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Correlation between CD16a binding and immuno effector functionality of an antigen specific immunoglobulin Fc fragment (Fcab)

Manuela Kainer [a], Bernhard Antes [a], Susanne Wiederkum [a], Gordana Wozniak-Knopp [b], Anton Bauer [a,1], Florian Rüker [b], Max Woisetschläger [a,*]

[a] f-star, Gastgebgasse 5-13, 1230 Vienna, Austria
[b] University of Natural Resources and Life Sciences, Vienna Department of Biotechnology Christian Doppler Laboratory for Antibody Engineering, Muthgasse 18, 1190 Vienna, Austria

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ABSTRACT

Antigen binding immunoglobulin Fc fragments (Fcab) are generated by engineering loop regions in the CH3 domain of human IgG1 Fc. Variants of an Fcab specific for Her-2 were designed to display either enhanced (S239D:A330L:I332E) or diminished (L234A:L235A) binding affinities to the Fc receptor CD16a based on mutations described previously. The two mutant Fcab proteins demonstrated the expected modulation of CD16a binding. Interaction with recombinant or cell surface expressed Her-2 was unaffected in both mutants compared to the parental Fcab. Binding affinities for CD16a correlated with the ADCC-potencies of the Fcab variants. Additional studies indicated that the L234A:L235A variant Fcab had equivalent structural features as the unmodified Fcab since their DSC profiles were similar and antigen binding after re-folding upon partial heat denaturation had not changed. Introduction of the S239D:A330L:I332E mutations resulted in a significant reduction of the CH2 domain melting temperature, a moderate decrease of the thermal transition of the CH3 domain and lower antigen binding after thermal stress compared to the parental Fcab. We conclude that the known correlation between CD16a binding affinity and ADCC potency is also valid in Fcab proteins and that antigen specific Fcab molecules can be further engineered for fine tuning of immuno effector functions.

Introduction

Antibody dependent cellular cytotoxicity (ADCC)[2] is being recognized as one of several molecular mechanisms by which monoclonal antibodies of the IgG isotype class can mediate their anti-tumor effects [1–3]. ADCC reactions are dependent on simultaneous binding of IgG1 molecules to tumor cells and to Fc receptors such as CD16a (Fc gamma RIIIa) expressed on a variety of immuno effector cells, like natural killer and monocytic cells [4,5]. It has been shown that the strength of antibody interaction with CD16a correlates with ADCC potencies [6–10]. For instance, patients harboring an allelic version of the CD16a gene (F176V) which allows for higher binding affinities of IgG1 antibodies respond better to treatment with the therapeutic antibody rituximab [11,12]. In addition, xenograft studies in Fc receptor deficient mice have demonstrated the requirement of these molecules for the anti-tumor activities of rituximab and trastuzumab [13]. Despite the success rates observed upon treatment with therapeutic antibodies in cancer indications, a significant percentage of patients do not respond adequately to mAb therapy most likely due to the multifactorial nature of cancer and also induction of drug resistance mechanisms in initially responsive patients. These shortcomings have led to an emergence of alternative antibody-based and other protein scaffolds as well as strategies to improve the efficacies of existing monoclonal antibodies, for example to improve ADCC potencies [14,15]. Antibody-based protein scaffolds, such as immunoglobulin Fc fragments containing antigen binding sites (Fcab) have been shown to hold significant promise as next generation protein therapeutics [16] since they combine all functional features of a standard monoclonal antibody in a smaller entity. In particular, Fcab molecules have been shown to mediate ADCC reactivity [16]. To establish proof of concept, that ADCC functionalities can be modulated in the context of an antigen specific Fcab, mutations described previously to affect ADCC were introduced into the Her-2 specific Fcab HAF3–4. The data demonstrated that binding to Her-2 is unaffected by these additional mutations while binding to CD16a was increased or diminished depending on the mutations introduced. More importantly, the binding affinities for CD16a correlated with the potencies of the mutants to elicit ADCC reactions.

* Corresponding author.
E-mail address: m.woisetschlaeger@kabsi.at (M. Woisetschläger).
[1] Present address: Intercell AG Campus Vienna Biocenter 3, 1030 Vienna, Austria.
[2] *Abberviation used:* ADCC, Antibody dependent cellular cytotoxicity; DSC, differential scanning calorimetry; SPR, surface plasmon resonance; Fcab, Fc fragments.

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Results

Fcab HAF3–4 is a homodimeric Fc protein derived from human IgG1 which contains high affinity binding sites for the Her-2 antigen in the C-terminal loop regions of the CH3 domains. A model of the structure of an Fcab and of the location of the antigen binding sites is shown in Fig. 1 HAF3–4 was used as parental molecule for the introduction of mutations previously published to affect binding to the Fc receptor CD16a. The L234A:L235A [17] mutations (called AA in this manuscript) were described to almost abolish CD16a binding of conventional antibodies [18–20]. Conversely, the S239D:A330L:I332E mutations (3M) were shown to increase binding to this Fc receptor [21]. As controls, the same mutations were introduced into wild-type Fc (Fcab wt). The impact of the mutations on Fc receptor binding was assessed by surface plasmon resonance experiments which are presented in Fig. 2 The data showed that CD16a binding of Fcab wt and Fcab HAF3–4 were identical indicating that the Her-2 binding sites in the loops of the CH3 domains did not negatively influence binding to this Fc receptor. By contrast, both proteins harboring the AA mutations were almost completely devoid of CD16a binding while the variants containing the 3M mutations both showed fast onset of binding and a slower off-rate compared to the proteins without mutations in the CH2 domain. Importantly, very similar binding kinetics to CD16a could be observed for wild-type Fc 3M and HAF3–4 3M (Fig. 2A). The same proteins were tested for their ability to bind to another Fc receptor, CD64. In these experiments, the 3M mutant proteins were indistinguishable from the Fc fragments without mutations in the CH2 domain indicating the specificity of the 3M changes for binding to CD16a. Both AA mutant proteins demonstrated a clear reduction in binding affinities to CD64 (Fig. 2B).

The HAF3–4 variants were analyzed for their capacity to elicit ADCC reactions. Calu-3 cells, which express high amounts of Her-2 on their cell surface, were mixed with Fcabs and purified primary natural natural killer cells as effector cells. After four hours of co-cultivation, dead cells were enumerated by flow cytometry after addition of 7-amino-actinomycin D. Fcab HAF3–4 led to potent killing of Calu-3 cells in a dose dependent fashion (EC_{50} = 0.9 nM). The HAF3–4 AA variant was considerably weaker and did not even reach maximal killing at the highest concentration used in these experiments. In contrast, Fcab HAF3–4 3M elicited much better

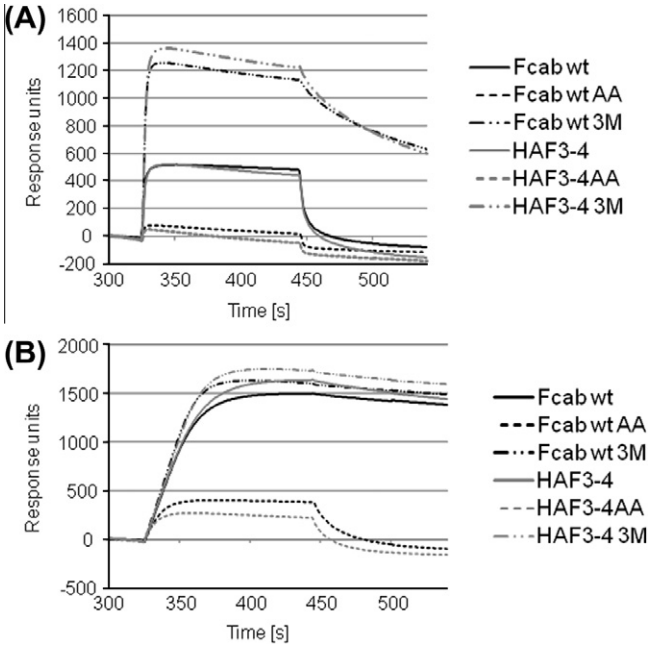

Fig. 2. (A) Binding of CD16a and (B) binding of CD64 to ProteinA captured Fcab variants.

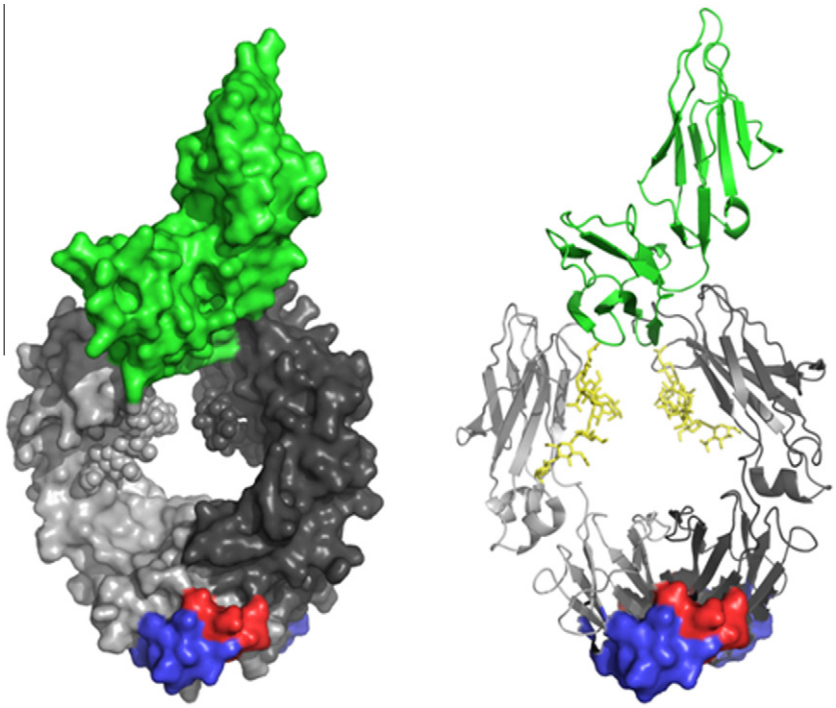

Fig. 1. Surface (left) and ribbon (right) presentation of a human Fc fragment of IgG1 (gray) in complex with CD16 (green). The location of the CD16 binding site is on the hinge-proximal end of the Fc, while engineered antigen binding sites like in Fcab HAF3–4 are located at the C-terminal tip of the Fc. Each of the two antigen binding sites in the Fc homodimer (dark and bright gray indicate the two chains) is composed of residues in the AB (red) and in the EF (blue) loop. Sugar residues in the N-linked glycosylation sites on the CH2 domain are indicated. The image was made on the basis of Protein Data Bank entry 1T83 [34].