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Characterisation and expression analysis of the chicken interleukin-7 receptor alpha chain

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ABSTRACT

Interleukin-7 (IL-7) is a central regulator of T cell survival and homeostasis and its expression is indicative for naïve and memory T cells. We cloned chicken IL-7Rα (*CHIL-7Rα*) and determined its expression profile in chicken lymphocyte subpopulations. The predicted protein sequence contained 460 amino acids. The extracellular domain exhibited features typical of a type I cytokine receptor; a fibronectin type III domain and the GXWSXWS motif were conserved. ChIL-7Rα mRNA is highly expressed in lymphoid organs and in CD4+, CD8α+ and CD8β+ cells. A monoclonal antibody was generated and expression of the protein investigated. ChIL-7Rα was expressed on CD4+ and CD8α+, but not CD8β+, T cells, in contrast to the high mRNA expression levels in all of these cells. Upon polyclonal stimulation with ConA, IL-7Rα was rapidly down-regulated on T cells, suggesting that in the chicken expression of this receptor might also be correlated to the T cell activation status.

1. Introduction

Mammalian interleukin-7 (IL-7) is a cytokine involved in the regulation of lymphopoiesis, acting primarily on cells of the lymphoid lineage. IL-7 was originally defined by its ability to stimulate the proliferation of pre-B cells. Cells of the T cell lineage also respond to IL-7; both foetal and adult thymocytes of all surface phenotypes proliferate in response to IL-7 [1,2].

More recently it has become evident that IL-7 is a prototypic homeostatic cytokine, produced constitutively by non-lymphoid cells [3]. The mammalian IL-7 receptor (IL-7R) is composed of two chains: the IL-7Rα-chain (CD127) and a common γ-chain (γ_c or CD132), shared by the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. IL-7Rα belongs to the type I cytokine receptor family. Structurally, two fibronectin-type III (FN) domains, four conserved cysteine residues and a WS motif in the extracellular domains characterise these receptors. The mature form of murine IL-7Rα consists of 439 amino acids (aa) and is a membrane glycoprotein with a single 25 aa transmembrane domain. The 195 aa cytoplasmic tail contains partially characterised regions; one region (A) that is rich in acidic residues, and one region (T) that contains three tyrosine residues that are conserved in mouse and man (Y401, Y449, and Y456 as numbered in the murine sequence). In addition, a small membrane-proximal domain termed "Box 1" is conserved throughout the class I cytokine receptor family [4].

Whereas the γ_c is expressed on most haematopoietic cells, IL-7Rα is almost exclusively expressed on cells of the lymphoid lineage in which it has crucial functions (reviewed in [3,5]). IL-7 has emerged as a key cytokine involved in controlling the survival of peripheral resting T cells and their homeostatic turnover. The effect of IL-7 on T cells is controlled by the expression of the IL-7R, the state of differentiation of the cell, the availability of the cytokine and whether there is concomitant T cell receptor signalling (reviewed in [3,5]). Expression of IL-7Rα is a marker of naïve and memory T cells and it may serve to distinguish memory from effector T lymphocytes at early phases of the immune response [5–7], especially when combined with the expression of CD62L. Cell subsets described as central memory T cells ($CD127^{hi}$ and $CD62L^{hi}$) and peripheral effector memory T cells ($CD127^{hi}$ and $CD62L^{lo}$) can be distinguished.

Moreover, surface expression of CD127 (IL-7Rα) combined with CD25 (IL-2Rα) can differentiate between human regulatory and conventional CD4+ T cells in adult peripheral blood [8]. $CD127^{lo}$ T regulatory cells and $CD127^{hi}$ conventional T cells can be distinguished within the CD25+ CD45RO+RA− effector/memory and CD25+ CD45RA+RO− naïve compartments. CD25+$CD127^{lo}$ cells have suppressive activity *in vitro* whereas CD25+ $CD127^{hi}$ cells do not [8].

For the class I cytokine receptor family in the chicken, all of the common signalling chains have either been cloned and characterised [9,10] or are present in the chicken genome sequence

Abbreviations: IL, interleukin; chIL-7Rα, chicken interleukin-7 receptor alpha chain.

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(reviewed in [11]). IL-7 is encoded in the chicken genome and is expressed [12]. In the chicken, naïve, effector and memory T cells cannot be distinguished yet and the presence of regulatory T cells has not been demonstrated, but tools and assays to dissect T cell subsets are becoming available [13]. Here, we describe the cloning and expression of the chIL-7Rα and the development of a monoclonal antibody specific for chIL-7Rα, which will facilitate future research in chicken T cell development and discrimination between functional T cell subsets.

2. Materials and methods

2.1. Animals and tissues

To study mRNA expression of IL-7Rα, liver, spleen, thymus, bursa of Fabricius and bone marrow were collected from broiler chickens (Ross) at embryonic days 7 and 20 (ED7 and ED20; $n = 4$). Peripheral blood and spleens from 4-day-old to 6-week-old broiler chickens were used to sort cell subpopulations. Peripheral blood leukocytes (PBL) were isolated by density gradient centrifugation for 20 min at $850 \times g$ using FICOLL-Hypaque 1.078. Subpopulations of leukocytes were obtained after staining of cells with mouse anti-chicken CD4-PE (CT-4), CD8α-FITC (CT-8), CD8β-PE (EP42), and Bu-1-FITC (ChB6 clone AV20; Southern Biotechnology (SB), USA). Cells were sorted using a FACS Vantage SE instrument (Becton Dickinson). For every cell subpopulation at least 3×10^4 PBL and 1×10^6 splenocytes were analysed. For total RNA extractions, organs and cells were immediately immersed in RNAlater (Ambion) and stored at −20 °C until RNA was extracted.

2.2. Isolation of chicken IL-7Rα cDNA

Total RNA was extracted in the presence of buffer containing β-mercaptoethanol and guanidine using an RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. RNA was eluted in 30 μl RNase-free water and stored at −20 °C until required. The extracted RNA was reverse-transcribed using an iScript cDNA Synthesis Kit (Bio-Rad), with an oligo dT primer. *CHIL-7Rα* was amplified using Kod Hot Start DNA Polymerase (Novagene) according to the manufacturer's protocol. Briefly, cDNA (1 μl) was mixed with 200 μM dNTPs, 1× PCR buffer for Kod Hot Start DNA Polymerase, forward and reverse primers (0.3 μM) and 1 unit Kod Hot Start DNA Polymerase in 50 μl final volume. PCR conditions were as follows, 1 cycle of 94 °C for 5 min followed by 30 cycles of 94 °C for 15 s, 61 °C for 30 s and 72 °C for 20 s followed by 1 cycle at 72 °C for 5 min, using an iCycler (Bio-Rad). All primers used for the PCR were designed to the EST sequence of chIL-7Rα in the chicken genome. Two primers, chIL-7R forward (5′-AGTATAAAGTCAGTGAGTGAGGGTTTC-3′) and chIL-7R reverse (5′-AATAGAGAGCTTCAGTATCCAGCTTG-3′) were designed outside the predicted chIL-7Rα coding sequence. To increase specificity of the PCR the first PCR product was used for a nested PCR. To obtain the full-length cDNA sequences for chIL-7Rα, two primers, chIL-7R-nested forward (5′-GGGGAATTCAGCATGCTCAGAATGACACG-3′) and chIL-7R-nested reverse (5′-GGGTCTAGAAACGCCTACT-GATTTTTGTAAAAGC-3′) were used in PCR. The forward primer contains an EcoRI restriction site at the 5′-end and the reverse primer contains a XbaI restriction site at the 3′-end. These restriction sites were used to ligate the PCR product into pCI-neo (Promega) and pcDNA3.1 myc-his (Invitrogen). Library-competent *Escherichia coli* DH5α cells (Invitrogen) were transfected with the plasmids using FuGene-6 Transfection Reagent (Roche) according to the manufacturer's protocol. Plasmid DNA was extracted with a Quantum Prep Plasmid Maxiprep Kit (Bio-Rad). The insert DNA was sequenced using the primers T7 promoter (5′-TAATACGACT-CACTATAGGG-3′) and BGH-Reverse (5′-TAGAAGGCACAGTCGAGG-3′) for pcDNA3.1 myc-his and T7EEV (5′-AAGGCTAGAGTACTTAA-TACGA-3′) and T3 (5′-ATTAACCCTCACTAAAGGGA-3′) for pCI-neo.

Alignment of various IL-7Rα aa sequences was performed using the ClustalW Chicken FN_III domain prediction was performed using Pfam (http://www.sanger.ac.uk/software.pfam/). The growth factor and cytokine receptors family signature 2 (GFCRFS2) motif was predicted by Prosite motif search (http://www.expasy.ch/prosite). Similarity searches were performed using Blast Alignment (NCBI: http://www.ncbi.nlm.nig.gov/).

2.3. Chicken IL-7Rα expression analysis by real-time quantitative RT-PCR

IL-7Rα mRNA levels in different organs and cell subpopulations were quantified using real-time quantitative RT-PCR (qRT-PCR). For both cytokine receptor- (Accession No. EF116487) and 28S rRNA-specific amplification, primers and probes were designed using the Primer Express software program (PE Applied Biosystems). Details of probes and primers are given in Table 1.

QRT-PCR was performed using the TaqMan Universal PCR Master Mix (PE Applied Biosystems). Amplification and detection of specific products were performed using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems) with the following amplification profile: 1 cycle of 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 20 s, 59 °C for 1 min. Primers were used at 600 nM and probes at 100 nM. Quantification was based on the increased fluorescence detected by the ABI-PRISM 7700 Sequence Detection System due to hydrolysis of the target-specific probes by the 5′ nuclease activity of the AmpliTaq Gold DNA Polymerase during PCR amplification. A passive reference dye ROX (present in the TaqMan Universal PCR Master Mix), which is not involved in amplification, was used to correct for fluorescent fluctuations resulting from changes in the reaction conditions, for normalisation of the reporter signal.

Results are expressed in terms of the threshold cycle value (Ct), the cycle at which the change in reporter dye passes the significance threshold (ΔRn). Corrections for variations in RNA preparation and sampling were performed according to Eldaghayes et al. [14]. Results were then expressed as 40-Ct.

2.4. Expression chIL-7Rα on leukocytes

Two Balb/c mice were immunised three times with 50 μg plasmid DNA, pCI-neo-chIL-7Rα, at three-week intervals. After

Table 1
Real-time qRT-PCR probes and primers.

RNA target	Probe/primer sequence (5′-3′)		Accession No.
IL-7Rα	Probe	(FAM)-TCGTGTGGCCAAACCTTCCTGATCAT-(TAMRA)	EF116487
	F	ATTCTGGGAAAGCAGGATCAAG	
	R	CTTACACAGTCGCTCCAGAGTTATTT	
28S	Probe	(VIC)-AGGACCGCTACGGACCTCCACCA-(TAMRA)	X59733
	F	GGCGAAGCCAGAGGAAACT	
	R	GACGACCGATTTGCACGTC	

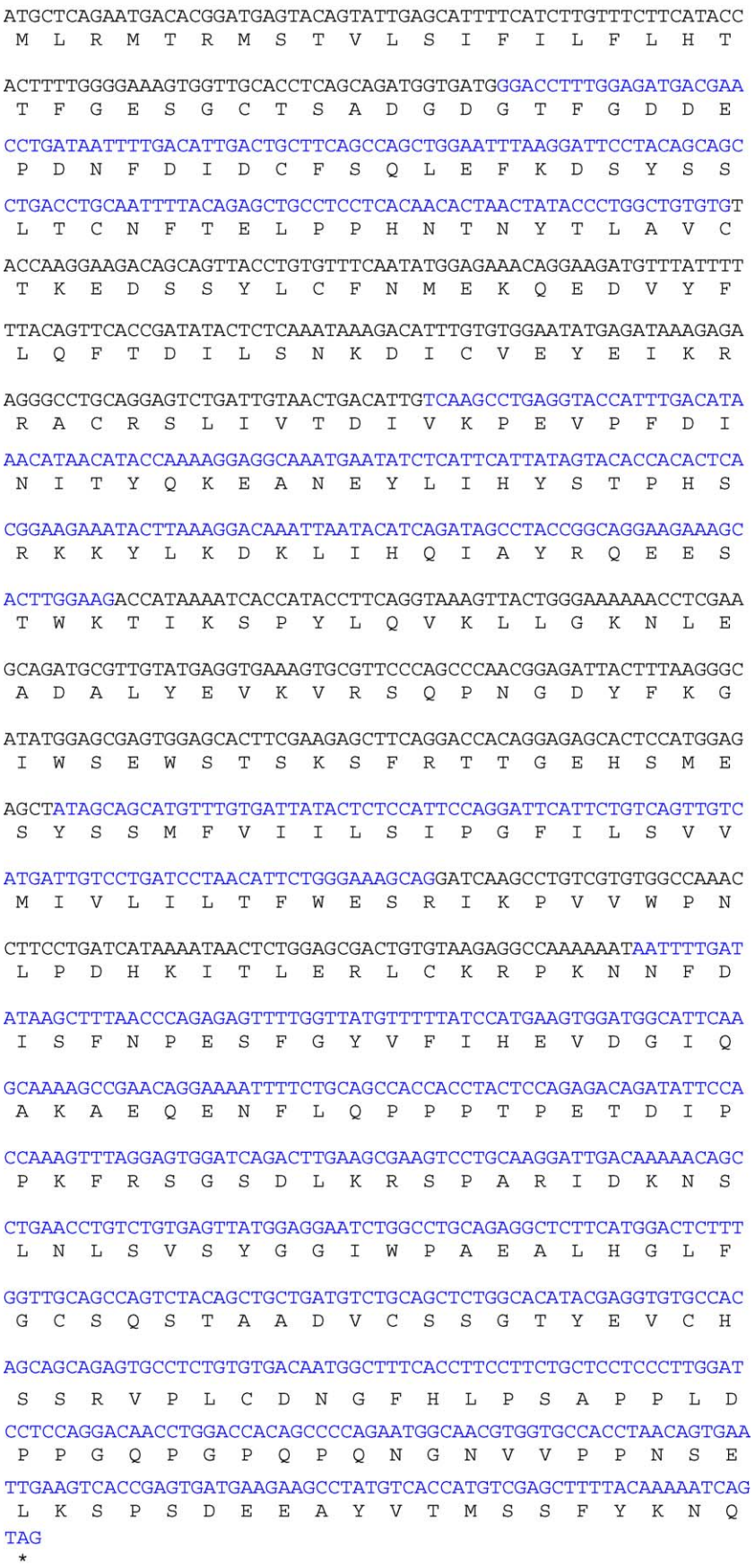

Fig. 1. Nucleotide sequence and predicted amino acid sequences of chicken IL-7Rα Different font colours indicate the different exons.

three weeks, mice were boosted with 2×10^7 chicken thymocytes. Hybridomas were generated by fusion of splenic lymphocytes, two weeks after the last boost, with mouse myeloma Sp2/0–Ag14 cells, according to the method of Segers et al. [15]. After fusion, the cells were distributed in flat-bottomed 96-well plates. Supernatants from wells containing growing colonies were tested by indirect immunofluorescence on chicken PBL and measured by flow cytometry. The second round of selection also included reactivity on spleen cryosections using immunohistochemistry [16] and on COS cells expressing recombinant chIL-7Rα. Positive wells were cloned by single cell limiting dilution on the FACSVantage cell sorter (BD Biosciences, San Jose, CA) using CellQuest software (BD Biosciences). Immunoglobulin subclass was determined using a Beadlyte Mouse Immunoglobulin Isotyping Kit (Upstate) using the Luminex 100 System.

IL-7Rα expression at the protein level was measured on PBL and splenocytes from Heisdorf & Nelson Silver Nick layer birds of one, three and six weeks of age. Blood was diluted in pre-warmed

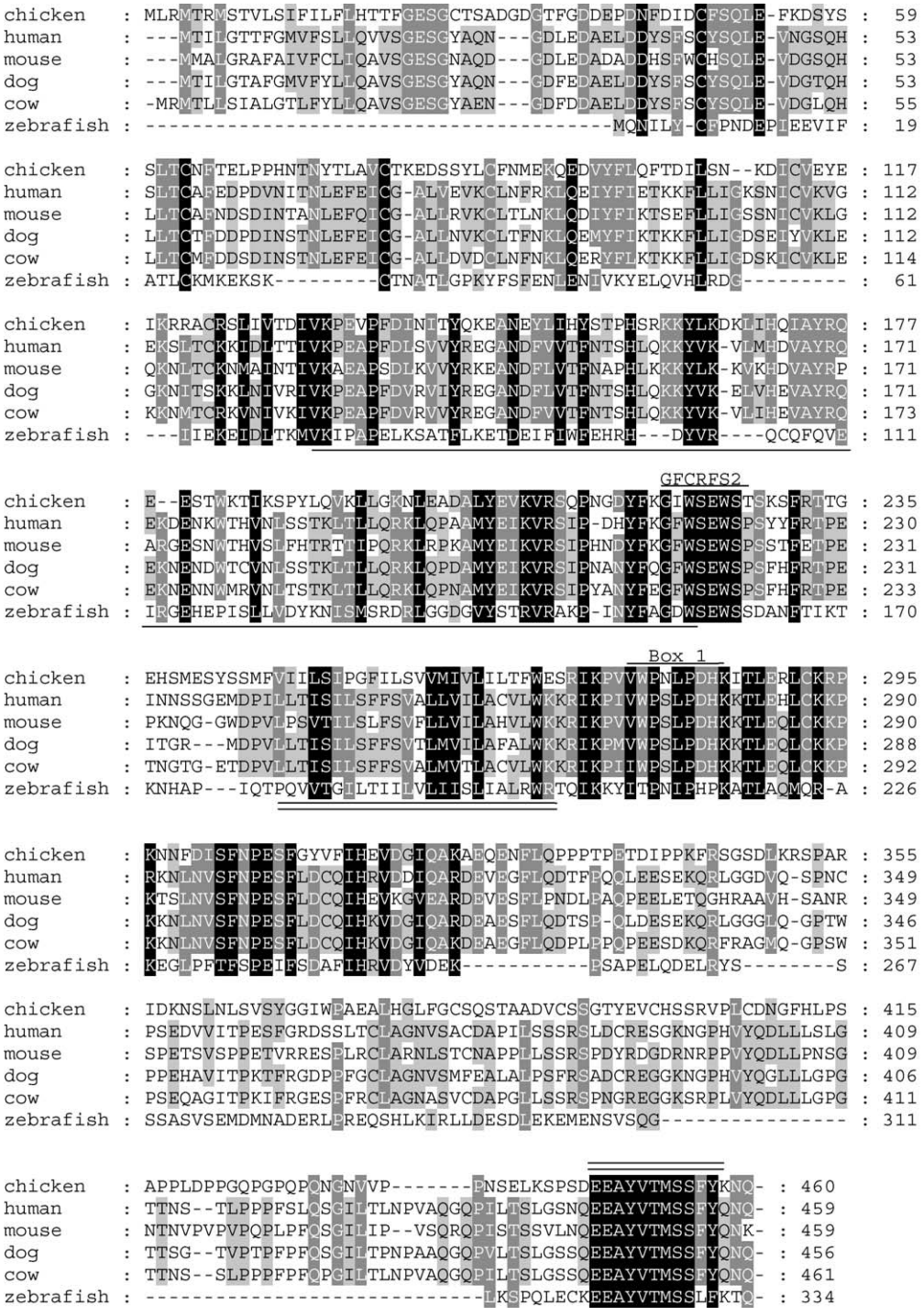

Fig. 2. Comparison of amino acid sequences between chicken IL-7Rα and human, mouse, dog, cow and zebrafish IL-7Rα (Accession numbers EF116487, NP_002176, EDL03312, XP_855408, XP_599818 and NP_001106979 respectively). The predicted Fn_III domain is underlined. The conserved GFCRFS2 (growth factors and cytokine receptors family signature 2) domain (GXWSXWS motif) in the Fn_III domain is overlined and annotated. The predicted trans-membrane domain is double-underlined. In the cytoplasmic tail, a small membrane-proximal domain, termed "Box 1" (Val^{277} to His^{284}) is also overlined and annotated. A conserved 11 aa motif at the COOH-terminal of the protein, containing two tyrosine residues (Tyr^{450} and Tyr^{457}), is double-overlined.

Hanks Balanced Salt Solution and lymphocytes purified after slow-spin centrifugation (62 × *g*, 20 min, RT). PBL and splenocytes were triple-stained with mouse anti-chicken IL-7Rα and goat anti-mouse (IgM)-APC (SB), fluorescein (FITC)-labeled or phycoerythrin (PE)-labeled mouse anti-chicken CD8α, CD8β, CD4, ChB6 (Bu-1-FITC), or KUL01-FITC (monocyte/macrophages; SB) and with T cell receptor-specific antibodies TCR-γδ-FITC (TCR1), TCR-αβ1-FITC (TCR2), or TCR-αβ2-FITC (TCR3; SB). Cells were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analysed using CellQuest software.

Splenocytes were stimulated for 24 h at 41 °C with 5 or 10 μg/ml ConA or medium (RPMI-1640, 5% FCS, penicillin, streptomycin) and triple-stained for chIL-7Rα and T cell markers as described above.

Immunocytochemical stainings were performed on cryosections of spleen and thymus. Sections were acetone fixed and incubated with monoclonal antibodies specific for chIL-7Rα (biotin labeled) and ChB6 (Bu-1), CD4, CD8α, CD8β, TCR-αβ1, TCR-αβ2, TCR-γδ or KUL01 (monocytes and macrophages). Slides were washed and incubated with avidin–enzyme complex (Vectastain® Elite ABC kit) and goat anti-mouse IgG–AP. Alkaline phosphatase activity was developed with Fast Blue BB salt and then the peroxidase activity was developed with AEC [17].

3. Results

3.1. Cloning and characterisation of full length chIL-7Rα

Using a PCR-based approach with primers designed to the predicted sequence in the chicken genome, we cloned the *CHIL-7Rα* chain cDNA (Fig. 1). The predicted protein sequence of chIL-7Rα contained 460 aa residues (Fig. 1), and included a predicted cleavable signal peptide (Met^{1} to Gly^{23}), and a calculated molecular mass of 52.2 kDa. The predicted aa sequence of chIL-7Rα was compared with those of human, mouse, rat, dog, tetraodon and fugu (Fig. 2). Three cysteine residues and the GXWSXWS motif (aa 220–226) located in the fibronectin type III domain (Pro^{134} to Ser^{223}) were conserved in all species. Another four cysteine residues were conserved with mammalian species, except the dog, where only two were conserved. ChIL-7Rα showed 34.4% identity to human IL-7Rα at the aa level. The predicted transmembrane domain was 24 aa in length, from Val^{247} to Glu^{270}. The cytoplasmic tail contains regions that are partially characterised in human and mouse. A small membrane-proximal domain, termed "Box 1" (Val^{277} to His^{284}), was highly conserved. An 11 aa motif at the COOH-terminal of the protein, containing two tyrosine residues (Tyr^{450} and Tyr^{457}), was completely conserved between the chicken and mammalian species. Four potential N-linked glycosylation sites (NXS/T) were located at aa 64–66, 74–76, 141–143, and 362–364 in chIL-7Rα, compared to nine in human IL-7Rα.

3.2. Expression of chIL-7Rα mRNA

Quantification of RNA levels was performed using real-time qRT-PCR and normalised against 28S rRNA expression levels. Expression of chIL-7Rα mRNA was analysed in organs of ED20 embryos and in chickens post-hatch (Fig. 3A). In both embryos and chickens post-hatch, expression was seen in all organs, with highest levels of chIL-7Rα mRNA detected in lymphoid organs which contain a high number of T cells. Expression of chIL-7R mRNA was detected as early as ED7 in pooled lymphoid organs and in the yolk sac, though expression levels were lower than at ED20 (data not shown). Lymphocyte subpopulations from blood and spleen of 6-week-old birds were sorted and analysed (Fig. 3B). In

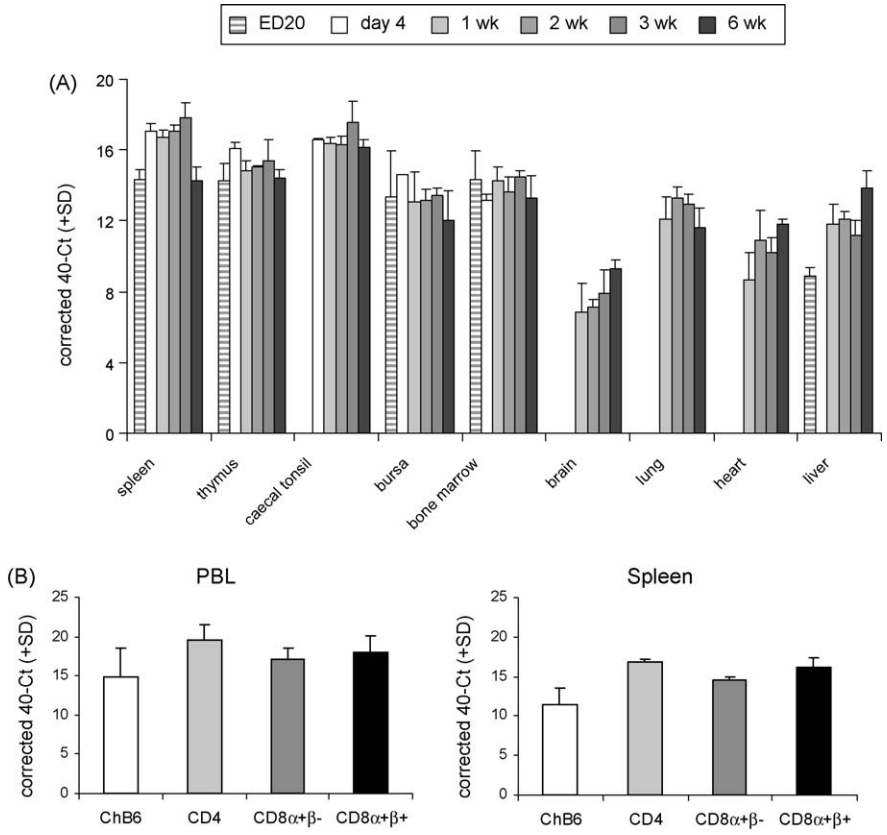

Fig. 3. A. Quantification of chIL-7Rα mRNA extracted from lymphoid and non-lymphoid organs of birds of different ages. Results are expressed as the mean corrected 40-Ct values +SD of 4 birds per time-point. B. PBL and splenocyte subpopulations were sorted using a FACSVantage and chIL-7Rα mRNA expression levels in the different subpopulations were quantified. Results are expressed as the mean corrected 40-Ct values +SD of at least 3 chickens.

the spleen, expression of chIL-7Rα did not vary much between chickens and significantly higher expression of chIL-7Rα mRNA was found in T cells compared to B cells ($p < 0.05$).

3.3. Expression of chIL-7Rα on leukocytes

After several rounds of selection, one hybridoma (8F10E11) was selected that reacted with PBL using flow cytometry, on cryosections of spleen using immunohistochemistry, and on transfected COS cells. This clone was found to be secreting an IgM antibody with a kappa light chain and was used for further characterisation and expression of chIL-7Rα on leukocytes.

Age-related expression of chIL-7Rα was determined on PBMC and splenocytes of birds of one, three and six weeks of age. Although chB6+ B cells expressed chIL-7Rα at the mRNA level, the percentage of chB6+ B cells that expressed the protein at the membrane surface was very low (<1%; data not shown). On monocytes and macrophages (KUL01+ cells), chIL-7Rα expression was also rare (<1%; data not shown). No changes with age in blood and spleen were found for either cell population.

T cells were triple-stained for chIL-7Rα; CD4, CD8α or CD8β; and T cell receptors (Fig. 4A). In peripheral blood, 90% of the CD4+ cells expressed chIL-7Rα of which 70–75% were TCR-αβ1+ (TCR2) and 25–30% TCR-αβ2+ (TCR3). In the spleen, 70–80% of the CD4+ cells expressed chIL-7Rα, with a similar distribution of the TCRs as on CD4+ cells in blood. ChIL-7Rα was not preferentially expressed on TCR-αβ1+ or on TCR-αβ2+ cells. The percentage of CD4+ cells that expressed chIL-7Rα in both blood and spleen was not significantly affected by age.

In peripheral blood, the percentage of CD8α+ cells expressing chIL-7Rα decreased with age from 60% to 30%. Of the CD8α+ IL-7Rα+ cells in blood, 75% of cells co-expressed TCR-αβ1 and 25% TCR-αβ2, with no significant changes with age. Fewer than 1% of the cells were TCR-γδ+ (data not shown). In the spleen the percentage of CD8α+ cells that expressed chIL-7Rα was much lower than in blood, but a similar decrease with age was found, ranging from 30% to 10%. With age the percentage of CD8α+ chIL-7Rα+ cells that co-expressed TCR-αβ1 decreased whereas the percentage of cells that co-expressed TCR-αβ2 increased. The percentage of CD8α+ chIL-7Rα+ TCR-γδ+ cells was higher in the spleen than in the blood, increasing from 1% to 10% with age (data not shown).

CD8α expression could be subdivided into high and intermediate expression (Fig. 4B). The expression of chIL-7Rα in blood and spleen was mostly confined to the CD8α^{int} cells. These CD8α^{int}

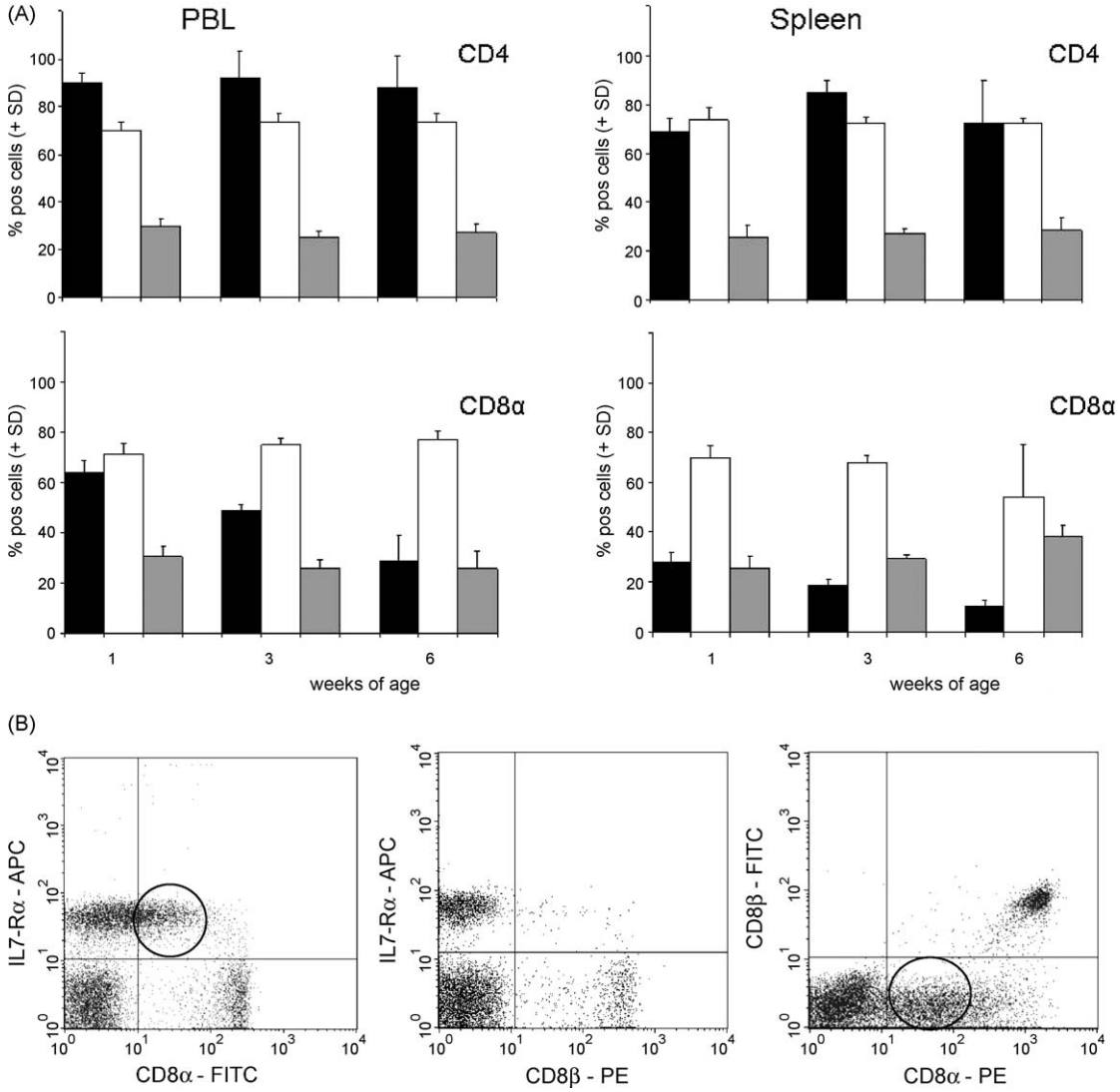

Fig. 4. A. Expression of chIL-7Rα on PBL and splenocytes of 1-, 3- or 6-week-old birds, as measured by FACS analysis. Black bars show the percentage of T cells (CD4 or CD8α) that express chIL-7Rα. Grey bars show the percentages of CD4+ IL-7Rα+ or CD8α+ IL-7Rα+ cells that express TCR-αβ1 (TCR2; white bars) or TCR-αβ2 (TCR3; grey bars). Bars represent percentage positive cells within the live cell gate +SD ($n = 4$). B. Expression of chIL-7Rα on CD8α+ PBL. Cells of a 3-week-old bird were triple-stained for chIL-7Rα, CD8α, and CD8β and FACS analysis indicated that CD8α^{int} cells (circled) are CD8β- and chIL-7Rα^{+}, whereas CD8α^{hi} cells co-express CD8β but rarely chIL-7Rα.

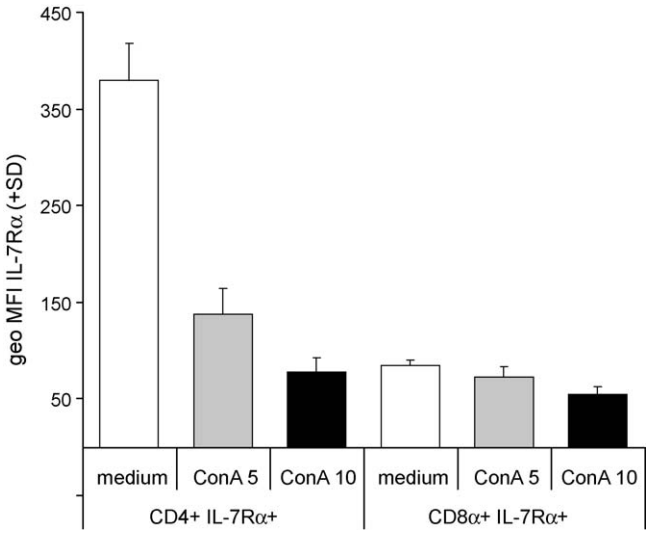

Fig. 5. Splenocytes of 3-week-old birds ($n = 4$) were stimulated with 5 or 10 μg/ml ConA for 24 h. The cells were double-stained for expression of chIL-7Rα and the T cell markers CD4 or CD8α. ChIL-7Rα expression levels are given as geometric mean fluorescent intensity (geometric MFI) +SD.

cells rarely expressed CD8β, which was in sharp contrast to the expression of chIL-7Rα at the mRNA level. Changing the order in which the monoclonal antibodies were used to stain the cells did not affect the result, suggesting that the lack of staining of CD8β+ cells was not due to steric hindrance of the monoclonal antibodies that were used.

Splenocytes were stimulated with a polyclonal T cell stimulus, ConA, in order to study changes in expression of chIL-7Rα upon activation. Stimulation for 24 h resulted in a tremendous down-regulation of chIL7Rα on CD4+ and only a slight down-regulation on CD8α+ T cells (Fig. 5). The number of CD4+ and CD8α+ cells increased after stimulation and the geometric MFI of CD4+ and CD8α+ cells slightly decreased (data not shown).

The expression of chIL-7Rα in thymus and spleen was studied using immunocytochemistry. Cortical thymocytes mainly represent double-positive cells expressing both CD4 and CD8. ChIL-7Rα was expressed on most cortical thymocytes except for the outer 2–5 cell layers in the subcapsular sinus, where the differentiation of the precursor cells starts. These thymocytes do not express CD4 or CD8. During migration from the cortex to the medulla, the thymocytes mature into single-positive T cells [18]. In the medulla most CD4+ and CD8+ cells co-expressed chIL-7Rα. Differences in staining were found when cells were stained for TCR-γδ and TCR-αβ1 (TCR1 and TCR2 respectively). Almost all αβ1-TCR+ cells co-expressed IL-7Rα in the medulla, but many TCR-γδ+ cells did not co-express chIL-7Rα (Fig. 6A). In the spleen, the FACS analysis results were confirmed. Many CD4+ cells but fewer CD8α+ cells co-expressed chIL-7Rα, whereas most TCR-αβ1+ cells also co-expressed IL-7Rα (Fig. 6B). Few TCR-γδ+ cells expressed the chIL-7Rα (Fig. 6).

4. Discussion

Herein we describe the cloning, characterisation, and expression patterns of the chicken IL-7Rα. Like mammalian IL-7Rα, the chIL-7Rα protein sequence contains a GXWSXWS motif located in the FN_III domain. This motif is conserved among type I cytokine receptor family members. Moreover, a small membrane proximal domain termed "Box 1" (Val^{277} to His^{284}) and an 11 aa motif containing two tyrosine residues (Tyr^{450} and Tyr^{457}) were also conserved. All these features strongly suggested that the cDNA described in this paper encodes the alpha chain of chIL-7R. The estimated molecular mass of chIL-7Rα was 52.2 kDa.

The IL-7R consists of two components, the IL-7Rα and a γc. Both chains are essential for the biological effects of IL-7 [19], as homodimerisation of chimeric IL-7Rα is not sufficient for signalling [20]. The model of IL-7R signalling is presumed to resemble that of other γc family cytokines, with IL-2Rβ being best studied [21,22]. IL-7 first binds to IL-7Rα and then recruits γc, bringing together their intracellular domains bearing Jak1 and Jak3. After mutual phosphorylation, these kinases acquire much higher activity and phosphorylate the critical Y449 (as numbered in the murine sequence) site on the IL-7Rα. This site binds STAT5 and possibly other adaptors and in turn becomes phosphorylated by Jak1 and/or Jak3. Jiang et al. [23] showed that deletion of Box 1 eliminated Jak1 phosphorylation and mutation of Y449 eliminated

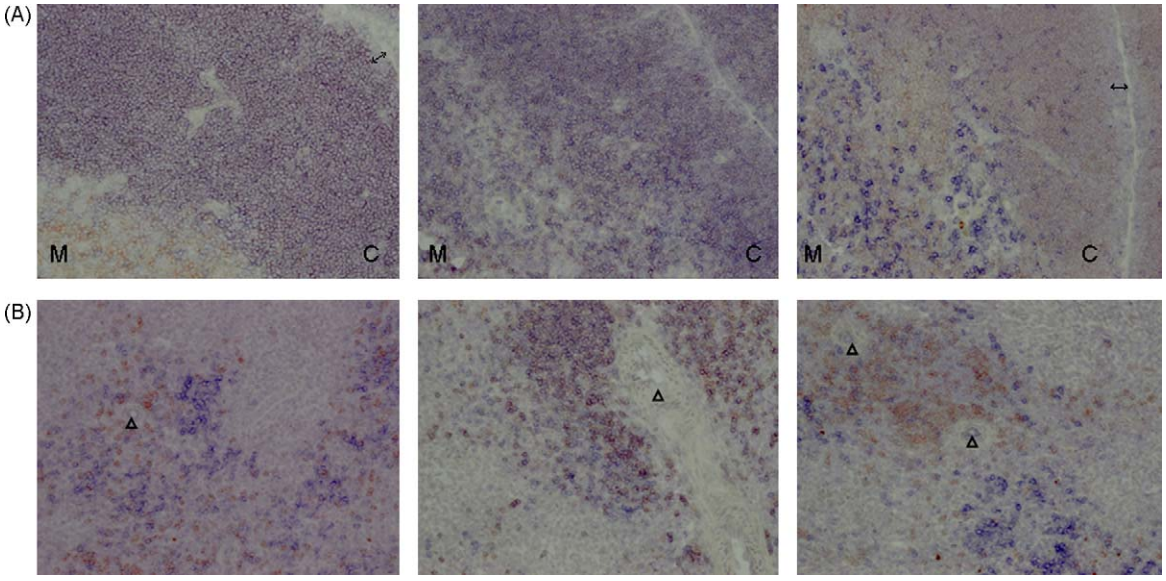

Fig. 6. Immunocytochemical double-staining of chIL-7Rα (red) and T cell markers (blue) in cryosections of thymus (A) and spleen (B). Double-staining with anti-chIL-7Ra and CD8a (panel 1), TCR-ab1 (panel 2, or TCR-gd (panel 3). A. Except for the outer cell layers (↔), most thymocytes in the cortex (C) and in the medulla (M) co-expressed chIL-7Rα and CD8α. TCR-αβ1+ cells co-expressed the chIL7Rα, whereas TCR-γδ+ cells showed lower levels of co-expression with chIL-7Rα. B. In the T cells areas around the arterioles (triangle) of the spleen, some CD8α+ cells, most TCR-αβ1+ cells and only few TCR-γδ+ cells co-expressed chIL-7Rα.

STAT5 phosphorylation. Both Box 1 and Y449 played a critical role in generation of thymocytes *in vivo* [23]. The chIL-7Rα also contains a Box 1 domain and a tyrosine-containing motif with two tyrosines (Y450 and Y457) with conserved spacing between them when compared to the same motifs in mammalian and fish IL-7Rα. Though not proven, based on the sequence homology of the IL-7Rα it is likely that these signalling pathways will be conserved in the chicken, especially as Jak1, Jak3 and STAT5 are all present in the chicken genome.

In the chicken, IL-7Rα mRNA expression in primary and secondary lymphoid organs of embryos increased during development (data not shown) and at ED20 high mRNA expression levels were seen, especially in lymphoid organs rich in T cells. In splenic cell subpopulations, the highest mRNA expression levels were seen in T cell subpopulations, in CD4+, CD8α+ and CD8β+ cells, which coincides with the expression pattern of the IL-7Rα in mammals.

Subsequently, expression of the chIL-7Rα protein was investigated on PBMC and splenocytes using a newly developed monoclonal antibody, 8F10E11. In blood, most CD4+ cells expressed chIL-7Rα independent of the age of the birds. In contrast, the percentage of CD8α+ cells expressing chIL-7Rα in blood decreased with increasing age. On these cells chIL-7Rα was mostly expressed on CD8α^{int} cells. Interestingly, these cells rarely co-expressed CD8β, indicating that although IL-7Rα mRNA was expressed in CD8β+ cells, the protein was not expressed on the surface of the cells. Stimulation of splenocytes did not induce expression of chIL-7Rα on CD8β+ cells (data not shown). The finding that mRNA transcripts are found in CD8β+ cells, but extracellular protein expression cannot be detected, needs further investigation, which will be focussed on intracellular expression of IL-7Rα and antigen-specific stimulation of CD8β+ cells. The expression of chIL-7Rα on splenic CD4 and CD8α cells was comparable to PBMC, except for the percentage of positive cells. A significantly lower percentage of CD4+ and CD8α+ spleen cells co-expressed the receptor, with no effect of age on the CD4+ IL-7Rα+ cells and a decreasing percentage of CD8α+ IL-7Rα+ cells with increasing age. In both peripheral blood and spleen there was no preferential expression of chIL-7Rα on either TCR-αβ1+ or TCR-αβ2+ cells. The lower percentage of chIL-7Rα+ T cells in the spleen might indicate that a higher percentage of T cells are activated in this organ.

Levels of CD8α expression inversely correlated with expression levels of IL-7Rα (Fig. 4B), in that CD8α^{int}+ cells expressed chIL-7Rα and most CD8α^{hi}+ cells did not. The CD8α^{int}+ cells mostly express the CD8αα homodimer and not the CD8αβ heterodimer. Especially in blood, some of the CD8α^{int} cells were CD4+ CD8αα+. In chickens, CD8αα homodimers have to date been described on NK cells [24] and intestinal intraepithelial lymphocytes [25]. In mice, CD8αα is constitutively expressed on intraepithelial lymphocytes [26], but not uniquely on mucosal T cells. CD8αα is not expressed on resting naïve T cells in the periphery, but co-expression of CD8αα and CD8αβ on activated conventional T cells has been reported [27,28]. The induction of CD8αα on primary effector cells *in vivo* is not an activation marker, but CD8αα plays a role during initial survival and differentiation of the memory precursor. Consistent with memory precursors, these CD8αα+ cells co-expressed high levels of IL-7R, which is characteristic for memory T cells and their predecessors [29,30]. Using cytotoxic T cell lines, the expression of both CD8 isoforms was shown to be differentially regulated. In response to TCR activation *in vitro* CD8αβ is down-regulated and internalised together with the activated TCR, whereas CD8αα is up-regulated [30]. Although it is unclear as to what occurs to CD8 isoforms upon TCR stimulation in the chicken, the CD8αα+ cells in blood and spleen are relatively more intracellular IFN-γ^{+} than the CD8α^{hi}+ population [13], which could indicate that these cells have a memory or predecessor phenotype in the chicken too. Luhtala et al. [31] demonstrated that chicken CD4+ CD8αα+ cells are functionally normal T cells, since they proliferate in response to mitogens and signals delivered via the TCR-αβ as well as via the CD28 co-receptor.

In mammals, IL-7Rα transcription is suppressed in response to IL-7, to other pro-survival cytokines (IL-2, IL-4, IL-6, and IL-15) and upon antigen encounter. Consequently, IL-7R expression is reduced and these T cells undergo massive clonal proliferation and differentiation into effector cells, and do not compete with unstimulated T cells for any remaining IL-7. Chicken splenocytes were polyclonally stimulated with ConA and the expression of chIL-7Rα decreased significantly thereafter. Considering that the number of CD4+ and CD8α+ cells increased after stimulation and the geometric MFI of CD4+ and CD8α+ staining slightly decreased, the down-regulation of chIL-7Rα cell surface expression was unlikely to be due to loss of CD4+ and CD8α+ cells.

The immunocytochemical data confirmed the chIL-7Rα expression patterns shown by FACS analysis, indicating that we developed a useful tool to detect chicken IL-7Rα that can be applied in different techniques, thereby expanding the chicken's "immunological toolbox". Future research will focus on chicken T cell development and discrimination between functional T cell subsets.

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