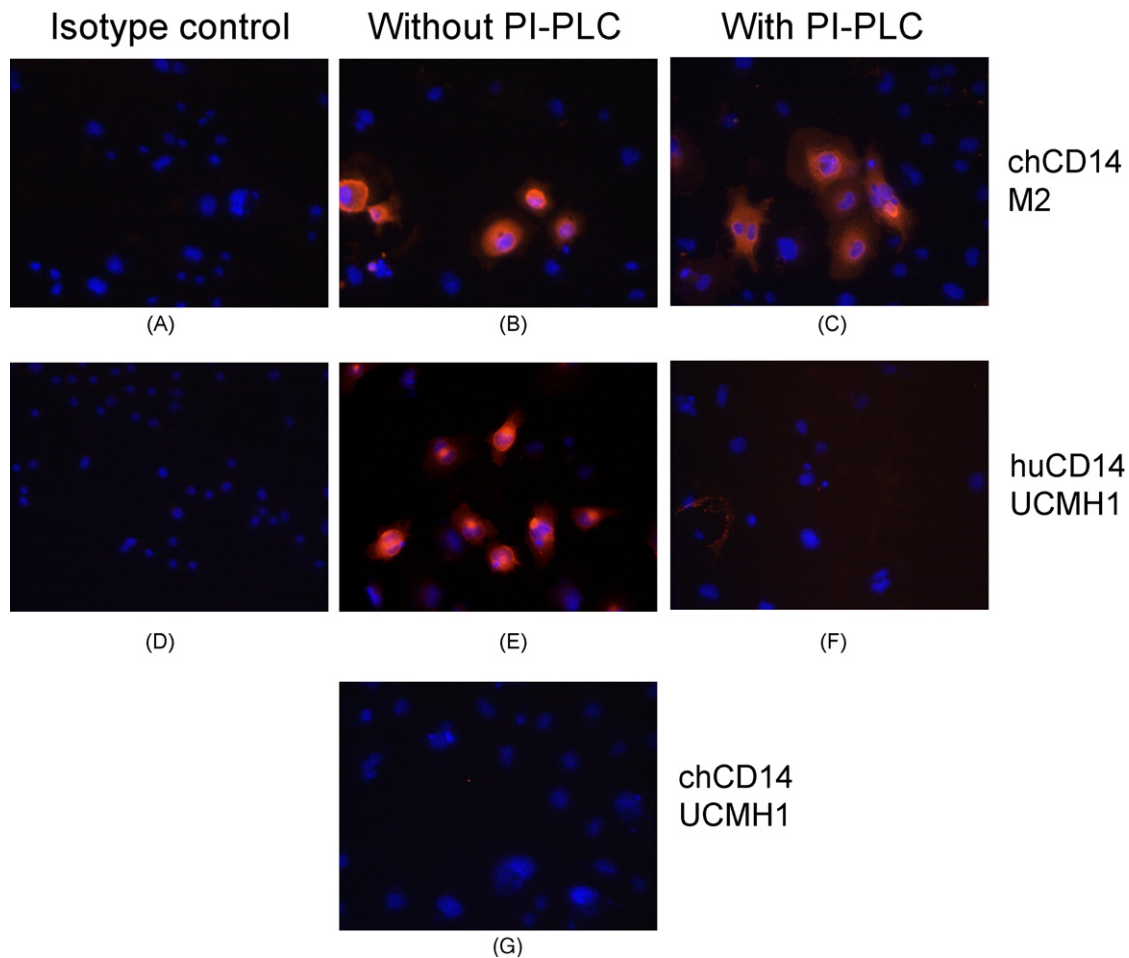
ChCD14 mRNA was detected in all splenocyte subsets. The general distributions of CD14 mRNA that we found in chicken tissues and cells were in accordance with the expression patterns of CD14 mRNA in mammals [13,34,35]. However, KUL01<sup>+</sup> splenocytes showed high expression of chCD14, as did blood monocytes/macrophages, and LPS stimulation significantly ( $P < 0.05$ ) up-regulated the expression of chCD14 in those cells. Up-regulated expression of CD14 mRNA by LPS stimulation is also seen in mammals [36].

### 3.3. Transient expression of chicken CD14 in COS cells

To detect if chCD14 is a GPI-anchored protein as it is in mammals, COS-7 cells were transfected with p3XFLAG-CMV<sup>TM</sup>-8::chCD14 or pCDM8::huCD14, and incubated with or without PI-

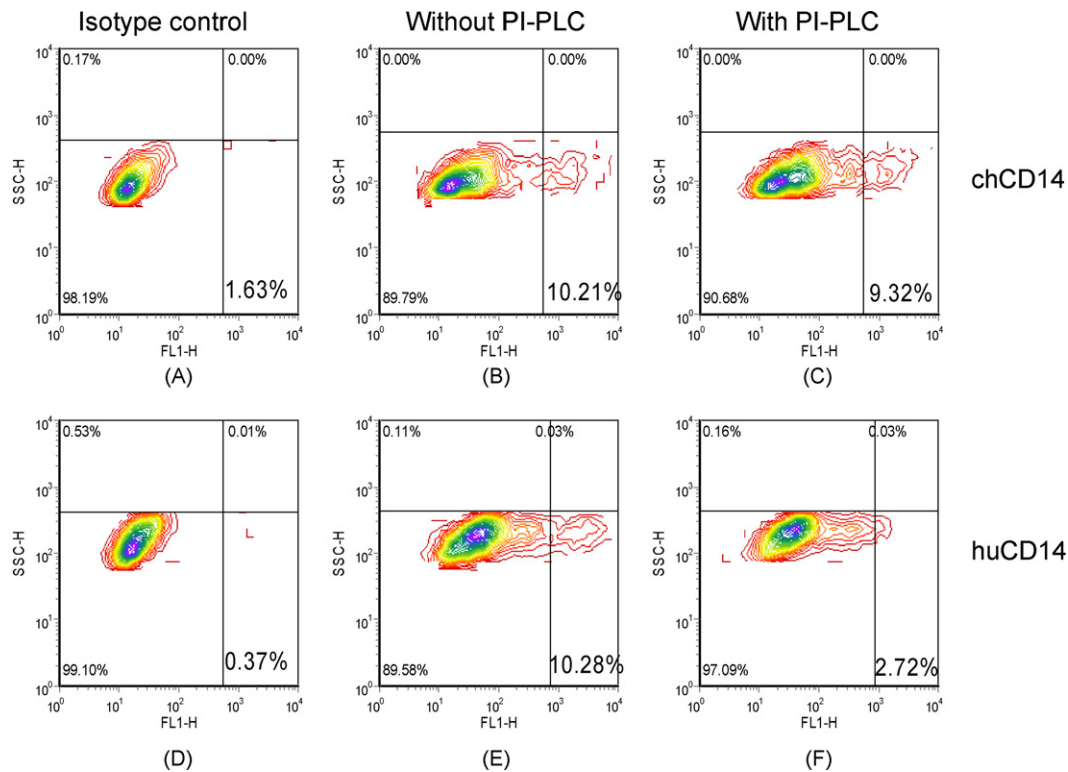
PLC, before staining with an anti-FLAG or anti-huCD14 mAb as appropriate. Fig. 4 clearly shows that COS-7 cells transfected with huCD14 stained with the anti-human CD14 mAb (Fig. 4E), and this staining was lost when the transfected cells were pre-treated with PI-PLC (Fig. 4F), which releases GPI-anchored proteins. In contrast, COS-7 cells transfected with FLAG-tagged chCD14 stained with the anti-FLAG mAb (Fig. 4B), and this staining was retained following PI-PLC pre-treatment (Fig. 4C), suggesting that chCD14 is not GPI-anchored. Fig. 5 shows FACS analysis of COS-7 cells transfected and treated as described previously. Without PI-PLC treatment, approximately 10% of COS-7 cells were positive for either huCD14 or chCD14. Following PI-PLC treatment, only 2.7% of COS-7 cells were positive for huCD14 but a similar proportion (9.3%) to non-treated cells were positive for chCD14. These results again suggest that chCD14 is not GPI-anchored.

Although there is broad species-overlapping reactivity of mAbs directed against CD14, anti-huCD14 mAbs (clones CAM36A, UCHM1, TÜK4 and M5E2) did not cross-react with chicken cells, as tested by Saalmuller et al. [37]. To confirm this lack of reported cross-reactivity, chCD14 was expressed in COS-7 cells and stained with an anti-huCD14 antibody (UCHM1) (Fig. 4G). There was no apparent cross-reactivity. Either the anti-huCD14 antibody does not cross-react with chCD14, or the binding affinity is low. We also used UCHM1 to stain chicken



**Fig. 4.** Detection of chCD14 expressed by COS-7 cells. (A–C and G) COS-7 cells transfected with p3XFLAG-CMV<sup>TM</sup>-8::chCD14. (A) Isotype control; (B) without PI-PLC incubation, stained with anti-FLAG mAb M2; (C) with PI-PLC incubation, stained with anti-FLAG mAb M2; (G) without PI-PLC incubation, stained with anti-huCD14 mAb UCMH1. (D–F) COS-7 cells transfected with pCDM8::huCD14. (D) isotype control; (E) without PI-PLC incubation, stained with anti-huCD14 mAb UCMH1; (F) with PI-PLC incubation, stained with anti-huCD14 mAb UCMH1.





**Fig. 5.** Detection of chCD14 expressed by COS-7 cells by flow cytometry. (A–C) COS-7 cells transfected with p3XFLAG-CMV<sup>TM</sup>-8::chCD14 and stained with anti-FLAG mAb M2; (D–F) COS-7 cells transfected with pCDM8::huCD14 and stained with anti-huCD14 mAb UCMH1. (A and D) Isotype control; (B and E) without PI-PLC incubation; (C and F) with PI-PLC incubation.

spleen cryo-sections, and again did not find any cross-reactivity (data not shown).

### 3.4. Summary

In summary, chCD14 was cloned and molecularly characterized. Analysis of chCD14 mRNA expression levels in chicken tissues and cells showed a higher expression in chicken blood-derived monocytes, monocyte-derived macrophages and KUL01<sup>+</sup> splenocytes.

ChCD14 shares many structural features with mammalian CD14, including eight conserved cysteines with the potential to form four disulphide bridges, and eleven conserved LRRs (and an extra potential chicken-specific LRR). Unlike mammalian CD14, chCD14 appears to have trans-membrane and cytoplasmic domains, suggesting that it is not GPI-anchored. This has important implications for the role of CD14 in the chicken's response to LPS. A GPI-anchored protein in general is more mobile in the cell membrane than one that is trans-membrane. In mammals, and presumably in the chicken, CD14 is a co-receptor for LPS with LBP, MD-2 and TLR4. The kinetics of such interactions, and therefore presumably the response to LPS, may well be compromised by chCD14 being trans-membrane. We have here provided evidence that chCD14 is not GPI-anchored and this may, in part, explain anecdotal reports that chicken cells respond less well to LPS than the equivalent mammalian cells.

### Acknowledgements

This research was undertaken with the financial support of Intervet. The authors would like to thank Lai Shan Kwong (Institute for Animal Health, Compton, UK) for kindly providing the

pCDM8::huCD14 plasmid, and Mike Kogut (USDA, College Station, Texas, USA) for providing the heterophil mRNA.

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