Immunobiology 224 (2019) 316–324

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Interactions of ficolin-3 with ovarian cancer cells

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ARTICLE INFO

Keywords:
Ficolin-3
H-ficolin
Complement
Ovarian cancer
Innate immunity

ABSTRACT

Background: Ficolin-3 is a pattern-recognition molecule with the ability to activate the lectin pathway of complement. It is found in lung, liver and blood, but its physiological role is unclear. We have investigated interaction of recombinant ficolin-3 with malignant cells and tissues.
Material and Methods: Cells of various lines of human origin as well as ovarian tissue sections have been studied with the use of flow cytometry and immunohistochemistry.
Results: Recombinant (but not serum-derived) ficolin-3 was found to bind strongly to the ovarian cancer cell lines, SKOV-3, OVCAR-3 and ES-2, at concentrations of 2.5 μg/ml and above. Moreover, His-tagged recombinant ficolin-3 (10 μg/ml) preferentially stained ovarian tissue sections from patients with malignant tumours compared with those from patients without. Binding to cell lines was inhibited by EDTA and specific carbohydrate ligands, indicating involvement of the fibrinogen-like domain. Binding was enhanced under mildly acidic conditions and at physiological pH after pre-incubation of cells with mildly acidic buffer.
Conclusion: Basing on data concerning recombinant protein, it may be suggested that ficolin-3 is involved in immune response in ovarian cancer. However, unidentified serum factor(s) seem(s) to protect cancer cells from recognition by natural or rficolin-3.

1. Introduction

Changes in protein glycosylation are common features of tumour cells (Hakomori and Handa, 2002). Moreover, many glycosyl epitopes constitute tumour-associated antigens. Clinical studies have revealed that high expression of some glycans may be associated with promotion of tumour invasion and metastasis while others with suppression of cancer progression. However, little is known about how specific glycans influence tumour fate. Some glycoproteins are established cancer biomarkers. The cancer antigen 125 (CA-125, mucin 16), a highly glycosylated protein with carbohydrate chains composed of D-galactose (D-Gal), *N*-acetyl-D-glucosamine (D-GlcNAc) and *N*-acetyl-D-galactosamine (D-GalNAc), is considered the main ovarian cancer biomarker (Bouanene and Miled, 2010). Its carbohydrate-rich structures may be a target for endogenous pattern recognition molecules, including collectins and ficolins. Although knowledge about the interactions of these proteins with cancer cells is limited, several reports regarding mannose-binding lectin (MBL) brought interesting findings.

Ma et al. (1999) first reported that MBL recognized and bound specifically to D-mannose (D-Man) or D-GlcNAc-terminated oligosaccharides found on the surfaces of human colorectal carcinoma SW1116 cells. Later, a large N-glycan with highly fucosylated polylactosamine-type structures having Lewis antigens ($Le^{(b)}$-$Le^{(a)}$ or tandem repeats of the $Le^{(a)}$) at the non-reducing end was identified as the ligand (Terada et al., 2005). Finally CD26/dipeptidyl peptidase IV (DPPIV) and CD98 heavy chain (CD98hc)/4F2hc were proposed as the major structures decorating those cells, and were targets for MBL (Kawasaki et al., 2009). Moreover, it was demonstrated that MBL also recognizes human primary and metastatic colorectal carcinoma cells and partially overlaps with $Le^{(b)}$ expression in carcinoma tissues (Nonaka et al., 2014). MBL staining inversely correlated with C19-9 (α2,3-sialyl-Le, pancreatic and gastrointestinal cancers biomarker) expression, but positively with favourable survival rate. Evidence of anticancer activity of MBL was demonstrated by injection of vaccinia virus-carrying human wild-type MBL-specific cDNA into the tumour mass induced in athymic nude mice from subcutaneous injection of SW1116

Abbreviations: C, control; OC, ovarian cancer; BT, benign tumours; MBL, mannose-binding lectin

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https://doi.org/10.1016/j.imbio.2019.01.002
Received 4 October 2018; Received in revised form 25 January 2019; Accepted 25 January 2019
Available online 30 January 2019
0171-2985/

cells. This led to MBL synthesis, a marked reduction in tumour size, and prolonged life span. Although the mechanism of MBL anti-tumour action remained unexplained, it was thought not to be associated with complement activation and instead attributed to MBL-dependent cell-mediated cytotoxicity (MBL-DCC) (Ma et al., 1999).

Taking into account the structural and functional similarities between collectins and ficolins it may be assumed that interaction of ficolin-3 with cancer cells is also possible. Ficolin-3 (H-ficolin, Hakata antigen) is a pattern recognition molecule (PRM) with the ability to recognize acetylated groups and sugar residues [for example the O-specific polysaccharide (O-PS) of *Hafnia alvei* PCM 1200 LPS (Swierzko et al., 2012)], activate the lectin pathway of complement, and enhance phagocytosis. Although some antibacterial activity of ficolin-3 has been described (Michalski et al., 2015; Tsujimura et al., 2002), little is known about any interaction(s) of ficolin-3 with host cells. Its recombinant form has only been reported to interact with apoptotic cells (Honore et al., 2007) suggesting that ficolin-3 might help protect against autoimmune diseases.

Association of ficolin-3 with carcinogenesis or its direct interaction with tumour cells has not been extensively investigated. Andersen et al. (2010), using differential in-gel electrophoresis (DIGE), found its overexpression in sera from ovarian cancer (OC) patients relative to controls and suggested that it could be an useful biomarker of that disease. Later, we showed that median ficolin-3 concentration in pre-operative serum samples from OC patients was significantly higher than in women with benign ovarian tumours or normal ovaries (Szala et al., 2013). In contrast, *FCN3* mRNA local relative expression level was significantly lower in patients with ovarian cancers than in controls (Szala et al., 2013), but it was impossible to distinguish between disease stages, histological types of tumour, or tumour grade. Decreased ficolin-3 expression was also observed in hepatocellular carcinomas and squamous cell lung carcinoma (Luo et al., 2006; Shi et al., 2011). It is unclear whether local under-expression of ficolin-3 is a risk factor for, or a result of, carcinogenesis.

Here we report the binding of recombinant ficolin-3 to the cells of various human-derived cell lines. To our knowledge, this is the first example of viable cells of human origin being recognized by ficolin-3.

2. Materials and methods

2.1. Cell lines

HeLa, HepG2, Huh7, A549, ES-2, SKOV-3, OVCAR-3, U-937, Mono Mac 6, THP-1, HEK293, SW-620, Jurkat, HT-29, CCRF, Ramos, MOLT-4, Daudi and HL-60 and HUVEC cell lines came from the collection of the Institute of Medical Biology Polish Academy of Science (IMB PAS, Lodz, Poland). The cells were routinely grown in DMEM (Gibco, USA) or RPMI (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), L-glutamine/penicillin/streptomycin (Gibco) (37 °C, 5% CO_2).

2.2. Preparation of ficolin-3 ligands

LPS was extracted from *Hafnia alvei* PCM1200 bacteria by the hot phenol/water method (Westphal and Jann, 1965) and purified as described previously (Petersson et al., 1997). O-specific polysaccharide (O-PS) was isolated by mild acidic hydrolysis and fractionated as described elsewhere (Dag et al., 2004). *H. alvei* PCM1200 was obtained from the Polish Collection of Microorganisms (PCM) at the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy (Wroclaw, Poland). The acetylated BSA (Ac-BSA) was prepared as described by Munthe-Fog et al. (2009).

2.3. Histological sections

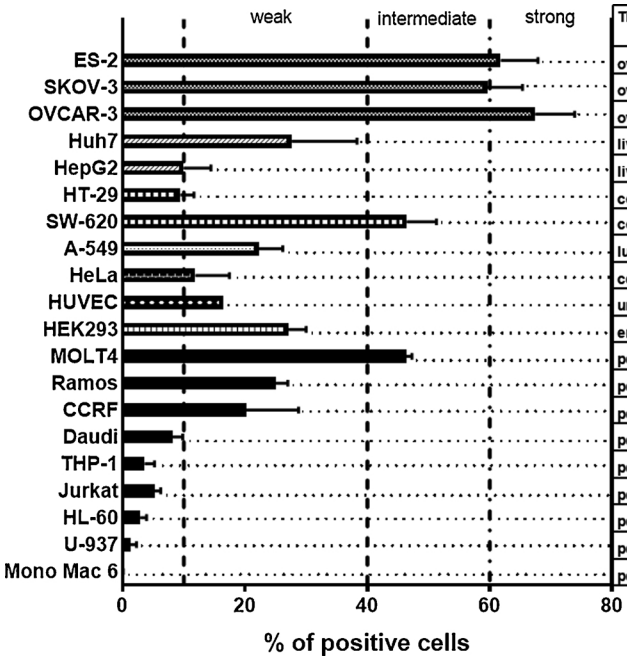
The ovarian tissue samples were obtained from 103 patients from the Department of Gynaecology, Oncologic Gynaecology and Gynaecologic Endocrinology, Medical University of Gdansk, Poland, during realization of our previous project (Szala et al., 2013). Among them, 48 patients were diagnosed with primary ovarian cancer (OC group). Two groups of women undergoing surgery for reasons other than malignancies were collectively classified as controls (C): 40 were diagnosed with benign tumours of the ovary (BT), while 15 were operated on because of leiomyomas or dysfunctional uterine bleeding but without pathological changes in the ovaries (NO group). The BT group included patients with ovarian serous cysts, adenomas, fibromas, endometriosis and teratomas. Approval of the local ethical committee (Independent Bioethics Commission for Research of Gdansk Medical Academy (NKEBN/3/2007), was obtained, as was written informed consent of patients.

2.4. Immunostaining of tissue sections

Paraffin embedded tissue sections were treated with xylene, hydrated and pretreated in microwave in citrate buffer. After incubation for 1 h at RT in 150 mM Tris-buffered saline, supplemented with 5 mM $CaCl_2$ and 1% BSA (pH 7.4) (1% BSA/TBS-Ca), the sections were incubated with 500 ng of His-tagged recombinant ficolin-3 (rficolin-3) (R &D Systems, USA) in 50 µl of TBS-Ca buffer (10 µg/ml) at 4 °C, overnight. After washing, biotinylated anti His-tag antibody (Abcam, UK) were added (1:500) in TBS-Ca and incubated overnight at 4 °C. After washing, the tissue sections were incubated with HRP-streptavidin (Dako, Denmark) and HRP substrate (DAB-chromogen, Dako) for 30 min at RT. The tissues were counterstained with hematoxylin according to standard procedures. To exclude nonspecific binding of recombinant ficolin-3 *via* its His-tag, His-tagged irrelevant protein was used in the preliminary experiments (recombinant His-tagged *Mycobacterium tuberculosis* RecA protein expressed in *E. coli* (not shown). All tissue sections were evaluated in a coded manner without knowledge of the clinical and pathological parameters. By taking into account the staining intensity of samples with recombinant ficolin-3 added or without (control), results were scored from 0 to 3 (0 - no staining; 1 - weak staining; 2 - moderate staining; 3 - strong staining). Assessment was performed by four independent investigators and the means of intensity of staining were calculated.

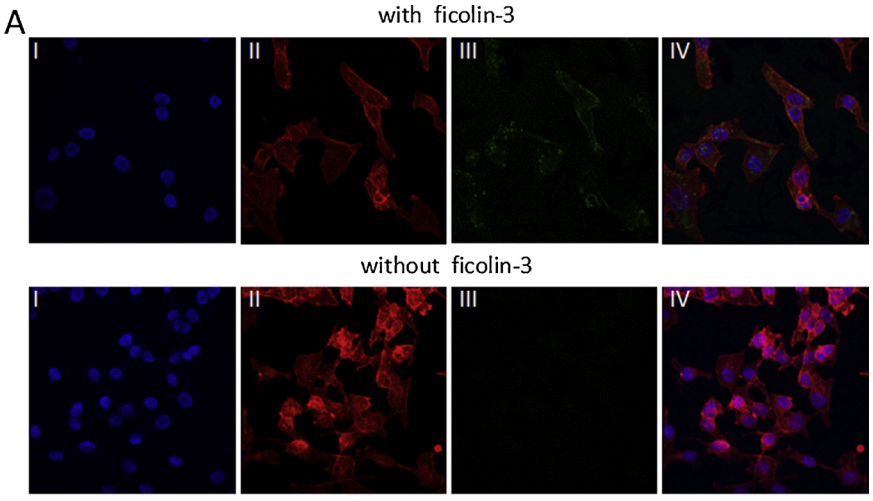
2.5. Flow cytometry

5×10^5 cells were incubated for 30 min at 4 °C in 1 ml veronal buffered saline (pH 7.5) supplemented with gelatin (0.1%), divalent cations (0.3 mM $CaCl_2$ and 2 mM $MgCl_2$) and 1% BSA (1% BSA/GVB^{2+}), followed by incubation with 500 ng of recombinant ficolin-3 in 200 µl of 0.1% BSA/GVB^{2+} (2.5 µg/ml) (pH 7.5) for 1 h at 37 °C. After that, the bound protein was detected with mouse anti-ficolin-3 (0.5 µg/ml, 40 min, 4 °C) (clone FCN334, BioPorto, Denmark) and AlexaFluor488-labelled secondary antibodies (0.5 µg/ml, 40 min, 4 °C) (ThermoFisher Scientific, USA). The cells were washed twice with PBS with 0.05% Tween-20 after each step and analyzed in a BD LSRII flow cytometer (Becton Dickinson, USA). Cells were detected using forward and side scatter dot plot. A total of 10,000 events were acquired. The ficolin-3 binding was calculated as percent of cells exceeding the peak for corresponding negative control (without added ficolin-3). While this procedure was used to screen the binding of ficolin-3 to various cell lines, some slight modifications were applied for further experiments: (i) inhibition assays: ficolin-3 (500 ng) alone or with LPS *H. alvei* PCM 1200 (1 µg) or its O-PS (1 µg) or Ac-BSA (1 µg) in 0.1% BSA/GVB^{2+} (pH 7.5) was incubated for 30 min at 37 °C, and then added to cells; (ii) pH-dependent binding assay: the binding was performed in two buffers: 0.1% BSA/GVB^{2+} (pH 7.5) and 0.1% BSA /GVB^{2+} (pH 6.5); (iii) the binding to pH treated cells: cells were incubated with 0.01% sodium azide for 30 min at 4 °C and then, after washing, treated over night at 37 °C in TBS-Ca buffer with various pH. Ficolin-3 binding was tested as



Tissue:	Morphology:	Disease:
ovary	fibroblast	clear cell carcinoma
ovary	epithelial	adenocarcinoma
ovary	epithelial	adenocarcinoma
liver	epithelial	carcinoma
liver	epithelial	hepatocellular carcinoma
colon	epithelial	colorectal adenocarcinoma
colon	epithelial	Dukes' type C, colorectal adenocarcinoma
lung	epithelial	carcinoma
cervix	epithelial	adenocarcinoma
umbilical vein/vascular endothelium	endothelial	-
embryonic kidney	epithelial-like	-
peripheral blood	lymphoblast	acute lymphoblastic leukemia
peripheral blood	lymphoblast	Burkitt's lymphoma (American)
peripheral blood	lymphoblast	acute lymphoblastic leukemia
peripheral blood	lymphoblast	Burkitt's lymphoma (American)
peripheral blood	monocyte	acute monocytic leukemia
peripheral blood	lymphocyte	acute T Cell Leukemia
peripheral blood	myeloblastic	acute promyelocytic leukemia
peripheral blood	monocyte	histiocytic lymphoma
peripheral blood	monocyte	acute monocytic leukemia

Fig. 1. Recombinant ficolin-3 binding to cells of various lines. The graph shows the mean (± SEM) of percentage of positive cells (data from at least four independent experiments).



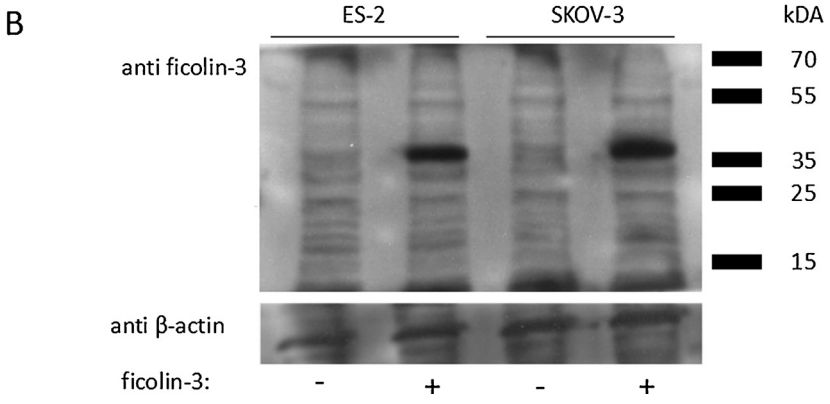


Fig. 2. Recombinant ficolin-3 binding to cells of ovarian cancer lines detected in confocal microscopy (A) and Western blot (B). SKOV-3 cells were seeded on the chamber slide and then incubated with recombinant ficolin-3 and detection antibodies (monoclonal anti-ficolin-3 antibody and AlexaFluor488- marked secondary antibodies). An upper panel (part A) shows data from cells incubated with rficolin-3 while the lower one - without. The order of pictures: I: nuclear staining by DAPI; II: actin staining by Texas Red-labelled phalloidin; III: ficolin-3 staining; IV: merged channels. For Western blot experiment (part B), recombinant ficolin-3 was added to ES-2 and SKOV-3 cells and, after incubation and washing (as detailed in methods) SDS-PAGE was performed and target protein and control β-actin were visualised by specific antibodies. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

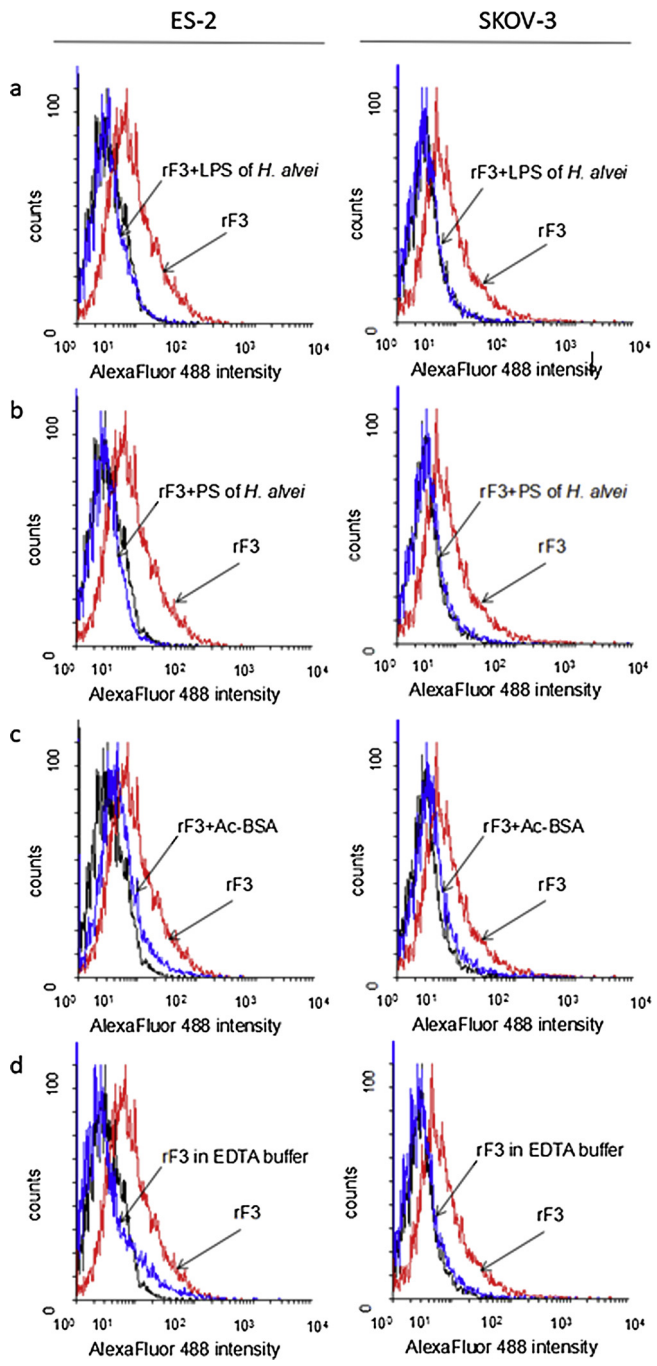


Fig. 3. Inhibition of ficolin-3 binding. Cells were incubated with rficolin-3 and with rficolin-3 preincubated with LPS of *H. alvei* PCM 1200 (A), its O-PS (B), Ac-BSA (C) or EDTA containing buffer (D) to ES-2 (left panel) or SKOV-3 (right panel). Unmarked lines (black) indicate controls without recombinant protein. Blue and red lines indicate samples of rficolin-3 with and without inhibitors, respectively. Data from one of three experiments are presented. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

described above, in GVB^{2+} (pH 7.5); (iv) cells were preincubated (1 h, 4 °C) with 10% NHS or TBS followed by incubation with rficolin-3 or rficolin-3 and 10% NHS.

Detection of apoptosis and necrosis was performed flow cytofluorometrically using Annexin V-FITC Apoptosis Staining/Detection Kit according to manufacturer's protocol (Abcam).

2.6. Confocal microscopy

SKOV-3 cells (2×10^4 per chamber) were cultured overnight as described above on chamber slides (Nunc™ Lab-Tek™ II Chamber Slide™ System, ThermoFisher Scientific) and, after blocking with 1% BSA/GVB^{2+}, recombinant ficolin-3 (500 ng) (2.5 μg/ml) in the same buffer was added and incubated for 1 h at 37 °C, followed by the incubation with anti-ficolin-3 monoclonal antibody (clone FCN334, BioPorto) (30 min, 37 °C and 30 min, 4 °C) and AlexaFluor488-labelled anti-mouse Ig. After cell fixation with BD Cytofix and permeabilisation with 0.1% Triton X-100 (Sigma-Aldrich), nucleus- and β-actin stainings were performed with DAPI (Life Technologies) and Texas Red-X Phalloidin (Life Technologies), respectively. The coverslips were mounted on glass slides using Mowiol (Sigma-Aldrich). The images were taken with the help of a confocal microscope (Nikon D-Eclipse C1 with EZ-C1 version 3.6 software).

2.7. Western blot

10^6 cells of tested lines were incubated with recombinant ficolin-3 (2.5 μg/ml) in GVB^{2+} buffer (pH 7.5) for 1 h at 37 °C. After washing in TBS-Ca supplemented with 0.05% Tween-20, cell lysates were separated on 10% polyacrylamide gels under reducing conditions and then transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, USA). Membranes were blocked with 1% BSA/TBS-Ca. Then, primary goat anti-ficolin-3 antibody (Santa Cruz Biotechnology, USA) was added, followed by the HRP-conjugated rabbit anti-goat Ig (Dako), as a secondary Ab. Membranes were incubated with electrochemical luminescence (ECL) substrate (Santa Cruz Biotechnology) and then exposed to an X-ray film. Anti-β-actin antibodies (Abcam) were used for loading control.

2.8. Statistical analysis

The distribution of staining intensity was verified by comparison with the normal distribution Shapiro–Wilk test in all subgroups. The distribution in OC group was not normal. The intensity of tissue staining was compared using *U*-Mann-Whitney test (2-sided). In analysis where more than two groups were analyzed, the Anova Kruskal-Wallis test and Dunn's post-hoc test were used. The frequencies of patients with ovarian cancer regarding positive staining were compared using the χ^2 test. 2×2 contingency tables were created to match OC and C groups with no staining or weak or intermediate or strong in relation to remaining patients. The 95-month survival was calculated using the Kaplan-Meier method, and the groups were compared with the use of a log-rank (Mantel-Cox) test. P values < 0.05 were considered statistically significant.

3. Results

3.1. Recombinant ficolin-3 strongly interacts with cells of ovarian cancer lines

Binding of recombinant ficolin-3 to cells of human-derived cell lines was analyzed by flow cytometry. Twenty cell lines originating from different tissues (ovary, liver, colon, lung, cervix, embryonic kidney, peripheral blood) were tested. The bound protein was detected with specific monoclonal antibodies. The strongest interaction of ficolin-3 at 2.5 μg/ml (> 60% of cells positive) was found for ovarian adenocarcinoma (SKOV-3 and OVCAR-3) and clear cell carcinoma (ES-2) cell lines (Fig. 1). Slightly stronger binding was obtained at 5 μg ficolin-3/ml (Supplementary data, Fig. S1), but since satisfactory results were obtained at the lower concentration, 2.5 μg/ml was chosen. Weaker binding (40–60% of cells positive) was observed for colorectal adenocarcinoma SW-620 and acute lymphoblastic leukemia MOLT-4 cell lines. The weakest binding (10–40% of cells positive) was detected with

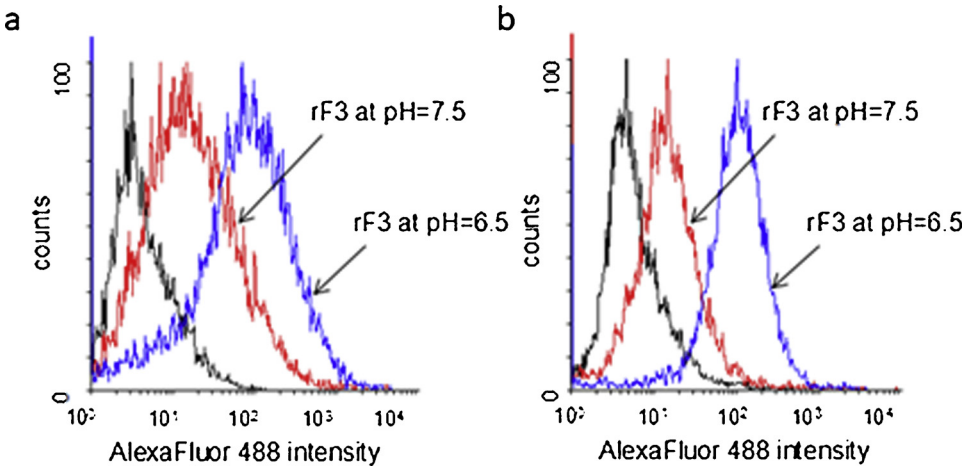


Fig. 4. Ficolin-3 binding, depending on pH. The binding of ficolin-3 was performed in GVB^{2+} with pH 7.5 or pH 6.5. Unmarked lines (black) indicate the control sample without ficolin-3 [pH 7.5 (similar result was for pH 6.5 data not shown)]. Blue and red lines indicate samples incubated at pH 6.5 and pH 7.5, respectively. (A) ES-2 cells, (B) SKOV-3 cells. Data from one of three experiments are presented. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Huh7, A-549, HeLa, RAMOS and CCRF cancer cell lines as well as HUVEC and HEK293 cells. Other cell lines tested were not recognized by recombinant ficolin-3 (< 10% of cells positive). Recombinant ficolin-3 interaction with SKOV-3 cells was confirmed by confocal microscopy and Western blot (Fig. 2A, B). We have not found any binding of rMBL (R&D), rficolin-1 (R&D) or rficolin-2 (R&D) to SKOV-3 and ES-2 (data not shown).

3.2. Recombinant ficolin-3 interacts with cells of ovarian cancer lines via its fibrinogen-like (FBG) domain

The ovarian cancer cell lines ES-2 and SKOV-3 were chosen for further experiments. Since < 10% of positive cells were apoptotic or necrotic, it may be concluded that binding of ficolin-3 is not restricted to cells undergoing programmed death or damage. To identify the ficolin-3 domain responsible for the interaction with the target on the surface of ovarian cancer cells, rficolin-3 was preincubated with its specific ligand, *H. alvei* PCM 1200 LPS (or its O-PS) or Ac-BSA before adding to SKOV-3 or ES-2 cells (Fig. 3A–C and Supplementary Fig. S2A and B). The observed inhibition of binding suggests the involvement of the fibrinogen-like domain of ficolin-3. Moreover, the binding of ficolin-3 with the cells of ovarian cancer ES-2 line was calcium- and/or magnesium-dependent since it was prevented in the presence of EDTA (Fig. 3D).

3.3. Lowering pH increases recombinant ficolin-3 binding to cells of ovarian cancer lines

Since inflammation is usually associated with lowering of pH, binding of recombinant ficolin-3 under physiological (pH 7.5) and pathophysiological (pH 6.5) conditions was compared. There was markedly enhanced binding at lower pH (Fig. 4 and Supplementary Fig. S3). However, lowering the pH again to 5.5 had no further impact (data not shown).

3.4. Acidic environment uncovers ligands for recombinant ficolin-3

Since acidic extracellular pH is a major feature of tumour tissue, the binding of recombinant ficolin-3 (at pH 7.5) was also tested after overnight incubation of cells in TBS-Ca buffers of varying pH. Lowering pH to 4.5 markedly improved ficolin-3 binding to both ES-2 and SKOV-3 cells in comparison with neutral pH (Fig. 5A–D). In contrast, preincubation of cells at pH 11 almost abolished ficolin-3 binding (Fig. 5E).

3.5. Recombinant ficolin-3 interacts with ligands in ovarian cancer tissue sections

The ability of recombinant ficolin-3 to recognize cells of ovarian cancer-derived cell lines prompted us to investigate the binding of this protein to ovarian sections. We compared sections from normal organs (NO) and those affected by benign tumours (BT) or malignant tumours (OC). Because the NO group included preparations from 15 patients only, it was combined with the BT group as the control group (C). OC was more frequent than NO/BT in preparations with strong ficolin-3 binding [82% *vs.* 18%, $p = 0.002$; OR = 6.15; 95%CI (1.25–29.91)] (Fig. 5). Conversely, among samples with weak or no staining, OC was observed less frequently. Moreover, median intensity of staining was higher in the OC group than in controls ($p < 0.0001$) (Fig. 6). We have not found any relationship between staining intensity and 95-month survival in the OC group ($p = 0.45$), however, it has to be taken into account that seven deaths among those patients were recorded only. Similarly, no difference was noted in median staining intensity between patients who survived and those who died ($p = 0.98$).

The intensity of staining correlated inversely with the expression of *FCN3* mRNA ($r = -0.32$, $p = 0.005$) and positively with *MASP2* mRNA ($r = 0.23$, $p = 0.046$) in ovarian sections [reported previously by Szala et al. (2013) and Swierzko et al. (2014)]. No correlation was found for *MBL2* or *FCN2*-specific mRNA.

3.6. Serum factor(s) abolish(es) ficolin-3 interaction with cancer cells

The interaction of serum ficolin-3 with cells of ovarian cancer lines was also tested. ES-2 or SKOV-3 cells were incubated with 10% serum from healthy donor with normal level of biologically active ficolin-3 (25 μg/ml). Unexpectedly, no binding was observed whereas recombinant ficolin-3 (positive control) binding was still detectable (Fig. 7A, B). Similar results were obtained when sera from other healthy donors were used. Moreover, no activation of complement C4 was also observed (data not shown). The applying of various buffers (TBS-Ca, GVB++, imidazole buffer, MBL-binding buffer), lowering of pH, increased/decreased temperature and time of incubation resulted in no improvement of serum ficolin-3 binding to ES-2 and SKOV-3 cells. Moreover, the presence of 10% human serum (normal as well as ficolin-3 or MASP-2 deficient) abolished the binding of recombinant ficolin-3 to ovarian cancer cells (Fig. 7B). In contrast, preincubation of cells with 10% human serum, followed by washing and addition of recombinant ficolin-3 led to cell labelling by recombinant ficolin-3 (Fig. 7C).

4. Discussion

Although microbial ligands are considered the main target for

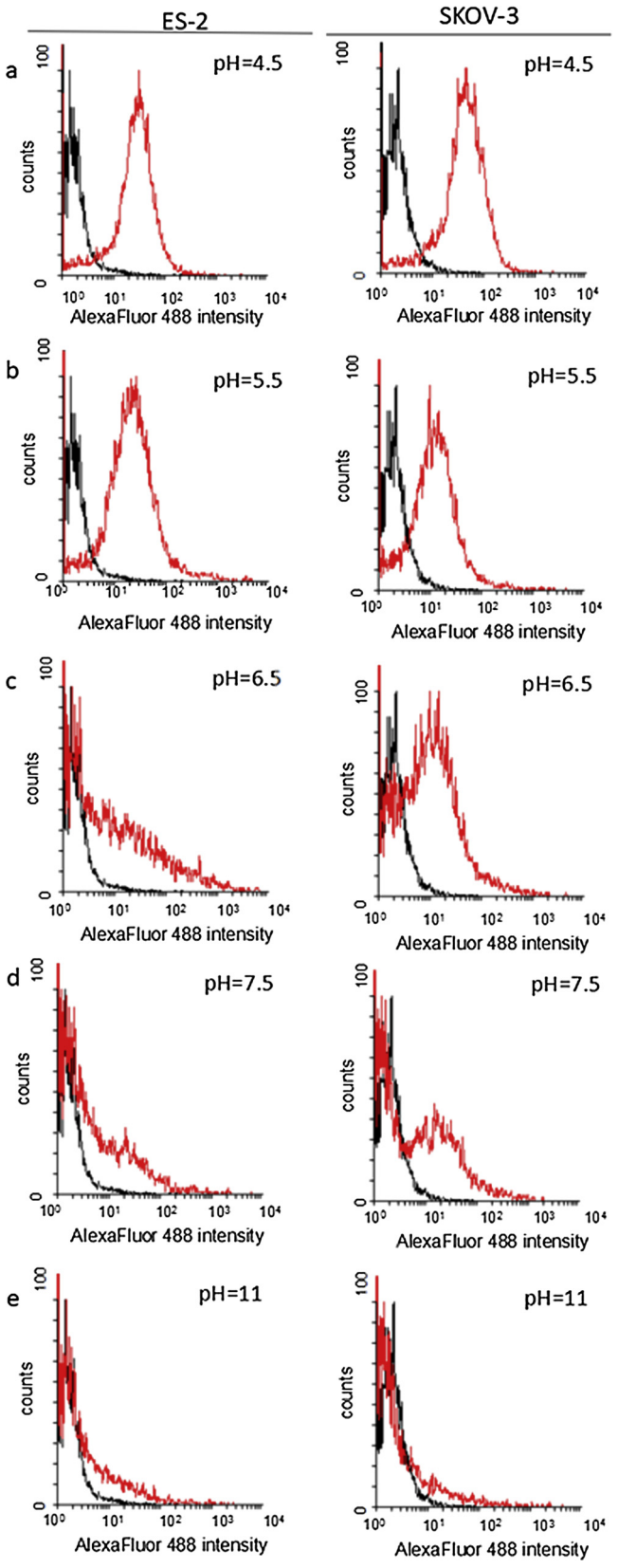


Fig. 5. Ficolin-3 binding after treatment of cells at various pH values. Cells were incubated in TBS-Ca buffers ranging from pH 4.5 to 11, and then, ficolin-3 binding in GVB^{2+} (pH 7.5) was tested. Black lines indicate the control sample without ficolin-3. Data from one of three experiments are presented.

ficolins and collectins, several reports have described their interactions with eukaryotic cells. Recombinant MBL, ficolin-2 and -3 were shown to interact with apoptotic/necrotic cells and are believed to participate in their clearance (Honore et al., 2007; Jensen et al., 2007; Kuraya et al., 2005; Nauta et al., 2003). Moreover, it was demonstrated that MBL and ficolin-2 may have the potential to function as soluble recognition molecules for scavenging apoptotic materials *via* direct interaction with CD91 or its partner- calreticulin (Duus et al., 2010; Pagh et al., 2008). It has also been suggested that detection of ficolin-2 and -3 in apoptotic trophoblasts indicates involvement in the development of clinical manifestations of preeclampsia (Wang et al., 2007).

Here, we describe the interactions of recombinant ficolin-3 with another type of eukaryotic cell – present in human ovarian cancer sections or coming from ovarian cancer derived cell lines. Weaker interactions were also observed for colorectal adenocarcinoma (SW-620) and acute lymphoblastic leukemia (MOLT-4) cell lines. It was previously reported that rficolin-3 is able to bind necrotic and apoptotic but not viable cells of JURKAT cell line (Honore et al., 2007) and we have confirmed the latter finding. However, this is the first report demonstrating direct interactions between recombinant ficolin-3 and viable cancer cells although corresponding results were published for MBL. MBL was found to bind to cells of six glioma lines (T98 G, A-172, U-87MG, U251, A7, 1321N1) (Fujita et al., 1995) and three colon adenocarcinoma lines (Colo205, Colo201 and DLD-1) (Muto et al., 1999). The binding of MBL to colon adenocarcinoma cells was sugar-specific and calcium-dependent, since it was almost completely inhibited in the presence of mannose or EDTA (Muto et al., 1999). Our observations with ficolin-3 were similar since the interactions required the presence of divalent cations and were completely inhibited by known ligands for ficolin-3: Ac-BSA, *H. alvei* PCM 1200 LPS or its O-PS (Fig. 3). These results indicate that the fibrinogen-like domain (determining lectin properties of ficolins) is responsible for binding to cancer cells. The target structures on the surface of ovarian tumour cell lines have not been determined, but binding was enhanced under acidic conditions (Fig. 4) and even above neutral pH when the cells had previously been exposed to an acidic environment (Fig. 5). These findings suggest the target cell ligand(s) may be more fully exposed by mildly acidic conditions. It is important to emphasize that due to excessive lactic acid secreted by solid tumors, the tumour extracellular environment shows a lower pH in comparison with normal physiological conditions (Jähde et al., 1982; Tannock and Rotin, 1989; Cardone et al., 2005). The acidic microenvironment mandates the development of pH-dependent anti-cancer drugs to improve selectivity (Lee et al., 2008; Liang et al., 2014; Han et al., 2015; Yao et al., 2017).

Previously, Lei et al. (2015) tested cytotoxic activity of 1000 sera from healthy donors against cells of several cancer lines. Even among AB0 compatible sera, there were some (depending on cell line – 12–53% of sera) able to kill cancer cells thanks activation of complement system *via* lectin pathway ("positive human sera", PHS). Ficolin-3 complexed with a subset of IgM was suggested to be responsible for complement activation, whereas IgM were shown to be involved in the direct interaction with cancer cells. We have demonstrated direct (without participation of immunoglobulins) binding of recombinant ficolin-3 to ovarian cancer cells (Figs. 1 and 2). On the other hand, we have not found serum ficolin-3 binding (Fig. 7A) [also when isolated from plasma and purified ficolin-3-MASP-Ig complexes were used (data not shown)]. Additionally, no deposition of complement C4 activation products on tested cancer cell lines was found, when even 50% normal human serum was used as ficolin-3 source (data not shown). It may suggest that sera used (also pooled plasma used for isolation of mentioned complexes) did not contain IgM subset able to interact with tested cancer cells. As mentioned, Honore et al. (2007) demonstrated recombinant ficolin-3 interaction with apoptotic cells, however serum-derived ficolin-3 was not tested.

Performing additional experiments, we noticed, that binding of recombinant ficolin-3 is inhibited when added contemporaneously with

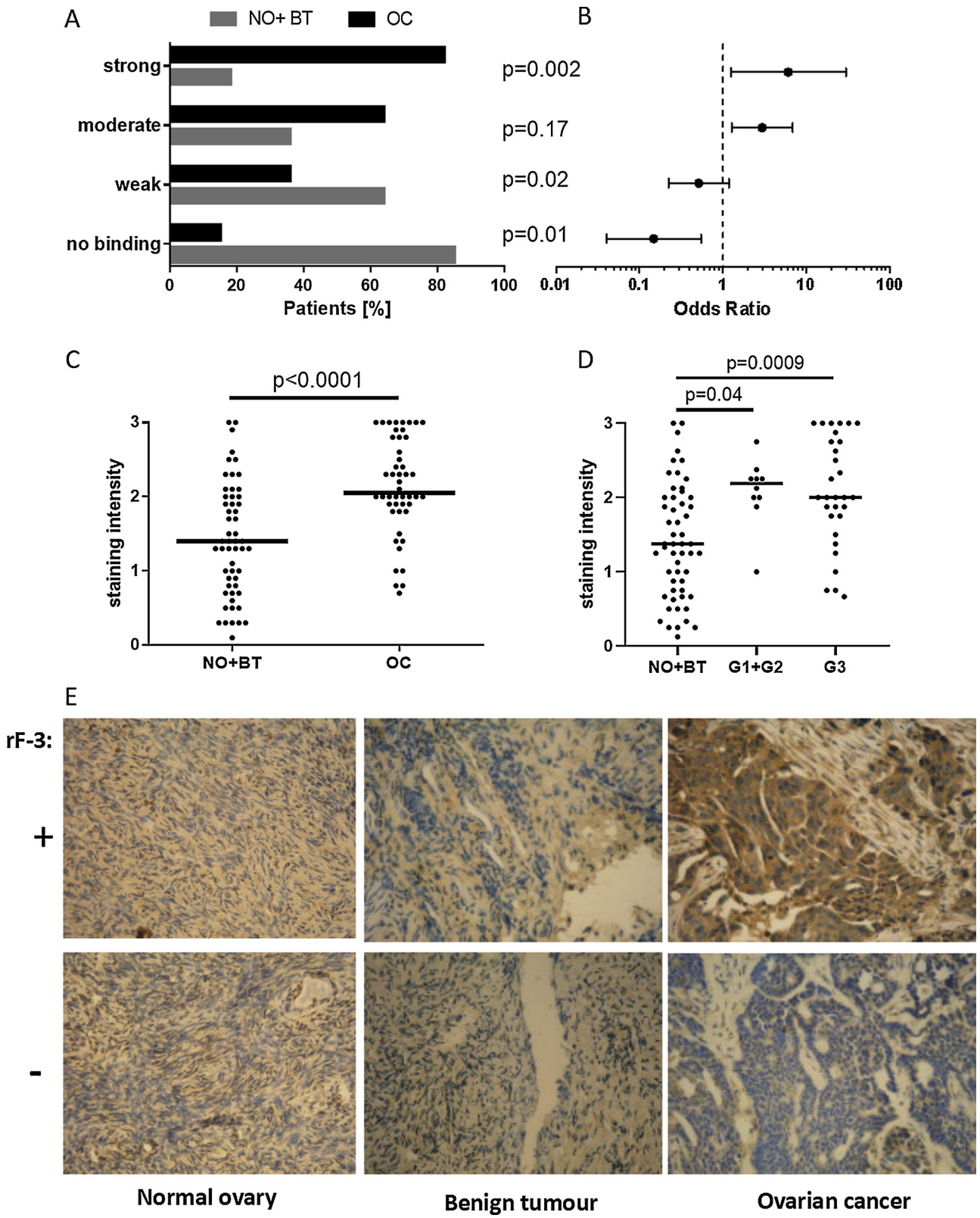


Fig. 6. Ficolin-3 binding to tissue sections. A - percent of preparations depending on staining intensity. B - odds ratio with confidential intervals. C - tissue staining intensity in controls (NO + BT) and patients (OC). D - tissue staining intensity in controls (NO + BT) and patients, depending on tumour grade (G1 + G2; G3). E - examples of ficolin-3 binding in tissue sections of normal ovary, benign tumour and ovarian cancer.

normal serum to the cells (Fig. 7B). Similar results were obtained when ficolin-3 or MASP-2 deficient serum was used. We suppose that some serum factor(s) inhibit(s) rficolin-3 interaction with cells. No cell structures are blocked since rficolin-3 was still able to interact with cells, pre-incubated with serum (Fig. 7C). It may be speculated that free MASP (or other, unidentified factor) present in normal serum associates with rficolin-3 and thus inhibits its binding to the cells. Previously, Pagh et al. (2008) described binding of recombinant but not plasma-

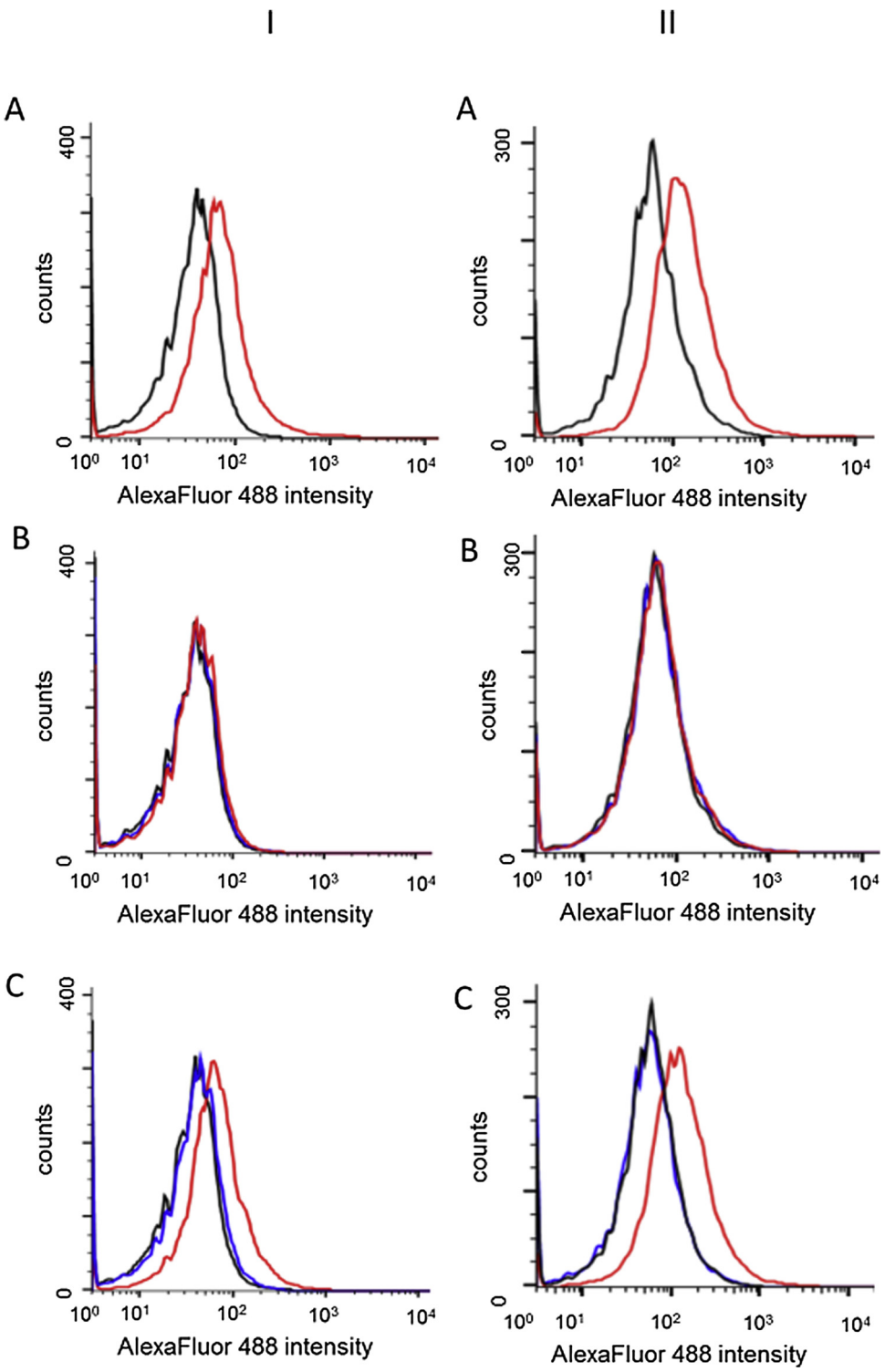


Fig. 7. Recombinant and serum ficolin-3 binding to cells of ES-2 (I) and SKOV-3 (II) lines. Cells were preincubated (1 h, 4 °C) with TBS (A and B) or 10% NHS (C) followed by incubation with rficolin-3 (A and C) or rficolin-3 and 10% NHS (B). Black lines indicate control samples without recombinant ficolin-3 or primary antibody in samples with serum, red lines indicate samples with addition of recombinant ficolin-3 and blue lines show detection of serum ficolin-3. Similar result were obtained in three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

derived MBL to calreticulin and demonstrated that MASP-binding site in MBL is responsible for this interaction. Interestingly, the interaction of recombinant ficolin-3 with calreticulin was described by Honore et al. (2007).

The specificity of ficolin-3 for malignant cells was confirmed by its preferential binding to thin sections of malignant ovary compared with tissue derived from patients without malignant disease. It is worth noting that elevated expression of calreticulin mRNA in ovarian cancer samples in comparison to normal ovaries or benign tumours was described by Vera et al. (2012). It may suggest its involvement in interaction of ficolin-3 with ovarian cancer cells.

Ficolin-3 is the most abundant plasma ficolin and the most potent activator of complement (Hummelshoj et al., 2008; Sallenbach et al., 2011; Troldborg et al., 2017), yet it does not appear to recognize common pathogens. Here we have shown that ficolin-3 recognizes ligands associated with viable malignant cells, and binds strongly at a concentration lower than that typically found in bile or plasma. Basing on data concerning recombinant protein, it may be suggested that ficolin-3 is involved in immune response in ovarian cancer. However, unidentified serum factor(s) seem(s) to protect cancer cells from

recognition by natural or rficolin-3.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This study was supported by the National Science Centre (Poland), grant no. UMO-2014/13/N/NZ5/03665. The authors thank Prof. Łukasz Pułaski (Laboratory of Transcriptional Regulation, IMB PAS) for providing CCRF, Ramos, MOLT-4, Daudi and HL-60 cell lines; Dr Izabela Papiewska-Pająk (Laboratory of Cellular Proteomics, IMB PAS) for providing HT-29 and HUVEC lines, Prof. Magdalena Klink (Laboratory of Experimental Immunology, IMB PAS) for providing OVCAR-3 line and Prof. Anna Brzostek (Laboratory of Mycobacterium Genetics and Physiology, IMB PAS) for providing recombinant His-tagged *Mycobacterium tuberculosis* RecA protein. We are very grateful Dr David C. Kilpatrick for critical reading of the manuscript and helpful discussion. Author contributions: MM was the main researcher for this study and contributed to write this manuscript. MM, ASŚ and MC designed study. SS and DW collected tissue samples. AK analyzed tissue samples. JŁ and AM prepared LPS.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2019.01.002.

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