The Pekin duck programmed death ligand-2: cDNA cloning, genomic structure, molecular characterization and expression analysis

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ABSTRACT

Programmed death-1 (PD-1), upon engagement by its ligands, programmed death ligand-1 (PD-L1) and programmed death ligand-2 (PD-L2), provides signals that attenuate adaptive immune responses. Here we describe the identification of the Pekin duck PD-L2 (duPD-L2) and its gene structure. The duPD-L2 cDNA encodes a 321 amino acid protein that has an amino acid identity of 76% and 35% with chicken and human PD-L2, respectively. Mapping of the duPD-L2 cDNA with duck genomic sequences revealed an exonic structure similar to that of the human P*dcd1lg2* gene. Homology modelling of the duPD-L2 protein was compatible with the murine PD-L2 ectodomain structure. Residues known to be important for PD-1 receptor binding of murine PD-L2 were mostly conserved in duPD-L2 within sheets A and G and partially conserved within sheets C and F. DuPD-L2 mRNA was constitutively expressed in all tissues examined with highest expression levels in lung, spleen, cloaca, bursa, cecal tonsil, duodenum and very low levels of expression in muscle, kidney and brain. Lipopolysaccharide treatment of adherent duck PBMC upregulated duPD-L2 mRNA expression. Our work shows evolutionary conservation of the PD-L2 ectodomain structure and residues important for PD-1 binding in vertebrates including fish. The information provided will be useful for further investigation of the role of duPD-L2 in the regulation of duck adaptive immunity and exploration of PD-1-targeted immunotherapies in the duck hepatitis B infection model.

1. Introduction

The inhibitory receptor programmed death-1 (PD-1, CD279) [1] and its ligands programmed death ligand-1 (PD-L1, B7-H1, CD274) [2] and programmed death ligand-2 (PD-L2, B7-DC, CD273) [3] play a key role in the attenuation of adaptive immune responses and peripheral tolerance. The B7 family members PD-L1 and PD-L2 are currently the only known ligands for PD-1. However, PD-L1 can also engage B7-1 (CD80) [4] and PD-L2 has recently been shown to interact with repulsive guidance molecule B (RGMb), thereby regulating respiratory T cell immunity [5] beyond the currently known co-inhibitory pathways of the B7: CD28 family [6].

The two PD-1 ligands are immunoglobulin superfamily (IgSF) members that contain tandem IgV-and IgC-like IgSF domains and a cytoplasmic tail of unknown function. PD-L2 is encoded by the P*dcd1lg2* gene on human chromosome 9 in close proximity to the P*dcd1lg1* gene

that encodes PD-L1 [7]. The human P*dcd1lg2* gene is comprised of seven exons and six introns, whereas the murine P*dcd1lg2* gene is comprised of 6 exons and 5 introns and its exon 5 contains a stop codon that results in a truncated cytoplasmic domain of 5 aa. The crystal structures have been determined for the complexes formed by the ectodomains of murine PD-L2 (mPD-L2) and murine PD-1 (mPD-1) [8], human PD-L1 (hPD-L1) and mPD-1 [9], and those of hPD-1 and hPD-L1 [10].

PD-L1 is constitutively and inducibly expressed in a wide range of hematopoetic and non-hematopoetic cells, whereas PD-L2 expression was initially thought to be more restricted and mainly found on antigen presenting cells, such as activated dendritic cells (DCs) and macrophages, and murine B1 B cells [11]. Additionally, PD-L2 has been shown to be expressed on various other human cells including endothelial cells, epithelial cells, fibroblasts and T cells; and PD-L2 is inducibly expressed on monocytes and macrophages by T-helper

Table 1
PCR primers and cycle conditions.

Name(position)	Sequence(5′→ 3′)
FF (1–24)	ATGTTCCAAATCCTGACACTGCTG
FFB(1–20)	CGggatccATGTTCCAAATCCTGACACT
F1 (735–758)	ATCCCTGATCTTATTTATCATCGC
F2 (774–795)	AACAGTCCTTCTCTCTGCCTTA
FR (966–946)	TCACAGACTCACACTCCCACT
FRE(966–946)	CGgaattcTCTCAGACTCACACTCCCACT
R1 (385–367)	CACCCTGGTAGTCAATAAG
R2 (252–235)	AGATGGAGGGAGTGCTTT
R3 (150–133)	CAACGAGCCATTCACAGG

Denaturation – 94 °C, 3 min; amplification 1–35 cycles – 94 °C, 30 s; 58 °C, 30 s; 72 °C, 60 s; final extension – 72 °C, 5 min.

Table 2
Real-time PCR primers, probes and cycle conditions.

Name(position)	Sequence(5′→ 3′)
qF-PDL2(14–35)	TGACACTGCTGTTGCTGGAAAT
qR-PDL2(99–77)	CACAGCAACATACAGCTGCTGAG
qP-PDL2(38–72)	/6-FAM/ AGCTCTGGGTGGTTTCAGGTTTATTTACAGTTGAA/ TAMRA-sp/
qF2-GAPDH (284–305)	TCCACCGGTGTCTTCACCA
qR2-GAPDH (308–333)	GAGATGATGACACGCTTAGCACC
qP2-GAPDH (359–337)	/6-FAM/TGGAGAAGGCTGGTGCTCACCTGAAG/TAMRA-sp/

50 °C 2 min; 95 °C 10 min; 95 °C 15 s and 60 °C 1 min for 45 cycles. Primers and probes are based on duck PD-L2 (GenBank ID, KU674826), and duck GAPDH sequence deposited by BGI (ENSAPLT00000014139, Pre Ensembl release 63 - September 2011).

cytokines, common γ-chain cytokines, GM-CSF and TLR ligands, and upon T-cell activation [12].

Inhibitory signaling through PD-1 is recognized for its important role in T cell exhaustion in persistent viral infections and tumor immunity [13]. Recently, therapeutic targeting of the PD-1: PD-L1 pathway has led to breakthroughs in the treatment of cancer [14]. To date, three humanized monoclonal antibodies (Mabs), nivolumab and pembrolizumab targeting PD-1 and atezolizumab targeting PD-L1, have obtained FDA approval. Nivolumab has been explored as a treatment for chronic hepatitis C [15]. Albeit in this small pilot study antiviral effects were highly variable, further development of therapeutic targeting of co-stimulatory pathways is of interest for chronic viral infections for which the induction of a functional cure or a sustained viral clearance remain an unattained treatment goal. Blockade of PD-1 has been shown to reverse the exhausted T cell phenotype in patients with chronic hepatitis B in *in vitro* studies [16]; and to enhance viral clearance in woodchucks chronically infected with the woodchuck hepatitis B virus [17].

Ducks infected with duck hepatitis B virus (DHBV) serve as a valuable natural disease model for hepatitis B infection amenable for the exploration of novel therapies for chronic hepatitis B [18,19]. In spite of the recent sequencing of the duck genome [20], studies on viral immunopathogenesis remain hampered by a limited repertoire of characterized immune genes and reagents available to study duck immune responses. Here we describe the cloning, sequence analyses and 3D modelling of the Pekin duck PD-L2 (duPD-L2). Furthermore, we analyze duPD-L2 mRNA expression in tissue and in lipopolysaccharide (LPS)-treated adherent and non-adherent peripheral blood mononuclear cells (PBMC). The identification of duPD-L2 will facilitate further work on the role of PD-1 signaling in the regulation of duck adaptive immunity relevant to studies on animal and human health.
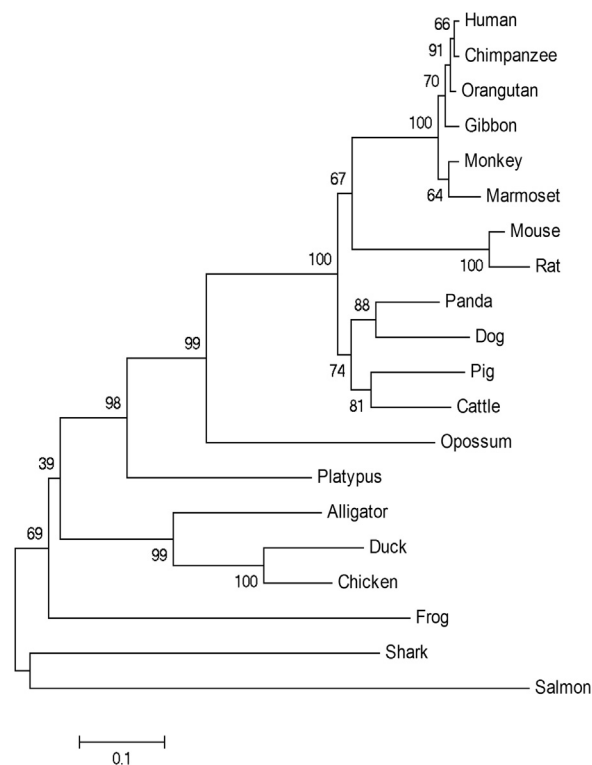

Fig. 1. Phylogenetic tree of PD-L2 proteins. Amino acid sequences were aligned using ClustalW and the phylogenetic tree was generated with MEGA 4 using the neighbor-joining method. Branches were validated by bootstrap analysis from 500 repetitions and are represented by numbers at the branch nodes. Bar indicates 0.1 substitutions per site. Protein sequences were extracted from GenBank with the following accession numbers: Human (*Homo sapiens*, NP_079515); chimpanzee (*Pan troglodytes,* XP_001140776); orangutan (*Pongo abelii*, XP_002819857); gibbon, (*Nomascus leucogenys*, XP_003273875); monkey (*Macaca mulatta*, NP_001077068); marmoset (*Callithrix jacchus*, XP_002742957); mouse (*Mus musculus*, NP_067371); rat (*Rattus norvegicus*, NP_001101052); panda (*Ailuropoda melanoleuca*, XP_002923722); dog (*Canis familiaris*, XP_852105); pig (*Sus scrofa*, NP_001020391); cattle (*Bos taurus*, BAO74173); opossum (*Monodelphis domestica*, XP_001371408); platypus (*Ornithorhynchus anatinus*, XP_001506123); chicken (*Gallus gallus*, XP_004949124.1); alligator (*Alligator mississippiensis*, XP_006268964); frog (*Xenopus tropicalis*, XP_012811615); shark (*Callorhinchus milii*, XP_007904718); salmon (*Salmo salar,* XP_013980940). The sequence for duPD-L2 is deposited in GenBank under accession number KU674826.

2. Materials and methods

2.1. Animals, tissues and cell cultures

Pekin ducks (*Anas platyrhynchos*) were from a flock maintained at the University of Alberta. Animals were housed, maintained and handled according to the guidelines of the Health Sciences Laboratory Animal Services (HSLAS), University of Alberta. Tissues from two-month-old Pekin ducks were resected at necropsy, snap-frozen in liquid nitrogen and stored at −70 °C. PBMC from healthy ducks were isolated by using Ficoll-Hypaque (GE Healthcare, Baie d'Urfe, Quebec, Canada) and centrifugation, suspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin, streptomycin and sodium bicarbonate and cultured at 37 °C with 5% CO_2 in air. Adherent cells were generated from PBMC cultured in 6-well plates for 6 h, that were then washed three times with PBS and adherent cells were then cultured for 18 h with or without LPS (final concentration 5 µg/ml; Sigma, Oakville, Ontario, Canada) and then harvested in Trizol (Invitrogen, Burlington, Ontario, Canada). Scrapings of frozen tissues were placed directly in Trizol and homogenized with a micropestle before RNA isolation [21]. Total RNA was isolated from tissues and PBMC using Trizol reagent according to the manufacturer's protocol.
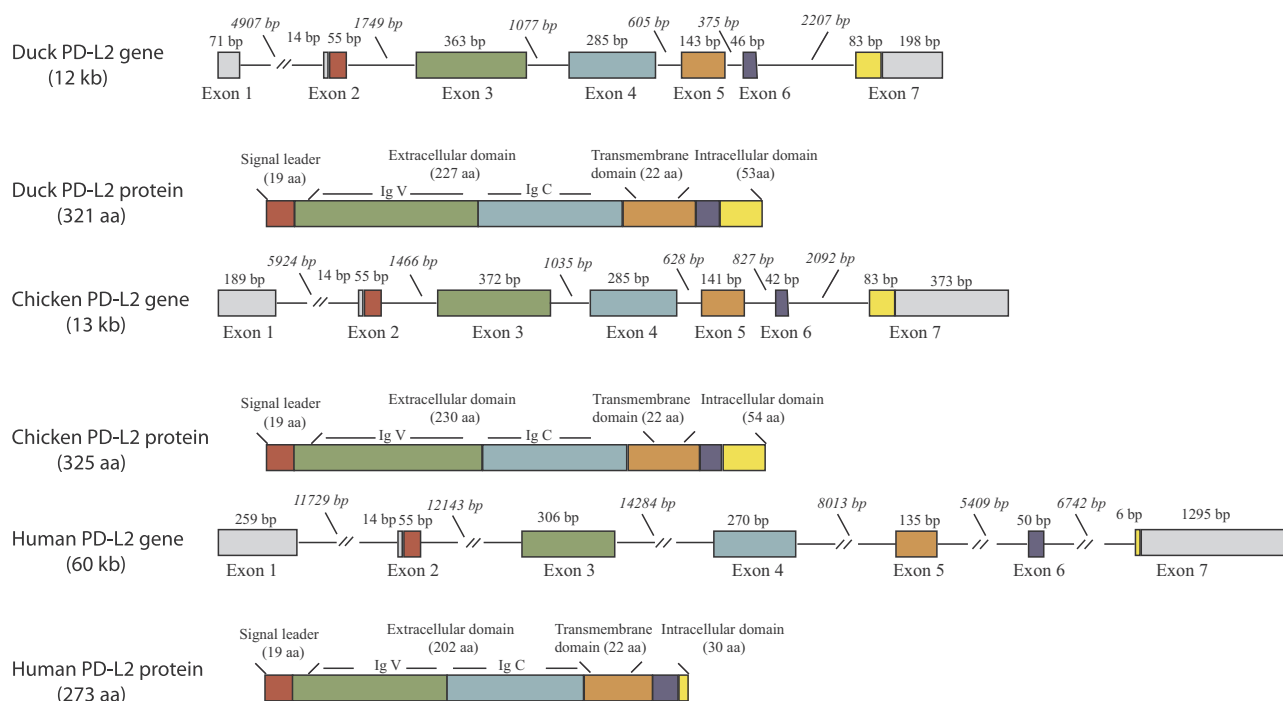
Fig. 2. Exonic structure and domain organization of duck, chicken and human PD-L2. Chicken and human genomic accession numbers obtained from the GenBank database are provided under materials and methods. Splign available at http://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi was used to analyze duck PD-L2 genomic sequences. Distances indicated are not drawn to scale.

2.2. Identification and cloning of the duck PD-L2 cDNA and genomic sequences

A partial coding sequence of duPD-L2 (Beijing Genomic Institute (BGI) accession no., ENSAPL00000004103) lacking 5′ terminal and 3′ terminal sequence was found by blastP using human PD-L2 (GenBank ID: NP_079515) within the BGI duck genomic database (http://uswest.ensembl.org/Anas_platyrhynchos/Tools/Blast?db=core). The nucleotide sequence at the 5′ and 3′ ends of the duPD-L2 ORF and untranslated regions were obtained by RACE using commercially available kits from Invitrogen and RT-PCR. Total RNA from duck lung was used as source material. First-strand cDNA synthesis was performed using 1 μg of RNA, Super Script II reverse transcriptase (Invitrogen) and primer R1 (Table 1) followed by polymerase chain reaction using Taq polymerase (Invitrogen), according to the manufacturer's protocols. Two internal 5′ RACE primers R2 and R3 (Table 1) were used for 1st and 2nd round PCR to amplify duPD-L2 in a reaction containing 0.1 μg of cDNA and PCR buffer (Invitrogen) containing 1.5 mM MgCl₂ and 0.2 mM dNTP. Primers F1 and F2 were used for 3′RACE. PCR parameters were as follows: Initial denaturation at 94 °C for 3 min followed by 35 cycles at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min, and at the end of the thermal cycling, reactions were incubated at 72 °C for 5 min. Following agarose electrophoresis (1%), amplicons were isolated using a gel extraction kit (Qiagen Inc., Mississauga, Ontario) and cloned into the plasmid pCR4-TOPO (Invitrogen) according to the manufacturer's protocol. Clones bearing inserts were sequenced, and BLAST comparison identified clones having an ORF showing amino acid homology with the human PD-L2. The duPD-L2 ORF was amplified by FF and FR (Table 1). Amplicons were cloned into pCR4-TOPO and three additional clones were sequenced to derive the consensus sequence for duPD-L2 (GenBank ID: KU674826).

2.3. Design of primer and probes for quantitative real-time PCR (qRT-PCR)

The design of primers and probes (Table 2) was carried out using Primer Express 3.0 Software (Applied Biosystems). The primer set (qF-PDL2, qR-PDL2) amplifies a 86-base pair fragment of duPD-L2. The probe of duPD-L2 (qF-PDL2) was designed to anneal across the exon 2 / exon 3 junction. The primer set (qF2-GAPDH, qR2-GAPDH) used for amplification of duck glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, labeling of probes and PCR conditions were identical as previously described [22,23]. Cycle threshold (Ct) values were calculated with sequence-detection system software v2.3 (Applied Biosystems). The data was normalized using GAPDH as an internal standard and expressed as expressed as $2^{-\Delta Ct}$ where ΔCt = (Ct gene of interest – Ct GAPDH).

2.4. Bioinformatic analysis

Sequences were aligned using ClustalW. The phylogenetic tree was generated with MEGA 4 using the neighbor joining (NJ) method [24]. The branches were validated by bootstrap analysis from 500 repetitions, which are represented by numbers at the branch nodes. The transmembrane domain predicted is based on protein hydrophobicity analysis using the statistical analysis of TM base, a database of naturally occurring transmembrane proteins at http://www.ch.embnet.org/software/TMPRED_form.html. The chicken genomic sequence (GenBank ID: NC_006127.3) and duck genomic sequence (http://www.ensembl.org/Multi/blastview) were used to determine genomic organization by Splign available at http://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi. Signal peptides were predicted by SignalP3.0 at http://www.cbs.dtu.dk/services/SignalP/.

2.5. Modelling of the duck PD-L2 extracellular domain structure

As a suitable template for the duPD-L2 extracellular domain structure, mPD-L2 (PDB: 3BP5) was identified by a BLAST search as implemented in the SWISS-MODEL protein-modelling server and the automatic sequence alignment obtained was used for homology modelling [25]. Model pictures were obtained using the PyMOL program (http://www.pymol.sourceforge.net/index.html).
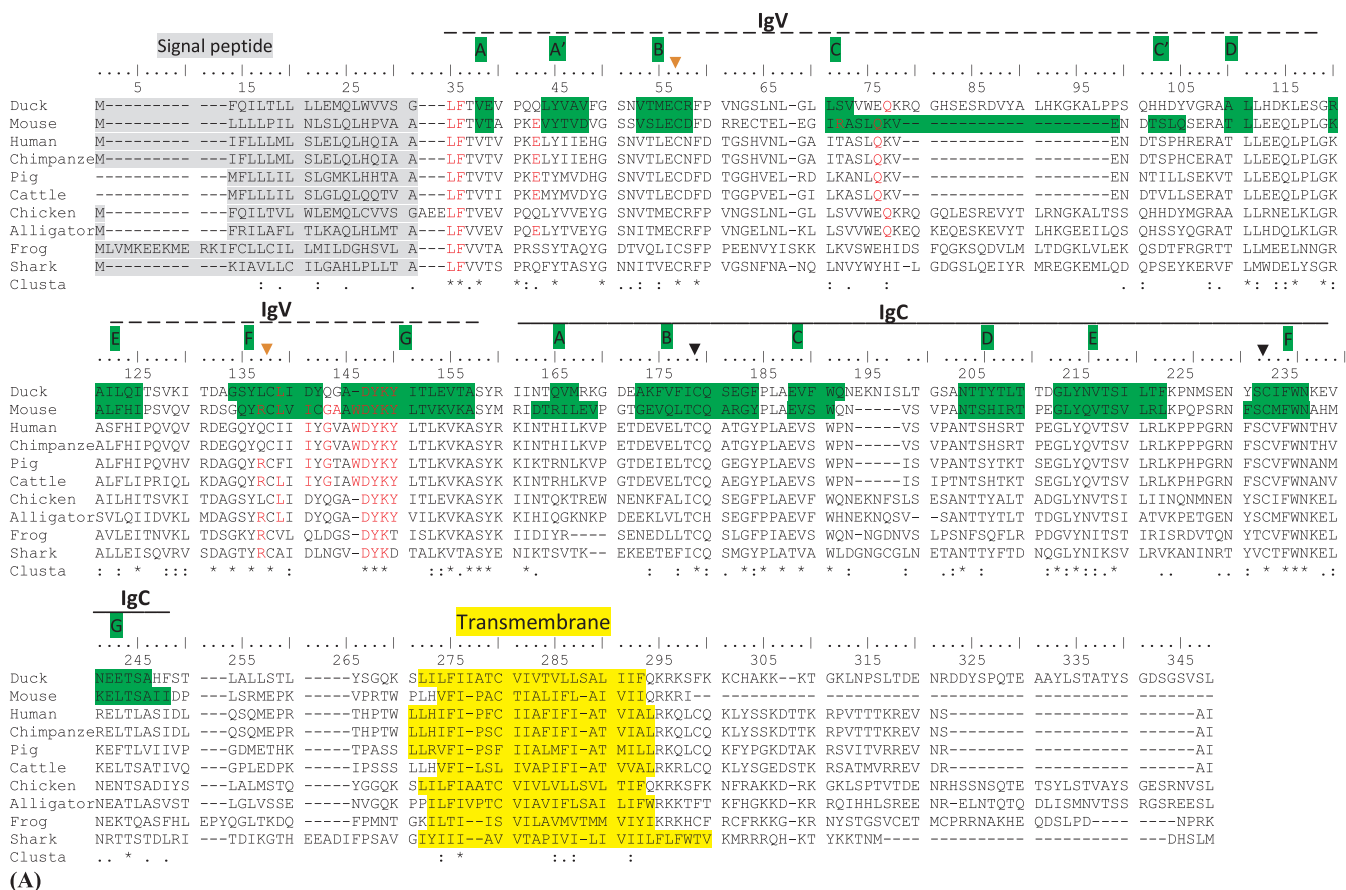
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                                                                                IgV
                                              _____
                    Signal peptide             [A]        [A]      [B]▼             [C]                                  [C]    [D]
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
                  5         15        25        35        45        55        65        75        85        95       105       115
Duck       M--------- ---FQILTLL LLEMQLWVVS G---LFTVEV PQQLYVAVFG SNVTMECFFP VNGSLNL-GL LSVVWEQKRQ GHSESRDVYA LHKGKALPPS QHHDYVGRAA ILHDKLESGF
Mouse      M--------- ---LLLLPIL NLSLQLHPVA A---LFTVTA PKEVYTVDVG SSVSLECLFD RRECTEL-EG ITASLQKV-- ---------- --------EN DTSLQSERAT LLEEQLPLGK
Human      M--------- ---IFLLLML SLELQLHQIA A---LFTVTV PKELYIIEHG SNVTLECNFD TGSHVNL-GA ITASLQKV-- ---------- -------EN  DTSPHRERAT LLEEQLPLGK
Chimpanze  M--------- ---IFLLLML SLELQLHQIA A---LFTVTV PKELYIIEHG SNVTLECNFD TGSHVNL-GA ITASLQKV-- ---------- --------EN DTSPHCERAT LLEEQLPLGK
Pig        ---------- ---MFLLLIL SLGMKLHHTA A---LFTVTV PKETYMVDHG SNVTLECDFD TGGHVEL-RD LKANLQKV-- ---------- --------EN NTILLSEKVT LLEEQLPLGK
Cattle     ---------- ---MFLLLIL SLGMKLHQIA A---LFTVTI PKEMYMVDVG SNVTLECDFD TGGPVEL-SL LKASLQKV-- ---------- --------EN DTVLLSERAT LLEEQLPLGK
Chicken    M--------- ---FQILTVL WLEMQLCVVS GAEELFTVEV PQQLYVVEYG SNVTMECRFP VNGSLNL-GL LSVVWEQKRQ GQLESREVYT LRNGKALTSS QHHDYMGRAA LLRNELKLGR
Alligator  M--------- ---FRILAFL TLKAQLHLMT A---LFVVEV PQELYTVEYG SNITMECRFP VNGELNL-KL LSVVWEQKEQ KEQESKEVYT LHKGEEILQS QHSSYQGRAT LLHDQLKLGR
Frog       MLVMKEEKME RKIFCLLCIL LMILDGHSVL A---LFVVTA PRSSYTAQYG DTVQLICSFP PEENVYISKK LKVSWEHIDS FQGKSQDVLM LTDGKLVLEK QSDTFRGRTT LLEQLKLGR
Shark      M--------- ---KIAVLLC ILGAHLPLLT A---LFVVTS PRQFYTASYG NNITVECRFP VGSNFNA-NQ LNVYWYHI-L GDGSLQEIYR MREGKEMLQD QPSEYKERVF LMWDELYSGR
Clusta                :         :    .    **.*   *:. *     *  .: : *  *       :         . :              .           .:    :.  ::*  *:
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                            IgV                                                                           IgC
         _____          _____
             [E]      [F]▼            [G]        [A]          [B]▼          [C]             [D]           [E]                [F]▼
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
                125       135       145       155       165       175       185       195       205       215       225       235
Duck      AILQITSVKI TDAGSYLCLI DYQGA-QYKV ITLEVTASYR IINTQVMRKG DEAKFVFICQ SEGFPLAEVF WQNEKNISLT GSANTTYTLT TDGLYQVNTSI LTFKPNMSEN YSCIFWNKEV
Mouse     ALFHIPSVQV RDSGQYRCLV ICGAAWDYKY LTVKVKASYM RIDTRILEVP GTGEVQLTCQ ARGYPLAEVS WQN-----VS VPANTSHIRT PEGLYQVTSV LRLKPQPSRN FSCMFWNAHM
Human     ASFHIPQVQV RDEGQYQCII IYGVAWDYKY LTLKVKASYR KINTHILKVP ETDEVELTCQ ATGYPLAEVS WPN-----VS VPANTSHSRT PEGLYQVTSV LRLKPPPGRN FSCVFWNTHV
Chimpanze ALFHIPQVQV RDEGQYQCII IYGVAWDYKY LTLKVKASYR KINTHILKVP ETDEVELTCQ ATGYPLAEVS WPN-----VS VPANTSHSRT PEGLYQVTSV LRLKPPPGRN FSCVFWNTHV
Pig       ALFHIPQVHV RDAGQYRCFI IYGTAWDYKY LTLKVKASYK IKTRNLKVP  GTDEIELTCQ GEGYPLAEVS WPN-----IS VPANTSYTKT SEGLYQVTSV LRLKPHPGRN FSCVFWNANM
Cattle    ALFLIPRIQL KDAGQYRCLI IYGIAWDYKY LTLKVKASYK KINTRHLKVP GTDEVQLTCQ AEGYPLAEVS WPN-----IS IPTNTSHRIT SEGLYQVTSV LRLKPHPGRN FSCVFWNANV
Chicken   AILHITSVKI TDAGSYLCLI DYQGA-QYKV ITLEVKASYK IINTQKTREW NENKFALICQ SEGFPLAEVF WQNEKNFSLS ESANTTYALT ADGLYNVTSI LIINQNMNEN YSCIFWNKEL
Alligator SVLQIIDVKL MDAGSYRCLI DYQGA-DYKY VILKVKASYK IKIHQGKNKP DEEKLVLTCH SEGFPPAEVF WHNEKNQSV- -SANTTYTLT TDGLYNVTSI ATVKPETGEN YSCMFWNKEL
Frog      AVLEITNVKL TDSGKYRCVL QLDGS-DYKT ISLKVKASYK IIDIYR---- KEENEDLLTCQ SLGFPIAEVS WQN-NGDNVS LPSNFSQFLR PDGVYNITST IRISRDVTQN YTCVFWNKEL
Shark     ALLEISQVRV SDAGTYRCAI DLNGV-DYKD TALKVTASYE NIKTSVTK-- EKEETEFICQ SMGYPLATVA WLDGNGCGLN ETANTTYFTD NQGLYNIKSV LRVKANINRT YVCTFWNKEL
Clusta     : : *  ::: * * * * :  ***  :***  *.         :  :  :.  . :  *:  . *.*  * *  *      :: *    . *  : :::*  .  .   .. :  * *** .:
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           IgC
         _____
            [G]                                    [Transmembrane]
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
                245       255       265       275       285       295       305       315       325       335       345
Duck      NEETSAHFST ---LALLSTL -----YSGQK LILFIIATC VIVTVLLSAL IIFQKRKSFK KCHAKK--KT GKLNPSLTDE NRDDYSPQTE AAYLSTATYS GDSGSVSL
Mouse     KELTSAIIDP ---LSRMEPK -----VPRTW PLHVFI-PAC TIALIFL-AI VIIQRKRI-- ---------- ---------- ---------- ---------- --------
Human     RELTLASIDL ---QSQMEPR -----THPTW LLHIFI-PFC IIAFIFI-AT VIALRKQLCQ KLYSSKDTTK RPVTTTKREV NS-------- ---------- ------AI
Chimpanze RELTLASIDL ---QSQMEPR -----THPTW LLHIFI-PSC IIAFIFI-AT VIALRKQLCQ KLYSSKDTTK RPVTTTKREV NS-------- ---------- ------AI
Pig       KEFTLVIIVP ---GDMETHK -----TPASS LLRVFI-PSF IIALMFI-AT MILLRKQLCQ KFYPGKDTAK RSVITVRREV NR-------- ---------- ------AI
Cattle    KELTSATIVQ ---GPLEDPK -----IPSSS LLHVFI-LSL IVAPIFI-AT VVALRKRLCQ KLYSGEDSTK RSATMVRREV DR-------- ---------- ------AI
Chicken   NENTSADIYS ---LALMSTQ -----YGGQK LILFIAATC VIVLVLLSVL TIFQKRKSFK NFRAKKD-RK GKLSPTVTDE NRHSSNSQTE TSYLSTVAYS GESRNVSL
Alligator NEATLASVST ---LGLVSSE -----NVGQK PPILFIVPTC VIAVIFLSAI LIFWRKKTFT KFHGKKD-RK RQIHHLSREE NR-ELNTQTQ DLISMNVTSS RGSREESL
Frog      NEKTQASFHL EPYQGLTKDQ -----FPMNT GKILTI--IS VILAVMVTMM VIYIKRKHCF RCFRKKG-KR NYSTGSVCET MCPRRNAKHE QDSLPD---- ----NPRK
Shark     NRTTSTDLRI ---TDIKGTH EEADIFPSAV IYIII--AV VTAPIVI-LI VIILFLFWTV KMRRRQH-KT YKKTNM---- ---------- ---------- ---DHSLM
Clusta     .. * . .            :  *          :  .:   :
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(A)

Fig. 3. Comparison of PD-L2 proteins. (A) Multiple alignment of amino acid sequences of duck PD-L2 with other PD-L2 proteins. Alignment was generated with ClustalW. Amino acids conserved among all species are indicated by: (*) identical, (:) highly conserved, (.) weakly conserved. Predicted secretory signal peptides are in grey shadow. β-sheets are shadowed green and transmembrane domains are shadowed yellow. Cysteine residues shown to form intramolecular disulfide bridges in mPD-L2 are indicated by triangles in the same color. PD-L2 contact residues based on the crystal structure of the complex formed by the ectodomains of mPD-L2 and mPD-1 (PDB: 3BP5) are highlighted in red. (B) 3D homology modelling of the duck PD-L2 ectodomain based on the structure of mPD-L2. (C) Spatial position of residues within sheets A, C, F and G. Contact residues indicated above that are conserved in duPD-L2 are in magenta and non-conserved residues are in yellow. Amino acid residues are numbered starting from the first methionine residue.

3. Results

3.1. Identification of the duck PD-L2 cDNAs and mapping of the duck PD-L2 gene

A partial coding sequence of duck PD-L2 (duPD-L2) lacking 5′ and 3′ terminal sequence was identified within the BGI duck genomic database and the sequences of full-length duPD-L2 and untranslated regions were determined by RACE. The duPD-L2 cDNA encodes a 321-amino acid protein that is predicted to have an N-terminal signal peptide of 19 amino acids. The duPD-L2 protein has an amino acid identity of 76% with chicken PD-L2, whereas the amino acid identities with mammalian PD-L2 proteins decreased to approximately 35%. The duck, human and mouse PD-L2 and PD-L1 proteins share an amino acid identity of 45%, 39%, 36% with each other, respectively. A phylogenetic tree constructed from the amino acid sequences of predicted PD-L2 proteins extracted from GenBank is shown in Fig. 1. Avian PD-L2 proteins comprise a grouping close to reptile, while distinct from those of mammalian species, frog and fish.

The sequence identity between the identified duPD-L2 cDNA and genomic sequences submitted to GenBank (Gene ID: 101798906) was 99%. The duck, chicken and human PD-L2 genes have a similar seven-exon-six intron structure with a non-coding exon 1 (Fig. 2) The coding sizes of the respective exons two, four, five and six of the duck, chicken and human PD-L2 genes are very similar, whereas the number of amino acids encoded by the respective exons three and seven of the avian PD-L2 genes is higher compared to those of the human PD-L2 gene. The human PD-L2 gene spans 60 kb, while the duck and chicken PD-L2 genes span only 12 kb and 13 kb, respectively (Fig. 2).

3.2. Comparative amino acid sequence analysis

A comparison of the amino acid sequence of duck PD-L2 with those reported for mammalian and other non-mammalian vertebrates is illustrated in Fig. 3.A. Residues predicted to be important for the ectdomain structure of mPD-L2 based on the crystal structure of the complex formed by the ectodomains of mPD-L2 and mPD-1 [8] were highly conserved. This includes cystines C42–C102^{mPD-L2} and C143–C192^{mPD-L2} that link strands B and F within the IgV-like and IgC-like domains, respectively, residues 122SYM124^{mPD-L2} within the short interdomain linker, and residues predicted to stabilize the interdomain interface via hydrophilic and hydrophobic interactions (V94^{mPD-L2}, V119^{mPD-L2}, K120^{mPD-L2}, A121^{mPD-L2}, S122^{mPD-L2}, Y148^{mPD-L2}, E201^{mPD-L2}). Predicted PD-1 binding residues 20LF21^{mPD-L2} within sheet A and 111DYK113^{mPD-L2} within sheet G were highly conserved, whereas residues Q60^{mPD-L2} within sheet C, L103^{mPD-L2} within sheet F and Y114^{mPD-L2} within sheet G were conserved in duck and chicken PD-L2 proteins but not in those of frog and fish (Fig. 3A).

3.3. 3 D modelling of the duck PD-L2 extracellular domain structure

The duPD-L2 extracellular domain structure as predicted by homology modelling using mPD-L2 (PDB: 3BP5) as a template (Fig. 3B) is largely compatible with the structure of mPD-L2 [8]. However, the
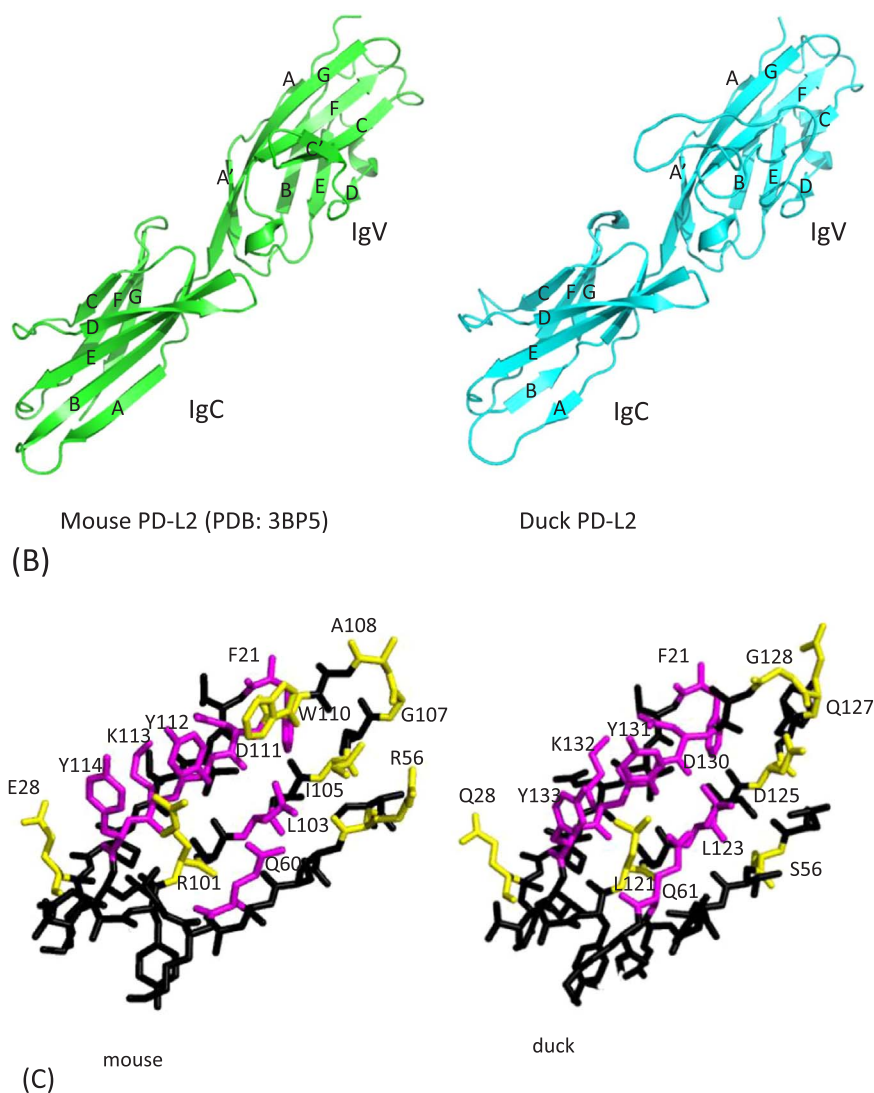
(B)

(C)

Fig. 3. (*continued*)

duPD-L2 model obtained lacks a β sheet C′ in the IgV-like domain and predicts a loop between β sheets C and D that contains an additional 20 amino acids compared to mammalian PD-L2 homologues, and predicts a shortened β sheet A within the IgC-like domain (Fig. 3B). The positions of conserved and non-conserved binding residues are shown in Fig. 3C.

3.4. Duck PD-L2 mRNA expression in tissues and PBMC

Tissue distribution of duPD-L2 mRNA was analysed by real-time PCR in two two-months-old ducks (Fig. 4A). Transcripts were most abundant in immune-cell-rich tissues such as spleen and bursa, cecal tonsil, cloaca and lung, while very low expression levels were found in kidney, muscle and brain. In freshly isolated duck PBMC and in adherent, monocyte-enriched, PBMC duPD-L2 mRNAs was readily detected whereas induction of duPD-L2 transcript expression by LPS was only observed in adherent PBMC up to 7–8 fold by 18 h (Fig. 4C,B).

4. Discussion

Engagement of coinhibitory receptors of the CD28 family, such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and PD-1 by B7 family members provides signals that are crucial for the establishment of peripheral tolerance and attenuation of adaptive immunity against

pathogens and tumors. This is the first report on the cloning of duck PD-L2 and comparative sequence analysis with mammalian and nonmammalian PD-L2 proteins. The duck PD-L2 gene shares a similar exonic structure with the chicken and human PD-L2 genes, but the avian PD-L2 genes are more compact. The predicted duPD-L2 protein has an amino acid identity of 76%, 35% and 34% with chicken, human and mouse PD-L2, whereas the amino acid identities shared between PD-L2 and PD-L1 of the human, mouse and duck proteins are 45%, 39% and 36%, respectively.

Despite this low amino acid identity, residues predicted to be critical for the maintenance of the ectodomain structure and PD-1 receptor binding are evolutionary conserved [8]. Cysteines shown to form disulfide bonds within the IgV- and IgC-like domains of mPD-L2 are retained in all PD-L2 proteins examined; and these cysteines are also retained in PD-L1 [2,23,26]. The N-terminal contact residues $20LF21^{mPD-L2}$ within sheet A and residues $111DYK113^{mPD-L2}$ within sheet G are highly conserved. Two of these, $D111^{mPD-L2}$ and $K113^{mPD-L2}$, have previously been shown to be critical for binding of mPD-L2 to mPD-1 based on relative affinities of alanine substitution mutants [8,27]. Furthermore, most of these highly conserved binding residues are also retained in PD-L1 ($F21^{mPD-L1}$, $D111^{mPD-L1}$, $Y112^{mPD-L1}$ and $K113^{mPD-L1}$) [9,23,27]. Hence, the majority of important contact residues are centered on the binding pocket formed by sheet G and the N-terminus and are evolutionary conserved in both PD-1 ligands.
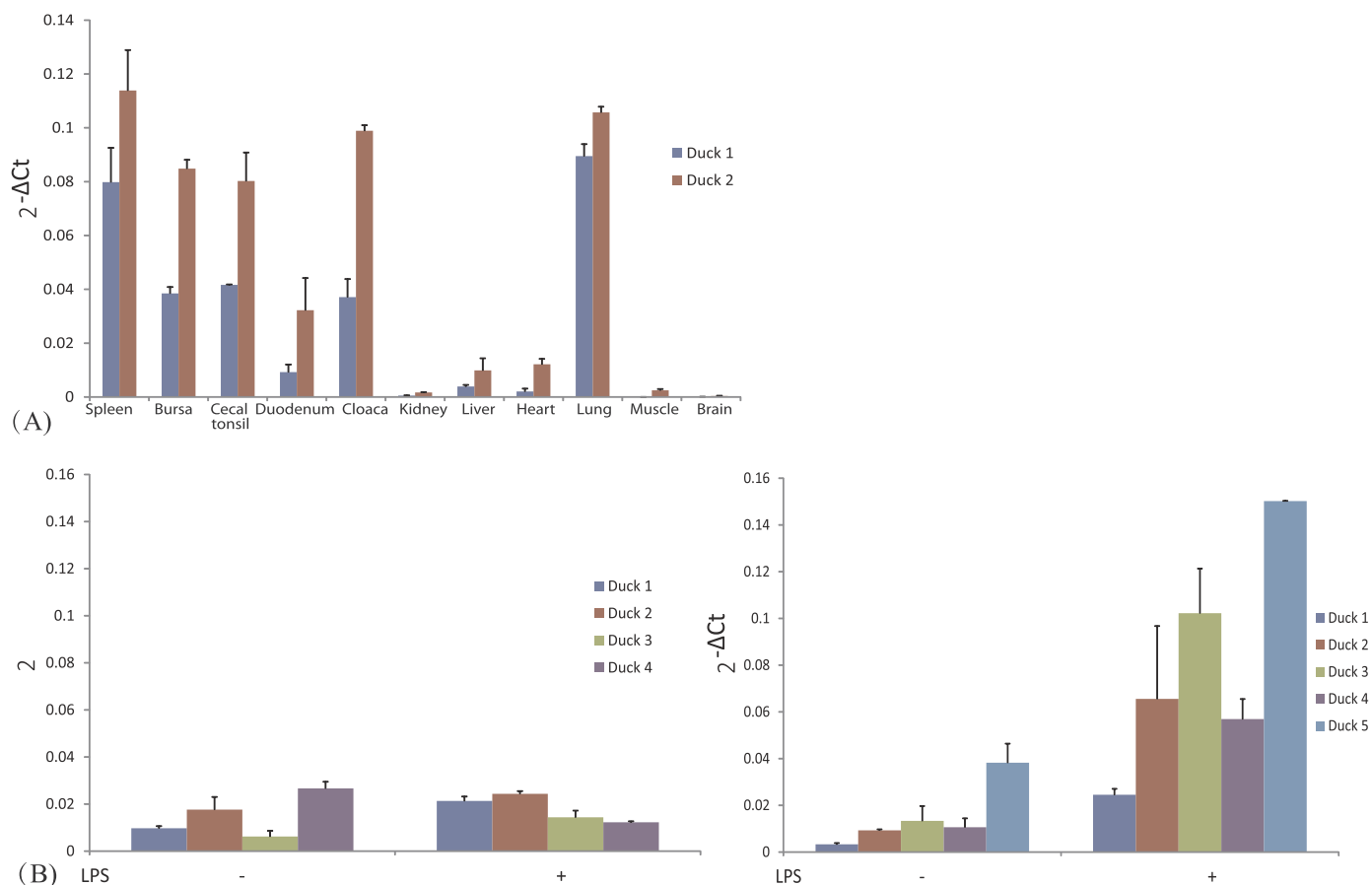
Fig. 4. Duck PD-L2 mRNA expression in tissue and PBMC. (A) Constitutive expression in tissues from two-month-old Pekin ducks. (B) Expression in freshly isolated duck PBMC (left panel) and adherent PBMC (right panel) cultured for 18 h in the absence (-) and presence (+) of LPS (5 μg/ml). Expression levels for duPD-L2 were determined by real-time quantitative RT-PCR relative to GAPDH mRNA levels. The Y-axis represents $2^{-\Delta Ct}$, where ΔCt = (Ct interest gene – Ct GAPDH). Error bars indicate standard deviation of two technical replicates.

The cytoplasmic domains of duPD-L2 and non-mammalian PD-L2 proteins are longer than those of most mammalian PD-L2 homologues but do not contain a recognized signaling domain. Messal and colleagues found that when PD-L2 on isolated human T cells was ligated with anti-PD-L2 antibody, T-cell proliferation and production of IL-2, IL-10 and IFNγ was decreased [28], and therefore raised the question whether this effect might be mediated by its cytoplasmic domain through a putative class I PDZ motif. However, the identified carboxy-terminal motif appears not evolutionary conserved beyond primates and there is no report on the effect of deletion or substitution mutants in support of a loss or gain of function. Hence, its functional role remains to be elucidated.

We observed abundant expression of duPD-L2 mRNA in spleen and lung and other immune-cell-rich tissues, while low expression levels were found in kidney, liver, muscle and brain. This is similar to initial work by Latchman and colleagues that showed PD-L2 transcript expression in several human tissues including spleen lymph nodes, pancreas, liver, lung and heart based on northern blot analysis, albeit expression of PD-L2 transcripts in murine tissues was exceedingly low [3]. However, using real time PCR the PD-L2 expression pattern in murine tissues [29] was similar to that previously described for human tissues [3] and observed in this report. It will be of interest to examine the level of PD-L2 protein expression in duck tissues as PD-L2 protein expression is much lower in murine compared to human tissues suggestive of differences in the immune-homeostatic roles of PD-L2 in tissues of different species [30].

In freshly isolated duck PBMC and adherent PBMC low levels of duPD-L2 transcripts were readily detected. However, induction of duPD-L2 expression by LPS was only observed in adherent PBMC

suggesting a TLR-mediated up-regulation of PD-L2 mRNA expression in duck PBMC enriched for monocytes/macrophages. In contrast mitogen activation of freshly isolated duck PBMC that constitute mainly lymphocytes [18] did not induce duPD-L2 expression (data not shown). In future studies it will be of interest to study duPD-L2 expression in response to other TLR-ligands and viral infections.

This analysis of a non-mammalian PD-L2 protein demonstrates considerable evolutionary conservation of the ectodomain structure and residues implicated in PD-1 receptor binding in mammalian and non-mammalian vertebrates including fish; and the mRNA expression pattern found for duPD-L2 is similar to that observed in mammalian counterparts. The work will permit the development of tools required to further study its role in the regulation of duck adaptive immunity, mediated through PD-1 or other putative receptors, such as RGMb. Of particular interest will be to reveal the role of PD-1 and other co-inhibitory signaling pathways, such as CTLA-4, in chronic DHBV infections, and to explore their potential for therapeutic targeting as the development of novel therapies that aim at a functional cure for chronic hepatitis B remains a priority.

Acknowledgements

References

[1] Y. Ishida, Y. Agata, K. Shibahara, et al., Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death, EMBO J. 11 (1992) 3887–3895.

[2] H. Dong, G. Zhu, K. Tamada, et al., B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion, Nat. Med. 5 (1999) 1365–1369.

[3] Y. Latchman, C.R. Wood, T. Chernova, et al., PD-L2 is a second ligand for PD-1 and inhibits T cell activation, Nat. Immunol. 2 (2001) 261–268.

[4] M.J. Butte, M.E. Keir, T.B. Phamduy, et al., Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses, Immunity 27 (2007) 111–122.

[5] Y. Xiao, S. Yu, B. Zhu, et al., RGMb is a novel binding partner for PD-L2 and its engagement with PD-L2 promotes respiratory tolerance, J. Exp. Med. 211 (2014) 943–959.

[6] F.A. Schildberg, S.R. Klein, G.J. Freeman, et al., Coinhibitory pathways in the B7-CD28 ligand-receptor family, Immunity. 44 (2016) 955–972.

[7] S.Y. Tseng, M. Otsuji, K. Gorski, et al., B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells, J. Exp. Med. 193 (2001) 839–846.

[8] E. Lazar-Molnar, Q. Yan, E. Cao, et al., Crystal structure of the complex between programmed death-1 (PD-1) and its ligand PD-L2, Proc. Natl. Acad. Sci. USA 105 (2008) 10483–10488.

[9] D.Y. Lin, Y. Tanaka, M. Iwasaki, et al., The PD-1/PD-L1 complex resembles the antigen-binding Fv domains of antibodies and T cell receptors, Proc. Natl. Acad. Sci. USA 105 (2008) 3011–3016.

[10] K.M. Zak, R. Kitel, S. Przetocka, et al., Structure of the complex of human programmed death 1, PD-1, and its ligand PD-L1, Structure. 23 (2015) 2341–2348.

[11] M.E. Keir, M.J. Butte, G.J. Freeman, et al., PD-1 and its ligands in tolerance and immunity, Annu. Rev. Immunol. 26 (2008) 677–704.

[12] E.N. Rozali, S.V. Hato, B.W. Robinson, et al., Programmed death ligand 2 in cancer-induced immune suppression, Clin. Dev. Immunol. 2012 (2012) 656340.

[13] K.E. Pauken, E.J. Wherry, Overcoming T cell exhaustion in infection and cancer, Trends Immunol. 36 (2015) 265–276.

[14] M.K. Callahan, M.A. Postow, J.D. Wolchok, Targeting T cell co-receptors for cancer therapy, Immunity 44 (2016) 1069–1078.

[15] D. Gardiner, J. Lalezari, E. Lawitz, et al., A randomized, double-blind, placebo-controlled assessment of BMS-936558, a fully human monoclonal antibody to programmed death-1 (PD-1), in patients with chronic hepatitis C virus infection, PLoS One 8 (2013) e63818.

[16] T. Watanabe, A. Bertoletti, T.A. Tanoto, PD-1/PD-L1 pathway and T-cell exhaustion in chronic hepatitis virus infection, J. Viral Hepat. 17 (2010) 453–458.

[17] J. Liu, E. Zhang, Z. Ma, et al., Enhancing virus-specific immunity in vivo by combining therapeutic vaccination and PD-L1 blockade in chronic hepadnaviral infection, PLoS Pathog. 10 (2014) e1003856.

[18] U. Schultz, E. Grgacic, M. Nassal, Duck hepatitis B virus: an invaluable model system for HBV infection, Adv. Virus Res. 63 (2004) 1–70.

[19] L. Cova, Advances and challenges in the development of therapeutic DNA vaccines against hepatitis B virus infection, Curr. Gene Ther. 14 (2014) 149–160.

[20] Y. Huang, Y. Li, D.W. Burt, et al., The duck genome and transcriptome provide insight into an avian influenza virus reservoir species, Nat. Genet. 45 (2013) 776–783.

[21] Q. Yao, K.P. Fischer, B. Motyka, et al., Identification of cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) isoforms in the Pekin duck, Dev. Comp. Immunol. 34 (2010) 749–758.

[22] Q. Yao, K.P. Fischer, D.L. Tyrrell, et al., CDNA cloning, genomic structure, molecular characterization and mRNA expression analysis of the Pekin duck interleukin-10 receptor 1, Int. J. Immunogenet. 39 (2012) 55–67.

[23] Q. Yao, K.P. Fischer, D.L. Tyrrell, et al., The Pekin duck programmed death-ligand 1: cdna cloning, genomic structure, molecular characterization and mRNA expression analysis, Int. J. Immunogenet. 42 (2015) 111–120.

[24] K. Tamura, J. Dudley, M. Nei, et al., MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0, Mol. Biol. Evol. 24 (2007) 1596–1599.

[25] K. Arnold, L. Bordoli, J. Kopp, et al., The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling, Bioinformatics 22 (2006) 195–201.

[26] G.J. Freeman, A.J. Long, Y. Iwai, et al., Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation, J. Exp. Med. 192 (2000) 1027–1034.

[27] S. Wang, J. Bajorath, D.B. Flies, et al., Molecular modeling and functional mapping of B7-H1 and B7-DC uncouple costimulatory function from PD-1 interaction, J. Exp. Med. 197 (2003) 1083–1091.

[28] N. Messal, N.E. Serriari, S. Pastor, et al., PD-L2 is expressed on activated human T cells and regulates their function, Mol. Immunol. 48 (2011) 2214–2219.

[29] K.S. Gorski, T. Shin, E. Crafton, et al., A set of genes selectively expressed in murine dendritic cells: utility of related cis-acting sequences for lentiviral gene transfer, Mol. Immunol. 40 (2003) 35–47.

[30] S.C. Liang, Y.E. Latchman, J.E. Buhlmann, et al., Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses, Eur. J. Immunol. 33 (2003) 2706–2716.