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Characterisation of chicken OX40 and OX40L☆

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ABSTRACT

The Tumour Necrosis Factor superfamilies of receptors and ligands play a crucial role in the regulation of effective immune responses against pathogens and malignant cells. In chickens, only few members have been identified. Here, we characterise the chicken homologues for mammalian costimulatory molecules OX40 and OX40L, which are involved in sustaining T cell responses. Both genes were identified by virtue of their genomic localisation close to highly conserved genes and their structural relationship to their mammalian homologues. Following cloning and expression of soluble and cell-associated chicken OX40 and OX40L, we confirmed their mutual interaction via ELISA and flow cytometric analyses. In addition, we showed the application of soluble OX40-Fc in staining of chicken cells. Whereas non-activated cells did not express OX40L, activation by IL-2 and IL-12 resulted in upregulation of OX40L on αβ and γδ T cell populations. Our results demonstrate the existence of the costimulatory OX40-OX40L system in the chicken and provide the basis for further investigations of chicken T cell responses.

1. Introduction

T cell activation is a complex process involving various forms of signals and receptors. Besides TCR triggering, binding of costimulatory molecules is pivotal to obtain a complete activation state in terms of cell differentiation, proliferation, longevity and cytokine production.

The classic costimulatory receptors belong to three superfamilies: the Immunoglobulin (Ig) superfamily (e.g. CD28 or CD2), the family of cytokine receptors (e.g. IL-2R or IL-7R) and the Tumour Necrosis Factor Receptor Superfamily (Croft, 2003). In the past decades, the focus of research of the T cell activation process was dominated by the investigation of CD28 signals. Only more recently the Tumour Necrosis Factor (TNF) superfamily, comprising receptors (TNFRSF) and their respective ligands (Tumour Necrosis Factor Superfamily, TNFSF), was studied more intensively (Croft, 2003). The family of TNFRSF receptors comprises 30 members (Gaur and Aggarwal, 2003), which can be divided into three functional groups depending on their intracellular signalling pathways (Ward-Kavanagh et al., 2016): so called decoy receptors without a cytoplasmic domain are distinguished from receptors containing a death domain (DD) in their cytoplasmic region which induces apoptosis. The third group comprises family members, which contain an intracellular motif for binding to adaptor molecules of the signalling cascade, called TRAFs (TNF Receptor-Associated Factors), promoting activation, survival and differentiation. Several TNF family members play major roles in sustaining T cell activation subsequent to the primary costimulatory effect of the CD28-B7 system. Among these receptors (CD27, CD30, GITR, 4-1BB, HVEM) (Watts, 2005), OX40 (CD134) and its ligand OX40L (CD252) hold a prominent position, as they ensure the longevity of T cell response by supplying anti-apoptotic signals (Rogers et al., 2001). During an immune response, the OX40-OX40L system regulates the functions of $CD4^+$ and $CD8^+$ T cells, regulatory T cells, Natural Killer (NK) and Natural Killer T (NKT) cells and interacts with Antigen Presenting cells (APCs) and tissue cells like endothelial or smooth muscle cells (Croft, 2010).

The receptor OX40 is a TRAF binding receptor and is reported to be a key mediator of costimulatory signals in mammalian T cells (Croft, 2010). OX40 belongs to the group of type I transmembrane proteins exhibiting an extracellular N terminus and an intracellular C terminus (Bodmer et al., 2002). Its extracellular domain comprises four Cysteine-Rich Domains (CRDs) which are the characteristic feature of TNFRSF receptors, but whose number may differ between family members (Ware, 2003). Due to these cysteine residues, the ectodomain of OX40 exhibits a stretched shape which allows for ligand binding (Banner et al., 1993).

Abbreviations: TNF, Tumour Necrosis Factor; TNFRSF, Tumour Necrosis Factor Receptor Superfamily; TNFSF, Tumour Necrosis Factor Superfamily.

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In mouse and human, the OX40 molecule is absent on naïve T cells, but is upregulated during the activation process as early as 12 h up to 5–6 days after antigen contact and TCR stimulation (Croft, 2010; Latza et al., 1994; Rogers et al., 2001). It can be detected on activated $CD4^+$ (Latza et al., 1994; Mallett et al., 1990) and on $CD8^+$ T cells (Bansal-Pakala et al., 2004), as well as on Th1, Th2, Th17 subsets of T helper cells and on regulatory T cells (Croft, 2010; Redmond et al., 2009). In addition, NK cells (Liu et al., 2008), NKT cells (Zaini et al., 2007) and neutrophils (Baumann et al., 2004) express the receptor at lower densities. The time course of OX40 expression depends on different factors like the nature of the antigen, its persistence, the evolving proinflammatory milieu and the affected T cell subset (Rogers and Croft, 2000). The involvement of other costimulatory molecules such as CD28, IL-2R or the presence of cytokines like IL-1, IL-4 and TNF also contributes to an optimal and prolonged expression (Croft, 2010; Redmond et al., 2009).

OX40L, like the other ligands of this family, is a type II transmembrane protein which exhibits an intracellular N terminus and an extracellular C terminus with a TNF Homology Domain (THD) (Ware, 2003). The THD is formed by two antiparallel β sheets which rejoin with the respective THDs of two other protomers to form non-covalent homotrimers. To date, the OX40 receptor is known only to bind its ligand OX40L (Croft, 2010) while other members of the TNFRSF family can interact with different ligands (Idriss and Naismith, 2000). As reported for other ligands like TNF, BAFF or FasL, OX40L also exists as a soluble variant, cleaved from the cell surface probably after the interaction with its receptor (Croft, 2010).

Human and murine OX40L molecules are expressed by a broader range of cell types, particularly on different kinds of activated APCs comprising B cells (Linton et al., 2003), macrophages (Karulf et al., 2010), dendritic cells (Jenkins et al., 2007) and Langerhans cells (Sato et al., 2002). OX40L expression is promoted by various stimuli like signalling through Toll-like receptors, the B cell receptor and the CD40 molecule or the presence of IL-18, TSLP (Thymic Stromal Lymphopoietin), IFNγ or prostaglandin E2 (Croft, 2010; Ito et al., 2005; Krause et al., 2009; Kurche et al., 2012; Maxwell et al., 2006). Furthermore, OX40L was observed on activated $CD4^+$ and $CD8^+$ T cells, suggesting an additional way of sustaining T cell response via T cell-T cell interactions (Mendel and Shevach, 2006). The expression of OX40 and OX40L in such a wide range of cell types affirms the manifold and extensive influence of the OX40-OX40L costimulatory system in mammals.

In chickens and other birds, information about representatives of the TNF superfamily, particularly about OX40 and OX40L, remains scarce. Although the genomic location of several members of the TNF superfamily has already been identified, the trace of others remains obscure, partly due to the incomplete annotation of the chicken genome (Kaiser, 2012). To date, chicken CD30 (Burgess et al., 2004) and CD30L (Abdalla et al., 2004) are the only TNF members involved in T cell activation, which were characterised in chickens. In our study, we describe the molecular cloning and expression of the chicken TNF receptor-ligand pair OX40-OX40L, characterise their structural properties, their ability of mutual recognition and their possible application in the cell staining of primary chicken cells. As OX40 and OX40L represent key costimulatory molecules in the activation process of T cells, our results will advance the understanding of the T cell response in chickens.

2. Materials and methods

2.1. Animals

Chicken line M11 was kindly provided by S. Weigend (Federal Research Institute for Animal Health, Mariensee, Germany). Fertilised eggs were incubated and hatched at the Institute for Animal Physiology, University of Munich, Germany. The chickens were reared under conventional conditions and they were eight weeks old when experiments were performed.

2.2. Cell culture and cell preparations

Human Embryonic Kidney cells (HEK) 293 were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen life technologies, Carlsbad, CA, USA) containing 10% Fetal Bovine Serum (FBS) (Biochrom GmbH, Berlin, Germany) in a 5% CO_2 incubator at 37 °C. To obtain chicken splenocytes, a single cell suspension was prepared by passing the spleen through a stainless steel mesh and mononuclear cells were segregated by consecutive density centrifugation on Biocoll Separating Solution (Biochrom GmbH). For stimulation, chicken splenocytes were plated at a density of 10^6 cells per well in a 96-well flat bottom plate and maintained at 40 °C and 5% CO_2 in RPMI 1640 medium supplemented with 10% FBS, 1% Penicillin/Streptomycin (Biochrom GmbH) and a combination of recombinant chicken IL-2 and IL-12. Chicken cytokines were obtained as previously described (Fenzl et al., 2017). Every second day, cultures were diluted by the substitution of 50% of the volume per well for fresh stimulation medium.

2.3. Database search and sequence analysis

ChOX40 and chOX40L were identified by keyword search using the gene database of NCBI (https://www.ncbi.nlm.nih.gov/) and the Ensembl database (https://www.ensembl.org/index.html). To confirm their correct annotation, mRNA and protein sequences of chOX40 and chOX40L were compared to their mammalian equivalents in the database using the Basic Local Alignment Search Tool (BLAST). Presumed protein sequences were structurally analysed by SMART (smart.embl-heidelberg.de). Protein sequences were aligned using the Clustal Omega method in the DNASTAR Lasergene Software package. Phylogenetic analysis was performed using the software MEGA5 (Tamura et al., 2011). Sequences of human, mouse and turkey equivalents of OX40, CD30 and their ligands OX40L, CD30L and of chicken TACI and BAFF were obtained by keyword search in the database mentioned above. The amino acid sequences of receptors or ligands were separately aligned by Muscle and Maximum Likelihood trees were generated using the following settings. For the data set of receptors, the JTT + G substitution model was used, whereas the sequences of ligands were analysed via the JTT model. To account for gaps in sequence alignment, the partial deletion option was selected and the tree was constructed with 1000 bootstrap replicates.

2.4. Cloning procedures

The sequences of chOX40 (accession no. NM_001354724.1) and chOX40L (accession no. XM_430147.4) were used for primer design. All oligonucleotides were obtained from Eurofins, Ebersberg, Germany. Primer sequences for the generation of the different constructs are displayed in Table 1. All constructs were verified by sequencing (GATC, Konstanz, Germany). To generate soluble forms of the ectodomains of OX40 and OX40L, either tagged by a C-terminal human IgG1-Fc domain (henceforth “OX40-Fc”) or by an N-terminal FLAG-tag (henceforth “OX40L-FLAG”), we applied the following cloning strategy. We employed vectors derived from variants of the pCR3 (Invitrogen life technologies) provided by P. Schneider (University of Lausanne, Epalinges, Switzerland). They either encoded an Ig signal peptide followed by a thrombin cleavage site and the human IgG1-Fc domain at 3’ (vector PS229) or a signal peptide of Hemagglutinin of Influenza A virus (HA) and a