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Chicken CD300a homolog is found on B lymphocytes, various leukocytes populations and binds to phospholipids

Beatrice Sperling, Birgit C. Viertlboeck, Thomas W. Göbel *

Institute for Animal Physiology, Department of Veterinary Sciences, University of Munich, Veterinärstr. 13, Munich 80539, Germany

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ABSTRACT

The chicken CD300 cluster contains three genes that encode inhibitory, activating and soluble forms. In the present study, we have generated a monoclonal antibody against the inhibitory CD300L-B1 molecule. The mab 1D4 was specific for the CD300L-B1 form and showed no crossreactivity with the related CD300L-X1. Virtually all bursal cells expressed CD300L-B1, whereas only a small positive subset was found in thymus that was identified as thymic B cell subpopulation. In peripheral tissues, CD300L-B1 was found to be expressed on lymphocyte subpopulations in blood and spleen. Double immunofluorescence analysis with B- and T-cell specific markers identified these subsets as B lymphocytes. In addition, analysis of PBMC revealed that CD300L-B1 was also present on monocytes, heterophils, blood NK cells and in vitro differentiated macrophages. We utilized a reporter cell line in order to identify potential ligands of CD300L-B1. When several phospholipids were tested, only phosphatidylserine and phosphatidylethanolamine were found to trigger strong reaction of the reporter cells. The two phospholipids elicited a response only in CD300L-B1 reporter cells, but not in CD300L-X1 reporter cells. Moreover the interaction could be blocked with the specific mab. In conclusion, we provide evidence for the expression of chicken CD300L-B1 on immature and mature B cells, monocytes, heterophils, macrophages and NK cells and identify phosphatidylserine and phosphatidylethanolamine as CD300L-B1 ligands.

1. Introduction

Immune responses can be modulated by a variety of Ig like receptor families. They are defined as receptors encoded by gene clusters on small chromosomal regions. The receptors have single or multiple Ig domains which can share a significant degree of homology. The transmembrane and cytoplasmic regions of the individual receptors within a family identify either prototypic activating receptors or inhibitory forms. Activating receptors have only short cytoplasmic tails and are further characterized by a basic transmembrane residue that allows the association of an adaptor molecule such as DAP-10, DAP-12 or the common Fcγ chain. Ligand binding of the activating receptors therefore mediates cellular activation via these adaptor molecules by phosphorylation of cytoplasmic ITAM with subsequent downstream signaling. Although highly homologous in the extracellular ligand binding domain, the inhibitory receptor partners display long cytoplasmic domains containing inhibitory motifs known as ITIM or ITSM (Bryceson and Long, 2008; Parham, 2005; Ravetch and Lanier, 2000; Vivier et al., 2008). Examples of these paired receptor families with immunomodulatory capacity include KIR, LILR, LAIR, CD300, TREM gene families among others (Borrego, 2013; Colonna, 2003; Lanier, 2005; Meyaard, 2008). It is still not completely resolved how these receptor pairs of inhibitory and activating forms are involved in modulation of immune responses. Ligands are only known for some of the receptors and although the extracellular domains share high homology, ligands of activating receptors not necessarily bind to the corresponding inhibitory forms.

In the chicken genome several of these receptor families have been described, including CHIR, TREM, SIRP, CD200R, CD300L families (Viertlboeck and Göbel, 2011; Viertlboeck et al., 2006, 2008, 2013). Thus, many of the Ig receptor families have been well conserved throughout vertebrate evolution. The number of individual receptor genes within a given family, however, shows high variability between different vertebrates. For instance the leukocyte receptor complex has been vastly expanded in chickens encoding a large number of CHIR that are not found in mammals (Lochner et al., 2010). In contrast, the chicken TREM, CD200R, SIRP and SLAM loci show the opposite tendency with only a few receptors encoded (Straub et al., 2014; Viertlboeck et al., 2013).

The CD300 gene family is also heterogeneous regarding the number of genes in different mammals with 7 human and 9 mouse

Abbreviations: PE, phosphatidylethanolamine; PS, phosphatidylserine; ITIM, immunoreceptor tyrosine-based motif; ITSM, immunoreceptor tyrosine-based switch motif.

* Corresponding author. Institute for Animal Physiology, Department of Veterinary Sciences, University of Munich, Veterinärstr. 13, 80539 Munich, Germany. Tel.: +49 89 2180 3827; fax: +49 89 2180 2552.
E-mail address: goebel@lmu.de (T.W. Göbel).

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CD300 members, respectively (Borrego, 2013; Clark et al., 2009). CD300 receptors are type I transmembrane proteins with a single IgV-like extracellular domain that has an extra pair of disulphide bonds. In human and mouse, CD300a and CD300f are classified as inhibitory due to their cytoplasmic ITIM or ITSM motifs, whereas the other genes lack inhibitory motifs. The complexity of the various CD300 receptors is potentiated by their propensity to form heterodimers (Martinez-Barriocanal et al., 2010).

The expression patterns of individual CD300 receptors vary and there are differences between mice and human, too. In general, most of the receptors are expressed by different types of myeloid cell types (Borrego, 2013). CD300a/c is also found on lymphocyte subsets including all human NK cells, as well as subsets of B and T cells.

Assigning protein expression data of CD300a and CD300c has been complicated by cross reactive mab. Only recently, two groups produced specific anti-human CD300 antibodies. Lankry et al. showed CD300a and CD300c expression on NK cells (Lankry et al., 2010). Takahashi et al. tested specific CD300a and CD300c mab on human blood cells and detected CD300a expression on monocytes, NK cells, PDCs, and basophils, whereas CD300c was expressed by monocytes. Moreover, both were expressed on mast cells (Takahashi et al., 2013). In an additional study, a single commercial anti-CD300 mab, staining monocytes, was reported to be CD300c specific (TX45), whereas the rest showed crossreactivity (Simhadri et al., 2013).

Recently ligands of CD300a and CD300c have been identified with the help of Fc fusion proteins. Initially it was found that these fusion proteins bound to dead cells from various species. The molecular identity of the ligands was subsequently found to represent phospholipids that are exposed on the outer membrane of cells during early stages of apoptosis. Although there are controversial results regarding individual specificity of different CD300 receptors, it has been demonstrated that the human CD300a reacts with both phosphatidylserine (PS) and phosphatidylethanolamine (PE) (Nakahashi-Oda et al., 2012a, 2012b; Simhadri et al., 2012). Moreover, one group also reported binding of PS and PE to CD300c, but got weaker signals compared to CD300a (Takahashi et al., 2013). The specificity for phospholipids is highly conserved, since binding to phospholipids has also been demonstrated for teleost CD300 homologs (Cannon et al., 2012).

The chicken CD300 gene family located on chromosome 18 was originally described in 2006 (Viertlboeck et al., 2006, 2013). It consists of three genes designated CD300L-B1, CD300L-X1 and CD300L-S1. A fourth gene CD300L-A1 most likely resembles a pseudogene. All genes encode receptors with a single Ig domain with four cysteins forming two intrachain disulfide bridges. The CD300L-S1 lacks a transmembrane and cytoplasmic domain and thus may resemble a soluble CD300 form. CD300L-B1 is characterized by a long cytoplasmic domain with one ITIM and one ITSM. It is thus most homologous to human CD300A. The CD300L-X1 is a potential activating receptor with homology to human CD300C.

In this report we have produced a novel mab that is specific for chicken CD300L-B1. Analysis employing this mab revealed expression on various myeloid cells including heterophils, monocytes, and macrophages. CD300 is also found on NK cells and it is expressed by virtually all immature and mature B cells. Chicken CD300L-B1, but not the related CD300L-X1, binds to the two phospholipids PE and PS. These findings raise interesting new questions regarding CD300 function on B cells as a receptor for apoptotic cells.

2. Materials and methods

2.1. Animals

Fertilized eggs from chicken strain M11 (RSV RES Line Mr, MHC:B2), a kind gift of S. Weigend, Institute for Animal Breeding, Mariensee, Germany, were hatched at the Institute of Animal Physiology, University of Munich. The chickens were used at the age of 3–5 months. Balb/c mice were purchased from Charles River (Sulzfeld, Germany) and housed in the institute facilities. All experimental procedures were in accordance with institutional, state and federal guidelines on animal welfare.

2.2. Cell preparation

Single cell suspensions of bursa, spleen and thymus were obtained by passage through a stainless steel mesh, followed by density centrifugation on Biocoll-Hypaque (Biochrom, Berlin, Germany). Peripheral blood lymphocytes (PBL) were prepared by slow-speed centrifugation as described. Peripheral blood mononuclear cells (PBMC, including lymphocytes, thrombocytes, monocytes, blood NK cells and heterophils) were obtained by density centrifugation. In detail heparinized blood was mixed with an equal volume of PBS and carefully layered above a double volume of Biocoll-Hypaque (Biochrom AG, Berlin, Germany). After centrifugation at $650 \times g$ for 12 minutes at room temperature, the PBMC in the interphase were obtained and washed with PBS prior further analysis.

2.3. Cloning, cell lines and transfections

For reporter gene constructs of CD300L-B1 and CD300L-X1 the respective full length plasmids were used as templates (GenBank accession No. AM076725 and AM076726) (Viertlboeck et al., 2006). In case of the CD300L-B1 reporter gene construct sense primer GAATTCCACCCCGGCTGGGCG and antisense primer GAATTC GCACGGGATGAGGAT were used to amplify the extracytoplasmic region. The PCR product was cloned in a pcDNA3.1/V5/His/Topo TA Vector (Invitrogen, Karlsruhe, Germany), EcoRI digested, gel extracted and ligated in a modified pcDNA3.1/V5-His expression vector (Invitrogen, Karlsruhe, Germany) (Viertlboeck et al., 2007), resulting in an N-terminally FLAG-tagged extracellular region of CD300L-B1 fused to the transmembrane region of chicken CD8α and the cytoplasmic domain of murine CD3ζ. The CD300L-X1 reporter gene construct was cloned similarly into this modified vector, but in this case we used the Gibson Assembly Method (Gibson Assembly™ Master Mix, New England BioLabs Inc., Massachusetts, USA) with antisense primer CGATGACGATAAGGAATTCCACCCCGGCTGGGCGG and sense primer GTGCTGGATATCTGCAGGGCAGCGCGCCGCG as described previously (Straub et al., 2014).

The mouse thymoma reporter cell line BWZ.36 (a kind gift of N. Shastri, Berkeley University of California, USA) was used for immunizations and binding assays. Cells were maintained in RPMI 1640 medium supplemented with 10% FCS (both Biochrom AG, Berlin, Germany) at 37 °C in a 5% CO_2 incubator. To generate reporter cell lines, cells were stably transfected with either CD300L-B1 or CD300L-X1 reporter gene construct by electroporation as described (Viertlboeck et al., 2007). After 10–14 days of selection with 800 μg/ml G418 (Biochrom, Berlin, Germany) single colonies were screened by flow cytometry (FACS Canto II, Beckton Dickinson, USA) for FLAG expression. Reporter cells that displayed high FLAG levels were further used and are described as BWZ.36 CD300L-B1 and BWZ.36 CD300L-X1 throughout the paper.

The macrophage cell lines HD11 and BM2 were cultured at 41 °C, 5% CO2 in RPMI containing 8% FCS and 2% chicken serum (Beug et al., 1979; Symonds et al., 1984).

2.4. Generation of specific mab and staining procedures

For generating a specific mouse monoclonal antibody (mab) against CD300L-B1, we used the stably expressing BWZ.36 CD300L-B1 cells for immunization followed by standard fusion protocols. Hybridoma supernatants were screened on either transfected BWZ.36