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The functions of the avian receptor activator of NF-κB ligand (RANKL) and its receptors, RANK and osteoprotegerin, are evolutionarily conserved

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ABSTRACT

A new member of the chicken TNF superfamily has recently been identified, namely receptor activator of NF-κB ligand (RANKL), as have its signalling receptor, RANK, and its decoy receptor, osteoprotegerin (OPG). In mammals, RANKL and RANK are transmembrane proteins expressed on the surface of Th1 cells and dendritic cells (DC) respectively, whereas OPG is expressed as a soluble protein from osteoblasts and DC. Recombinant soluble chicken RANKL (chRANKL) forms homotrimers whereas chicken OPG (chOPG) forms homodimers, characteristic of these molecules in mammals. ChRANKL, chRANK and chOPG are expressed at the mRNA level in most tissues and organs. ChRANKL is transcriptionally regulated by Ca^{2+} mobilisation and enhances the mRNA expression levels of pro-inflammatory cytokines in bone marrow-derived DC (BMDC); this is inhibited by both chOPG-Fc and soluble chRANK-Fc. However, chRANKL does not enhance the expression of cell surface markers in either BMDC or BM-derived macrophages (BMM). Furthermore, chRANKL enhances the survival of APC similar to its mammalian orthologue.

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1. Introduction

Members of the TNF superfamily are key regulators of the activity of a variety of pathways associated with the regulation and modulation of the immune system. The TNF superfamily is made up of TNF ligands and their respective receptor(s). The signals from the receptor members of the TNF (TNFR) superfamily control survival versus death signals but also have a role in regulatory events, such as controlling cytokine and chemokine expression (Locksley et al., 2001). Members of the TNFR superfamily are characteristically type I transmembrane proteins that have low degrees of homology and are grouped due to the presence of conserved cysteine-rich domains (CRD) in their extracellular ligand-binding domains (Naismith and

Sprang, 1998). The CRD are typically defined by three intra-chain disulphide bridges generated between highly conserved cysteine residues that act as a scaffold to produce an elongated structure protruding from the cell (Smith et al., 1994). The TNFR superfamily can be further categorised by the expression of either TNFR-associated factors (TRAF)-binding motifs or death domains (DD). The DD receptors are characterised by the presence of an ~80 amino acids (aa) cytoplasmic sequence necessary for apoptosis (Nagata, 1997). TNF superfamily ligands are mostly type II transmembrane proteins with an extracellular site for proteolytic cleavage to release a soluble protein from the membrane. All TNF superfamily ligands share a common structural core, a scaffold of ten hydrogen bonds that assumes a jelly-roll β-sandwich fold (Lam et al., 2001).

Analysis of the chicken genome has identified a reduced repertoire for the TNF superfamily compared to those of mammals (Kaiser et al., 2005). Other cytokine families, including the type I and type III IFN, IL-1, IL-10, IL-12 and chemokine families (Kaiser, 2010, 2012) also have a reduced repertoire compared to their mammalian equivalents. For the TNF superfamily, 19 ligands and 29 receptors have been identified in mammals, but only 10 ligands and 15 receptors in the chicken genome. When both a ligand and its respective receptor are not present in the chicken genome, we hypothesise that these members are really not present rather than not annotated (Kaiser, 2012). In mammals and chickens, the TNF superfamily members are mainly found in small sub-families of syntenic genes on different

chromosomes. Chickens lack certain of these sub-families in their genome and this has implications for their biology. For example, the lack of an equivalent of the canonical mammalian lymph node in chickens may be linked to the absence of the sub-family of genes encoding the lymphotoxins (LT-α and LT-β) and TNF-α in their genome. LT-α$^{-/-}$ mice have defects in lymph node development and splenic architecture (Banks et al., 1995). TNF-α$^{-/-}$ mice develop normally but are highly susceptible to infectious agents (Pasparakis et al., 1996). However, two receptors for TNF-α – a partial clone of TNFR1 (Bridgham and Johnson, 2001) and TNFR2 (Abdalla et al., 2004b) – have been identified in the chicken genome. Of the ten TNF ligands identified in the chicken genome, the biological activities of five have so far been characterised – CD30 and TNF-related apoptosis inducing ligand (TRAIL) (Abdalla et al., 2004a), B cell activating factor (BAFF) (Schneider et al., 2004), CD40L (Tregaskes et al., 2005) and vascular endothelial growth inhibitor (VEGI) (Takimoto et al., 2005). Overall, the functions of these chicken TNF family members are evolutionarily conserved.

Mammalian receptor activator of NF-κB ligand (RANKL), a ligand member of the TNF superfamily, is a transmembrane protein of 316 aa containing a COOH-terminal receptor-binding domain, a 20 aa hydrophobic transmembrane domain and a relatively long extracellular domain that contains a TNF-homologous domain that is the active receptor-binding site (Anderson et al., 1997; Lam et al., 2001). Its expression in the immune system is limited to the thymus and lymph nodes (Anderson et al., 1997; Wong et al., 1997b) a restricted pattern of expression not usually seen for TNF superfamily members. RANKL has two receptors, a signalling receptor, RANK, and a decoy receptor, osteoprotegerin (OPG), a secreted TNF-related protein that inhibits the RANKL–RANK interaction (Simonet et al., 1997). RANK is a type I transmembrane protein of 616 aa, with the longest extracellular domain (residues 234–616) of all TNFR members identified so far, containing four CRD (Anderson et al., 1997). It is the only known signalling receptor for RANKL (Anderson et al., 1997; Wong et al., 1997b). Originally identified in a bone marrow-derived myeloid DC cDNA library, RANK mRNA expression is widely detected in the lungs, spleen, skeletal muscle, brain, liver, kidney and surface expression is detected in cells of the myelomonocytic lineage ranging from osteoclast progenitor cells to DC (Anderson et al., 1997; Wong et al., 1997b). OPG was identified through sequence homology with the TNF superfamily (Simonet et al., 1997). It contains two C-terminal homologous DD of TNFR1 that are not functional. OPG is a naturally secreted protein which can form disulphide-linked dimers of 110 kDa. Transgenic mice overexpressing OPG suffered from osteopetrosis and osteoclastogenesis could be inhibited by the addition of OPG to cell cultures (Simonet et al., 1997). RANKL$^{-/-}$ and RANK$^{-/-}$ deficient mice had entirely unexpected phenotypes: early defects in T and B cell development, complete absences of lymph nodes, severely reduced osteoclastogenesis and failure to develop mammary glands (Dougall et al., 1999; Kong et al., 1999). RANKL, RANK and OPG have been implicated in a number of biological processes, such as bone metabolism (Kong et al., 1999; Simonet et al., 1997; Yasuda et al., 1998), lymph node development (Kim et al., 2004), thymocyte development (Hikosaka et al., 2008; Rossi et al., 2007), M cell differentiation and maintenance (Knoop et al., 2009) and immunity (Anderson et al., 1997).

Although RANKL$^{-/-}$ or RANK$^{-/-}$ mice expressed normal DC and macrophages numbers and functions (Darnay et al., 1998; Kong et al., 1999) they were deficient in production of the cytokines that drive pro-inflammatory immune responses. RANKL induces pro-inflammatory cytokine expression in monocyte-derived and BM-DC, driving predominantly expression of Th1 effector cytokines (Anderson et al., 1997; Josien et al., 1999; Schiano de Colella et al., 2008; Wong et al., 1997b). Strong RANKL surface expression is predominantly on activated murine Th1 cell clones rather than Th2 cell clones (Josien et al., 1999). RANKL$^{-/-}$ T cells produce higher levels

of IL-4 and IL-5 compared to wild-type cells (Kong et al., 1999). These studies strongly suggested that RANKL is a predominant Th1 surface marker. RANKL transcription is upregulated within 2 h of TCR stimulation. Its expression on T cells is regulated by calcium ion (Ca^{2+}) mobilisation and is enhanced by protein kinase C (PKC) activation (Bishop et al., 2011; Fionda et al., 2007; Wang et al., 2002).

Various members of the TNF superfamily can upregulate the expression of costimulatory molecules on the surface of target cells. Mammalian RANKL did not significantly increase the surface expression of MHC class II, CD80 and CD86 on DC but did upregulate CD40 expression (Anderson et al., 1997; Wong et al., 1997b). RANKL induced partial maturation of monocyte-derived DC and enhanced the levels of CD83 and CD86 expression (Schiano de Colella et al., 2008). Macrophages upregulate MHC class II and CD86 when stimulated with RANKL and can further increase surface expression by synergising with LPS and IFN-γ (Park et al., 2005). Mammalian RANKL is a survival factor for antigen presenting cells (APC), encouraging cell survival through the upregulation of the anti-apoptotic molecule, Bcl-X$_L$ (Wong et al., 1997a).

Here we describe the cloning and molecular characterisation of chicken RANKL (chRANKL) and its receptors, chRANK and chOPG. The main objective of this study was to characterise the role of chRANKL in the function of avian APC, by analysing its ability to enhance pro-inflammatory cytokine mRNA expression levels, alter APC phenotype and enhance cell survival.

2. Materials and methods

2.1. Identification and molecular cloning of chRANKL, chRANK and chOPG

The full-length cDNA of chRANKL and chRANK were cloned using primers designed to the predicted sequences of the genes in the Ensembl and NCBI databases (see Table 1). ChOPG has previously

Table 1
Primers and probes.

Name	Target	Sequence (5′–3′)
RANKL	Full length	F: ATGCGGCGCGCCAGCCG
		R:TCAGTCTAAATCCCTTACTTTAAATGCCCCA
RANK	Full length	F: TTGTCACTACAAAGCACTCTG
		R: ACAAACTGCATCGGACTTATC
OPG	Full length	F: ATGAACAAGTTCCTGTGCTGCA
		R: TTAGACACATCTTACTTTCACTGATTTCA
sRANKL	Extracellular domain	F: TGAAGAAGATGGACCCTAGTA
		R: TCAGTCTAAATCCCTTACTTTA
sRANK	Extracellular domain	F: ACAAACTGCATCGGACTTATC
		R: TCAGTTCAACACCTTCTCA
28S	Taqman	F: GGCGAAGCCAGAGGAAACT
		R: GACGACCGATTTGCACGTC
		P: AGGACCGCTACGGACCTCCACCA
IL-1β	Taqman	F: GCTCTACATGTCGTGTGTGATGAG
		R: TGTCGATGTCCCGCATGA
		P: CCACACTGCAGCTGGAGGAAGCC
IL-6	Taqman	F: GCTCGCCGGCTTCGA
		R: GGTAGGTCTGAAAGGCGAACAG
		P: AGAGAAATGCCTGACGAAGCTCTCCA
IL-12α	Taqman	F: TGGCCGCTGCAAACG
		R: ACCTCTTCAAGGGTGCACTCA
		P: CCAGCGTCCTCTGCTTCTGCACCTT
RANKL	Taqman	F: CTGGAACTCGCAAAGTGAACCT
		R: TTTCCCATCACTGAACGTCATATTT
		P: TGGCATCATAACAAAGGCCAGGCAAAC
RANK	Taqman	F: GCCATGTCCC AGAGGATACT
		R: GCCAATCCCGAGCTGAACA
		P:TGCTTCATCCACTGATGAGTGTAAATCCTGGACCA
OPG	Taqman	F: ACAGCCAGGCACTCCTGAGA
		R: GCTTTTGACAGACTGCTTTGGA
		P: CCCAGAGGGGATTTTTCTCCAATGAAACG

been cloned (Hou et al., 2011) and used to designed primers (Table 1). Total RNA was extracted from either ConA (Sigma-Aldrich, Poole, UK)-stimulated chicken splenocytes for 4 h or bone marrow cells using an RNeasy mini kit (QIAGEN, Crawley, UK) following the manufacturer's instructions. First strand synthesis used Superscript III (Invitrogen). After denaturation of the reverse transcriptase at 70 °C for 15 min, 1 µl of the reaction was used in a 50 µl volume polymerase chain reaction (PCR) containing 10 pmol of each primer, 1 µM dNTP and 0.625 U of *Taq* DNA polymerase (Invitrogen). Each cDNA was cloned into pGEM-T Easy and sequences confirmed from three independent clones on each strand. The cDNA sequences of chRANKL and chRANK were submitted to Ensembl (Accession nos. LM999949 and LM999950). Sequences from species containing orthologues of RANKL, RANK and OPG were aligned using CLUSTALX v2.1. The secondary structure of chRANKL was predicted using PSIPRED v3.0 (http://bioinf.cs.ucl.ac.uk/psipred/).

2.2. Sub-cloning of chRANKL, chRANK and chOPG into expression vectors

To obtain soluble forms of chRANKL (schRANKL) and chRANK (schRANK), their extracellular domains were identified using their human orthologues and the SMART prediction program (http://smart.embl-heidelberg.de/). Primers containing restriction enzyme sites were designed to flank the extracellular domains of chRANKL and chRANK (Table 1). To obtain schRANKL, the extracellular domain was amplified and sub-cloned into the *Nho*I and *Nhe*I sites of a modified pCI-neo vector (Promega, Southampton, UK) expressing the mouse CD8 signal peptide, an isoleucine zipper and an NH$_2$-terminal FLAG-tag, named pCI-IZFLAG-schRANKL (generous gift from John Young, the former Institute for Animal Health, UK). The obtained schRANK and chOPG, the extracellular domain of chRANK and the full-length chOPG cDNA were directionally sub-cloned into two similar plasmids, Signal-pKW06 and pKW06 (Staines et al., 2013), to generate COOH-human Fc-tagged recombinant proteins. All sub-cloning was verified by sequencing.

2.3. Chicken tissues and cells

Tissues were removed from three 6-week-old J-line chickens (The Roslin Institute, UK) *post-mortem*. Tissues taken were the heart, kidney, liver, muscle, lung, brain, skin, spleen, thymus, bursa of Fabricius, caeca, bone marrow, crop, gizzard, lower and mid-gut, caecal tonsils, Meckel's diverticulum and Harderian gland. Tissues were homogenised using matrix tubes containing 1.4 mm ceramic spheres (MD Biomedicals). Primary cell populations were prepared from the spleen, bursa of Fabricius and thymus of three individual birds. Cells were purified by passing the tissues through 70 µm mesh cell strainers (Fisher Scientific) and splenocytes were collected over Histopaque-1077 (Sigma-Aldrich) followed by two washes with PBS. Cell numbers were adjusted to 5×10^6 cells/ml with pre-warmed complete RPMI (10% chicken serum (ChS), 2% L-glutamine, 1 U/ml penicillin and 1 µg/ml streptomycin (Gibco Life Technologies)) and cultured in 25 mm² flasks for 24 h either unstimulated or stimulated as follows: splenocytes, 1 µg/ml ConA (Sigma-Aldrich); bursal cells, 500 ng/ml PMA (Sigma-Aldrich) and 1 µg/ml ionomycin (Sigma-Aldrich); thymocytes, 25 µg/ml PHA (Sigma-Aldrich). Lymphocyte subsets (CD4$^+$, CD8β$^+$, TCRγδ$^+$, TCRαβ1$^+$ and TCRαβ2$^+$) were isolated from total splenocytes by positive selection using an AutoMACS Pro Separator (Miltenyi Biotech). Purity of the isolated populations was verified by FACS analysis (BD FACSCalibur, BD Biosciences). To determine the transcriptional regulation of chRANK in avian splenocytes, cells were unstimulated or stimulated with ionomycin (3 µg/ml) or ionomycin and PMA (500 ng/ml) for 2, 4 or 18 h. To determine if Ca^{+2} modulation was linked with chRANKL transcriptional regulation, cells were either stimulated with ionomycin alone or in combination with various amounts of the

pharmacological inhibitor of Ca^{2+} mobilisation, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) (Sigma-Aldrich) for 18 h.

2.4. Protein production

COS-7 cells were routinely grown and passaged in complete DMEM (10% FCS, 2% L-glutamine, 2% non-essential aa, 1 U/ml penicillin and 1 µg/ml streptomycin). To produce recombinant protein, pCI-IZFLAG-schRANKL, Signal-pKW06-schRANK and pKW06-chOPG were transfected into COS-7 cells using DEAE-dextran (Avery et al., 2004). Cell supernatants (ex-COS-7) were collected 72 h later and stored at 4 °C until use.

2.5. Western blot analysis

The supernatants from COS-7 cells transfected with pCI-IZFLAG-schRANKL or pKW06-chOPG-Fc were treated with loading buffer with or without β-mercaptoethanol and boiled for 5 min at 95 °C. Samples were then loaded on a 4–12% gradient SDS–PAGE gel (Bio-Rad) and transferred onto nitrocellulose membrane. Samples were blocked with 2% casein for 1 h at room temperature. FLAG tagged schRANKL protein was detected using a mouse anti-FLAG mAb (Sigma-Aldrich, M2) and a goat anti-mouse IgG HRP-conjugated secondary antibody (SouthernBiotech, 1070-05). ChOPG-Fc was detected using a goat anti-human HRP-conjugated antibody (SouthernBiotech, 2040-05). Recombinant protein was detected using an ECL™ Western Blotting Detection Reagents system (GE Healthcare Life Sciences, Little Chalfont, UK).

2.6. BMDC and BMM preparation

Femurs and tibias were collected aseptically from 4 to 6-week-old J-line chickens. Bone marrow-derived DC (BMDC) and macrophages (BMM) were expanded *in vitro* as described in Wu et al. (2010) and Garceau et al. (2010), respectively. In brief, bones were flushed with PBS and single cell suspensions were collected using Histopaque-1077 (Sigma-Aldrich) followed by two washes with PBS. BMDC were seeded at 1×10^6 cells/ml in 6-well plates in complete RPMI and cultured with appropriate dilutions of exCOS-7 supernatants of chIL-4 and chCSF-2. For BMM, cells were seeded at 1×10^6 cells/ml in complete BMM RPMI (2% ChS, 3% FCS, 2% L-glutamine, 1 U/ml penicillin and 1 µg/ml streptomycin) and appropriate dilutions of exCOS-7 supernatants of chCSF-1 in 25-well chamber plates (Thermo Scientific). BMDC and BMM were cultured for 6 days at 41 °C, 5% CO$_2$.

2.7. Bioactivity of schRANKL on BMDC and BMM

BMDC and BMM were unstimulated or stimulated with LPS (1–2 ng/ml), schRANKL (1:50 exCOS-7 supernatant) or both for 3 and 6 h. To determine the antagonistic effects of schRANK-Fc and chOPG-Fc (1:5 exCOS-7 supernatant), either was pre-incubated with schRANKL for 3 h prior to cell treatment. The resulting mRNA expression levels of pro-inflammatory cytokines were analysed by qRT-PCR.

2.8. Fluorescent activated cell sorting

On day 6 of BMDC and BMM cultures, cells were unstimulated or stimulated with LPS (1–2 ng/ml), schRANKL (1:5 exCOS-7 supernatant) or both for 24 h. Cells were removed from culture plates using 0.05 mM EDTA and resuspended in PBS with 0.5% BSA and 0.001% azide (FACS buffer). Immunofluorescence labelling was carried out using mouse anti-chicken MHC class II (IgG1, 2G11), mouse anti-chicken CD40 (IgG2a, Serotec, MCA2836) and mouse anti-chicken KUL01 (IgG1, SouthernBiotech, 8420-01) mAbs on ice for 20 min. A FITC-conjugated goat anti-mouse IgG1 antibody (SouthernBiotech,

1070-02) was used as a secondary antibody to detect MHC class II and KUL01 and a PE-conjugated goat anti-mouse IgG2a antibody (SouthernBiotech, 1080-09S) to detect CD40, incubated on ice for 20 min. Negative/isotype controls were used for each sample to control for non-specific binding. Cells were stained with 7-AAD prior to analysis and gated using FSC and SSC using a BD FACSCalibur instrument (BD Biosciences). Data were analysed using FlowJo v7.6.5 software (Tree Star, Ashland, USA).

2.9. Flow cytometer-based survival assay

BMDC and BMM were treated with fresh complete media or schRANKL (1:5 exCOS-7 supernatant) for 24 and 48 h. Cells were harvested at indicated time-points and stained with biotin-conjugated recombinant Annexin-V according to the manufacturer's instructions (eBioscience). Conjugated streptavidin-Alexa 657 (Molecular Probes, S-21374) was incubated at RT for 20 min. Propidium iodide (PI) was added to the cells and immediately analysed using a BD FACSCalibur instrument.

2.10. Quantitative real-time RT-PCR analysis

RNA from the tissues and cells were extracted using RNeasy Mini kit (Qiagen, Crawley, UK) following manufacturer's instructions. ChRANKL, chRANK and chOPG and pro-inflammatory cytokine mRNA expression levels were quantified by TaqMan real-time quantitative RT-PCR (qRT-PCR) using a well described method (e.g. Avery et al., 2004; Eldaghayes et al., 2006). Primers (Sigma) and probes (Eurogentec, Belgium) (Table 1) were designed using Primer Express (Applied Biosystems). TaqMan assays were performed using the One-Step RT-PCR Master Mix reagents and amplification and detection were performed using the FAST Universal PCR Master Mix (Applied Biosystems). Data are expressed in terms of the cycle threshold (Ct) value, which is normalised using the Ct values of 28S rRNA for each sample using the formula; $Ct + (N't-C't)*(S/S')$ where $N't$ is the mean Ct value for 28S RNA among all samples, $C't$ is the mean value for 28S RNA in the sample and the S and S' are the slopes of regression of the standard plots for the cytokine mRNA and the 28S RNA, respectively. Normalised Ct values were corrected against the negative association of the log concentration of mRNA detected using the formula 40 – normalised Ct value of the cytokine of interest. Final results are shown as corrected 40 – Ct values or as fold difference from levels in untreated control cells.

2.11. Statistical analysis

Statistical analyses were carried out using the Mann–Whitney U test in Minitab 16.1.0 (State College, USA). Statistical significance was determined as significant when ($p < 0.05$).

3. Results

3.1. Cloning and analysis of chRANKL, chRANK and chOPG

A multiple alignment of the aa sequences of human, mouse and chicken RANKL is shown in Fig. 1A. ChRANKL shares ~59–62% identity with mammalian RANKL. The extracellular domains of TNF superfamily members express the highly conserved TNF homology domain (residues 163–318 in the chicken molecule) which is required for receptor interaction and trimerisation. This domain is composed of β-strands and loops connected in a "jelly-roll" fold (Lam et al., 2001). To investigate the conservation of these β-strands in chRANKL, each was identified in mammalian RANKL (Fig. 1A) using PSIPRED. ChRANKL has conserved residues at the positions of the β-strands and loops found in mammalian counterparts suggesting it has the potential to fold into a trimeric protein. There

are four conserved cysteines between mammalian and chicken RANKL, and three potential N-glycosylation sites in the chRANKL extracellular domain, one of which is conserved with mammalian RANKL.

A multiple alignment of the aa sequences of human, mouse and chicken RANK is shown in Fig. 1B. ChRANK has ~40–42% sequence identity with mammalian RANK. The TNFR superfamily members have conserved CRD in their extracellular ligand-binding domains (Naismith and Sprang, 1998), which are typically defined by three intra-chain disulphide bridges between highly conserved cysteine residues that act as a scaffold to produce an elongated structure protruding from the cell (Smith et al., 1994). All four CRD are present in chRANK (labelled I–IV) and all of the cysteine residues in the CRD are conserved between the three species. There are two potential N-glycosylation sites in the extracellular domain of chRANK, both conserved in the mammalian molecules. All TNFR lack catalytic domains and require adaptor proteins for downstream signalling, called TRAFs, which for mammalian RANK include TRAF1-3, TRAF5-6. Two binding motifs for TRAF1-3 and TRAF5 in TNFR have been identified; a major one, (P/S/A/T)X(Q/E)E, and a minor one, PXQXXD (X being any aa) (Devergne et al., 1996; Ishida et al., 1996). The TRAF6 specific-binding motif is the peptide PXEXX (X being an aromatic or acidic residue). Four of the five mammalian RANK TRAF-binding sites (Cremer et al., 2002; Darnay et al., 1998; Wong et al., 1998) are conserved in chRANK (Fig. 1B). The "missing" TRAF-binding motif is TRAF6-specific (residues 457–463 in huRANK). ChRANK also contains the highly conserved region (HCR; between Pro[508] and Gly[546] in muRANK), which is vital for osteoclastogenesis in mammals (Taguchi et al., 2009).

To produce soluble protein the extracellular domain of chRANKL was cloned as a 729 bp cDNA fragment corresponding to 243 aa (chRANKL[75–318]) and is henceforth called schRANKL. The extracellular domain of chRANK was cloned as a 498 bp cDNA fragment which corresponded to 166 aa (chRANK[24–190]) and is henceforth called schRANK.

The full-length chOPG cDNA was cloned using primers designed based on the NCBI database sequence. Total RNA was extracted from bone marrow and used to amplify a cDNA fragment of 1.2 kb, corresponding to 401 aa. A multiple alignment of the aa sequences of human, mouse and chicken OPG is shown in Fig. 1C. ChOPG shares high identity with mammalian OPG (60–64%). It possesses four CRD domains (labelled I–IV) with 17 out of 18 cysteine residues conserved and two homologous DD which are conserved between the three species. ChOPG contains a penultimate cysteine residue at position 401 which is vital for homodimerisation in mammalian OPG (Luan et al., 2012).

3.2. Soluble chRANKL and chOPG form homo-trimeric and homo-dimeric protein structures

The hallmark of TNF ligand and TNFR interactions is their threefold-symmetry. Three subunits of RANKL are required for the activation of the receptor, RANK. To enhance the formation of trimeric schRANKL protein, a modified plasmid was designed to express an in-frame isoleucine zipper sequence upstream of the chRANKL sequence with an NH₂-terminal FLAG-tag, called pCI-IZFLAG-schRANKL. SchRANKL protein was generated in COS-7 cells and protein structures were analysed by SDS–PAGE under reducing and non-reducing conditions and western blot using antibodies against the FLAG-tag. In reducing conditions, schRANKL appears as predominantly a monomeric protein at ~37 kDa with a weak band of ~75 kDa which is possibly dimeric protein (Fig. 2). Proteins analysed under non-reducing conditions produced bands at ~75, ~140 and ~200 kDa, suggesting the formation of dimeric, trimeric and possibly tetrameric protein structures (Fig. 2A).

To analyse the ability of chOPG to form homodimeric protein, COS-7 cells were transfected with pKW06-chOPG to produce soluble
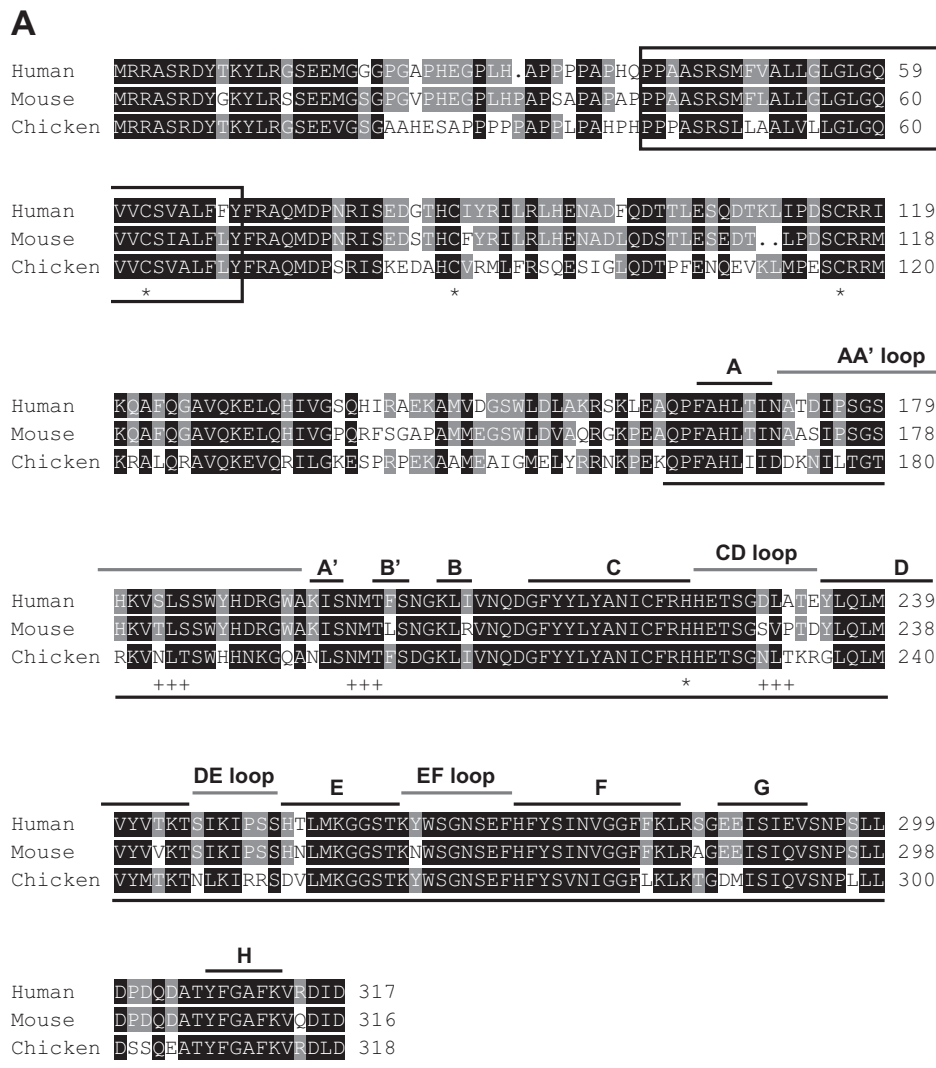
A

```
Human    MRRASRDYTKYLRGSEEMGGGPGAPHEGPLH.APPPPAPHQPPAASRSMFVALLGLGLGQ   59
Mouse    MRRASRDYGKYLRSSEEMGSGPGVPHEGPLHPAPSAPABAPPPPAASRSMFLALLGLGLGQ   60
Chicken  MRRASRDYTKYLRGSEEVGSGAAHESAPPPPPAPPLPAHPHPPPASRSLLAALVLLGLGQ    60
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```
Human    VVCSVALFFYFRAQMDPNRISEDGTHCIYRILRLHENADFQDTTLESQDTKLIPDSCRRI   119
Mouse    VVCSIALFLYFRAQMDPNRISEDSTHCFYRILRLHENADLQDSTLESEDT..LPDSCRRM   118
Chicken  VVCSVALFLYFRAQMDPSRISKEDAHCVRMLFRSQESIGLQDTPFENQEVKLMPESCRRM   120
                 *                           *                        *
```

A   AA' loop

```
Human    KQAFQGAVQKELQHIVGSQHIRAEKAMVDGSWLDLAKRSKLEAQPFAHLIINATDIPSGS   179
Mouse    KQAFQGAVQKELQHIVGPQRFSGAPAMMEGSWLDVAQRGKPEAQPFAHLIINAASIPSGS   178
Chicken  KRALQRAVQKEVQRILGKESPRPEKAAMBAIGMELYRRNKPEKQPFAHLIIDDKNILTGT   180
```

A'   B'   B   C   CD loop   D

```
Human    HKVSLSSWYHDRGWAKISNMTFSNGKLIVNQDGFYYLYANICFRHHETSGDLATEYLQLM   239
Mouse    HKVTLSSWYHDRGWAKISNMTLSNGKLRVNQDGFYYLYANICFRHHETSGSVPTDYLQLM   238
Chicken  RKVNLTSWHHNKGQANLSNMTFSDGKLIVNQDGFYYLYANICFRHHETSGNLTKRGLQLM   240
             +++          +++                          *    +++
```

DE loop   E   EF loop   F   G

```
Human    VYVTKTSIKIPSSHTLMKGGSTKYWSGNSEFHFYSINVGGFFKLRSGEEISIEVSNPSLL   299
Mouse    VYVVKTSIKIPSSHNLMKGGSTKNWSGNSEFHFYSINVGGFFKLRAGEEISIQVSNPSLL   298
Chicken  VYMTKTNLKIRRSDVLMKGGSTKYWSGNSEFHFYSVNIGGFLKLKTGDMISIQVSNPLLL   300
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H

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Human    DPDQDATYFGAFKVRDID   317
Mouse    DPDQDATYFGAFKVQDID   316
Chicken  DSSQEATYFGAFKVRDLD   318
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Fig. 1. Alignment of the sequences of RANKL, RANK and OPG. Amino acid sequence alignment of (A) chRANKL, (B) chRANK and (C) chOPG with their mammalian orthologues. Shaded areas represent conservation of aa; the darker the shading, the more conserved the residue across species. Dots indicate gaps in the alignment. Asterisks indicate conserved cysteine residues. Predicted N-glycosylation sites in any molecule are indicated by +++. (A) RANKL is a type II membrane protein. The box indicates the transmembrane domains. The TNF homology domain is underlined. Black lines above the sequences indicate the location of the ten conserved β-strands (labelled A, A′, B′, B, C, D, E, F, G and H) involved in the "β-jelly" roll structure, and the surface loops are shown as grey lines between the β-strands, labelled AA′, CD, DE and EF (based on the mouse RANKL crystal structure (Lam et al., 2001). (B) The four CRD domains are overlined and numbered I–IV, the black box indicates the transmembrane domain and the light grey box the HCD. TRAF-binding motifs are overlined and labelled (6 = TRAF6, 1/2/3/5 = TRAF1, TRAF2, TRAF3, TRAF5 binding motifs). (C) The four CRD domains are overlined and numbered I–IV, the DD are overlined and the triangle indicates the conserved cysteine residue in mammals not conserved in the chicken.

chOPG-Fc. Protein structures were analysed under reducing and non-reducing conditions and visualised by western blot using antibodies against the Fc tag (Fig. 2). The chOPG-Fc monomer has a predicted molecular mass of ~72 kDa. In reducing conditions, a ~ 75 kDa band was just evident whereas in non-reducing conditions there was a strong band at ~160 kDa, suggesting the formation of homodimers (Fig. 2B).

3.3. Tissue distribution and cellular mRNA expression levels of chRANKL, chRANK and chOPG

The mRNA expression profiles of chRANKL, chRANK and chOPG were determined in a broad range of non-lymphoid and lymphoid tissues by qRT-PCR. Primers and probes for chRANKL and chRANK were designed against their extracellular domains. In both non-lymphoid and lymphoid tissues, mRNA transcripts for each gene were detected, suggesting each is ubiquitously expressed in chicken tissues (Fig. 3).

In non-lymphoid tissues, chRANKL and chRANK mRNA expression levels are highest in muscle, which had the lowest levels of chOPG mRNA expression (Fig. 3A). The lowest levels of chRANKL and chRANK mRNA expression were in the heart. Levels of mRNA expression of all three cytokine were consistent in the liver, kidney and lung and slightly lower in the skin and brain (Fig. 3A). In chicken lymphoid tissues, the highest levels of mRNA expression for all three cytokines were in the thymus, followed by the upper-gut and the bone marrow (Fig. 3B). In the crop, chRANKL mRNA expression levels were higher (~16-fold) than those of chRANK and chOPG. In the Harderian gland, chRANKL was more highly expressed than chRANK.

The mRNA expression levels of chRANKL, chRANK and chOPG were also examined in ConA stimulated splenocytes, PMA and
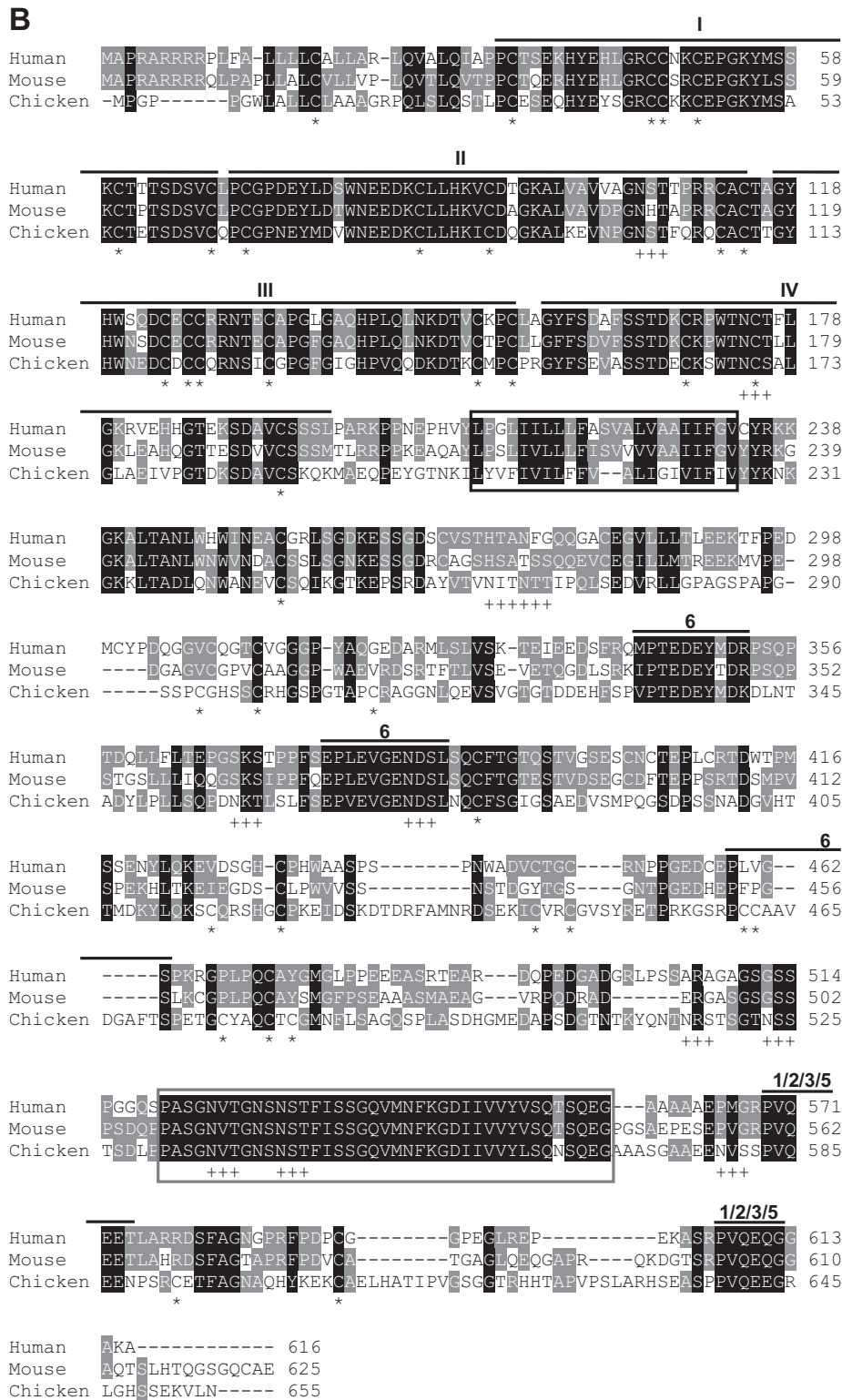
B

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                                                          I
Human    MAPRARRRRPLFA-LLLILCALLAR-LQVALQIAPPCTSEKHYEHLGRCCNKCEPGKYMSS  58
Mouse    MAPRARRRRQLPAPLLALCVLLVP-LQVTLQVTPPCTQERHYEHLGRCCSRCEPGKYLSS  59
Chicken  -MPGP------PGWLALLCLAAAGRPQLSLQSTLPCESEQHYEYSGRCCKKCEPGKYMSA  53
              *              *           **  *

                               II
Human    KCTTTSDSVCLPCGPDEYLDSWNEEDKCLLHKVCDTGKALVAVVAGNSTTPRRCACTAGY  118
Mouse    KCTPTSDSVCLPCGPDEYLDTWNEEDKCLLHKVCDAGKALVAVDPGNHTAPRRCACTAGY  119
Chicken  KCTETSDSVCQPCGPNEYMDVWNEEDKCLLHKICDQGKALKEVNPGNSTFQRQCACTTGY  113
            *        *  *            *         *      +++        *  *

                   III                                    IV
Human    HWSQDCECCRRNTECAPGLGAQHPLQLNKDTVCKPCLAGYFSDAFSSTDKCRPWTNCTFL  178
Mouse    HWNSDCECCRRNTECAPGFGAQHPLQLNKDTVCTPCLLGFFSDVFSSTDKCKPWTNCTLL  179
Chicken  HWNEDCDCCQRNSICGPGFGIGHPVQQDKDTKCMPCPRGYFSEVASSTDECKSWTNCSAL  173
           *  **   *       *               *  *             *   *
                                                            +++

Human    GKRVEHGTEKSDAVCSSSLPARKPENEPHVYLPGLIILLLFASVALVAAIIFGVCYRKK  238
Mouse    GKLEAHQGTTESDVVCSSSMTLRRPEKEAQAYLPSLIVLLLFISVVVVAAIIFGVYYRKG  239
Chicken  GLAEIVPGTDKSDAVCSKQKMAEQPEYGTNKILYVFIVILFFV--ALIGIVIFIVYYKNK  231
           *                           *

Human    GKALTANLWHINEACGRLSGDKESSGDSCVSTHTANFGQQGACEGVLLLTLEEKTFPED  298
Mouse    GKALTANLWWVNDACSSLSGNKESSGDRCAGSHSATSSQQVECEGILLMTREEKMVPE-  298
Chicken  GKKLTADLQNWANEVCSQIKGTKEPSRDAYVTVNITNTTIPQLSEDVRLLGPAGSPAPG-  290
           *                                        ++++++

                                                      6
Human    MCYPDQGGVCQGTCVGGGP-YAQGEDARMLSLVSK-TEIEEDSFRQMPTEDEYMDRPSQP  356
Mouse    ----DGAGVCGPVCAAGGP-WAEVRDSRTFTLVSE-VETQGGLSRKIPTEDEYTDRPSQP  352
Chicken  -----SSPCGHSSCRHGSPGTAPCRAGGNLQEVSVGTGTDDEHFSPVPTEDEYMDKDLNT  345
                 *       *                           *

                            6
Human    TDQLIFLTEPGSKSTPPFSEPLEVGENDSLSQCFTGTQSTVGSESCNCTEPLCRTDWTPM  416
Mouse    STGSLLLIQQGSKSIPPFQEPLEVGENDSLSQCFTGTESTVDSEGCDFTEPPSRTDSMPV  412
Chicken  ADYIPLLSQPDNKTLSLFSEPVEVGENDSLNQCFSGIGSAEDVSMPQGSDPSSNADGVHT  405
              +++              +++         *

                                                          6
Human    SSENYLQKEVDSGH-CEHWAASPS-------PNWADVCTGC----RNPPGEDCEPLVG--  462
Mouse    SPEKHLTKEIEGDS-CLPWVVSS---------NSTDGYTGS----GNTPGEDHEPFPC--  456
Chicken  TMDKYLQKSCQRSHGCEKEIDSKDTDRFAMNRDSEKICVRCGVSYRETPRKGSRPCCAAV  465
            *         *                         *  *        **

Human    -----SPKRGPLPQCAYGMGLPPEEEASRTEAR---DQPEDGADGRLPSSARAGAGSGSS  514
Mouse    -----SLKCGPLPQCAYSMGFPSEAAASMAEAG---VRPQDRAD------ERGASGSGSS  502
Chicken  DGAFTSPETGCYAQCTCCMNFLSAGQSPLASDHGMEDAPSDGTNTKYQNLNRSTSGTNSS  525
              *     *   *                              +++     +++

                                                      1/2/3/5
Human    PGGQSPASGNVTGNSNSTFISSGQVMNFKGDIIVVYVSQTSQEG---AAAAAEPMGRPVQ  571
Mouse    PSDQEPASGNVTGNSNSTFISSGQVMNFKGDIIVVYVSQTSQEGPGSAEPESEPVGRPVQ  562
Chicken  TSDLEPASGNVTGNSNSTFISSGQVMNFKGDIIVVYLSQNSQEGAAASGAAEENVSSPVQ  585
              +++    +++                                   +++

                                                      1/2/3/5
Human    EETLARRDSFAGNGPRFPDPCG--------GPEGLREP----------EKASRPVQEQGG  613
Mouse    EETLAHRDSFAGTAPRFPDVCA--------TGAGLQEQGAPR----QKDGTSRPVQEQGG  610
Chicken  EENPSRCETFAGNAQHYKEKCAELHATIPVCGSGGTRHHTAPVPSLARHSEASPPVQEEGR  645
              *                    *                        *

Human    AKA------------  616
Mouse    AQTSLHTQGSGQCAE  625
Chicken  LGHSSEKVLN-----  655
```

Fig. 1. (*continued*)

ionomycin stimulated bursal cells and PHA stimulated thymocytes (Fig. 4A). After 24 h of stimulation the mRNA expression levels of chRANKL, chRANK and chOPG were not statistically significantly altered compared to levels in unstimulated cells (Fig. 4A). At a cellular level, the mRNA expression levels of all three molecules were detected in CD4^{+}, CD8β^{+}, TCRγδ^{+} and TCRαβ^{+} cells (Fig. 4B).

3.4. ChRANK and chOPG mRNA are differentially expressed in mature BMDC

In mammals, RANK surface expression is predominantly found on mature DC and osteoclasts (Anderson et al., 1997; Wong et al., 1997b) while OPG expression is in osteoblasts (Yasuda et al., 1998),
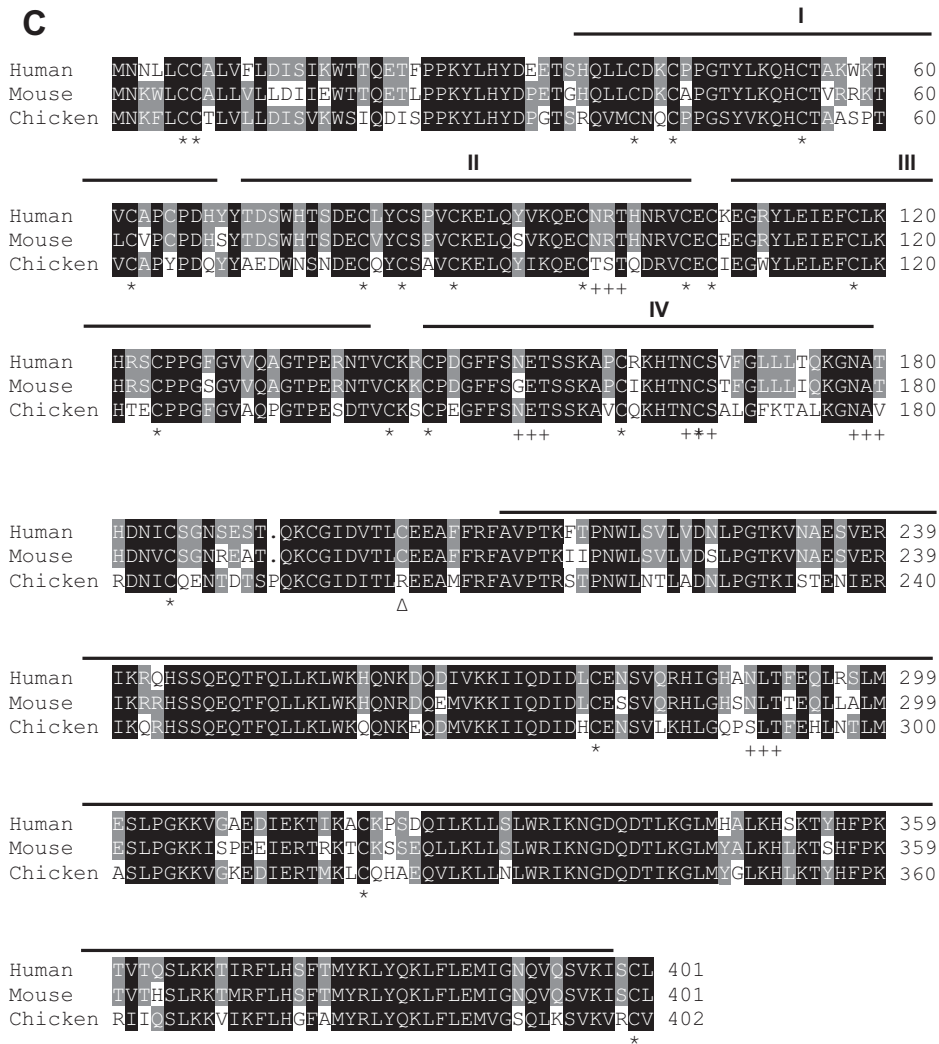
C

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                                                                    I
Human     MNNLLCCALVFLDISIKWTTQETFPPKYLHYDEETSHQLLCDKCPPGTYLKQHCTAKWKT  60
Mouse     MNKWLCCALLVLLDIIEWTTQETLPPKYLHYDPETGHQLLCDKCAPGTYLKQHCTVRRKT  60
Chicken   MNKFLCCTLVLLDISVKWSIQDISPPKYLHYDPGTSRQVMCNQCPPGSYVKQHCTAASPT  60
                **                           *   *          *         III

Human     VCAPCPDHYYTDSWHTSDECLYCSPVCKELQYVKQECNRTHNRVCECKEGRYLEIEFCLK  120
Mouse     LCVPCPDHSYTDSWHTSDECVYCSPVCKELQSVKQECNRTHNRVCECEEGRYLEIEFCLK  120
Chicken   VCAPYPDQYYAEDWNSNDECQYCSAVCKELQYIKQECTSTQDRVCECIEGWYLELEFCLK  120
              *       *        *   *        *+++    *  *           *
                                            IV
Human     HRSCPPGFGVVQAGTPERNTVCKRCPDGFFSNETSSKAPCRKHTNCSVFGLLLTQKGNAT  180
Mouse     HRSCPPGSGVVQAGTPERNTVCKKCPDGFFSGETSSKAPCIKHTNCSTFGLLLIQKGNAT  180
Chicken   HTECPPGFGVAQPGTPESDTVCKSCPEGFFSNETSSKAVCQKHTNCSALGFKTALKGNAV  180
             *       *        *  *        +++      *    +*+        +++

Human     HDNICSGNSEST.QKCGIDVTLCEEAFFRFAVPTKFTPNWLSVLVDNLPGTKVNAESVER  239
Mouse     HDNVCSGNREAT.QKCGIDVTLCEEAFFRFAVPTKIIPNWLSVLVDSLPGTKVNAESVER  239
Chicken   RDNICQENTDTSPQKCGIDITLREEAMFRFAVPTRSTPNWINTLADNLPGTKISTENIER  240
             *                    Δ

Human     IKRQHSSQEQTFQLLKLWKHQNKDQDIVKKIIQDIDLCENSVQRHIGHANLTFEQLRSLM  299
Mouse     IKRRHSSQEQTFQLLKLWKHQNRDQEMVKKIIQDIDLCESSVQRHLGHSNLTTEQLLALM  299
Chicken   IKQRHSSQEQTFQLLKLWKQQNKEQDMVKKIIQDIDHCENSVLKHLGQPSLTFEHLNTLM  300
                                              *           +++

Human     ESLPGKKVGAEDIEKTIKACKPSDQILKLLSLWRIKNGDQDTLKGLMHALKHSKTYHFPK  359
Mouse     ESLPGKKISPEEIERTRKTCKSSEQLLKLLSLWRIKNGDQDTLKGLMYALKHLKTSHFPK  359
Chicken   ASLPGKKVGKEDIERTMKLCQHAEQVLKLINLWRIKNGDQDTIKGLMYGLKHLKTYHFPK  360
                                *

Human     TVTQSLKKTIRFLHSFTMYKLYQKLFLEMIGNQVQSVKISCL  401
Mouse     TVIHSLRKTMRFLHSFTMYRLYQKLFLEMIGNQVQSVKISCL  401
Chicken   RIIQSLKKVIKFLHGFAMYRLYQKLFLEMVGSQLKSVKVRCV  402
                                                   *
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Fig. 1. (continued)

suggesting a role for the latter in regulating the interaction between RANKL–RANK in bone metabolism. OPG in human Mo-DC has a similar role, where OPG expression levels increase as DC mature (Schoppet et al., 2007), suggesting that OPG is also a molecular brake for RANKL–RANK interactions in the immune system. Before examining the bioactivity of schRANKL, the mRNA expression levels of both of its receptors were analysed in BMDC. BMDC were unstimulated or stimulated with various concentrations of LPS for 3, 6 and 9 h and mRNA expression levels of the two receptors analysed by qRT-PCR.

At 3 h, LPS-stimulated (200 ng/ml) cells had a ~ 2-fold increase in chRANK and chOPG mRNA expression levels which dropped to ~1-fold at the lower amounts of LPS used, compared to levels in unstimulated cells (Fig. 5). At 6 h post-stimulation with high LPS concentrations, chOPG mRNA expression levels were increased by nearly 3-fold while levels of chRANK mRNA expression were only increased by ~0.5-fold. At lower concentrations of LPS, chRANK mRNA expression levels decreased to basal unstimulated levels or lower (Fig. 5). Cells stimulated with 100 ng/ml of LPS increased chOPG mRNA expression levels statistically significantly compared to chRANK mRNA expression levels ($p < 0.05$). At 9 h, chOPG mRNA expression levels were statistically significantly higher compared to chRANK mRNA expression levels in all LPS-stimulated cells ($p < 0.05$).
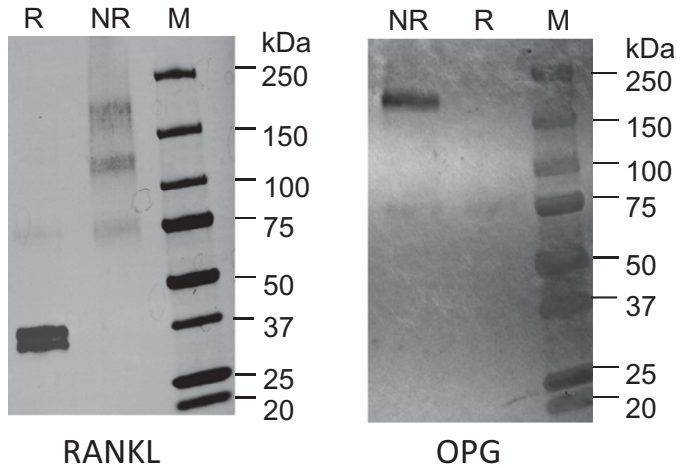
Fig. 2. Western blot analyses of schRANKL and chOPG proteins. The extracellular soluble domain of chRANKL (schRANKL) was sub-cloned into a modified pCI-neo vector, expressing an NH_2-terminal FLAG-tag and an in-frame isoleucine zipper sequence to encourage trimer formation. ChOPG was sub-cloned into a pKW06 vector and expressed as a human Fc-tagged protein. Samples were analysed under reducing (R) and non-reducing (NR) conditions. Marker (M) = protein precision plus (BioRAD).
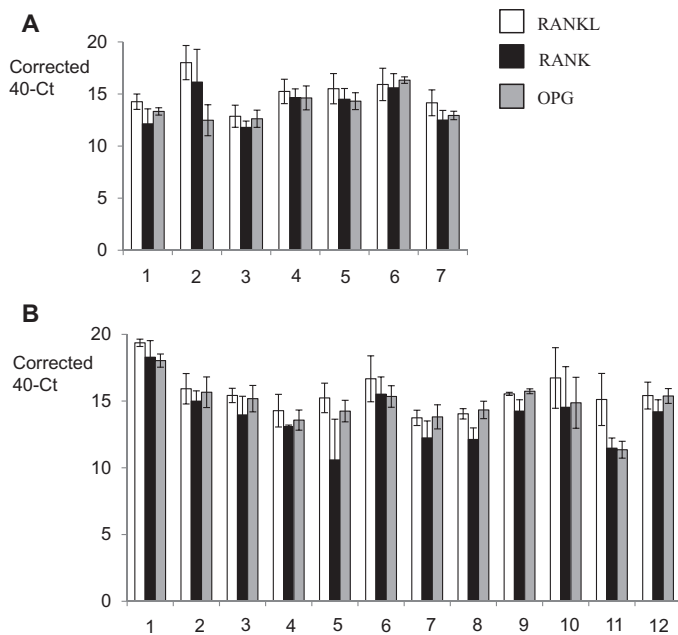
Fig. 3. ChRANKL, chRANK and chOPG mRNA expression in chicken tissues. Expression profiles of chRANKL, chRANK and chOPG mRNA in non-lymphoid and lymphoid tissues, as measured by qRT-PCR. (A) Non-lymphoid tissues: (1) brain, (2) muscle, (3) heart, (4) liver, (5) kidney, (6) lung, (7) skin. (B) Lymphoid tissues: (1) thymus, (2) spleen, (3) bursa of Fabricius, (4) caecal tonsil, (5) Harderian gland, (6) bone marrow, (7) Meckel's diverticulum, (8) caeca, (9) mid-gut, (10) upper-gut, (11) crop, (12) gizzard. Data are presented as the average corrected 40-Ct values of triplicate assays on each of three individual birds ± SEM.

Fig. 4. ChRANKL, chRANK and chOPG mRNA expression in chicken immune cells. ChRANKL, chRANK and chOPG mRNA expression in lymphoid cells, as measured by real-time qRT-PCR. (A) Expression levels of chRANKL, chRANK and chOPG in ConA stimulated splenocytes, PMA and ionomycin stimulated bursal cells and PHA stimulated thymocytes for 24 h. Data are presented as fold change in comparison to levels in unstimulated control cells of triplicate assays on each of three individual birds ± SEM. (B) Sorted splenocytes. Data are presented as the average corrected 40-Ct values of triplicate assays on each of three individual birds ± SEM.

3.5. Transcriptional control of chRANKL mRNA expression in avian splenocytes

Our previous data have shown that ConA stimulation has no effect on chRANKL mRNA expression in chicken splenocytes. Therefore, to further understand the regulation of chRANKL transcription, splenocytes were activated with the Ca^{2+}-mobiliser/inducer, ionomycin or ionomycin and the PKC activator, PMA, to mimic the signals initiated by TCR activation for 2, 4 and 18 h. Levels of chRANKL mRNA expression were analysed by qRT-PCR (Fig. 6).

At 2 h post-stimulation with ionomycin alone, chRANKL mRNA expression levels were unaltered compared to those in unstimulated cells whereas costimulated cells had a ~ 3-fold, but not significant increase in chRANKL mRNA expression levels (Fig. 6A). At 4 h, ionomycin-stimulated cells statistically significantly increased chRANKL mRNA expression levels by ~5-fold compared to levels in unstimulated cells ($p < 0.05$). The addition of PMA in costimulated cells did not increase chRANKL mRNA expression levels any further. At 18 h, chRANKL mRNA expression levels in ionomycin-stimulated cells again were not statistically significantly different from levels in unstimulated controls. This could suggest that ionomycin-mediated Ca^{2+}-mobilisation declines over time and no longer induces chRANKL mRNA expression. At 18 h, chRANKL mRNA expression levels were statistically significantly increased in costimulated cells compared to ionomycin-only stimulated cells and unstimulated cells ($p < 0.05$), indicating that the concomitant activation of Ca^{2+}-mobilisation and the PKC pathway are necessary to drive the transcription of chRANKL in chicken splenocytes, after at least 4 h of co-stimulation.

To verify that the transcriptional regulation of chRANKL was linked to the activation and mobilisation of Ca^{2+}, splenocytes were stimulated with ionomycin or ionomycin with various concentrations of the Ca^{2+} channel and PKC inhibitor, TMB-8. The levels of

chRANKL mRNA expression were analysed in these cells after 18 h of treatment by qRT-PCR (Fig. 6B), and the addition of TMB-8 at all three concentrations reduced chRANKL mRNA expression levels to those in unstimulated cells.

3.6. The effects of chRANKL on mRNA expression levels of pro-inflammatory cytokines in BMDC and BMM

To examine the bioactivity of schRANKL on BMDC, recombinant protein (schRANKL fused to FLAG) was produced in COS-7 cells and its effect on pro-inflammatory cytokine expression by BMDC was determined. On day 6 of culture, BMDC were unstimulated or stimulated with LPS, schRANKL or both, for 3 and 6 h. mRNA expression levels of the pro-inflammatory cytokines, IL-1β, IL-6 and IL-12α, were analysed by qRT-PCR (Fig. 7A).

At both time-points, LPS-stimulated cells had upregulated IL-12α mRNA expression levels. Stimulation with schRANKL on its own did not induce IL-12α mRNA expression and costimulation with LPS did not affect IL-12α mRNA expression levels compared to those in cells stimulated only with LPS. IL-12α mRNA expression was not detected in unstimulated cells, and therefore these data are presented as corrected 40-Ct and not as fold change.

At 3 h, IL-1β mRNA expression levels were significantly increased (26-fold) in LPS-stimulated cells (Fig. 7A) compared to those in unstimulated cells ($p < 0.05$). Costimulated cells also had a significant increase in IL-1β mRNA expression levels compared to those in unstimulated cells ($p < 0.05$) but were not statistically significantly
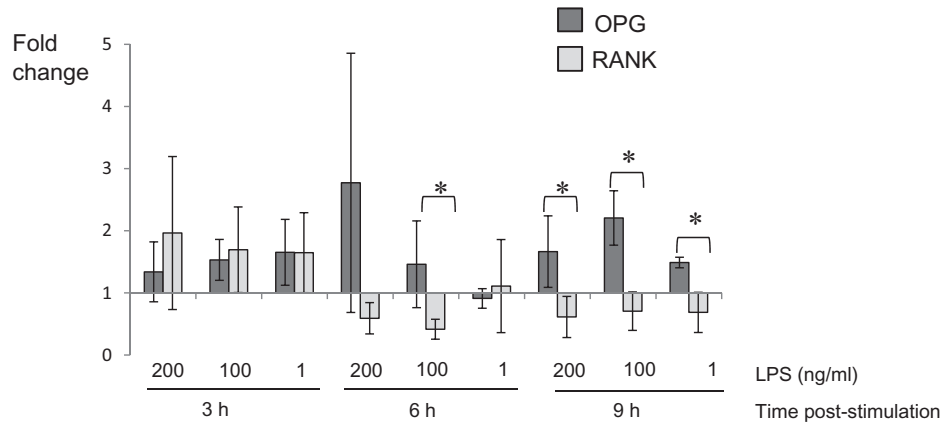
Fig. 5. Expression of chOPG and chRANKL in stimulated BMDC. Expression levels of chOPG and chRANK mRNA in BMDC following stimulation with LPS (200, 100 and 1 ng/ml) for 3, 6 and 9 h. Data are presented as fold change in comparison to levels in unstimulated control cells of triplicate assays on each of three individual birds ± SEM. *$p < 0.05$ as indicated (Mann–Whitney U test).

higher than those in LPS-stimulated cells (Fig. 7A). At 6 h, LPS-stimulation led to a significant (~52-fold) increase in IL-1β mRNA expression levels which were further statistically significantly increased to ~79-fold in co-stimulated cells compared to levels in unstimulated cells ($p < 0.05$) and statistically significantly higher than levels in LPS-stimulated cells ($p < 0.05$) (Fig. 7A). Treatment with schRANKL alone increased the mRNA expression levels of IL-1β at 6 h but this was not statistically significant compared to levels in unstimulated cells.

At 3 and 6 h, IL-6 mRNA expression levels were significantly increased in LPS- and co-stimulated cells compared to levels in

Fig. 6. Transcriptional regulation of chRANKL in chicken splenocytes. ChRANKL mRNA expression levels were analysed by qRT-PCR in (A) splenocytes stimulated with ionomycin (3 μg/ml), or ionomycin and PMA (500 ng/ml) for 2, 4 and 18 h and (B) splenocytes stimulated with ionomycin (3 μg/ml) or ionomycin and TMB-8 (10, 20 or 50 μM) for 18 h. Data are presented as fold change in comparison to levels in unstimulated control cells of triplicate assays on three individual birds ± SEM. *$p < 0.05$ compared to unstimulated control cells and #$p < 0.05$ as indicated (Mann–Whitney U test).

unstimulated cells ($p < 0.05$) (Fig. 7A). At 6 h, IL-6 mRNA expression levels in co-stimulated cells were significantly increased compared to those in LPS-stimulated cells ($p < 0.05$), suggesting that schRANKL enhances IL-6 mRNA expression in BMDC. Cells treated with schRANKL alone had a non-significant increase (~5-fold) in IL-6 mRNA expression levels at 3 h but there was no increase at 6 h (Fig. 7A).

Antagonistic effects of schRANK-Fc and chOPG-Fc were determined by their ability to inhibit the schRANKL-mediated upregulation of pro-inflammatory cytokines in BMDC. SchRANKL was pre-incubated with schRANK-Fc or chOPG-Fc for 3 h prior to stimulation of cells and IL-1β, IL-6 and IL-12α mRNA expressions levels were analysed (Fig. 7A). Incubation of schRANKL with either schRANK-Fc or chOPG-Fc prior to co-culture with LPS resulted in statistically significantly lower pro-inflammatory mRNA expression levels than those induced by co-culture with LPS and schRANKL (Fig. 7A). Therefore, pro-inflammatory cytokine mRNA expression levels induced by schRANKL in costimulated cells were inhibited by the addition of schRANK-Fc and chOPG-Fc.

The bioactivity of schRANKL on BMM was also determined by examining pro-inflammatory cytokine mRNA expression levels (Fig. 7B). Cells were unstimulated or simulated with LPS, schRANKL or both, for 3 and 6 h, similarly to BMDC. IL-12α mRNA expression levels were not detected in any sample (data not shown). At 3 h, LPS-stimulated cells increased IL-1β mRNA expression levels significantly by ~50-fold compared to those in unstimulated cells ($p < 0.05$) (Fig. 7B). SchRANKL treatment alone had no effect. Co-stimulation with LPS and schRANKL did not increase IL-1β mRNA expression levels further than LPS stimulation alone. At 6 h, IL-1β mRNA expression levels were still upregulated in LPS-stimulated cells but at a lower level than at 3 h. SchRANKL treatment alone again had no effect. Co-stimulated cells increased IL-1β mRNA expression levels but levels were not statistically significantly higher than those in unstimulated or LPS-stimulated cells.

IL-6 mRNA expression levels were increased in LPS and co-stimulated cells at both 3 and 6 h but levels were not statistically significantly compared to unstimulated cells, and co-stimulation did not increase IL-6 mRNA expression levels higher than those in LPS-stimulated cells (Fig. 7B).
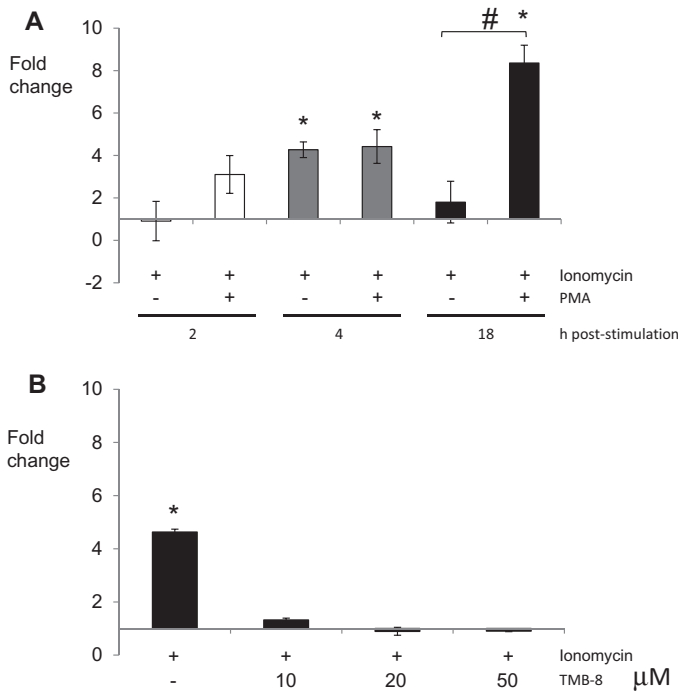
3.7. Cell surface marker expression of BMDC and BMM treated with schRANKL

To investigate the ability of schRANKL to enhance the expression of cell surface markers on BMDC and BMM, cells were treated
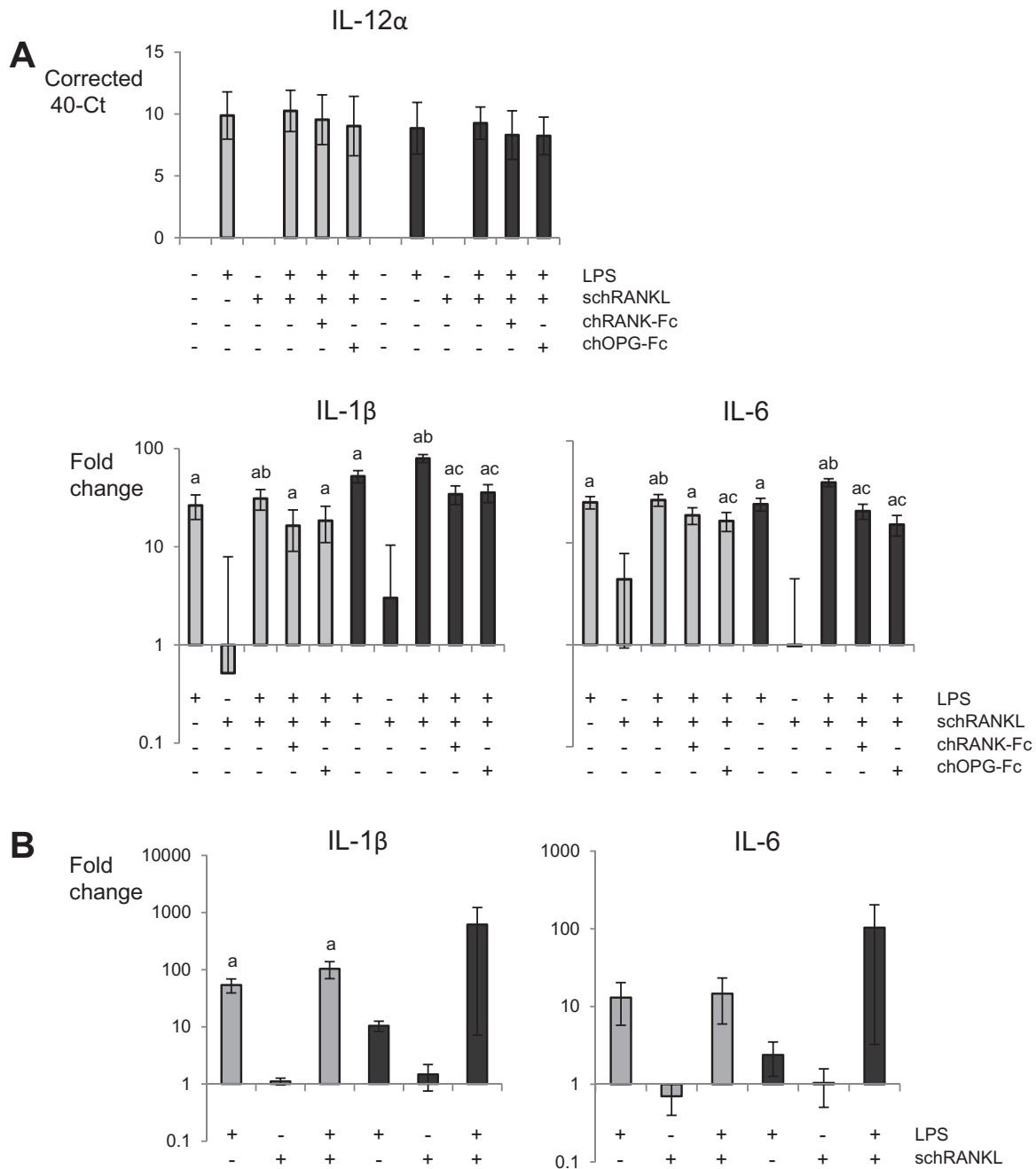
Fig. 7. Effect of chRANKL on pro-inflammatory cytokine expression in chicken BMDC and BMM. Pro-inflammatory cytokine mRNA expression levels in (A) BMDC and (B) BMM stimulated with schRANKL, as measured by qRT-PCR. (A) BMDC were unstimulated or stimulated with LPS (2 ng/ml), schRANKL (1:50 ex-COS) or co-stimulated with both for 3 (grey bars) or 6 h (black bars). To determine schRANKL bioactivity, schRANK-Fc or chOPG-Fc (both 1:5 ex-COS) were pre-incubated with schRANKL for 3 h prior to cell stimulation. (B) BMM were unstimulated or stimulated with LPS (1 ng/ml), schRANKL (1:50 ex-COS) or costimulated with both for 3 (grey bars) or 6 h (black bars). IL-12α mRNA expression levels (expression only seen in BMDC) are presented as corrected 40-Ct as expression was undetectable in unstimulated cells, IL-1β and IL-6 mRNA expression levels are presented as fold change in comparison to levels in unstimulated control cells. Data are presented as the average of between three (BMM) and six (BMDC) independent experiments ± SEM. (a) Data are statistically significant ($p < 0.05$) compared to unstimulated cells; (b) data are statistically significant ($p < 0.05$) compared to LPS-stimulated cells; (c) data are statistically significant ($p < 0.05$) compared to co-stimulated cells (Mann–Whitney U test).

with LPS (1–2 ng/ml), schRANKL (1:5 exCOS-7) or both, for 24 h (Fig. 8). Cells were removed from culture plates and stained for the chicken mononuclear phagocyte marker, KUL01 (Mast et al., 1998), and activation markers of chicken APC: MHC class II and CD40 (Wu et al., 2010).

Co-stimulation of BMDC with LPS, schRANKL or both did not increase the cell surface expression levels of MHC class II. CD40 cell surface expression levels were increased in LPS-stimulated cells but were not enhanced by addition of schRANKL (Fig. 8A). The

expression of KUL01 on the surface of BMDC decreases as the cells mature (Wu et al., 2010) and this is evident in the LPS-stimulated BMDC where there were lower cell surface expression levels of KUL01 than on the control cells (Fig. 8B). SchRANKL had no effect on cell surface expression of KUL01 when compared to the control BMDC. Co-stimulated cells had similar cell surface expression levels of KUL01 as the LPS-stimulated cells (Fig. 8A).

Similarly to BMDC, MHC class II expression levels were not increased by stimulation of BMM with LPS (1 ng/ml) or schRANKL,
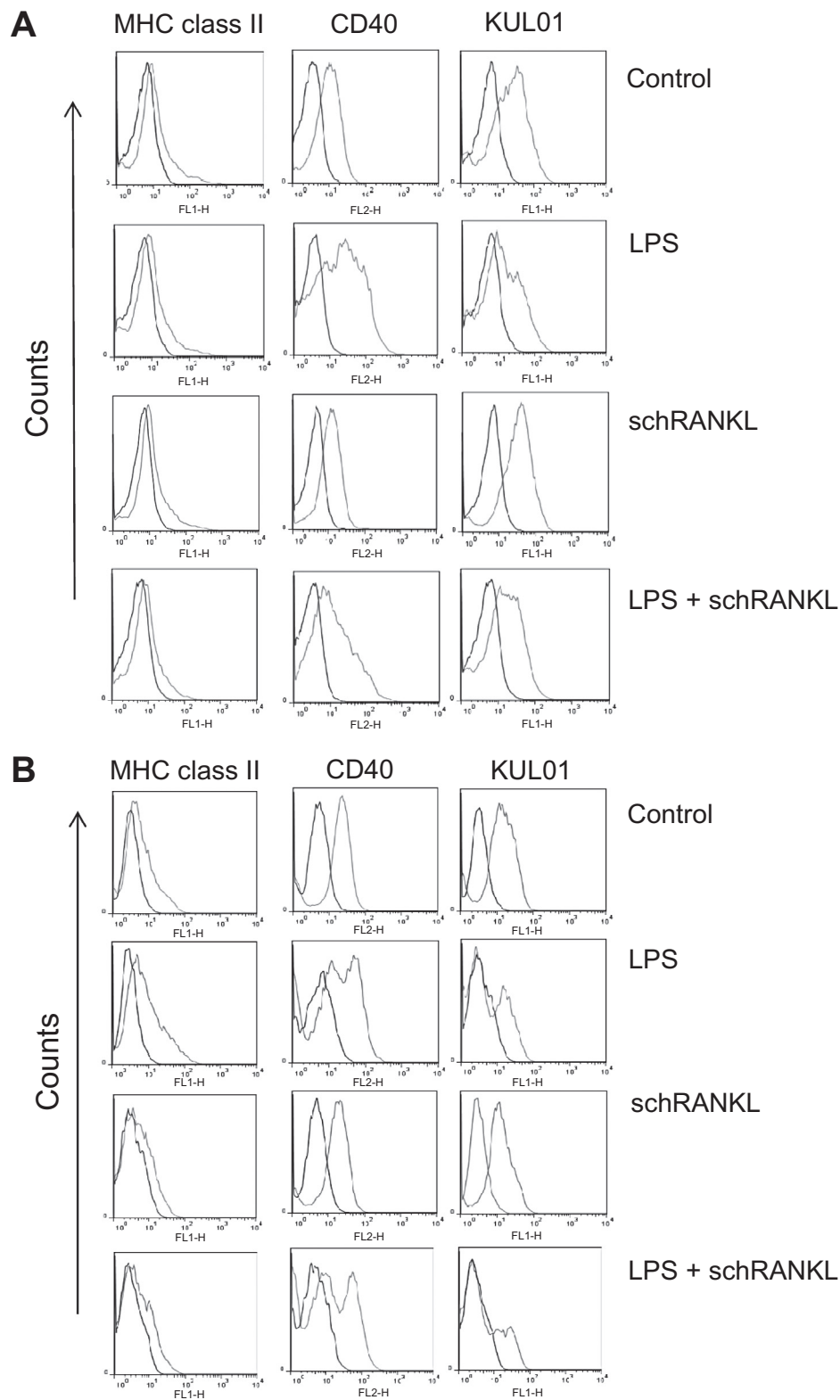
Fig. 8. Effect of schRANKL on the phenotype of chicken BMDC and BMM. (A) BMDC were unstimulated or stimulated with LPS (2 ng/ml), schRANKL (1:5 exCOS) or both for 24 h. (B) BMM were unstimulated or stimulated with LPS (1 ng/ml), schRANKL (1:5) or both for 24 h. Cell surface expression of MHC class II, CD40 and KUL01 were analysed by FACS. Data are represented as histograms comparing isotype controls (black lines) to surface staining (grey lines) and are representative of between four and six independent experiments with similar results.
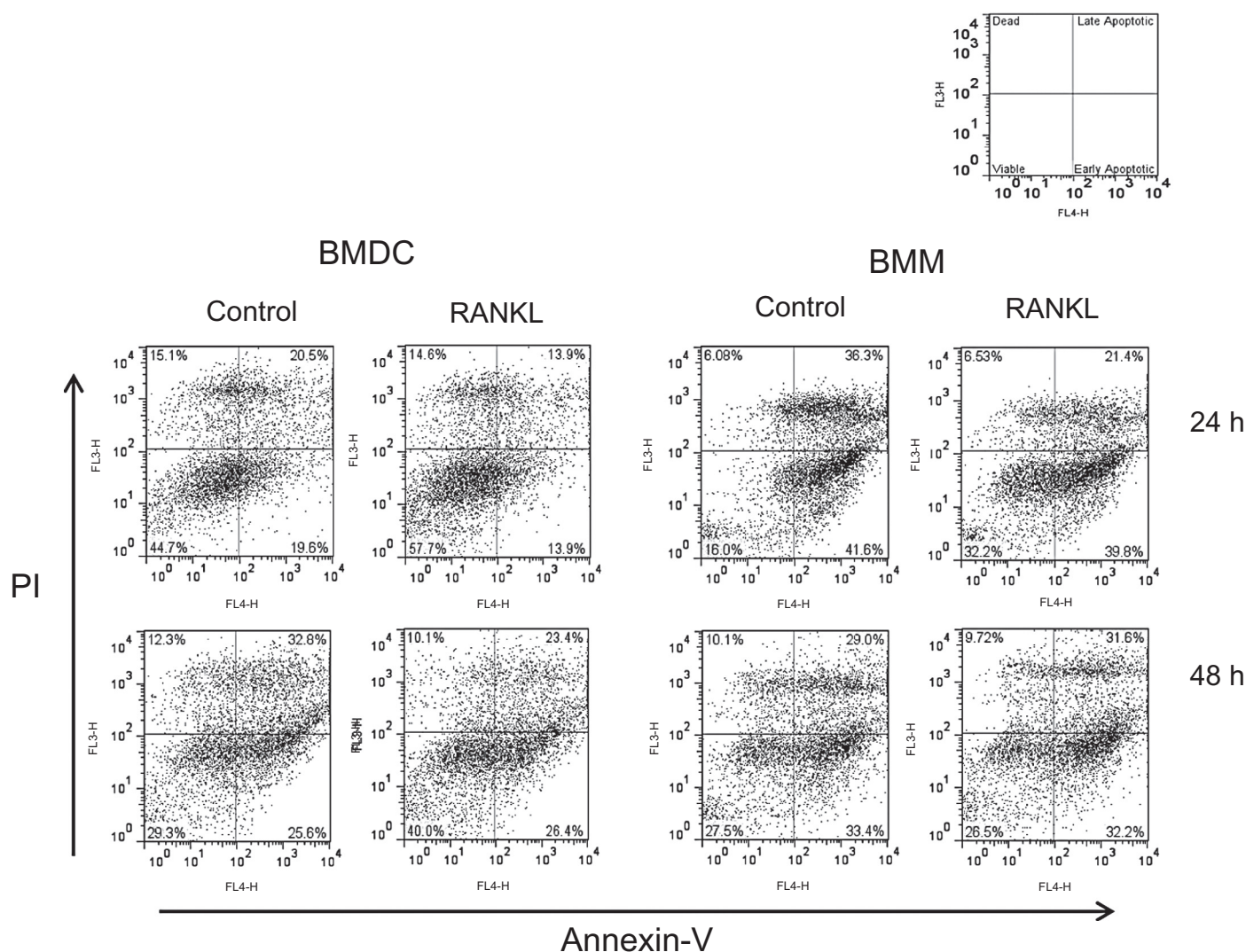
Fig. 9. Survival of BMDC and BMM treated with schRANKL. Cell survival was measured by staining for annexin-V and PI and analysed by FACS. BMDC and BMM were untreated or treated with schRANKL (1:5 ex-COS) for 24 and 48 h. Data are representative of three independent experiments with similar results.

either alone or in combination (Fig. 8B). CD40 cell surface expression levels were increased in LPS-stimulated BMM but were not enhanced by the addition of schRANKL (Fig. 8B), which by itself had no effect. KUL01 cell surface expression levels were decreased in LPS-stimulated and co-stimulated BMM compared to unstimulated or schRANKL-stimulated BMM (Fig. 8B).

3.8. Survival of BMDC and BMM treated with schRANKL

To examine whether chRANKL was a survival factor for BMDC and BMM, cells were treated with or without schRANKL (1:5 exCOS-7) for 24 and 48 h (Fig. 9). Cell death and apoptosis were measured by annexin-V and PI staining and analysed by FACS.

After 24 h, 44.7% of untreated BMDC were viable (annexin-V⁻ PI⁻) and 55.3% positive for annexin-V (annexin-V⁺ PI⁺) (Fig. 9). This indicates that over half of the cell population analysed under normal conditions was undergoing apoptosis. In contrast, schRANKL-treated cells had 57.7% viable cells and 27.8% undergoing apoptosis. After 48 h, the number of viable untreated BMDC fell to 29% in comparison to schRANKL-treated cells where 40% were viable (Fig. 9). Cells undergoing apoptosis in untreated cells increased to 58.4% but for schRANKL-treated cells apoptotic cells were 49.8% (Fig. 9). Overall

the data suggest that schRANKL acts as a survival factor for chicken BMDC.

After 24 h, untreated BMM had a very low number of viable cells (16.1%) whereas for schRANKL-treated BMM viability numbers were 32.2%. Cells undergoing apoptosis were lower in chRANKL-treated cells (61.2%) compared to untreated cells (77.9%) (Fig. 9). A further 24 h of cell culture resulted in similar numbers of viable and apoptotic cells between untreated and schRANKL-treated BMM. SchRANKL can enhance the survival of BMM at 24 h of stimulation but this enhanced survival is short-lived, as cells begin to undergo similar rates of apoptosis after 48 h.

4. Discussion

In mammals, the majority of TNF superfamily members are present as small loci of two or three genes on different chromosomes – the chicken lacks three of these small sub-families: LT-α, TNF-α and LT-β (TNFSF1-3); 4-1BBL, CD27L and LIGHT (TNFSF9, 7 and 14); TWEAK and APRIL (TNFSF12 and 13) (Kaiser et al., 2005). It has been hypothesised that the lack of lymph nodes in the chicken is due to the absence of the lymphotoxin genes. Mice lacking some of the other missing genes in the chicken have defective T and B

cell responses (Locksley et al., 2001). The absence of the other TNF superfamily members has not yet been linked to any abnormal development or obvious difference in the chicken's ability to mount an immune response. However, it is therefore important to study the biological function of those chicken TNF superfamily members that are present in more detail.

Mammalian RANKL is a type II transmembrane protein that can function as either a membrane-bound or secreted form (Ikeda et al., 2001; Walsh et al., 2013), with no known functional difference between the two (Nakashima et al., 2000). An aa alignment of full-length chRANKL with those of human and mouse showed high conservation across all species. The RANKL monomer consists of two anti-parallel β-sheets which are formed by various β-strands (Lam et al., 2001). Using the PSIPRED prediction program, the locations of the residues involved in β-sheet formation in chRANKL were found to be conserved. SchRANKL contains ten β-strands and the four loops required for assembly of a homotrimer (Fig. 1A). To investigate the ability of schRANKL to form homotrimers, the extracellular domain of chRANKL was sub-cloned into a modified vector expressing an in-frame isoleucine zipper previously shown to enhance the bioactivity of members of the TNF superfamily by encouraging and stabilising trimeric protein structures (Kim et al., 2004; Morris et al., 1999). SchRANKL protein structures were analysed under both reducing and non-reducing conditions (Fig. 2) and the data suggest that schRANKL forms homotrimeric protein structures, a signature of TNF superfamily members.

Human and mouse OPG cDNAs encode a 401 aa protein that is further processed to form a 380 aa mature protein. OPG is synthesised as a glycosylated 55 kDa protein which self-associates to form disulphide-linked dimers prior to secretion from cells. ChOPG was previously cloned by Hou et al. (2011). However, no sequence alignment or analysis was presented in that study to investigate the conservation of the CRD, DD and penultimate cysteine residue required for OPG dimer assembly (Simonet et al., 1997). An aa alignment of chOPG with those of human and mouse showed it to be highly conserved (64–65% aa identity). ChOPG, similar to mammalian OPG, naturally forms soluble homodimeric proteins (Fig. 2).

ChRANKL, chRANK and chOPG mRNA expression levels were ubiquitous across a range of lymphoid and non-lymphoid tissues (Fig. 3) and immune cells (Fig. 4) in the chicken. In the bone marrow, all three molecules were highly expressed at the mRNA level, which was not surprising as all three have vital roles in bone metabolism in mammalian species (Simonet et al., 1997; Yasuda et al., 1998). In non-lymphoid organs, chRANKL and chRANK mRNA expression levels were highest in muscle, similarly to mammalian RANKL and RANK (Anderson et al., 1997; Wong et al., 1997a). The heart had the lowest mRNA expression levels of all three molecules amongst the non-lymphoid tissues examined.

In the upper gut, levels of chRANKL mRNA expression were high. In the mammalian gut-associated lymphoid tissues (GALT), sampling of lumenal pathogens is achieved by M cells (Mabbott et al., 2013). M cells are highly efficient phagocytic cells that uptake bacterial antigen from the lumen into the Payer's patches, priming CD4+ T cells and IgA+-committed B cells (VanCott et al., 1996). In mammals, RANKL is necessary for the differentiation of RANK-expressing enterocytes into M cells (Knoop et al., 2009). Recently the generation of CSF1 transgenic chickens identified the large number of macrophages in the chicken gut (Balic et al., 2014) therefore it would be interesting to determine if the high levels of chRANKL mRNA expressed in the upper gut correlated with the presence of large numbers of M cells in this tissue.

The expression of both RANK and OPG has been observed on mature DC in mammals (Anderson et al., 1997; Wong et al., 1997a; Yasuda et al., 1998). To investigate the expression patterns of the chRANKL receptors on chicken BMDC, BMDC were generated and

then unstimulated or stimulated with various concentrations of LPS and the levels of chRANK and chOPG mRNA expression analysed by qRT-PCR (Fig. 5). The data suggest that the mRNA expression levels of chRANK and chOPG in BMDC are both dose- and time-dependent. The more the BMDC matured, the lower the levels of chRANK mRNA expression and the higher the levels of chOPG mRNA expression observed. Mammalian OPG expression is dependent on activation of the NF-κB pathway and increases as DC mature (Schoppet et al., 2007). It is therefore possible that mature chicken BMDC increase levels of chOPG expression to regulate the magnitude of chRANKL+ T cells interacting with chRANK+ DC and chOPG is therefore a potential marker for mature chBMDC.

The expression of RANKL by mammalian T cells is induced by their activation and is dependent on Ca2+ mobilisation (Wang et al., 2002). To understand whether the mechanism of chRANKL expression was conserved between mammals and birds, chicken splenocytes were stimulated with the intracellular calcium modulator, ionomycin, and the PKC activator, PMA, which mimic TCR activation, for 2, 4 and 18 h and levels of chRANKL mRNA expression were analysed by qRT-PCR (Fig. 6A). In co-stimulated cells the levels of chRANKL mRNA expression were significantly increased after 4 and 18 h indicating that ionomycin and PMA work in synergy to induce chRANKL mRNA expression. In murine T cells, ionomycin alone was sufficient to induce RANKL expression and no synergy was found when cells were co-stimulated with ionomycin and PMA (Wang et al., 2002). In similar studies, murine T cell hybridomas weakly induced RANKL after PMA stimulation but a combination of PMA and ionomycin synergised and induced higher levels of RANKL expression (Bishop et al., 2011; Fionda et al., 2007). The transcriptional control of chRANKL and mammalian RANKL by Ca2+ modulation and PKC activation, as verified by inhibition studies (Fig. 6B), is conserved between mammals and chickens.

The ability of schRANKL to induce or enhance pro-inflammatory cytokine expression in immature or mature chicken APC was analysed by qRT-PCR (Fig. 7). APC were either unstimulated or stimulated with LPS, schRANKL or both for 3 and 6 h. Costimulation of BMDC with schRANKL and LPS led to a significant increase in pro-inflammatory cytokine mRNA expression levels compared to those in unstimulated BMDC and, in some cases, LPS-stimulated BMDC. SchRANKL-treatment alone did not significantly alter pro-inflammatory cytokines mRNA expression levels. BMM costimulated with LPS and schRANKL significantly increased IL-1β mRNA expression levels in comparison to unstimulated cells but not LPS-stimulated cells. Pre-incubation with both schRANK-Fc and chOPG-Fc decreased the schRANKL-mediated increase in pro-inflammatory cytokine mRNA expression levels (Fig. 7). This also indicated that the soluble receptors of chRANKL were capable of interacting with their ligand.

SchRANKL treatment did not affect the expression levels of MHC class II either alone or in combination with LPS (Fig. 8). CD40 expression levels were increased in LPS-stimulated cells but were not enhanced by the addition of schRANKL. The expression pattern of KUL01 was also analysed; although it is not an activation marker like CD40 and MHC class II, KUL01 expression decreases as chicken APC mature (Wu et al., 2010). This was therefore a good marker to identify the ability of schRANKL to induce cell maturation. As expected, at 24 h LPS-treated cells had decreased KUL01 expression but expression levels thereof were unaltered by schRANKL treatment. Overall schRANKL does not mediate the upregulation of cell surface activation markers in chicken BMDC, similarly to mammalian RANKL (Anderson et al., 1997; Wong et al., 1997a).

Studies on the biological effect of mammalian RANKL on DC have produced contrasting evidence as to its effect on their phenotype. One of the first studies examining the biological role of mammalian RANKL proposed that RANKL-treated murine BMDC did not significantly increase expression levels of MHC class II, CD80 or CD86

but did slightly (not significantly) upregulate the expression of CD40 (Anderson et al., 1997; Wong et al., 1997a). Monocyte-derived DC treated with RANKL increased surface expression of MHC class II, CD80 and CD86 (Seshasayee et al., 2004), suggesting that DC derived from different sources react differently to RANKL exposure. One study (Park et al., 2005) on the bioactivity of mammalian RANKL on macrophages suggested that RANKL-treated cells upregulated MHC class II expression which was enhanced when cells were co-stimulated with LPS but not with IFN-γ. It is important to note that DC may express RANK but react differently in their response to RANKL depending on their tissue of origin. Our data indicate that chRANKL has no effect on the phenotype of BMDC or BMM alone or in combination with LPS (Fig. 8).

The lifespan of DC can influence the duration of lymphocyte activation, thereby affecting the outcome of immune responses. Mammalian RANKL is a survival factor for DC and macrophages (Anderson et al., 1997; Cremer et al., 2002; Park et al., 2005; Wong et al., 1997a). To determine the ability of schRANKL to enhance the survival rates of BMDC and BMM, cells were untreated or treated with schRANKL for 24 and 48 h (Fig. 9). BMDC survival rates were increased in schRANKL-treated cells compared to untreated cells after 24 h and 48 h (Fig. 9). After 24 h, the number of viable cells was increased in schRANKL-treated BMM compared to untreated cells. However, after 48 h schRANKL-treated cells had similar numbers of viable cells as untreated cells. It seems that the ability of schRANKL to enhance the survival of BMM is short-lived. Various mammalian studies have shown that the upregulation of the Bcl-2 family members, Bcl-X_L and Bcl-2, are linked to RANKL-mediated cell survival (Izawa et al., 2007; Josien et al., 1999; Wong et al., 1997a) and genetic and molecular analysis from nematodes to humans indicates that programmed cell death is highly conserved (Ellis et al., 1991), so it is therefore plausible that chRANKL may upregulate the levels of anti-apoptotic chicken Bcl-2 family members in BMDC and BMM.

5. Conclusion

In summary, chRANKL, chRANK and chOPG mRNA expression has been analysed in different tissues and cells in the chicken. Expression of chRANKL mRNA and its receptors was detected across a wide range of tissues. Both of the receptors for chRANKL are differentially expressed in mature BMDC. The bioactivity of chRANKL has been analysed in both BMDC and BMM indicating conservation of RANKL bioactivities between mammalian and chicken in BMDC but not completely in BMM.

Acknowledgments

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