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Identification of high-affinity anti-CD16A allotype-independent human antibody domains

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ABSTRACT

CD16A (FcγRIIIA) is an activating receptor mostly expressed on natural killer (NK) cells and monocytes/macrophages. It can mediate antibody-dependent cell-mediated cytotoxicity (ADCC) through low-affinity interaction with human immunoglobulin G (IgG) Fc. It can also mediate cell lysis if NK cells are guided by bispecific killer cells engagers (BiKEs). BiKEs showed some success in clinical trials of cancer and are promising candidate therapeutics. However, currently reported BiKEs are based on antibody fragments (scFvs) of relatively large size. The CD16A-specific antibodies are also typically from animal origin. Decreasing the BiKE size could result in enhanced penetration into solid tumor and normal tissues, and using fully human antibodies could decrease the likelihood of immunogenicity. Here we report the identification and characterization of two antibody domains, D6 and E11, isolated from a very large human VH antibody domain library displayed on phage. D6 and E11 bound CD16A with EC_{50} of 4 nM and 8 nM, respectively, but not other Fc gamma receptors (FcγRs) such as CD64 (FcγRI), CD32 (FcγRII) and CD16B (FcγRIIIB). They bound to both CD16A allotypes (158F,V) with equal affinity and competed with each other as well as with human IgG1 and the mouse anti-CD16A antibody 3G8. These and other results were used to build a molecular docking model predicting that D6 and E11 may bind to the CD16A membrane proximal D2 domain by interacting with its BC, C′E and EF loops. Importantly, cross-linked (bivalent) D6 and E11 induced secretion of IL-2 after binding to CD16A-expressing Jurkat T cells. The small size of these antibody domains combined with their high-affinity, specific, allotype-independent, activating interactions with CD16A could allow generation of novel highly effective BiKEs and other candidate protein therapeutics.

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1. Introduction

CD16 (FcγRIII) was first identified by using the monoclonal antibody (mAb) 3G8 which inhibited binding of IgG complexes to neutrophils (Fleit et al., 1982). Several groups found that in humans CD16 can be expressed in two isoforms (CD16A and CD16B) which are highly homologous but CD16A has a transmembrane domain and a cytoplasmic tail while CD16B is glycosylphosphatidylinositol (GPI)-anchored (Selvaraj et al., 1988; Lanier et al., 1989; Edberg et al., 1989; Ravetch and Perussia, 1989; Scallon et al., 1989; Ueda et al., 1989; Simmons and Seed, 1988). CD16A is associated with the immune-receptor tyrosine-based activation motifs (ITAM)-bearing proteins ζ and FcεRIγ and is expressed mainly on NK cells and macrophages as well as on some subsets of monocytes and T cells (van Sorge et al., 2003). Cross-linking of CD16 on NK cells could result in the association of the receptor complex with the $p56^{lck}$ tyrosine kinase and tyrosine phosphorylation of ζ and FcεRIγ followed by a number of events finally resulting in degranulation and cytokine production or in some cases apoptosis of NK cells. A major function of CD16A is to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) through low-affinity interaction with human immunoglobulin G (IgG) Fc(Lanier, 2001). It can also mediate ADCC-independent cell lysis (Mandelboim et al., 1999).

The capability of NK cells to kill target cells specifically by using bispecific antibodies to both CD16 and target cells was demonstrated >30 years ago (Perez et al., 1985). However, difficulties in generating such antibodies and for other reasons it was not until relatively recently when such bispecific mAbs called Bispecific Killer cell Engagers (BiKEs) were successfully used in a clinical trial (Rothe et al., 2015).

A major component of a BiKE is the antibody that binds to CD6A. Most (but not all, e.g., (McCall et al., 1999)) previously reported antibodies (e.g., (Weiner et al., 1995)) are from animal origin. The animal antibodies can be humanized and some, e.g., from lamma (Behar et al., 2008), are similar in sequence to human antibodies; however, the probability for immunogenicity when administered in humans is still on average higher than that for fully human antibodies (Dimitrov, 2010). Here, we described two VH antibody domains (Ads) derived from a human library displayed on phage which bind to CD16A with high affinity, are highly specific and allotype independent. To our knowledge

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these are the first human Ads reported to bind CD16A. They could be used alone or as components of BiKEs and other fusion proteins for development of therapeutics.

2. Materials and methods

2.1. FcγRs, plasmids, antibodies and cells

Recombinant FcγRs ectodomains were purchased from Sino Biologic Inc. (North Wales, PA, USA). The pComb3X was kindly provided by Dennis Burton (Scripps Research Institute, La Jolla, CA, USA) and the pSecTag2 B was purchased from Invitrogen. IgG1 m336 and m912-mFc were produced in our group. The following antibodies were purchased: mouse anti-CD16A IgG1, 3G8 (Abcam, Cambridge, MA, USA); phycoerythrin (PE)-conjugate mouse anti-CD16A, PE conjugated mouse anti-FLAG (Miltenyi, Bergisch Gladbach, Germany); fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD64 (FcγRI) and CD32 (FcγRII) (Invitrogen); horseradish peroxidase (HRP) anti-M13 polyclonal (Pharmacia, Piscataway, NJ); HRP-conjugated mouse anti-FLAG tag, HRP conjugated goat anti-mouse IgG and HRP-conjugated goat anti-human IgG (Fc-specific) (Sigma-Aldrich). U937 cell was a gift from Anu Puri (National Cancer Institute, Frederick, MD). The following cell lines were purchased: Jurkat T (ATCC); 293 freestyle (Invitrogen) and Jurkat T over-expressing CD16A (Promega). Human blood was obtained from the NIH blood center.

2.2. Preparation of recombinant CD16A-mFc and its biotinylation

The CD16A gene was synthesized by Genescript (Piscataway, NJ). Its extracellular domain (ECD, Gly17-Gln208) was fused to monomeric Fc (mFc) which was generated in our group (Ying et al., 2012). The CD16A ECD was subcloned into modified pSecTag2 B vectors containing mFc by using the *Sfi*I endonuclease restriction site. The recombinant CD16A-mFc was expressed in 293 freestyle cells by transient transfection, and purified by Protein A Sepharose 4 Fast Flow (GE Healthcare, Piscataway, NJ) resin according to the manufacturer's protocols. The purity was checked by SDS-PAGE under both reducing and non-reducing conditions (Fig. 1A), and the homogeneity was confirmed by Superdex 200 chromatography (GE Healthcare). The protein was stored at −80 °C until use. We noticed that storage could change its properties but not to significant degree to affect conclusions. Biotinylation of CD16A-mFc was performed by EZ-Link Sulfo-NHS-Biotin reagent (Thermo Fisher) according to the manufacturer's protocols.

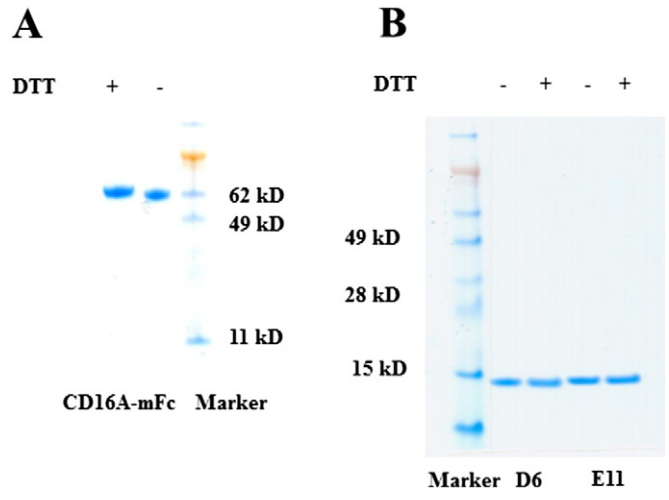

Fig. 1. SDS-PAGE of CD16A-mFc **(A)** used as the panning antigen, and the Ads, D6 and E11 **(B)** in presence or absence of reducing reagent, DTT. Under the reducing conditions, the samples were subjected to heating at 100°C for 10 min.

2.3. Panning, selection, expression and purification of binders

A large human antibody domain (Ad) phage library (size ~10^{11}) was constructed by grafting CDR loops from peripheral blood mononuclear cell (PBMC) cDNA of 40 healthy donors into human VH3-23 scaffold (T Ying and DS Dimitrov, unpublished). This library was panned against biotinylated CD16A-mFc in the presence of 10-fold excess of PD1-mFc as a competitive (depletion) protein. Amplified libraries of 10^{12} phage displayed Ads were incubated with 1, 0.5, 0.2, and 0.05 μg of CD16A-mFc during the first, second, third, and fourth rounds of panning, respectively. After four rounds of panning, the phage library was significantly enriched with CD16A binders as measured by polyclonal phage ELISA (ppELISA). Positive clones from the third and fourth rounds were selected by using supernatants from randomly picked clones which were incubated with immobilized antigens (semELISA) and measured. Then their DNA was sequenced. For protein preparation, these clones were transformed into HB2151 cells for expression, and proteins were purified by one-step Ni-NTA resin. Protein purity and homogeneity were analyzed by SDS-PAGE. Protein concentration was measured spectrophotometrically (NanoVue, GE Healthcare).

2.4. ELISA

96-well plates (Costar) were coated with CD16A-mFc protein at 50 ng/well in phosphate-buffered saline (PBS) overnight at 4 °C. Phage from each round of panning (ppELISA) or supernatants from randomly picked clones (semELISA) were incubated with immobilized antigens. Bound phage was detected with HRP-anti-M13-polyclonal Ab and bound proteins with HRP-conjugated mouse anti-FLAG mAb, which was also used for the determination of the binding EC_{50} to CD16A for the Ads. For evaluation of binding with other FcγRs, the commercial recombinant FcγRs were coated on plates and the bound Ads were detected by HRP-conjugated mouse anti-FLAG mAb. To determine whether the Ads obtained by panning against high-affinity CD16A-158V isoform could also bind to the low affinity CD16A-158F isoform, we performed ELISA using the two CD16A allotypes as coating antigens. For competition ELISA, 5 nM Ad was incubated with serially diluted competitors: E11-mFc; m336-IgG1-Fc and 3G8. For negative control, we used a mFc fusion protein, m912-mFc developed in our lab. After incubation with the coated antigen and extensive washing, the bound Ads were detected by HRP-conjugated mouse *anti*-FLAG tag Ab. The half-maximal binding (50% effective ELISA Concentration, EC_{50},) was estimated as the concentration at which OD is 50% of its maximal value.

2.5. Enrichment of NK cells from PBMC and flow cytometry (FACS)

PBMCs were isolated from peripheral blood of healthy donors by centrifugation on a Ficoll/Hypaque gradient (GE health). NK cells were enriched from human PBMC by using the NK cell isolation kit II in the negative selection modes (Miltenyi Biotec). Purified NK cells were detected by incubation with biotinylated anti-CD56 antibody (m909, developed by our group (Feng et al., 2016)) followed by streptavidin-FITC and PE conjugated mouse anti-human CD16 antibody. Ad binding to NK cells was measured by incubating 10^6 NK cells in 200 μL PBS containing 0.1% bovine serum albumin (BSA) (PBSA) with 10 nM of Ads for 30 min at room temperature. The cells were washed twice with 200 μL PBSA, followed by incubation with PE-conjugated-mouse anti-FLAG antibody (Miltenyi Biotec) for 30 min on ice. After washing, the cells were used for fluorescence-activated cell sorter (FACS) analysis. The second antibody alone was used as negative control, and the PE conjugated mouse anti-human CD16A antibody (Mouse IgMκ, clone VEP13) was used as a positive control. Jurkat T cells and phorbol myristate acetate (PMA) stimulated U937 cells, which express high levels of CD64 and CD32, but do not express CD16A were used as negative controls (Looney et al., 1986). For competition FACS, we incubated serially