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Bi-specific antibodies with high antigen-binding affinity identified by flow cytometry

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ABSTRACT

Using conventional approaches, the antigen-binding affinity of a novel format of bi-specific antibody (BsAb) cannot be determined until purified BsAb is obtained. Here, we show that new lipoprotein A (NlpA)-based bacteria display technology, combined with flow cytometry (FCM), can be used to detect antigen-binding affinity of BsAbs, in the absence of expression and purification work. Two formats of BsAb, scFv2-CH/CL and Diabody-CH/CL, specific for human interleukin 1β (hIL-1β) and human interleukin 17A (hIL-17A), were constructed and displayed in *Escherichia coli* using NlpA-based bacteria display technology. Conversion of these cells to spheroplasts, and their incubation with fluorescently conjugated antigens resulted in the selective labeling of spheroplasts expressing BsAb; enabling their antigen-binding affinity to be analyzed with FCM. The association and dissociation of BsAbs for binding to hIL-1β and hIL-17A were analyzed using FCM-based assays. The results showed that antigen-binding affinity of Diabody-CH/CL was significantly higher than that of scFv2-CH/CL. To confirm these results of FCM-based assays, BsAbs were expressed, purified and subjected to relative affinity measurements, *in vitro* and *in vivo* bioactivity analysis. The results showed that Diabody-CH/CL had greater relative affinities for both antigens, resulting in better blocking bioactivities on cellular level and effects on alleviating joint inflammation, and cartilage destruction and bone damage in collagen induced arthritis (CIA) mice model. These results indicate that BsAbs with good antigen-binding affinity can be identified by FCM-based assays without expression and purification work, and the indentified BsAb can serve as a lead compound for further drug development.

1. Introduction

Bi-specific antibodies (BsAbs) are human-made antibodies that can bind to two different epitopes [1]. The simultaneous recognition of two different molecules allows for dual targeting strategies; thereby increasing selectivity and functional affinity [2–6]. BsAbs cover a wide spectrum of therapeutic and diagnostic applications including the targeting of cancerous tumor cells with cytotoxic agents, the simultaneous targeting of two different tumor targets to enhance the biological activities of individual antibodies, and the targeting of different proinflammatory cytokines in the treatment of inflammatory diseases [7–10].

BsAbs are considered powerful therapeutics for a large number of diseases, and many BsAb formats assembling antigen-binding domains in various configurations have been generated [10–14]. The development of BsAbs exhibiting excellent antigen-binding properties presents an obvious challenge from the beginning [15]. Traditional methods for antigen-binding analysis for different formats of BsAb are time consuming and labor intensive, in part because antigen-binding affinity cannot be determined until purified BsAb is obtained, and immunological assays conducted. In contrast, the New lipoprotein (NlpA)-based bacteria display technology developed in the current study does not require expression and purification work to detect BsAb antigen-binding affinity.

NlpA is a nonessential *E. coli* lipoprotein that exclusively localizes to the inner membrane. NlpA is secreted across the membrane via the Sec pathway, and once in the periplasm a diacylglycerol group is attached through a thioether bond to a cysteine residue on the C-terminal side of the signal sequence. The signal peptide is cleaved by signal peptidase II, and the protein is acylated at the modified cysteine residue. The lipophilic fatty acid then inserts into the membrane to anchor the protein. The NlpA leader peptide with the first six amino acids of the mature NlpA, containing the putative fatty acylation and inner membrane targeting sites, were used for generating NlpA-based bacteria display technology [16,17]. Another signal peptide incorporated in this technology is the leader sequence of pectate lyase B (pelB). The pelB leader is a sequence of amino acids which when attached to a protein, directs the

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protein to the bacterial periplasm, where the sequence is removed by a signal peptidase. In this study, NlpA-based bacteria display technology relies on the anchored periplasmic expression of one chain of BsAb (bait) at the periplasmic cavity of the inner membrane of *Escherichia coli*, and the other chain (prey), a soluble periplasmic protein. Upon removal of the outer membrane by spheroplasting, any unbound protein is released into the extracellular fluid. However, if the prey binds to the membrane-anchored bait, BsAb will be formed and associated with the spheroplasts. The incorporation of fluorescently-labeled antigen enables interactions between BsAbs and their antigens to be quantified by flow cytometry (FCM)-based assays (Fig. 1). As model antibodies for this study we designed and constructed two formats of BsAb, Diabody-CH/CL and svFv2-CH/CL, from humanized mouse anti-human IL-1 β IgG4 and humanized mouse anti-human IL-17A IgG1. The two humanized IgG antibodies were proven not only can block hIL-1β and hIL-17A but also can block mouse IL-1β and hIL-17A [10,18]. The two chains of the designed BsAbs were co-expressed in the periplasm of *E. coli* as either bait or prey, depending on the assay format. The FCM results demonstrated that both format of BsAbs bound specifically to each of the antigens, and the antigen-binding affinity for the Diabody-CH/CL format was superior to that of scFv2-CH/CL. These results were confirmed *in vitro* and *in vivo*. This study demonstrates that NlpA-based bacteria display technology enables real-time visualization to identify BsAb with desired antigen-binding affinity [19,20] through FCM-based assays, and without the need for the expression and purification of BsAb.

2. Materials and methods

2.1. Materials

We used *E. coli* DH5α for displaying BsAbs, while *E. coli* Rosetta was used for expression of BsAbs. The pBFD vector used for BsAbs display was generated in our laboratory [20]. The FITC Protein Labeling Kit

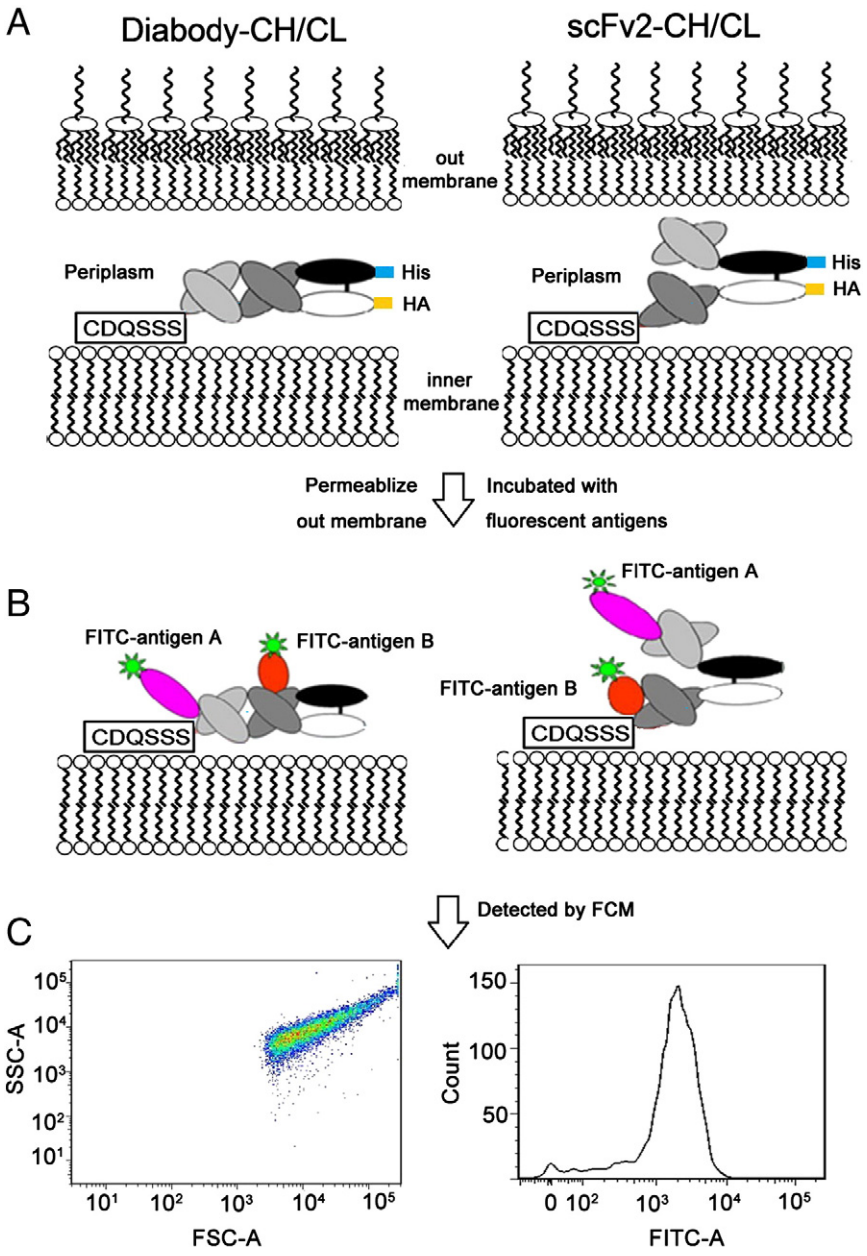

Fig. 1. NlpA-based bacteria display technology. A: Co-expression and assembly of two chains of BsAbs in the periplasm via NlpA-based bacteria display technology. B: Binding of BsAbs with fluorescence-labeled antigens. C: Analysis of antigen-binding affinity using FCM by gating the region defined by the distinct scatter of the spheroplasts (FSC and SSC) and the high FITC-A signal. FSC: forward scatter; SSC: side scatter; CDQSSS: NlpA amino acids 1–6.