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Cellular endocytic compartment localization of expressed canine CD1 molecules

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ABSTRACT

CD1 molecules are glycoproteins present primarily on dendritic cells (DCs), which recognize and present a variety of foreign- and self-lipid antigens to T-cells. Humans have five different CD1 isoforms that survey distinct cellular compartments allowing for recognition of a large repertoire of lipids. The canine CD1 family consists of seven functional CD1 molecules (canine CD1a2, CD1a6, CD1a8, CD1a9, CD1b, CD1c and CD1e) and one presumed non-functional isoform (canine CD1d) due to a disrupted gene structure. The aim of this study was to describe in vitro steady-state localization ptterns of canine CD1 isoforms and their correlation with endocytic organelles. GFP-fused canine CD1 293T cell transfectants were stained with markers for early endocytic compartments (EEA-1) and late endocytic/lysosomal compartments (LAMP-1), respectively, and analyzed by confocal microscopy. Canine CD1a molecules localized to the plasma membrane and partially to the early endocytic compartment, but not to late endosomes or lysosomes. In contrast, canine CD1b was highly associated with late endosomal/lysosomal compartments and showed a predominant intracellular expression pattern. Canine CD1c protein expression localized more promiscuously to both the early endosomal compartments and the late endosomal/lysosomal compartments. The canine CD1e molecule showed a strictly intracellular expression with a partial overlap with late endosomal/lysosomal compartments. Lastly, canine CD1d was expressed abnormally showing only a diminished GFP expression. In conclusion, canine CD1 transfectants show distinct localization patterns that are similar to human CD1 proteins with the exception of the canine CD1d isoform, which most likely is non-functional. These findings imply that canine CD1 localization overall resembles human CD1 trafficking patterns. This knowledge is important for the understanding of lipid antigen-receptor immunity in the dog.

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1. Introduction

CD1 molecules are antigen-presenting glycoproteins predominantly present on dendritic cells (DCs), which are responsible for foreign-and self-lipid antigen recognition and subsequent presentation to a number of CD1-restricted T cells (Adams, 2014; Barral and Brenner, 2007; Porcelli et al., 1989; Sugita and Brenner, 2000). The ability of CD1-lipid complexes to stimulate different subsets of T cell populations makes them important immune mediators not only in normal tissue homeostasis, but also in a number of diseases, including mycobacterial infections, atopic dermatitis, melanomas, carcinomas and epitheliotropic lymphoma (de Jong et al., 2014; Gerlini et al., 2004; Pigozzi et al., 2006; Shevchuk et al., 2014; Van Rhijn and Moody, 2015). The dog is prone to spontaneously developing a number of these diseases, and in many instances shares similar disease pathogenesis as the human disease forms (Malik et al., 2004, 2013; Marsella and Girolomoni, 2009; Moore et al., 2009; Simpson et al., 2014).

Much like the peptide-recognizing major histocompatibility complex molecules, CD1 molecules survey intracellular compart-

Abbreviations: GFP, green fluorescent protein; DC, dendritic cell; EEA-1, Early Endosome Antigen 1; LAMP-1, Lysosomal Associated Membrane Protein 1; ERC, Endocytic Recycling Compartment.

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ments of the antigen-presenting cell to sample for different antigenic targets that have been taken up by DCs (Cao et al., 2002; Dougan et al., 2007). After uptake, extracellular lipid cargo is generally distributed to different endocytic compartments including early endosomes, late endosomes and/or lysosomes depending on the composition of the lipid (Briken et al., 2000b; Krul et al., 1985; Moody et al., 2002; Mukherjee et al., 1999; van den Elzen et al., 2005).

To ensure the recognition of a broad variety of lipid antigens by the immune system, it is therefore highly important for CD1 molecules to reach these individual intracellular compartments wherein lipids are located. Thus, the five different isoforms seen in the human CD1 family (CD1a, CD1b, CD1c, CD1d and CD1e) each have the ability to traffic to distinct endocytic compartments based on the amino acid composition of their cytoplasmic tail (Briken et al., 2000a; Sugita et al., 1999). A tyrosine trafficking domain (YXXΦ, where Y is a tyrosine amino acid, X is any amino acid and Φ is a bulky hydrophobic residue) is found in human CD1b, CD1c and CD1d molecules, which allows these isoforms to survey deeper endocytic compartments such as late endosomes and lysosomes (Bonifacino and Traub, 2003; Jackman et al., 1998; Porcelli and Modlin, 1999; Sandoval and Bakke, 1994). In contrast, human CD1a cytoplasmic tail does not contain a trafficking motif, which results in localization within the cell membrane and the superficial endocytic system (Cernadas et al., 2010; Salamero et al., 2001; Sugita et al., 1999). The human CD1e molecule differs from the rest of the CD1 family in that it never reaches the cell surface, but stays strictly as an intracellular protein within DCs (Angenieux et al., 2000).

The canine CD1 family is larger than the human counterpart in that it consists of a total of eight CD1 isoforms (Looringh van Beeck et al., 2008; Schjaerff et al., 2016). It has four functional CD1a proteins (canine CD1a2, CD1a6, CD1a8 and CD1a9) followed by one functional canine CD1b, CD1c and CD1e protein. Although, the canine CD1 locus contains a *canCD1D* gene, a corresponding canine CD1d protein translation is believed to be impaired due to a disrupted gene structure (Looringh van Beeck et al., 2013). Similar to the human orthologs, the canine CD1 molecules share the same characteristics of their cytoplasmic tails. Thus, no tyrosine trafficking motif is seen in the cytoplasmic tails of canine CD1a's, whereas canine CD1b, CD1c and CD1d all possess a tyrosine trafficking signaling motif (Looringh van Beeck et al., 2008; Schjaerff et al., 2016). The cytoplasmic tail of canine CD1e has not previously been described in detail.

The goal of this study was therefore to characterize the steady-state cellular distribution patterns of canine CD1 molecules, as this has not previously been reported. Specifically, it was of interest to determine if canine CD1's follow similar intracellular endocytic localization patterns as human CD1's since this may reflect similar involvement in health and disease in the dog as is known in humans.

2. Materials and methods

2.1. Canine CD1 cell lines

Stable 293T cell lines expressing individual canine CD1 molecules fused with green fluorescent protein (GFP) at the C-terminal end were previously established (Schjaerff et al., 2016). Briefly, cDNA transcripts of *canCD1A2*, *canCD1A6*, *canCD1A8*, *canCD1A9*, *canCD1B*, *canCD1C*, *canCD1D* and *canCD1E* were amplified from canine thymus and from a canine histiocytic cell line (*canCD1D*), respectively. The transcripts were fused with GFP by cloning into a plasmid vector (pcDNA3.1/CT-GFP-TOPO, Invitrogen, Grand Island, NY, USA) and transformed into *E. coli* bacteria (Invitrogen). Subsequently, GFP-fused CD1 products were then transfected into human 293T cells (ATCC, Manassas, VA, USA) by jetPRIME Polyplus transfection reagent (Polyplus-transfection,Illkirch, France). Stable cell lines were selected for by the use of geneticin (G418) (EMD Millipore, Billereca, MA, USA). Two different clones were expressed for each isoform, except for canine CD1d, where only one full length clone was retrieved (Schjaerff et al., 2016).

2.2. Canine non-GFP fused clones and CD1a8:human cytoplasmic tail chimeric construct

To investigate whether GFP fused to the cytoplasmic tail influenced trafficking behavior, clones of canine CD1a8 and canine CD1b were additionally expressed without a GFP tag. Cloning and transfection was performed by similar methods as described above, except a stop codon was added at the end of each transcript prior to the GFP segment.

A chimeric clone of canine CD1a8 fused with the human CD1a cytoplasmic tail portion (CD1a8:huCFC_nonGFP) was constructed to test whether its localization patterns would differ from those of wild type (WT) canine CD1a8. In brief, a synthetic canine CD1a8 gene fragment (gBlock) containing the exchanged amino acid cytoplasmic tail portion (canine WKAH replaced by human CFC) was constructed and purchased through Integrated DNA Technologies (Coralville, IA, USA). Wild type canine CD1a8 and the gBlock fragment were enzymatically digested by *AasI* restriction enzyme (Thermo Fisher, Waltham, MA, USA) and gel separated. Products of interest were gel extracted by Qiagen MinElute PCR purification kit (Qiagen, Valencia, CA, USA) followed by a DNA ligation procedure using T4 DNA Ligase (Promega, Madison, WI, USA). The procedure was performed according to manufacturer's protocol. Finally, the fused product was then cloned and transfected into 293T cells similar to previously described methods.

2.3. Cell culture

Stable 293T canine CD1 transfectants were cultured on poly-L-lysine coated coverslips of 12 mm diameter and #1.5 thickness (Neuvitro, Vancouver, WA, USA). The cells were grown as a monolayer at 37 °C in DMEM media (Life Technologies, Grand Island, NY, USA) supplemented with 1% PenStrep (LifeTechnologies) and 10% Fetal Bovine Serum (FBS) (Cellgro, Manassas, VA, USA).

2.4. Staining protocols

All staining protocols were performed in triplicates. Coverslips were first fixed in 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 15 min followed by a permeabilization step using a 0.1% Triton X-100 solution (Sigma-Aldrich) for 5 min. Prior to antibody exposure, the cells were blocked with 10% horse serum for 40 min. Based on previous antibody validation (Schjaerff et al., 2016), the following primary antibodies were used to detect the expression of non-GFP tagged canine CD1a8 and CD1b proteins, respectively. Thus, FITC-conjugated anti-canine CD1a monoclonal antibody (mAb) (Ca13.9H11-FITC-conjugate) (P.F. Moore, Davis, CA, USA) was used to detect canine CD1a8_nonGFP and was incubated for 1 h at a concentration of 1:70. Non-conjugated anti-guinea pig CD1b mAb (gpCD1F2-1B12) (S.A. Porcelli, Albert Einstein College of Medicine, Bronx, NY, USA) was used to detect canine CD1b_nonGFP and was incubated for 1 h at a 1:10 dilution followed by incubation with secondary anti-mouse IgG Alexa 488 FITC-conjugate (Invitrogen) for 30 min.

Anti-human EEA-1 Alexa Fluor 647-conjugated mAb (Abcam, Cambridge, MA, USA) was used to detect early endosome compartments by binding Early Endosome Antigen 1 protein. Furthermore, anti-human LAMP-1 Alexa Fluor 647-conjugated mAb (Biolegend, San Diego, CA, USA) was used to detect late endosomes and lysosomal compartments expressing Lysosomal Associated Mem-

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