Epitope-dependent mechanisms of CD27 neutralization revealed by X-ray crystallography

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Short Title: Epitope-dependent mechanisms of CD27 antibodies

Abstract

CD27 is a T and B cell co-stimulatory protein of the TNF receptor superfamily dependent on the availability of the TNF-like ligand CD70. Two anti-CD27 neutralizing monoclonal antibodies were obtained from mouse hybridoma and subsequently humanized and optimized for binding the target. The two antibodies are similar in terms of their CD27-binding affinity and ability to block NF- κ B signaling, however their clearance rates in monkeys are very different. The pharmacokinetics profiles could be epitope dependent. To identify the epitopes, we determined the crystal structure of the ternary complex between CD27 and the Fab fragments of these non-competing antibodies. The structure reveals the binding modes of the antibodies suggesting that their mechanisms of action are distinctly different and provides a possible explanation of the *in vivo* data.

Keywords: CD27, antibody, crystal structure, epitope, mechanism

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; CRD, cysteine-rich domain; ECD, extracellular domain; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; PDB, Protein Data Bank; PEG, polyethyleneglycol; PK, pharmacokinetics; SEC, size exclusion chromatography; SEM, standard error of mean; SPR, surface plasmon resonance; TNF, tumor necrosis factor; RMSD, root-mean-square deviation.

1. Introduction

CD27 is a glycosylated, type I transmembrane protein of about 55 kDa expressed as a surface antigen on a majority of T cells, natural killer cells and antibody secreting plasma and memory B cells (Borst et al., 2005). CD27 is required for generation and long-term maintenance of T cell immunity. CD27 belongs to the tumor necrosis factor (TNF) receptor superfamily (Camerini et al., 1991). Similarly to the other members of the superfamily, the extracellular portion (ECD) of CD27 contains several cysteine-rich domains (CRD). Additionally it has a free cysteine proximal to the cell membrane that forms an interchain disulfide to yield a dimeric CD27 molecule.

CD27 exerts its function through binding to CD70, a trimeric type II transmembrane protein of the TNF ligand superfamily. CD70 expression is normally restricted to B cells, mature dendritic cells and activated T cells (Nolte et al., 2009; Denoeud and Moser, 2011). CD27:CD70 ligation results in activation of NF- κ B signaling pathways that in turn stimulates B- and T-cell proliferation, plasma cell differentiation and subsequent antibody secretion (Yamamoto et al., 1998). Studies in humans and animals suggest an important role of the CD27:CD70 pathway in various immune-related diseases including systemic lupus erythematosus (Dörner and Lipsky, 2004), rheumatoid arthritis (Tak et al., 1996) and multiple sclerosis (Hintzen et al., 1991). CD27 may also control the accumulation of CD4⁺ and CD8⁺ T cells at sites of infection (Hendricks et al., 2000). While agonist anti-CD27 antibodies may be useful for promoting T cell-mediated antitumor or antiviral immunity (He et al., 2013), antagonist antibodies can exert a clinically useful cytotoxic, cytostatic or immunomodulatory effect on CD27-expressing cells, particularly without causing undesirable agonist effects in the absence of CD70 (Makino et al., 2012).

Two anti-CD27 neutralizing monoclonal antibodies (mAbs), 2177 and 2191, were obtained from mouse hybridoma and subsequently humanized and optimized for binding the target. This report describes *in vitro* and *in vivo* characterization of the mAbs. A striking finding was the difference in their half-life times in monkeys. These mAbs are otherwise very similar suggesting that their different pharmacokinetics (PK) profiles could be epitope dependent. To identify the epitopes, we determined the crystal structure of the ternary complex between CD27 ECD and the Fab fragments of these non-competing antibodies. The structure reveals the binding modes of the mAbs suggesting that their mechanisms of action are distinctly different. The mechanistic model provides a possible explanation of the *in vivo* data.

2. Materials and Methods

2.1. Proteins

For mouse immunization, human CD27 ECD-Fc protein was purchased from R&D systems. For binding assays, amino acids 2-122 of the CD27 ECD (corresponding to residues 21-141 of UniProtKB entry CD27_HUMAN) with a C-terminal 6xHis tag were expressed in HEK 293 cells and purified by metal ion chromatography followed by size exclusion chromatography (SEC) on Superdex 75. For crystallization, amino acids 2-102 of the CD27 ECD (21-121 of CD27_HUMAN) with a C-terminal 6xHis tag were expressed in a baculovirus system and purified by metal ion chromatography (NiNTA) and SEC (Superdex 200) by Proteos, Inc. (Kalamazoo, MI). It was further purified on a MonoS column (GE Healthcare).

The human CD70 ECD (residues 39-193 of UniProtKB entry CD70_HUMAN) with an N-terminal FLAG tag was expressed in HEK 293 cells and purified by affinity and size exclusion chromatography.

Anti-CD27 mAbs M2177 and M2191 and their humanized versions H2177 and H2191 were expressed in HEK 293 cells and purified by affinity chromatography on a Protein A column. MAbs H2177 and H2191 have the IgG4-PAA (S228P, F234A, L235A) isotype characterized by the reduced antibody-dependent cell-mediated cytotoxicity (ADCC).

The Fab fragments of anti-CD27 mAbs M2177, M2191 and H2191 were constructed by fusing their variable domains (mouse or humanized) with human IgG1/kappa constant domains that contained the 6xHis tag at the C-terminus of the heavy chain. The Fabs were expressed in HEK 293 cells and purified by affinity (IMAC or HisTrap) and size exclusion (Superdex 200) chromatography.

2.2. Generation of antibodies

Murine anti-CD27 monoclonal antibodies (mAbs) were obtained by the hybridoma method (Köhler and Milstein, 1975). Ten 12-14 week old C3H/HeJ mice were obtained from Charles River Laboratories. The mice were immunized subcutaneously with 50 µg human CD27-Fc (R&D Systems) in combination with murine interferon- α and interferon- β (Biosource). In 2 weeks the mice were boosted with 50 µg CD27-Fc in combination with 50 µg anti-murine CD40 agonist mAb (MAB440, R&D Systems). Four days later, spleen cells from immunized mice were fused with myeloma fusion partner FO cells (ATCC #CRL-1646). Supernatants from

resulting hybridomas were screening by ELISA using human CD27 ECD (1-173). mAbs were purified on a protein A-sepharose column (GE Healthcare).

2.3. Affinity measurements

The affinities of the mAbs were measured by surface plasmon resonance (SPR) using a Biacore 3000 system (GE Healthcare). The biosensor surface was prepared by covalently coupling 60 RU goat anti-human Fc antibody (Jackson ImmunoResearch, Cat. No. 109-005-098). About 350 units of mAb were captured on the sensor chip. The kinetic experiments were performed at 25 °C in running buffer (PBS pH 7.4, 0.005% Tween 20, 3 mM EDTA) using serial dilutions of human monomeric CD27 ECD (residues 2-122) starting at 100 nM. Antigen injection (association phase) at 30 μ L/min for 3 min was followed by 10 min of buffer flow (dissociation phase). The data were processed using the instrument software. The double reference subtraction was applied by subtracting the curves generated by buffer injection from the reference-subtracted curves for analyte injections. A 1:1 Langmuir binding model with global fit was used for kinetics analysis.

The affinities to the cell-surface CD27 were measured on HEK 293 cells overexpressing human or cyno CD27 utilizing Meso Scale Discovery (MSD) technology. Serial dilutions of cells starting at 10^7 cells/mL were prepared and incubated for 24 h at 4 °C with a fixed concentration of mAb starting at 400 pM. After equilibrium was reached, the free fraction of mAb was separated by centrifugation, applied to the plates coated with biotinylated CD27 and detected by a Ru-labeled anti-IgG mAb. The K_D values are derived from 2 independent experiments.

2.4. Competition assay

Inhibition of sCD70 binding to human CD27 ECD-Fc by mAbs M2177 and M2191 was measured by ELISA. CD27 ECD-Fc was captured on the plate by coating the plate with a goat anti-human IgG antibody (Sigma-Aldrich). FLAG-tagged soluble human CD70 (sCD70) at 2 μ g/mL and antibody at 10 μ g/mL were added simultaneously. The binding was detected by an anti-FLAG mouse antibody covalently conjugated to horseradish peroxidase (Sigma-Aldrich) and measured as OD₄₅₀.

2.5. Functional assay

Inhibition of CD70-mediated NF- κ B signaling through the CD27 receptor was assessed in a reporter gene assay. The 1×10^5 stably-transfected CD70-expressing HEK cells (irradiated

with 4000 rads) were incubated with 5×10^4 transiently-transfected CD27/Luc-expressing cells for 18 hrs in Freestyle293 medium. Steady-Glo (Promega) was used to develop the assay and plates were read on a Victor using a luminescence protocol. The assay was performed two times with 4 replicates in each. The IC₅₀ values were calculated in GraphPad Prism 5.0 using a nonlinear curve fit.

2.6. Pharmacokinetics

Pharmacokinetics of the two antibodies were measured in cynomolgus monkeys (*Macaca fascicularis*). The monkeys were dosed with 5 mg/kg of each antibody and blood was drawn at the appropriated time points. Antibody levels were determined by ELISA. Briefly, blood was collected into a Vacutainer CP tube (BD) and spun at RT for 20 min, the resulting plasma was stored at -70 °C until time of assay. The ELISA plates were coated with a Penta-His antibody (Qiagen) that was used to capture the His-tagged CD27 protein. The plates were loaded with serum samples, incubated overnight at 4 °C and washed. The antibodies were detected by using a goat anti-human IgG antibody covalently conjugated to horseradish peroxidase (HRP) and Supersignal™ ELISA Pico Chemiluminescence Substrate (Thermo Fisher Scientific). Chemiluminescence was measured on a Victor Plate Reader (PerkinElmer). The data were plotted in GraphPad Prism 5.0 and $t_{1/2}$ values were determined in Phoenix.

2.7. Crystallization

CD27:M2177:M2191 complex was prepared by mixing CD27 ECD (2-102) with the excess of Fabs at a molar ratio 1:1.25:1.25 and incubated for 2 h at 4°C. The complex was separated from the unbound Fabs by SEC (Superdex 200) and concentrated to 12 mg/mL in 20 mM Tris, pH 8.5, 250 mM NaCl.

Crystallization of the complex was carried out by the vapor-diffusion method at 20°C using an Oryx4 robot (Douglas Instruments). The experiments were composed of equal volumes of protein and reservoir solution in a sitting drop format in 96-well Corning 3550 plates. The initial screening was performed with the in-house screens and the PEGs suite (Qiagen). A hit was observed in PEGs condition #B12 (100 mM Tris, pH 8.5, 25% polyethylene glycol (PEG) MME 2000) and was used to prepare seeds for microseed matrix screening (D'Arcy et al., 2007, Obmolova et al., 2010). The crystals suitable for X-ray analysis were obtained from 24% PEG 3350 and 0.2 M ammonium chloride in 0.1 M Tris buffer, pH 8.5.

CD27:M2177:H2191 complex was prepared and crystallized following the same procedure. The crystals used for X-ray analysis grew from 22% PEG 3350 in 0.1 M Tris buffer, pH 8.5. The crystal data for both complexes are given in Table 2.

2.8. X-ray data collection and structure determination

For X-ray data collection, one crystal of CD27:M2177:M2191 was soaked for a few seconds in a cryo-protectant solution containing mother liquor supplemented with 20% glycerol, and flash frozen in the stream of nitrogen at 100 K. X-ray diffraction data were collected in house using a Rigaku MicroMaxTM-007HF microfocus X-ray generator equipped with a Saturn 944 CCD detector and an X-streamTM 2000 cryocooling system (Rigaku). Diffraction intensities were detected over a 240° crystal rotation with 2-min exposures per 0.25°-image. The crystal of CD27:M2177:H2191 complex was frozen in liquid nitrogen and shipped to the Advanced Photon Source at the Argonne National Laboratory for X-ray data collection at the IMCA beamline 17-ID equipped with a Pilatus 6M detector. Both data sets were processed with the program XDS (Kabsch, 2010). X-ray data statistics are given in Table 2.

The structures were solved by molecular replacement with the program Phaser (McCoy et al., 2007) using the crystal structure of CD27:M2177 complex (PDB code 5TL5) as a search model. All crystallographic calculations were performed with the CCP4 suite of programs (Winn et al., 2011). Model adjustments were carried out using the program Coot (Emsley et al., 2010). The rigid-body and individual atomic refinement was performed with Refmac (Murshudov et al., 2011). Refinement statistics are given in Table 2. The atomic coordinates and structure factors were deposited in the Protein Data Bank under accession codes 5TLJ (CD27:M2177:M2191) and 5TLK (CD27:M2177:H2191).

2.9. Modeling of the CD27:CD70 complex

The model was built using the CD40:CD40L crystal structure (PDB code 3QD6; An et al., 2011) as a template. The crystal structure of CD27 from the CD27:M2177 complex (residues 1-101 of 5TL5) was superimposed on residues 26-120 of the CD40 molecule (chain Q of 3QD6) from the CD40:CD40L complex. Residues 102-139 of CD27 ECD missing in the present structure were replaced by residues 116-153 of TNFR1 from 1TNR (Banner et al., 1993). The CD40L trimer was substituted for the CD70 trimer in the model and the corresponding amino acid substitutions were managed with Coot (Emsley et al., 2010).

3. Results

3.1. Characterization of anti-CD27 mAbs *in vitro*

Mouse anti-CD27 mAbs M2177 and M2191 and their humanized versions H2177 and H2191 were tested for their ability to bind and neutralize human CD27 *in vitro*. The binding was measured on a monomeric variant of CD27 ECD (residues 2-122) to exclude the avidity effect. Affinities of the murine versions were in the nanomolar range (Table 1). Antibody humanization coupled with affinity maturation resulted in a significant improvement of the off-rate. The final versions of the mAbs have affinities in the picomolar range, 30 pM for H2177 and 130 pM for H2191. The affinity difference between the two mAbs is due to the on-rate, which is 4 times faster for H2177.

The binding of mAbs H2177 and H2191 to the cell-surface CD27 was measured on the HEK cells overexpressing the target receptor. H2177 binds both human and cyno CD27 on the cell with very high affinity (apparent $K_D = 7$ pM). H2191 binds cyno CD27 with lower affinity than human CD27 (Table 1).

In a competition experiment, mouse mAbs M2177 and M2191 were tested for their ability to bind CD27 in the presence of soluble CD70 (sCD70). M2191 blocks the CD27-CD70 interaction with the IC_{50} in the low nanomolar range. On the contrary, M2177 does not compete with sCD70 (Fig. 1).

The functional activity of the mAbs was tested in a CD70-mediated NF- κ B signaling assay. Both mAbs, H2177 and H2191, demonstrate a dose-dependent response with the EC_{50} in the sub-nanomolar range (0.04-0.06 μ g/mL) (Fig. 2).

3.2. Pharmacokinetics of anti-CD27 mAbs in non-human primates

Pharmacokinetics of mAbs H2177 and H2191 were measured in cynomolgus monkeys dosed with 5 mg/kg of each antibody. The observed clearance rates were very different. H2177 displayed a much faster clearance when compared to H2191. The calculated half-life ($t_{1/2}$) was about 12 h for mAb H2177 and 7 days for mAb H2191. The mAbs were tested using the IgG4-PAA silent isotype to avoid possible agonism due to Fc γ R interactions. The mAbs show no agonistic activity *in vitro* without cross-linking (Chen et al., 2015).

3.3. Crystal structure of the ternary complex

The crystal structure of the CD27:M2177:H2191 complex was determined at 2.7 Å resolution using the structure of the binary CD27:M2177 complex that had been determined previously (PDB code 5TL5). There are two ternary complexes in the asymmetric unit in space group P2₁. The electron density is good for all 4 Fab molecules and for both CD27 molecules excluding residues 85-101 that are completely disordered. Asn75 appears to be glycosylated, and tandem N-acetylglucosamine moieties are visible in the electron density in both CD27 molecules.

The crystal structure of the CD27:M2177:M2191 complex was determined at 3.5 Å resolution using the same search model. The crystals of the two complexes are nearly isomorphous with the unit cell variation of about 2% (see Table 2). The electron density is clear for all 4 Fab molecules but only for one CD27 molecule, whereas it is largely missing for the other although there is enough space between the Fabs. The CD27 molecule has good electron density for residues 5-67 and 74-84, but the rest of the structure (residues 68-73 and 85-101) is apparently disordered.

The CD27 ECD consists of cysteine-rich repeats, each cross-linked by three internal disulfides, which is typical for the TNFR superfamily. The number of such repeats in different members of the superfamily varies, usually between 3 and 4. CD27 contains only two complete repeats (residues 1-43 and 44-84) and one incomplete with 2 disulfides. Overall, CD27 ECD is an elongated molecule that lacks a secondary structure and folds into a number of disulfide-linked loops.

3.4. Antibody epitopes

The ternary complex, represented in Figure 4, reveals the binding mode of the two noncompeting antibodies. Fab 2177 binds CD27 on top of the molecule, i.e. at the end distal from the cell surface. The antibody-antigen interface covers 700 Å² of the surface of each molecule. The mAb epitope on CD27 is conformational and includes residues 5-17 and 36-37 from two stretches of the protein chain (the epitope is defined as the antigen residues within 4 Å from the antibody residues). His36 and Arg37 stack against two tyrosine residues of the antibody, Tyr31 of VL and Tyr102 of VH, and also form hydrogen bonds to Asp104 of VH and Asn96 of VL (Fig. 5A). Given the central position within the epitope and the number of contacts, these are likely the key interactions between mAb 2177 and CD27. Additionally, there are hydrophobic interactions between Trp13 of CD27 and Tyr101 of VH and between the aliphatic chains of Lys5 and Lys17 of CD27 and phenol groups of Tyr105 and Tyr52 of VH.

Fab 2191 binds at the side surface of the CD27 molecule (Fig. 4). The size of the epitope is about 800 Å², which is similar to the 2177 epitope. The principle interactions involve a single stretch of the CD27 chain that includes residues 43-49 (Fig. 5B). Most of these residues are hydrophobic and fit in a hydrophobic patch on the surface of the Fab. Pro44 and Pro47 of CD27 provide binding energy through, respectively, face-to-face and orthogonal interactions with Tyr34 and Phe36 of VL. Ile46 is sandwiched between Gly101 of VH and Trp100 of VL. Asp43, His60 and Ser63 at the periphery of the epitope form a number of hydrogen bonds.

Comparison of the M2191 and H2191 complexes shows that the humanized version of mAb 2191 occupies the same position on CD27 as the mouse parent. When the structures are superimposed on the variable domains of the Fabs, the RMSD for all atoms of the epitope residues is only 0.55 Å. This may be compared to the RMSD of 0.37 Å for the same atoms between two copies of the H2191 complex in the same crystal. In other words, the epitope of mAb 2191 does not shift upon humanization.

The epitope of mAb 2177 is completely conserved in cyno CD27, consistent with the identical affinity of H2177 for human and cyno CD27 (Table 1). In contrast, the epitope of mAb 2191 contains a single amino acid substitution at position 43, Asp43 in human versus His43 in cyno CD27 (Fig. 6). The structure reveals that Asp43 forms a short hydrogen bonds to Tyr53 (Fig. 5B) suggesting that His at position 43 would clash with the phenol group of Tyr53. Furthermore, Asp43 is sandwiched between Arg57 and Arg100 in an electrostatic environment that does not favor histidine. These observations may explain why mAb 2191 binds cyno CD27 substantially weaker than human CD27.

4. Discussion

4.1. Pharmacokinetics of anti-CD27 mAbs

Anti-CD27 neutralizing mAbs 2177 and 2191 were generated from mouse hybridoma and optimized for binding. Their affinities measured on the monomeric human CD27 ECD and the apparent affinities on CD27-expressing cells were in the picomolar range. The two mAbs recognize different epitopes on CD27 but demonstrate similar behavior in terms of the inhibition of CD70-mediated NF-κB signaling. However, the difference in their pharmacokinetics profiles in monkeys is striking. The half-life time for mAb 2191 is one week, whereas it is surprisingly short for mAb 2177, just about 12 hours.

There are two principal mechanisms of the antibody clearance from the blood (Tabrizi et al., 2006). One of them involves antibody uptake by fluid-phase pinocytosis into endothelial cells followed by the binding to FcRn in the more acidic environment of the endosomes and finally recycling of the antibody to the cell surface. Thus, FcRn functions as a salvage receptor, rescuing antibody from a lysosomal degradation pathway (Ghetie & Ward, 2000). While the interactions between Fc and FcRn influence the association and dissociation kinetics of the two mAbs in a similar way, it is possible that affinity or kinetic differences may also depend on the conformational effect of the Fab regions (Datta-Mannan et al., 2007). Specifically, in the two mAbs, the pI's of the variable domains are 3.5 units apart (5.5 for H2177, 9.0 for H2191) suggesting that the long-range electrostatic forces may asymmetrically affect interactions with FcRn. Since FcRn has a pI of about 6.0, H2177 may experience electrostatic repulsion that may impede the recycling process.

The second, antigen-specific, mechanism involves binding to the cell surface receptor and internalization of the complex by the cell (Kyriakos et al., 1992; Thurber et al., 2008). The higher-affinity antibodies may exhibit a greater degree of internalization if their dissociation rates are slower than the internalization rate of the cell surface antigen (Rudnick et al., 2011). MAb 2177 has a significantly higher affinity (7 pM) to cyno CD27 than mAb 2191 (220 pM) and it is possible that the difference comes from the slower dissociation rate, which may be below the internalization rate of CD27. However, the receptor-mediated endocytosis typically requires activation of the receptor (Doherty & McMahon, 2009), which is an unlikely event in the present case as both mAbs do not possess agonistic activity.

On the other hand, a number of studies suggest that the antibody-mediated internalization may depend on the epitope recognized, and in some cases even a monovalent antibody fragment (scFv or Fab) may act as a ligand mimetic resulting in conformational changes in the receptor that trigger endocytosis (Poul et al., 2000). Therefore, we cannot rule out that the difference in the pharmacokinetics profiles may be related to the location of the mAb epitopes on the antigen.

The binding epitopes were determined from the crystal structure of the ternary complex between CD27 ECD and the Fab fragments of mAbs 2177 and 2191. In one structure, both mAbs were murine (M2177 and M2191). In the other structure, M2177 and the humanized variant of mAb 2191 (H2191) were used. The structures are virtually identical as expected because CDR grafting does not change the binding site of the antibody.

The epitope of mAb 2177 is located in the N-terminal portion of CD27 and spans the first CRD. The epitope of mAb 2191 is located within the second CRD. The epitopes do not overlap and actually are quite remote from each other. MAb 2177 binds CD27 at the site most distant from the cell surface with the Fab approximately perpendicular to the surface. MAb 2191 binds CD27 in the middle of the ECD with the Fab approximately parallel to the cell surface. We hypothesize that the location of the mAb 2177 epitope on top of the CD27 molecule allows for a tighter clustering of the receptor chains that may mimic to some extent the clustering upon binding the trimeric ligand, CD70. On the contrary, mAb 2191 may be unable to bring the CD27 chains close enough. If so, the distinctly different binding geometries of the mAbs may reveal themselves in the remarkably different PK profiles.

It should be noted that other factors including structural characteristics of antibodies may impact the pharmacokinetics (Bumbaca et al., 2012). In particular, the Fc isotype and glycosylation, the net charge and charge distribution may play a role in the antibody clearance. For example, it was observed that lowering the pI by engineering the variable region could reduce the elimination of IgG antibodies (Igawa et al., 2010). Curiously, for the antibody pair in question, the relationship is inverse: mAb 2177 has a much lower pI than mAb 2191 (6.6 versus 8.2), but has a shorter half-life. Another factor is immunogenicity, specifically generation of anti-drug antibodies that is primarily sequence-dependent. The clearance of a therapeutic mAb within the immune complex may be much faster compared to unbound mAb (Vugmeyster et al., 2012).

4.2. Model of CD27-CD70 interaction

There is no 3D information on the complex between CD27 and its ligand CD70. However, a model of such a complex could be generated based on the following considerations. Firstly, the CD70/CD27 system is a structural analogue of the well-characterized TNF β /TNFR system, which also includes TRAIL/DR5, OX40L/OX40 and CD40L/CD40. The structures of all these complexes are available from the PDB (Banner et al., 1993; Hymowitz et al., 1999; Compaan and Hymowitz, 2006; An et al., 2011). In all cases the trimeric TNF β -like ligand binds the TNFR-like receptor at the side surface of the elongated molecule formed by the cysteine-rich repeats. The epitope is invariably centered on the second repeat.

Secondly, the alignment of CD27 sequences from various species shows one region of high conservation, which is located around the second cysteine-rich repeat. There is a single amino acid difference between human and mouse CD27 in the stretch of residues 50-70 (Fig. 6) whereas the overall identity in the extracellular domain is only 62%. The conservation of the

CD70-binding epitope is expected since human CD70 binds mouse CD27 and vice versa (Tesselaar et al., 1997).

Thirdly, mAb 2191 blocks binding of both the membrane-bound and the soluble form of CD70, indicating that it directly competes for the same epitope. This means that the mAb epitope revealed by the crystal structure at least partially overlaps with the CD70 epitope.

Taken together, these considerations lead to a model, in which the CD70 epitope on CD27 is located on the side surface, around the second cysteine-rich repeat, and likely includes residues 57-68 which would fit in between CD70 subunits. Additionally, the hydrophobic pocket on CD27 formed by Phe28, Ile46, Val49 and disulfide Cys45-Cys61 possibly anchors Gly78-Pro79 of CD70 (numbering of full-length CD70). In this arrangement, mAb 2191 would compete with CD70 since residues 46-49 and 60-66 form part of its epitope on CD27.

4.3. Mechanism of CD27 neutralization

The crystal structure of the ternary CD27:2177:2191 complex allows us to propose a mechanism of action of these antibodies that would explain the experimental data. Both mAbs are neutralizing as evidenced in the functional assays. MAb 2191 directly competes with CD70 for the overlapping epitopes on the side surface of the CD27 ECD. As such, mAb 2191 competes with both the soluble and the cell-bound forms of CD70. MAb 2177 binds CD27 on top of the molecule, at the membrane-distal end, far away from the putative CD70 epitope (Fig. 7). This means that mAb 2177 would not compete with the soluble form of CD70 which contains only the extracellular domain. Indeed, the competition experiment demonstrates that mAb 2177 does not prevent sCD70 from binding CD27 (Fig. 1).

While there is no competition between mAb 2177 and sCD70, this mAb does block the binding of the full-length CD70 to CD27 due to steric effects. The neutralizing mechanism employed by mAb 2177 is indirect since it does not involve direct competition for the overlapping epitope. The hypothesis is based on the following geometric considerations. On the one hand, the linker between the structured, CD27-binding portion of CD70 ECD and the transmembrane domain is short, only about 20 residues. Such a linker can span 70 Å when fully stretched, but probably much less in real conditions. On the other hand, the mAb molecule is bulky, over 70 Å for the Fab only. Thus, mAb 2177 bound on top of the CD27 ECD would prevent the cells bearing CD70 and CD27 from coming close enough for CD70 to engage CD27.

Antibodies that block signaling by preventing ligand-receptor association may employ quite different mechanisms of neutralization. The most typical mechanism involves competition for the overlapping epitope as shown here for mAb 2191. Another mechanism involves antibody-induced conformational changes in the target that disrupt the ligand epitope even though the antibody binding site may be remote (Roguin and Retegui, 2003). The unusual mechanism employed by mAb 2177 is neither of the first kind, nor of the second. This antibody does not compete for the same epitope, nor does it cause conformational changes. It simply works as a wedge between the ligand and the receptor. In this sense, one may say, the neutralization is achieved through a 'wedge' mechanism.

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Glossary

R-work: a measure of the agreement between the crystallographic model and the experimental X-ray diffraction data

R-free: a measure of how well the current atomic model predicts a subset of the measured reflection intensities that were not included in the refinement

R-merge: a measure of the agreement between multiple measurements of a given reflection

Table 1. Binding of anti-CD27 mAbs to the target.

mAb	monomeric human CD27 ECD			human CD27 cells K_D (pM)	cyno CD27 cells K_D (pM)
	k_a (1/Ms)	k_d (1/s)	K_D (pM)		
M2191	4.7×10^5	9.6×10^{-3}	20000	N/A	N/A
H2191	$5.0 \pm 0.2 \times 10^5$	$6.6 \pm 1.0 \times 10^{-5}$	130 $\pm$ 25	30 $\pm$ 8	220 $\pm$ 40
M2177	1.1×10^6	1.3×10^{-3}	1200	N/A	N/A
H2177	$1.9 \pm 0.3 \times 10^6$	$6.8 \pm 0.5 \times 10^{-5}$	36 $\pm$ 8	7 $\pm$ 2	7 $\pm$ 2

N/A, not assessed.

Table 2. Crystal data, X-ray data, and refinement statistics.

	C27:M2177:M2191		C27:M2177:H2191	
Source	MicroMax-007HF		APS IMCA 17-ID	
Detector	Saturn 944		Pilatus 6M	
Wavelength (Å)	1.5418		1.000	
Temperature (K)	100		100	
Distance (mm)	100/90		450	
Total rotation (°)	240		180	
Exposure per image	120 s per 0.25°		0.5 s per 0.5°	
<i>Crystal data</i>				
Space group	P2 ₁		P2 ₁	
Unit cell axes (Å)	141.08, 52.96, 143.41		139.71, 52.96, 140.60	
Unit cell angles (°)	90, 112.19, 90		90, 109.52, 90	
Molecules per asymmetric unit	2		2	
V _m (Å ³ /Da)	2.29		2.27	
Solvent content (%)	46		46	
<i>X-ray data</i>				
Resolution (Å)	30–3.5	(3.6–3.5)*	30–2.7	(2.8–2.7)
Number of measured reflections	111,342	(6,239)	181,629	(11,811)
Number of unique reflections	25,023	(1,599)	53,961	(3,556)
Completeness (%)	97.8	(85.5)	98.4	(88.3)
Redundancy	4.4	(3.9)	3.4	(3.3)
R-merge (I)	0.158	(0.489)	0.062	(0.487)
Mean I/σ(I)	9.6	(2.7)	14.2	(2.6)
B factor (Wilson plot) (Å ²)	41.7		57.0	
<i>Refinement</i>				
Resolution (Å)	20–3.5		20–2.7	
Number of all atoms	13,728		14,287	
Number of water molecules	0		75	
R-work (%)	28.3		21.3	
R-free (%)	35.6		26.3	
Bond lengths RMSD (Å)	0.008		0.007	
Bond angles RMSD (°)	1.3		1.2	
Mean B factor (Å ²)	61.4		61.2	

*Numbers in parentheses refer to the highest-resolution shell.

Figure legends

Figure 1. Inhibition of sCD70 binding to human CD27 ECD-Fc by mAbs M2177 and M2191.

Figure 2. Inhibition of CD70-mediated NF- κ B signaling by anti-CD27 mAbs H2177 and H2191. The CD70-expressing HEK cells were incubated with CD27-expressing cells for 18 hrs. The data points are the average of two experiments measured in quadruplicate; H2177 IC_{50} = 0.040 μ g/mL, H2191 IC_{50} = 0.063 μ g/mL.

Figure 3. Pharmacokinetics of mAbs H2177 and H2191 in naive monkeys. The data points indicate the mean of three monkeys $\pm$ SEM.

Figure 4. Ribbon presentation of the CD27 ECD:Fab M2177:Fab H2191 complex. Colors: Fab M2177 blue/cyan, Fab H2191 red/orange (heavy chains are darker, light chains are lighter), CD27 yellow (CRD1)/pale green (CRD2)/green (CRD3).

Figure 5. Key interactions between CD27 and antibodies: (A) Fab M2177; (B) Fab H2191. Orientation and colors as in Fig. 4. Hydrogen bonds are shown as dashed lines.

Figure 6. Amino acid sequence alignment of cyno, human and mouse CD27 ECD. The differences from human CD27 are indicated by vertical lines. The numbering is that of human CD27 ECD where the N-terminus corresponds to position 21 of UniProtKB entry CD27_HUMAN. The epitopes of mAb 2177 and mAb 2191 are indicated by pluses and stars, respectively (the epitopes are defined as the antigen residues in direct contact with the Fab using a 4-Å cutoff).

Figure 7. A space-filling model of the complex between CD70 ECD trimer (red), CD27 ECD (yellow) and Fab 2177 (blue/gray). The membrane-proximal C-terminal end of CD27 ECD is at the bottom of the picture. The membrane-proximal N-terminal end of CD70 ECD is at the top of the picture.

	1	2	3	4	5	6
	. 0	. 0	. 0	. 0	. 0	. 0
cyno	TPAPKSCPERHYWAQGKLCQMCPEPGTFLVKDCDQHRKAAQCHPCIPGVSFSPDHHTRPH					
human	TPAPKSCPERHYWAQGKLCQMCPEPGTFLVKDCDQHRKAAQCDPCIPGVSFSPDHHTRPH					
mouse	TLAPNSCPDKHYWTGGGLCCRMCEPGTFFVKDCEQDRTAQCDPCIPGTSFSPDYHTRPH					
	++	+	+	+	++	*
					++	** **** *

	7	8	9	10	11	12
	. 0	. 0	. 0	. 0	. 0	. 0
cyno	CESCRHCNSGLLIRNCTITANAVCACRNGWQCRDKECTECDPPPNPSLTTWPSQALGPHP					
human	CESCRHCNSGLLVRNCTITANAECACRNGWQCRDKECTECDPLPNPSLTARSSQALSPHP					
mouse	CESCRHCNSGFLIRNCTVTANAECSCSKNWQCRDQECTECDPPLNPALTRQPSETPSPQP					
	*	*				

	13	14	15	16	17
	. 0	. 0	. 0	. 0	. 0
cyno	QPTHLPHYVNEMLEARTAGHMOTLADFRHLPARTLSTHWPPQRS LCSSDFIR				
human	QPTHLPHYVSEMLEARTAGHMOTLADFRQLPARTLSTHWPPQRS LCSSDFIR				
mouse	PPTHLPHGTE-----KPSWPLHRQLPNSTVYSQRSSHRPLCSDCIR