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Ficolin-2 triggers antitumor effect by activating macrophages and CD8$^+$ T cells

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ABSTRACT

Ficolin-2 is an important serum complement lectin. Here, we describe novel findings indicating that serum ficolin-2 concentrations in multiple tumor patients are significantly lower than those in healthy donors. Administration of exogenous ficolin-2 or ficolin-A (a ficolin-2-like molecule in mouse), with only once, could remarkably inhibit the tumor cells growth in murine tumor models via early macrophages, dendritic cells (DCs) and CD8$^+$ T cells, but not CD4$^+$ T cells. Ficolin-A (FCN-A) knockout (KO) mice exhibits significantly increased tumor cell growth. Ficolin-2 induces macrophage activation, promotes M1 polarization and facilitates proliferation and antigen-specific cytotoxicity of CD8$^+$ T cells. Ficolin-2 binds to Toll-like receptor 4 (TLR4) on macrophages and DCs and promotes their antigen-presenting abilities to CD8$^+$ T cells. Our findings provide a new therapeutic strategy for tumors based on the triggering of immune-mediated antitumor effect by ficolin-2.

1. Introduction

Human serum ficolins are a group of complement lectins with an overall structure similar to that of mannose-binding lectin (MBL) and C1q. These oligomeric lectins play key roles in first-line host defense, including opsonization, phagocytosis, induction of apoptosis, and activation of complement cascades, and inflammation [1,2]. Ficolins act as soluble pattern-recognition molecules or receptors (PRRs) and recognize pathogen-associated molecular patterns (PAMPs) on the surfaces of pathogens and apoptotic cells. They also interact with a broad spectrum of infectious pathogens and can participate in microbe infection and apoptotic cell clearance by activating the complement lectin pathway or primitive opsonophagocytosis [3–5].

Three human ficolins, namely, L-ficolin/P35 (FCN-2 or ficolin-2), M-ficolin (FCN-1 or ficolin-1), and H-ficolin/Hakata antigen (FCN-3 or ficolin-3), and two mouse ficolins (ficolins-A and -B) have been identified [6,7]. Mouse ficolin-A is closely related to human ficolin-2, and they are mainly synthesized in the liver and then secreted into blood circulation. Ficolin-2 has a single-chain molecular weight of 35 kDa and shows lectin-like activity toward GlcNAc, 1,3-β-D glucan, lipoteichoic acid (LTA), and various acetylated compounds [7–9].

Increasing studies have shown that ficolin-2 can bind to viral and bacterial glycoproteins to inhibit viral and bacterial infections [10–16]. Furthermore, abnormal ficolin-2 expression plays a crucial role in various infectious diseases [13–17]. A recent report showed that ficolin-3 directly attacked cancer cells via a novel complement pathway [18]. Lower ficolin-2 protein expression was found in the tissue samples of HCC patients with HCC metastasis compared to non-metastasis patients [19]. Despite this, little is known about ficolin-2's functions during malignancy.

Generally, CD8$^+$ T cells, macrophages and dendritic cells (DCs) played important roles in human anti-tumor immunity [20–22]. Classical macrophages (M1 macrophages), which are known as proinflammatory immune cells, express high levels of proinflammatory cytokines (TNF-α, IL-1, IL-6, IL-12 or IL-23) and MHC-II molecules, induce nitric oxide synthase (iNOS) and are reported to be capable of killing pathogens and priming antitumor immune responses [21]. In contrast, alternative macrophages (M2 macrophages), which typically produce TGF-β and arginase 1 (Arg-1), are characterized as tumor-promoting cells [23]. However, the valuable insight of ficolin-2 in association with immune cells, especially M1 and M2 macrophages, in the context of cancer remains elusive. Therefore, in the present work, we investigated the immune roles of serum ficolin-2 in association with immune cells in several important cancers, including colon cancer, lung cancer and hepatocarcinoma.

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2. Materials & methods

2.1. Ethics statement and participant inclusion criteria

This study was approved by the ethics committee of Wuhan University School of Medicine. Written consent was obtained from all of the participants. Fresh serum samples were obtained from 346 cancer patients at Hubei Province Cancer Hospital, China, and Wuhu No. 2 People's Hospital of Anhui Province, China, between 2012 and 2015. All patients were confirmed with tumor marker evaluations, histopathologic diagnosis, chest computer tomography (CT) detection or other imagine techniques. Patients receiving immunosuppressive regimens or those known to be infected with human immunodeficiency virus or tuberculosis were excluded. Fresh serum samples from 143 healthy donors (HDs) were obtained from the medical examination center of Zhongnan Hospital at Wuhan University from 2012 to 2015, based on clinical and laboratory findings with no signs or symptoms of tumors and other diseases. Fresh serum samples were collected into the same type of blood collection tubes and tested timely [24,25]. All subjects were unrelated Chinese of Han ethnicity.

2.2. Animals

BALB/c, C57BL/6, TLR4$^{-/-}$ C3H/HeJ, TLR4$^{+/+}$ C3H/HeN, and nude mice (20–25 g, 6 to 8 weeks, SPF level) were purchased from the Animal Center of Wuhan University, China. FCN-A KO mice were produced by disrupting the FCN-A gene in C57BL/6 mice through homologous recombination [10]. IL-6$^{-/-}$ C57BL/6, IFN-γ$^{-/-}$ C57BL/6, TLR4$^{-/-}$ and TLR2$^{-/-}$ C57BL/6 mice were kindly provided by Dr. Zhinan Yin. OT-I (C57BL/6-Tg (TcraTcrb) 1100Mjb/J) mice were kindly provided by Prof. Jianqin Xu from the Shanghai Public Clinical Health Center (SPHCC) (with transgenic inserts for mouse Tcra-V2 and Tcrb-V5 genes). The transgenic T cell receptor in OT-I mice was designed to recognize ovalbumin (OVA) residues 257–264 in the context of H2Kb, and these mice have been used to study the response of CD8$^+$ T cells to antigens.

All care of laboratory mice and mouse experiments were carried out in accordance with the Code of Ethics of the World Medical Association (EU Directive 2010/63/EU relating to animal welfare) and the Guide for the Care and Use of Laboratory Animals from the National Academy of Science (http://www.nap.edu/readingroom/books/labrats/).

2.3. Cells

Mouse CT26 colon carcinoma cell line, Lewis lung carcinoma cell line and Hca-f hepatocarcinoma cell line were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) (HyClone, GE Healthcare Life Science, UT, USA) and 1% penicillin-streptomycin.

Bone marrow derived M0 macrophages (M0-BMDM) were differentiated from isolated mouse bone marrow cells with incubation of 10% FBS plus 40 ng/ml M-CSF for seven days. Bone marrow derived M2 macrophages (M2-BMDM) were induced from M0 macrophages via stimulating with 20 ng/ml of IL-4 and IL-13 for 24 h [26].

CD8$^+$ T cells and CD4$^+$ T cells were isolated from mouse splenocytes using the BD TM IMag Mouse CD8$^+$ T and CD4$^+$ T Lymphocyte Enrichment Set-DM (BD, Biosciences).

2.4. Measurement of serum ficolin-2 concentrations

Sandwich enzyme-linked immunosorbent assays (ELISAs) were used to measure the concentrations of serum ficolin-2 as previously described [10,17]. 96-well ELISA plates were coated with 100 μl of rabbit anti-ficolin-2 polyclonal antibody (pAb) (1:200 dilution) [17]. After incubation at room temperature (RT) for one hour, the solution was removed, and the plates were rinsed. After washing three times with 0.2% Tween-20 in phosphate-buffered saline (PBS), 100 μl of each serum samples were incubated at 37 °C for 2 h. The plates were washed three times and blocked with 5% bovine serum albumin (BSA) overnight. Subsequently, a monoclonal antibody (mAb) against human ficolin-2 GN5 (1:1000, HyCult Biotechnology b.v.) was added to each well and incubated at 37 °C for 1 h. The plates were washed three times and incubated with 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:2000, 10285-1-AP, Proteintech). Color development was achieved by adding 100 μl/well of tetramethylbenzidine (TMB) chromogen substrate (Sigma-Aldrich). The reaction was terminated by adding 100 μl of 0.5 M H_2SO_4, and the OD at 450 nm was measured using an ELISA reader. Data were obtained from at least three independent experiments. All statistical data shown represent the mean ± SEM. Concentrations of ficolin-2 were determined by generation of a standard curve.

2.5. Construction of recombinant plasmids

Full-length ficolin-2 (GenBank accession no. NM004108) and ficolin-A (GenBank accession no. NM007995) were amplified and subcloned in-frame into eukaryotic expression vectors pcDNA3.1(-) Myc-His (named as pc), pVAX-1 (named as pV) (Invitrogen, Carlsbad, CA) and prokaryotic expression vectors pET-28a and pGEX-KG to generate plasmids pcDNA3.1-ficolin-2, pVAX1- ficolin-A, pET-28a-ficolin-2 and pGEX-KG-ficolin-2, respectively. pET-28a-ficolin-2 was transformed into *Escherichia coli* (*E. coli*) BL21(DE3)[pLysS], and the ficolin-2-6xhis fusion protein was expressed in the *E. coli* after induction by Isopropyl β-D-1-Thiogalactopyranoside (IPTG). The expressed protein was purified over Ni-NTA Agarose (QIAGEN, Chatsworth, CA) according to the manufacturer's instructions. pGEX-KG-ficolin-2-D1 (113–313 aa), pGEX-KG-ficolin-2-D2 (201–313 aa) and pGEX-KG-ficolin-2-D3 (26–111 aa) were constructed and overexpressed in *E. coli* after induction by IPTG, and the proteins were purified using Glutathione Sepharose 4B (Amersham Biosciences). The purified proteins were further treated with endotoxin-removing resin (containing 50 μg/ml polymyxin B) (Sigma-Aldrich, St Louis, MO, USA) and examined by SDS- polyacrylamide gels (PAGE) and western blotting. The recombinant ficolin-2 protein was identified by native PAGE.

2.6. SDS-PAGE and western blotting analysis

Freshly mouse thigh muscle and liver tissues samples were harvested and frozen immediately in liquid nitrogen. Tissue samples were homogenized in lysis buffer using a tissue homogenizer. After measuring protein concentration (Bio-Rad, Hercules, CA, USA), equal protein amounts were loaded on 10% SDS- PAGE. Proteins were blotted onto polyvinylidene fluoride membranes, which were incubated with primary antibodies overnight at 4 °C. Anti-ficolin-2 monoclonal antibody (mAb) (FCN219, Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect ficolin-2 and rabbit anti-ficolin-A pAb [17] was used to detect ficolin-A. Subsequently, membranes were incubated with secondary antibodies anti-IgG for 30 min at 37 °C. The signals were visualized using the ECL system.

2.7. Native PAGE

Prepare 20 μg of ficolin-2-6×his in a final volume of 20 μl with PBS following by adding 2× native sample buffer (1.25 ml 0.5 M Tris-HCl pH 6.8, 3 ml glycerol, 2 ml 0.5% bromophenol blue, 5.5 ml dH_2O) to a final concentration of 1×. Load the samples on a native PAGE with 10% as stacking and 5% as resolving gel. Run the gel with pH 8.8 Tris-Gly buffer (3.03 g This-base, 14.4 g glycine to 1 L dH_2O).