Immunobiology 222 (2017) 807–813

Contents lists available at ScienceDirect

Immunobiology

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

Structural evaluation of a nanobody targeting complement receptor Vsig4 and its cross reactivity

Yurong Wen[a,1], Zhenlin Ouyang[a,1], Steve Schoonooghe[b,c], Siyu Luo[d], Patrick De Baetselier[b,c], Wuyuan Lu[a], Serge Muyldermans[b,c], Geert Raes[b,c,2], Fang Zheng[d,*,2]

[a] Center for Translational Medicine, The Key Laboratory of Biomedical Information Engineering of Ministry of Education, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an 710049, China
[b] Research Group of Cellular and Molecular Immunology, Vrije Universiteit Brussel, Brussels, Belgium
[c] Laboratory of Myeloid Cell Immunology, VIB Inflammation Research Center, Ghent, Belgium
[d] Department of Biochemistry and Molecular Biology, Key Laboratory of Environment and Genes Related to Diseases, Health Science Center, Xi'an Jiaotong University, Xi'an 710049, China

ARTICLE INFO

Article history:
Received 14 June 2016
Received in revised form
10 November 2016
Accepted 12 November 2016
Available online 18 November 2016

Keywords:
Vsig4
CRIg
Nanobody
Crystallography
B7 family
Cross reactivity
Complement receptor

ABSTRACT

Vsig4 is a recently identified immune regulatory protein related to the B7 family with dual functionality: a negative regulator of T cell activation and a receptor for the complement components C3b and C3c. Here we present a structural evaluation of a nanobody, Nb119, against the extracellular IgV domain protein of both mouse and human recombinant Vsig4, which have a high degree of sequence identity. Although mouse and human Vsig4 bind to Nb119 with a 250 times difference in dissociation constants, the interaction results in a highly identical assembly with a RMSD of 0.4 Å. The molecular determinants for Vsig4 recognition and cross reactivity unveiled by the atomic structure of Nb119 in complex with mVsig4 and hVsig4 afford new insights useful for the further optimization of the nanobody for potential use in humans. Additionally, structural analysis of the Vsig4-Nb119 complexes indicates that Nb119 occupies the interface on Vsig4 recognized by the macroglobulin-like domains MG4 and MG5 of C3b. Thus an affinity-improved Nb119 may have the potential to influence the activation of both T cells and complement.

1. Introduction

V-set and immunoglobulin domain-containing 4 (Vsig4, also known as Z39Ig or CRIg) is a novel immune regulatory protein related to the B7 family. Vsig4 is reported to be exclusively expressed on tissue resident macrophages such as peripheral tissue macrophages, synovial macrophages, Kupffer cells (KCs), foam cells in atherosclerotic plaques and interstitial macrophages in the heart (Helmy et al., 2006). Recently Vsig4 has been described as having a dual functionality, being involved in both T cell immunity and the alternative complement pathway.

Vsig4 negatively regulates the $CD4^+$ and $CD8^+$ T cell response with a concomitant *in vitro* reduction of IL-2 and IFN-γ. Soluble Vsig4-IgV can strongly attenuate the $CD4^+$ and $CD8^+$ T cell responses in the presence of an anti-CD3 and anti-CD28 signal. Furthermore, Vsig4 is as potent a negative regulator of T cell activation as other B7 family proteins, like PD-L1 and PD-L2 (Programmed Death Ligand 1 and 2) (Vogt et al., 2006; Zang and Allison, 2006). More recently, Vsig4 was found to be overexpressed on tumor-associated macrophages and may be involved as a promoter of non-small cell lung cancer development. Lewis lung carcinoma cells generated significantly smaller tumors in $Vsig4^{-/-}$ mice compared to wild type mice (Liao et al., 2014). Since effective immunotherapy of lung cancer is highly dependent on the efficacy of tumor-specific $CD8^+$ cytotoxic T lymphocytes, suppression of $CD4^+$ and proliferation of $CD8^+$ T cells by Vsig4 could promote cancer growth. Hence, Vsig4 emerges as a potential cancer immunotherapy target, whereby a Vsig4 neutralizing molecule would inhibit the T cell suppression.

Additionally, Vsig4 functions as a complement receptor, by binding specifically to C3b and C3c fragments, derived from complement component 3 (Helmy et al., 2006). The complement system

* Corresponding author.
E-mail addresses: Fang.Zheng@xjtu.edu.cn, geert70@telenet.be (F. Zheng).
[1] These authors have contributed equally.
[2] These authors have shared the senior author.

http://dx.doi.org/10.1016/j.imbio.2016.11.008
0171-2985/

plays a key role in the control of exogenous pathogens and clearance of self-antigens coming from apoptotic cells (Ram et al., 2010). KCs play a crucial role in liver homeostasis and pathogen clearance. Mouse KCs mainly express two complement receptors, CR3 and Vsig4, whereas human KCs also express CR1 and CR4. Interestingly, Vsig4$^{-/-}$ mice exhibited a significantly lower number of internalized bacteria after either *Listeria monocytogenes* or *Staphylococcus aureus* challenges, which indicated Vsig4 is required for C3b-mediated recognition and opsonization of the pathogen. In addition, Vsig4 was found to enhance peritoneal macrophage phagocytosis in a C3b-dependent manner. The C3b and Vsig4 binding is crucial for mediating macrophage cellular responses, therefore, a Vsig4 targeting method has great potential to enhance specifically the capture of circulating pathogens and macrophage phagocytosis. Moreover, the interaction between Vsig4 and C3b selectively abrogates the C3 and C5 mediated alternative complement pathways but not the classical complement pathway.

The aberrant regulation of the complement may contribute to various immune diseases. Uncontrolled activation and insufficient regulation of the complement pathway will lead to various immune disorders such as autoimmune, inflammatory and infection diseases. Hence, Vsig4 is an attractive therapeutic target which is highly selective for the alternative pathway of the uncontrolled activation of complement-mediated diseases such as arthritis, kidney disorder membranoproliferative glomerulonephritis (KDMG) and age-related macular degeneration (AMD). A Vsig4-Fc fusion protein was reported to quench the inflammation and reverse bone destruction in an experimental arthritis mouse model. Additionally, Vsig4 and the alternative pathway regulator factor H fusion protein prevents tissues from complement damage as was validated in a rat model (Qiao et al., 2014). So it seems that the obstruction of the C3b binding site of Vsig4 is a potential route to diminish complement activation. However, there are no functional Vsig4 targeting probes available.

A Nanobody is a single antigen-binding variable domain (VHH) of camelid heavy chain- only antibodies. Over the past years it became an attractive diagnostic and therapeutic agents supported by favorable properties such as nanometer size, economical production, stable and soluble behavior and a 'human-like' sequence (Muyldermans, 2013). Multiple Nanobodies, including those against the transmembrane receptors HER2 and EGFR, have been developed as specific and high affinity agents for cancer diagnosis and therapy (Vaneycken et al., 2011; Vosjan et al., 2012).

Previously, we identified a Nanobody, referred to as Nb119, targeting the mouse complement receptor Vsig4 that was useful in the diagnosis of arthritis and liver damage through molecular imaging (Zheng et al., 2015, 2014). Since Vsig4 is a potential therapeutic target in both, aberrant complement activation and cancer immunotherapy, we assessed the cross reactivity of Nb119 with human recombinant Vsig4 (hVsig4) with isothermal titration calorimetery. To guide (in later experiments) the affinity maturation of our Nb119 and to improve the cross-reactivity to mouse/human Vsig4 we decided to determine the high-resolution atomic structure of Nb119 in complex with mVsig4 or hVisg4. Furthermore, the crystal structure of the complex discloses the overlap between Nb119 and macroglobulin-like domain MG4 and MG5 region of complement fragment C3b and C3c for Vsig4 binding.

2. Materials and methods

2.1. Production of recombinant nanobody and mouse or human Vsig4 IgV domain

Mouse Vsig4 IgV domain (Amino acids 20–139) with C terminal His tag was cloned into pHEN6c plasmid and overexpressed in *E.coli* WK6 as host, the mVsig4 protein extracted from periplasm was purified by Ni-NTA chromatography followed by a size exclusion chromatography (SEC) purification on Superdex 75 column (GE). Detailed experimental procedures were reported as described previously Zheng et al. (2014). Human Vsig4 IgV domain (Amino acids 20–137) gene was inserted into the pET28a and transformed into *E.coli* BL21 (DE3) host cell, the hVsig4 was overexpressed as inclusion body, extracted and refolded following the protocol described in Helmy et al. (2006).

Nb119 was generated against recombinant mouse Vsig4 extracellular domain and screened as described before Zheng et al. (2014). In brief, the screened Nb119 gene sequence was inserted into the pHEN6c plasmid and transfected in the *E. coli* WK6 host. Nb119 was extracted from *E. coli* WK6 periplasmic space. All the extracted proteins were first loaded on an NI-NTA column and further buffer exchanged and purified by SEC to 20 mM Tris pH 7.5, 150 mM NaCl, 5% Glycerol for further usage.

2.2. Crystallization and data collection

All crystallization trials were carried out with the sitting-drop vapor-diffusion approach at 20 °C. Both mVsig4 and hVsig4 were mixed with Nb119 at a 1:1 ratio and purified by size exclusion chromatography on columns pre-equilibrated with 20 mM Tris pH 7.5, 150 mM NaCl and 5% Glycerol. The fractions containing the complex were pooled and concentrated to around 3–5 mg/mL. The crystallization trials were further carried out by mixing 1 μL protein complex with an equal volume of reservoir solution (Hampton). The mVsig4:Nb119 complex crystals, which were observed in: (1) 0.2 M Ammonium sulfate, 0.1 M BIS-Tris pH 5.5, 25% PEG-3350 and (2) 0.1 M Sodium citrate pH 5.5 and 20% PEG3000 resulted in two spacegroups P212121 and P3221, respectively. The hVsig4:Nb119 complexes crystals were grown in: (1) 0.1 M Sodium citrate tribasic dehydrate pH 5.6, 20% 2-Propanol, 20% PEG4000 in spacegroup C2; (2) 1.2 M sodium citrate tribasic dehydrate, 0.1 M BIS-TRIS propane (1,3-bis(tris(hydroxymethyl)methylamino)propane) pH 7.0 and (3) 0.17 M Ammonium sulfate, 15% Glycerol, 25.5% PEG4000 in spacegroup P212121. All the crystals were harvested from crystallization drop and flash frozen with a cryostream. Data were collected in the Shanghai Synchrotron Radiation Facility (SSRF) BL18U1 and BL19U1. Data were collected from single crystals, exposed to X-rays at a wavelength of 0.98 Å at 100 K. The mVsig4:Nb119 P212121 spacegroup crystal diffracted to 1.2 Å with $\alpha = \beta = \gamma = 90^\circ$, a = 29.6 Å, b = 50.3 Å, c = 163.2 Å and the P3221 spacegroup crystal diffracted to 2.0 Å with $\alpha = \beta = 90^\circ$, $\gamma = 120^\circ$, a = b = 49.9 Å c = 183.7 Å. The hVsig4:Nb119 complexes C2 crystal diffracted to 1.23 Å with $\alpha = \gamma = 90^\circ$, $\beta = 126.9^\circ$, a = 126.8 Å, b = 30.4 Å, c = 84.3 Å and the P212121 spacegroup crystal diffracted to 1.8 Å with $\alpha = \beta = \gamma = 90^\circ$, a = 29.3 Å, b = 50.0 Å, c = 160.6 Å.

2.3. Structure determination

Data were processed with XDS (Kabsch, 2010). The structures of both mVsig4:Nb119 and hVsig4:Nb119 complexes were determined via molecular replacement using Phaser implemented in the Phenix package (Adams et al., 2010). The mouse Vsig4 structure (PDB: 2PND) was used as the initial search template with all the residues truncated to alanine (Katschke et al., 2007). The final Vsig4 and nanobody complex forms a heterodimer containing 1 molecule Vsig4 and 1 molecule nanobody in the asymmetric unit cell. The model was manually improved with the COOT program (Emsley and Cowtan, 2004) and refinement was further done using Phenix refine (Adams et al., 2010). The interaction interface was calculated by PDB PISA (Krissinel and Henrick, 2007). Figures were generated from PyMOL program (Schrödinger, 2015). Data collection and refinement statistics are summarized in Table 1.