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Molecular cloning and characterization of DNGR-1 in rhesus macaques

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ABSTRACT

DC, NK lectin group receptor-1 (DNGR-1), also known as C-type lectin domain family 9 member A (CLEC9A), is a promising target for immunological therapeutics and vaccination against tumors and viruses. However, little is known about its property in rhesus macaques. In this study, we cloned rhesus macaque *DNGR-1* cDNA, and found that its coding region could encode a 241-amino acid polypeptide with 91.7% sequence identity and similar antigenicity to that of humans. Both free and cell surface rhesus macaque DNGR-1 expressed *in vitro* could bind to apoptotic/dead cells induced by serum deprivation or freeze-thaw, and to pyroptotic cells stimulated with PMA and LPS. We also demonstrated that rhesus macaque *DNGR-1* mRNA was present in all the examined tissues, with the highest in lymph nodes, spleen, blood, and thymus. The expression of DNGR-1 that is highly similar to that of humans warranted the usefulness of rhesus macaques in testing human therapeutics and vaccines targeting DNGR-1, especially those for HIV/AIDS.

1. Introduction

DC, NK lectin group receptor-1 (DNGR-1), also known as C-type lectin domain family 9 member A (CLEC9A), is a C-type lectin-like receptor from the Dectin-1 cluster of the natural killer gene complex (NKC) (Huysamen and Brown, 2009; Huysamen et al., 2008; Jongbloed et al., 2010; Poulin et al., 2010; Sancho et al., 2009; Sattler et al., 2010). In recent years, much attention has been focused on the structure and function of human and mouse DNGR-1, and the crucial role of DNGR-1 in specific immune responses to tumors and viral infections (Huysamen et al., 2008; Iborra et al., 2012; Piva et al., 2012; Sancho et al., 2008; Zelenay et al., 2012).

The DNGR-1 consists of an extracellular C-type lectin-like domain (CTLD), a stalk region, a transmembrane domain, and a cytoplasmic tail (Caminschi et al., 2008; Huysamen et al., 2008). The CTLD can serve as a probe to recognize the ligand through its critical ligand binding sites, *i.e.*, W131 and W227 in human DNGR-1 (Hanc et al., 2015; Zhang et al., 2012). The cytoplasmic tail with the immunoreceptor tyrosine-based activation motif-like (ITAM-like) motif promotes signaling *via* Syk (Caminschi et al., 2008; Huysamen et al., 2008; Kerrigan and Brown, 2010; Sancho et al., 2008). DNGR-1 can directly facilitate the cross-presentation of damaged or dead cell-associated antigens by sensing the filamentous actin (F-actin) exposed after the loss of membrane integrity, and DNGR-1 signaling through ITAM-like motif could promote NF-κB activation and induction of the proinflammatory cytokines similar to that induced by Dection-1 signaling (Ahrens et al., 2012; Geijtenbeek and Gringhuis, 2009; Huysamen et al., 2008; Kerrigan and Brown, 2010; Sancho et al., 2009; Zhang et al., 2012). Hanc et al. unequivocally demonstrated an in-depth structure of mouse DNGR-1 bound to F-actin at 7.7 Å resolution by electron cryomicroscopy and helical image analysis (Hanc et al., 2015). The current understanding of DNGR-1 ligands and functions provides the insights of its functional roles in the mechanisms of immunity and homeostasis, the development of certain autoimmune diseases, and $CD8^+$ T cell responses to viral infections (Ahrens et al., 2012; Huysamen and Brown, 2009; Sancho et al., 2009).

DNGR-1 mRNA is expressed in all the tissues examined, with the highest expression levels in brain, thymus, and spleen in both human and mouse (Caminschi et al., 2008; Huysamen et al., 2008; Sancho et al., 2008). Noticeably, different DNGR-1 isoforms are detected in mouse tissues (Quintana et al., 2015; Sancho et al., 2008). Cells expressing DNGR-1 are restricted to a small subset of DCs including mouse splenic $CD8\alpha^+$ DCs and peripheral $CD103^+$ DCs and human $BDCA3^+$ DCs sharing similar functional capacity to that of mouse (Caminschi et al., 2008; Huysamen et al., 2008; Jongbloed et al., 2010; Kelly et al., 2014; Martinez et al., 2015; Poulin et al., 2012; Poulin et al., 2010; Sancho et al., 2008), and DNGR-1 molecules are co-expressed with XCR1, Necl2, IRF8, and TLR3 molecules (Breton et al., 2015; Haniffa et al., 2012; Martinez et al., 2015; Poulin et al., 2010; Proietto et al., 2012). Recently, the equivalence of $CD8\alpha^+$ DCs and $BDCA3^+$ DCs in rhesus macaque has been identified (Dutertre et al., 2014).

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Rhesus macaques (*Macaca mulatta*), sharing genetic, physiological and immunological similarities with humans, are invaluable animal models for understanding the pathogenesis, immunity, and developing novel vaccine strategies for human infectious diseases (Messaoudi et al., 2011), especially the unquestionable utility of SIV/macaque model for AIDS researches (Evans and Silvestri, 2013; Hatziioannou and Evans, 2012; Hu, 2005; Sui et al., 2013). However, little is known about DNGR-1 in rhesus macaques. Here, we described the nucleotide sequence of rhesus macaque DNGR-1, characterized its binding activity *in vitro* to apoptotic/dead and pyroptotic cells, and its transcriptional levels in tissues. The presence of human-like DNGR-1 in rhesus macaque could promote significant advancement in the development of DNGR-1 targeting therapeutics and vaccines, and in the understanding of its role in human diseases, especially in the prevention and treatment of AIDS.

2. Material and methods

2.1. Cells, tissues, and animals

2.1.1. Blood cells

Blood samples from rhesus macaques were obtained commercially from an animal breeding facility (Sharing, Beijing). Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation on Ficoll-Paque PLUS (GE-healthcare, Sweden) (Lei et al., 2013).

2.1.2. Cell lines

The cell lines used in this study were THP-1, MT-4, TZM-bl, HTR-8, HeLa, 293T, and T-84. The cells were cultured either in RPMI1640 (THP-1, MT-4, TZM-bl, and HTR-8), DMEM (HeLa and 293T), or in DMEM plus FAM'S/F-12, 1% NEAA, and 1% sodium pyruvate (T-84). All the culture media were supplemented with 10% fetal bovine serum (FBS), L-glutamine (1%), penicillin (100 U/ml), and streptomycin (100 μg/ml). All cells were maintained at 37 °C in 5% CO_2.

2.1.3. Tissues

Lymphoid tissues from thymus, bone marrow, spleen, lymph nodes, and non-lymphoid tissues from organs of the digestive, urogenital, nervous, and cardiopulmonary systems were obtained from 5 normal rhesus macaques of Chinese origin. All the samples were archived frozen tissues that had been preserved at −80 °C after collecting immediately post euthanasia of the animals.

2.1.4. Animals

New Zealand white rabbits, all female and 2.0 kg weight, were purchased from the HFK (Beijing, China). All animal experiments were performed in accordance with the local and state laws and regulations and were approved by the Institutional Animal Care and Use Committee (IACUC) at China CDC.

2.2. RNA isolation

Total RNA was extracted using an RNAprep pure Tissue Kit (Tiangen, China) from frozen tissues or PBMCs as described previously (Wang et al., 2014). The extracted RNA was preserved as aliquots at −80 °C before use.

2.3. Rapid amplification of cDNA ends

Rapid Amplification of cDNA Ends (RACE) was performed using a 5′- and a 3′-Full RACE Kits, respectively (TaKaRa, Japan), to clone the *DNGR-1* gene of rhesus macaques, according to the manufacturer's instructions (Wang et al., 2014). The nucleotide sequences of the gene-specific primers were designed according to the nucleotide sequence of *DNGR-1* derived from Indian rhesus macaque genomic sequence (NM_001194664.1) and that of *Homo sapiens* (NM_207345.2). For 5′RACE, primers DNG 4 R and DNG 3 R were used; for 3′RACE, primers DNG 2 F, DNG 5 F, 3 DNG 3F, and 3 DNG 2R were used (Supplementary Table 1). The cDNAs synthesized were amplified by nested PCR and TaKaRa LA PCR™ Kit was used to set-up the reaction mixtures according to the manufacturer's recommedations. The PCR end-products were conducted as described previously (Wang et al., 2014), and the retrieved plasmids were commercially sequenced (Genewiz, Beijing, China).

The genomic localization of rhesus macaque *DNGR-1* was identified by alignment of the obtained nucleotide sequence to the Rhesus assembly [Oct.2010 (BGI CR_1.0/rheMac3)] on UCSC Genome Brower (available at: http://genome.ucsc.edu/). The complete nucleotide sequence of *DNGR-1* cDNA was submitted to GenBank (KT378619.1).

2.4. Real-time RT-PCR

RNA standards for quantitative RT-PCR were prepared, and TaqMan® probe RT-PCR methods for *DNGR-1*, *XCR1* and *Flt3L* genes were established as described previously (Wang et al., 2014). The gene-specific primers and probes were listed in Supplementary Table 1. The Real-time RT-PCR was performed on 7500 Real-time PCR system (ABI, USA) with One Step PrimeScript® RT-PCR Kit (TaKaRra, Japan) as per the manufacturer's instructions. The specific primers and probes were qClec F, qClec R, and probe Clec for *DNGR-1*, R-1E-F, R-3E-R, and M Probe XCR for *XCR1* and qFlt3L F, qFlt3L R, and probe Flt3L for *Flt3L*.

The levels of *GAPDH* mRNA in the same tissues were analyzed. The *DNGR-1* transcriptional levels of each tissue were determined using a standard curve and expressed as a ratio relative to per million copies of the *GAPDH* gene.

2.5. Preparation of polyclonal antibody against rhesus macaque DNGR-1

To generate polyclonal antibodies against rhesus macaque DNGR-1, DNA coding for rhesus macaque DNGR-1 CTLD amplified with 5′-GGAATTCCATATGCCTTGTCCAAACAATTG-3′ and 5′-CCGCTCGAG GACAGAGGATCTTAATGC-3′ primers was cloned into pET-32a(+) vector (Novagen, USA). The recombinant DNGR-1 CTLD proteins (rh-CTLD) tagged with 6× His tag were expressed in BL21 (*DE3*) cells (Novagen, USA) as described previously (Yao and Yang, 2014), and extracted with the BugBuster™ Protein Extraction Reagent (Novagen, USA), purified using Ni-Agarose Resin (CWBIO, China), refolded, and preserved in PBS at −80 °C before use.

New Zealand white rabbits were injected i.d. and s.c. with rh-CTLD and CFA at multiple sites for primary immunization. For the booster immunization, the animals were inoculated i.m. three times consecutively with rh-CTLD and IFA (Cooper and Patterson, 2008). The antiserum (against rhesus macaque DNGR-1, anti-rhDNGR-1) that tested positive by ELISA was purified using the MabAffinity rProtein A Agarose High Flow Kit (ACRO Biosystems, China), and was verified using Western blotting by comparing with that of a commercially available anti-human CLEC9A antibody (anti-CLEC9A, Abcam, UK).

2.6. Expression of rhesus macaque DNGR-1 in HeLa cells

The complete DNGR-1 ORF was amplified from rhesus macaque spleen RNA with PrimeSTAR® HS DNA Polymerase (TaKaRa, Japan) by RT-PCR using the primers 5′-CACCGCCGCCCACCATGGTGCATGA GGAAGAAATATAC-3′ and 5′-GACAGAGGATCTTAATGCATAC-3′, and cloned into the pcDNA™3.1D/V5-His TOPO® vector (Invitrogen, USA). The fidelity of all constructs was verified by sequencing. The desired construct and the pcDNA™3.1D/V5-His/*lacZ* control were transfected into HeLa cells using PEI (Polysciences, USA). The transfected cells were tested after 72–120 h by flow cytometry and confocal microscopy.