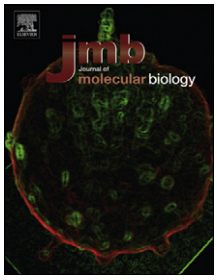

Robust Antibody–Antigen Complexes Prediction Generated by Combining Sequence Analyses, Mutagenesis, *In Vitro* Evolution, X-ray Crystallography and *In Silico* Docking

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Abstract

Hu 15C1 is a potent anti-human Toll-like receptor 4 (TLR4) neutralizing antibody. To better understand the molecular basis of its biological activity, we used a multidisciplinary approach to generate an accurate model of the Hu 15C1–TLR4 complex. By combining site-directed mutagenesis, *in vitro* antibody evolution, affinity measurements and X-ray crystallography of Fab fragments, we identified key interactions across the Hu 15C1–TLR4 interface. These contact points were used as restraints to predict the structure of the Fab region of Hu 15C1 bound to TLR4 using computational molecular docking. This model was further evaluated and validated by additional site-directed mutagenesis studies. The predicted structure of the Hu 15C1–TLR4 complex indicates that the antibody antagonizes the receptor dimerization necessary for its activation. This study exemplifies how iterative cycles of antibody engineering can facilitate the discovery of components of antibody-target interactions.

Introduction

Antibodies evolve to interact with high affinity and specificity to a wide variety of antigens. These properties, as well as the stability and relative ease of antibody production, have facilitated their development as a successful class of therapeutics. Often the mechanism of action of an antibody to mediate a specific biological activity is complex. Therefore, having additional and detailed information of the antibody–antigen interaction can be highly insightful. The methods of choice to obtain information at atomic resolution for antibody–antigen interactions are X-ray crystallography and nuclear magnetic resonance (NMR) [1–3]. However, experimental determination of antibody-target complexes using these technologies can be technically challenging and time consuming. Thus, other methods have been developed to understand antibody–antigen interactions, such as hydrogen–deuterium exchange mass spectrometry [4] and site-directed mutagenesis, but may have lower resolution and the data are difficult to interpret [5]. An alternative approach is to use the structure of each interacting protein and perform computational docking to generate the structure of the complex [6,7]. Models obtained with this method may not always be correct. However, the accuracy and, thus, the value of such models can be significantly increased if interaction points between the paired proteins have been derived experimentally and used as distance restraints during docking calculation [8,9].

The antibody, Hu 15C1, is a potent antagonist of the Toll-like receptor 4 (TLR4) pathway. The TLR4-MD2 heterodimer is a receptor of the innate immune system that senses pathogen-associated molecular patterns, such as lipopolysaccharide (LPS) from Gram-negative bacteria, to trigger a protective immune response. However, overactivation of TLR4 can lead to the systemic release of proinflammatory cytokines resulting in acute inflammatory disorder such as septic shock

[10]. This receptor can also be activated by endogenous proteins known as damage-associated molecular patterns that are associated with chronic diseases, including type 1 or 2 diabetes and rheumatoid arthritis [11,12]. Therefore, Hu 15C1 is being developed for clinical use.

Structural studies have shown that the binding of LPS to the hydrophobic pocket of MD2 induces conformational changes that drive the dimerization of the TLR4 receptor [13]. The formation of the receptor dimer allows the recruitment of intracellular adaptor molecules and initiates signal transduction [13]. Hu 15C1 is able to bind to TLR4 independently of MD2 and blocks the activation of the receptor independent of ligand type or concentration [14]. Using chimeric human–mouse receptors, we have mapped the antibody epitope to a region in close proximity to the dimerization interface of the receptor. Taken together, Hu 15C1 appears to prevent the activation of TLR4 by blocking receptor dimerization [15].

Given the difficulties of generating crystal of Hu 15C1 in complex with TLR4, an approach combining site-directed and combinatorial mutagenesis was successfully used. The species selectivity of the antibody was exploited to identify key interactions occurring at the interface between Hu 15C1 and TLR4 to determine distance restraints. Following the generation of X-ray crystallography data from the crystals of the Fab fragment of Hu 15C1, this information was incorporated in order to build a structural model of the antibody–TLR4 complex. The model was further tested by site-directed mutagenesis and its accuracy and predictive value was confirmed. The model revealed that Hu 15C1 can effectively block TLR4 dimerization by steric hindrance that further explains the ability of this antibody to prevent TLR4 activation by multiple ligands.

Results

Species specificity exploited to determine an epitope hot spot

Multiple alignment of TLR4 sequences from primates indicated that the regions of human TLR4 previously shown to be important for binding of Hu 15C1 were highly conserved except at positions 349 and 351 (Supplementary Fig. 1) [15]. The amino acids from these regions are clustered and exposed on the convex side of human TLR4 (Fig. 1a and c). As Lys349 and Lys351 of human TLR4 are substituted by glutamic acid in cynomolgus monkey (cyno) TLR4, we evaluated the binding of Hu 15C1 to the surface of CHO (*C*hinese *h*amster *o*vary) cell lines expressing similar levels of either human or cyno TLR4 (Supplementary Fig. 2a). Flow cytometry analysis showed that Hu 15C1 binds to human TLR4 but is not cross-reactive for cyno TLR4 (Fig. 1b). This result suggested that the presence of positively charged residues at position 349 or 351, or both, is important for binding. To further determine the relative contribution of these two positively charged residues, we introduced the single mutations K349E and K351E into human TLR4 using site-directed mutagenesis and we expressed the mutated forms of the receptor at the surface of CHO cells (Supplementary Fig. 2b). Similar signals were obtained with Hu 15C1 for either human WT (*w*ild *t*ype) or K351E TLR4 expressing cells, indicating that Lys351 did not contribute to the Hu 15C1–human TLR4 interaction (Fig. 1d). In contrast, binding to cells expressing human TLR4 K349E was drastically reduced compared to the WT receptor (Geo Mean = 15 and 2800, respectively) indicating that Lys349 is central for binding of Hu 15C1 to human TLR4. The importance of this position was further confirmed by substituting Glu349 in cyno TLR4 by a Lys residue and showing a strong binding of Hu 15C1 to cyno TLR4 E349K (Geo Mean = 1600) (Fig. 1d). Taken together, these results demonstrate that the residue located at position 349 on TLR4 is important for Hu 15C1 binding and drives the species specificity of this antibody.

In vitro antibody evolution leads to the identification of a pair of interacting residues at the Hu 15C1–TLR4 interface

As Lys349 is important for the binding of Hu 15C1 on human TLR4, we aimed at finding the interacting counterparts of this residue in the complementarity-determining regions (CDRs) of this antibody. In most cases, the third CDR of the heavy and light chains (CDRH3 and CDRL3, respectively) is located at the center of the paratope and provides most of the binding energy. We therefore hypothesized that residues making contact with TLR4 Lys349 are presented by these loops. For this reason, we introduced diversity into the CDRH3 and CDRL3 of Hu 15C1 to remodel the antibody paratope and identify variants capable of binding to cyno TLR4. After the generation of libraries diversified in either CDRH3 or CDRL3 of Hu 15C1, we performed phage display selections and identified single-chain variable fragments (scFv) that bind cyno TLR4. The final diversity obtained after this selection was small as only nine different scFv sequences were specifically obtained from the heavy chain libraries (Supplementary Fig. 3). To further study these positive variants, we reformatted the scFv into IgG but some of them appeared to be prone to aggregation. Therefore, only the 1A1, 1C10, 1C12 and 1E11 variants (Fig. 2a) could be tested for their binding to cyno and human TLR4 expressing cells by flow cytometry. All these variants are able to bind to cyno TLR4 and 1C12 and 1E11 were found to be cross-reactive with both cyno and human TLR4 as shown in Fig. 2b. The 1A1 and 1C10 cyno specific antibodies and the 1C12 and 1E11