Structural Insights into the Inhibitory Mechanism of an Antibody against B7-H6, a Stress-Induced Cellular Ligand for the Natural Killer Cell Receptor NKp30

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Abbreviations used: NK, natural killer; MHC, major histocompatibility complex; NCR, natural cytotoxicity receptor; Ig, immunoglobulin; V, variable; C, constant; L, light; H, heavy; Fab, antigen-binding fragment; CDR, complementarity-determining region; mAb, monoclonal antibody.

Abstract

Antibodies have been shown to block signaling through cell surface receptors using several mechanisms. The two most common are binding to the ligand-binding site of the receptor and, conversely, binding to the receptor-binding site of the ligand. Here we investigated the inhibitory mechanism of an antibody (17B1.3) against human B7-H6, a stress-induced cellular ligand for the natural killer (NK) cell receptor NKp30. Binding of this antibody to B7-H6, a transmembrane protein expressed on tumor and other stressed cells, but not on normal cells, prevents NK cell activation via NKp30. We determined the crystal structure of antibody 17B1.3 in complex with the ectodomain of B7-H6 to 2.5 Å resolution. Surprisingly, 17B1.3 binds to a site on B7-H6 that is completely distinct from the binding site for NKp30, such that 17B1.3 does not block the NKp30–B7-H6 interaction. We then asked whether 17B1.3 prevents signaling by binding to a putative site for B7-H6 dimerization. However, structure-based mutations designed to disrupt potential B7-H6 dimerization through this site did not diminish NKp30-mediated cell activation. We conclude that the bulky 17B1.3 antibody most likely acts by sterically interfering with close cell–cell contacts at the NK cell–target cell interface that are required for NK cell activation. A similar inhibitory mechanism may apply to other antibodies, including therapeutic antibodies, that block signaling through cell surface receptors whose ligands are also cell surface proteins.

Introduction

Natural killer (NK) cells are innate lymphoid cells that participate in the elimination of tumor cells and virally infected cells [1–3]. The activation of NK cells is controlled by a dynamic interplay between positive signaling activating receptors and negative signaling inhibitory receptors [4,5]. The dominant signal received by an NK cell through interaction with normal levels of major histocompatibility complex class I (MHC-I) molecules on target cells is inhibitory. If MHC-I expression is reduced by tumorigenic or infectious processes, this inhibitory signal is attenuated and the NK cell is activated. Inhibitory receptors specific for MHC-I include killer immunoglobulin (Ig)-like receptors (KIRs) in humans and C-type lectin-like Ly49 receptors in rodents [6].

Besides reduced inhibitory signals, activating signals are also necessary for NK cell triggering and target cell lysis [3–5]. These signals are transmitted by diverse activating receptors, including DNAM-1, NKG2D and the natural cytotoxicity receptors (NCRs), whose ligands comprise both MHC-like and non-MHC molecules. The NCR family includes NKp30, NKp44 and NKp46. These activating receptors are type I transmembrane glycoproteins comprising one (NKp30 and NKp44) or two (NKp46) Ig-like extracellular domains [6].

NKp30 recognizes the stress-induced cell surface protein B7-H6 [7]. B7-H6 is a member of the B7 family, which includes ligands (B7-1 and B7-2) for the T cell co-inhibitory receptor CTLA-4 and the co-stimulatory receptor CD28, as well as ligands (PD-L1 and PD-L2) for the T cell co-inhibitory receptor PD-1 [8]. Like other B7 family members, B7-H6 comprises a membrane-distal Ig variable (IgV) domain and a membrane-proximal Ig constant (IgC) domain [7]. We previously determined the crystal structure of B7-H6 bound to NKp30, showing how

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4 B7-H6 contacts NKp30 through the complementarity-determining region (CDR)-like loops of its
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7 IgV domain in an antibody-like interaction [9].
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9 B7-H6 is not expressed by normal human tissues, but is selectively expressed on stressed
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11 cells, including a wide variety of tumors, such as melanomas, T and B lymphomas,
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13 neuroblastomas, and carcinomas [7,10–12]. The interaction of B7-H6 on tumor cells with
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15 NKp30 on NK cells results in interferon- γ production and tumor cell killing. Thus, B7-H6
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17 functions as a tumor-induced self-molecule that alerts innate immunity to cellular transformation
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19 [7,10,12]. Furthermore, treatment of tumor cells with chemotherapeutic agents or radiation was
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21 found to up-regulate B7-H6 expression and enhance tumor sensitivity to NK cell cytotoxicity [11].
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23 These and related findings suggest that the NKp30–B7-H6 axis is a promising target for cancer
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25 immunotherapy [13–16]. In addition, B7-H6 can be expressed in inflammatory conditions, such
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27 as epithelial cells during Sjögren's syndrome [17] or inflammatory monocytes during sepsis [18].
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33 To investigate NK cell activation via NKp30, as well as to target tumors expressing B7-
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35 H6, we previously isolated a set of four mouse monoclonal antibodies (mAbs) specific for
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37 human B7-H6 [18]. Although all four antibodies blocked activation of NK cells by B7-H6, they
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39 appear to do so using different inhibitory mechanisms. Two antibodies directly blocked the
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41 binding of B7-H6 to NKp30 [18]. These antibodies likely recognize sites on the ligand that at
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43 least partially overlap the receptor-binding site, which is a commonly observed mechanism for
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45 antibodies that inhibit signaling through cell surface receptors [19]. By contrast, two other anti-
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47 B7-H6 antibodies did not block the interaction of B7-H6 with NKp30, implying that they employ
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49 a different strategy to prevent NKp30-mediated cell activation [18]. To obtain insights into this
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51 inhibitory mechanism, we determined the crystal structure of the antigen-binding fragment (Fab)
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53 of one of these antibodies (designated 17B1.3) in complex with B7-H6. The structure, in
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4 conjunction with mutational analysis using cell-based assays, revealed the relation between the
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6 binding sites on B7-H6 for NKp30 and 17B1.3, and suggested a novel mechanism to explain the
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8 inhibitory activity of this antibody.
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10 11 12 13 14 **Experimental Procedures**

15 16 17 **Protein expression, crystallization and data collection**

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20 Recombinant Fab 17B1.3 was produced by *in vitro* folding from inclusion bodies
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22 produced in *Escherichia coli*, as described previously [20]. The extracellular portion of B7-H6,
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24 comprising IgV and IgC domains, was expressed by secretion from baculovirus-infected Sf9
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26 insect cells [9]. The Fab 17B1.3–B7-H6 complex crystallized in 9% (w/v) polyethylene glycol
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28 (PEG) 8000, 100 mM Tris-HCl (pH 8.0), and 200 mM lithium sulfate [20]. For data collection,
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30 crystals were transferred to a cryoprotectant solution of mother liquor containing 30% (v/v)
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32 glycerol before flash-cooling in a nitrogen steam. X-ray diffraction data for the Fab 17B1.3–B7-
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34 H6 complex were collected at beamline X29 of the Brookhaven National Synchrotron Light
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36 Source (BNSLS) using a CCD ADSC detector. Diffraction data were indexed, integrated, and
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38 scaled with the program HKL2000 [21]. Data collection statistics are presented in **Table 1**.
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46 47 **Structure determination and refinement**

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49 The structure of the Fab 17B1.3–B7-H6 complex was solved by molecular replacement
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51 with Phaser [22] using the structures of B7-H6 (Protein Data Bank accession code 3PV7) [9],
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53 and of the variable ($V_L V_H$) and constant ($C_L C_H1$) domains of anti-lysozyme antibody HyHEL-63
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55 (1DQJ) [23]. There are two Fab 17B1.3–B7-H6 complexes per asymmetric unit. All refinements
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57 were carried out using the program CNS1.1 [24], including iterative cycles of simulated
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annealing, positional refinement, and *B* factor refinement, interspersed with model rebuilding into σ_A -weighted $F_o - F_c$ and $2F_o - F_c$ electron density maps using XtalView [25]. Refinement statistics are summarized in **Table 1**. Stereochemical parameters were evaluated with PROCHECK [26]. Hydrogen bonds and van der Waals contacts were assigned with the program CONTACT from the CCP4 suite [27] using cut-off distances of 3.4 Å and 4.2, respectively. Coordinates and structure factors for the Fab 17B1.3–B7-H6 complex have been deposited in the Protein Data Bank under accession code **4ZSO**.

BW cell lines

BW cells (a gift of Dr. T. Kitamura, University of Tokyo, Japan) expressing B7-H6 wild-type (wt), B7-H6 mut1 or B7-H6 mut2 were prepared by retrovirus gene transfer. B7-H6 ORF cDNA was obtained by RT-PCR from human myelomonocytic cell line MM6 [28] using the following primers: H6 ORF up 5' CAGTTGCCGAAGGGATCTG and H6 ORF dw 5' CCAGGTCCCCTCTTATTTGC. cDNAs encoding B7-H6 mut 1 (Asp84[GAT]→Arg[CGT], Arg99[AGG]→Glu[GAG], Lys101[AAG]→Glu[GAG], Ser102[AGT]→Arg[CGT]) and B7-H6 mut 2 (Asn41[AAT]→Arg[CGT], Trp67[TGG]→Arg[CGG], Lys74[AAA]→Glu[GAA], Glu115[GAG]→Arg[AGG]) were synthesized chemically (Life Technologies). cDNAs for B7-H6 wt, B7-H6 mut1 and B7-H6 mut2 were subcloned in pMXs-IG (IRES-GFP) retrovirus vector (kindly provided by Dr. T. Kitamura, University of Tokyo, Japan). The three constructs were transiently transfected into Plat-E packaging cell line using JetPEI (Polyplus-transfection) in order to generate viral particles that were used to infect BW cells. B7-H6 wt-positive cells were selected and enriched by staining with soluble NKp30-Fc receptor followed by microbeads coated with mouse anti-human IgG (Miltenyi Biotec), while BW-B7-H6-mut1 and -mut2

positive cells were sorted according to GFP expression. In order to obtain cell clones, B7-H6⁺ cell populations were subcloned by limiting dilution.

DOMSP30 assay

DOMSP30 and DOMSP46 reporter cell lines have been described previously [7]. Engagement of NKp30-CD3 ζ and NKp46-CD3 ζ chimeric proteins at the cell surface triggers IL-2 secretion. DOMSP30, DOMSP46, or DO.11.10 (30,000 cells/well in 96-well plates) were incubated with parental and transfected BW5147 cells (27,000 cells/well in 96-well plates). After 24 h, cell supernatants were assayed for the presence of mouse IL-2 in a standard CTLL-2 survival assay using Cell Titer-Glo Luminescent Cell Viability Assay (Promega).

Flow cytometry

For mAb stainings, 100,000 cells were collected and stained in PBS 2 mM EDTA 10% SVF for 30 min at 4 °C with 10 μ g/ml of 10E2.9-A647 [18], 17B1.3-A647 [18] and mIgG1-A627. For Fc stainings, 5 μ g/ml or otherwise indicated amount of NKp30-Fc or NKp44-Fc control (R&D Systems) were mixed to 2.5 μ g/ml of APC-conjugated anti-human Ig (Jackson ImmunoResearch Laboratories) for 45 min at 4 °C in PBS 2 mM EDTA 10% BSA (ID Bio). 50 μ l per sample was used to stain 100,000 cells for 30 min at room temperature. Fluorescent stainings were analyzed on a FACSCanto (BD) using the FlowJo software (Tree Star).

Results and Discussion

Overview of the Fab 17B1.3-B7-H6 complex

Antibody molecules are composed of light (L) and heavy (H) chains, each having variable (V) and constant (C) regions. We expressed the Fab of 17B1.3, comprising the V_LC_L and V_HC_H1 domains of this inhibitory mAb, by *in vitro* folding from inclusion bodies produced in *Escherichia coli* [20]. The extracellular portion of B7-H6 was expressed by secretion from baculovirus-infected Sf9 insect cells [9]. To investigate the mechanism by which 17B1.3 blocks NKp30-mediated NK cell activation [18], we determined the structure of the Fab 17B1.3–B7-H6 complex to 2.5 Å resolution (**Table 1** and **Fig. 1a**). The root mean square deviation (r.m.s.d.) in α-carbon positions for the two complex molecules in the asymmetric unit of the crystal was 1.42 Å. Based on this close similarity, the following description of Fab 17B1.3–B7-H6 interactions applies to both complexes in the asymmetric unit.

The 17B1.3 mAb uses V_LCDR1, V_LCDR2 and V_HCDR1–3 to bind B7-H6, which is composed of a membrane distal V-like domain and a membrane proximal C-like domain (**Fig. 1a**). The contacts are mainly with the CC', C''D, and EF loops of the V-like domain. Although the epitope recognized by 17B1.3 is located near the junction between the V-like and C-like domains of B7-H6 (**Fig. 1a**), the antibody contacts the V-like domain almost exclusively. The only exception resides in several interactions between the side chains of V_H Gln1 of 17B1.3 mAb and Met203 of the B7-H6 C-like domain. However, since glutamine is usually processed to pyroglutamate in the endoplasmic reticulum, these contacts would not occur *in vivo*. The 17B1.3 epitope is completely distinct from binding site for NKp30, which comprises strand C and loops BC, C'C'', and FG of the B7-H6 V-like domain [9]. Indeed, there are no steric clashes between NKp30 and 17B1.3 in a ternary 17B1.3–B7-H6–NKp30 complex constructed by superposing the 17B1.3–B7-H6 and NKp30–B7-H6 structures through their common B7-H6 component (**Fig. 1b**). Therefore, 17B1.3 would not block the interaction of NKp30 with B7-H6, in agreement with

previous binding experiments [18]. The conformation of B7-H6 bound to 17B1.3 is very similar to its conformation in complex with NKp30 (r.m.s.d. in α -carbon positions of 1.56 Å). This similarity extends to the nearly identical hinge angle between the V-like and C-like domains of B7-H6 in the two structures, suggesting limited conformational flexibility in the interdomain region.

The Fab 17B1.3–B7-H6 interface

The 17B1.3–B7-H6 complex buries a total solvent-accessible surface area of 1832 Å², with 15 antibody residues (5 V_L and 10 V_H) contacting 15 B7-H6 residues (**Table 2**). The interaction is dominated by V_H, which contributes 675 Å² to the buried surface on B7-H6 compared to 235 Å² by V_L. Overall, V_H makes 71 van der Waals contacts and 4 hydrogen bonds with B7-H6, compared with 24 van der Waals contacts and 4 hydrogen bonds by V_L. Only three B7-H6 residues, Phe72 (CC' loop), Lys74 (C' strand) and Glu75 (C' strand), are involved in interactions with V_L. Thus, V_LCDR1 Tyr31 contacts primarily the main chains of B7-H6 Lys74 and Glu75, and forms a hydrogen bond with Glu75 (V_LCDR1 Tyr31 O_H–O Glu75 B7-H6) (**Table 2** and **Fig. 2**). The side chains of B7-H6 Phe72 and Lys74 contact V_LCDR2 Tyr48, Leu49, Ser51 and Ser52, with B7-H6 Lys74 making three hydrogen bonds via its N ζ atom with V_LCDR2 Leu49, Ser51 and Asn52. The 17B1.3–B7-H6 complex is very unusual among antibody–antigen complexes in that V_LCDR3 does not engage the ligand [29]. In contrast, all three V_HCDR loops contact B7-H6 (**Table 2**). The side chains of V_HCDR1 Thr28 and Asn33 interact with B7-H6 Asn41 (AB loop) and Pro91 (C'D loop), respectively, while those of V_HCDR1 Ser31 and Tyr32 contact B7-H6 Gly111, Gln113 and Glu115 (EF loop) (**Fig. 2**). These interactions are reinforced by three hydrogen bonds: V_HCDR1 Ser31 O_Y–O Gly111 B7-H6,

V_HCDR1 Ser31 O_γ-Nε2 Gly113 B7-H6, and V_HCDR1 Tyr32 O_η-Oε2 Glu115 B7-H6. Only one V_HCDR2 residue, Tyr52, contacts the ligand (**Table 2**). By contrast, V_HCDR3 interacts extensively with B7-H6 loops CC', C'D and EF (**Fig. 2**). In particular, V_HCDR3 Leu105 and Arg106 pack tightly against B7-H6 Phe89, with formation of a hydrogen bond linking the side-chain N_η1 atom of V_HCDR3 Arg106 to the main-chain O atom of B7-H6 Phe89.

Mutational analysis of NKp30 activation by B7-H6

Since the inhibitory activity of antibody 17B1.3 cannot be explained by disruption of the NKp30-B7-H6 interaction (**Fig. 1b**), we investigated another possible mechanism involving B7-H6 dimerization. Crystal structures have been reported for human CTLA-4 in complex with B7-1 and B7-2, which, like B7-H6, belong to the B7 family [30,31]. In both cases, an alternating network of bivalent CTLA-4 and B7-1 or B7-2 homodimers was observed in the crystal, whereby bivalent CTLA-4 homodimers bridge bivalent B7-1 or B7-2 homodimers. It was proposed that similar, if not identical, periodic arrays form at the interface between T cells and antigen-presenting cells, and that these arrays promote signaling by stabilizing CTLA-4-B7-1 and CTLA-4-B7-2 complexes within the immunological synapse [30,31]. Indeed, mutagenesis of the putative B7-1-B7-1 dimer interface supports such a signaling mechanism [32,33], which could also apply to other B7 family members, including B7-H6. Although B7-H6 is monomeric in the NKp30-B7-H6 crystal [9], we hypothesized that B7-H6 might nevertheless dimerize on the cell surface, like B7-1 or B7-2.

B7-1 and B7-2 exhibit roughly similar dimeric arrangements in their complexes with CTLA-4, with the dimer interface formed by the back sheet of the IgV domains [30,31]. However, the B7-1 and B7-2 dimers have somewhat different orientations. Accordingly, we used

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4 both B7-1 and B7-2 to model the putative B7-H6 dimer. If we construct the B7-H6 dimer by
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6 superposing B7-H6 in the 17B1.3–B7-H6 structure onto the B7-1 dimer through the IgV
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8 domains, it can be seen that 17B1.3 would not interfere with potential B7-H6 dimer formation,
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10 since the antibody binding site does not overlap the dimerization site (**Fig. 3a**). Therefore, the
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12 inhibitory activity of 17B1.3 cannot be explained by prevention of potential B7-H6 dimerization,
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14 if B7-H6 dimerizes similarly to B7-1 [31]. If, however, B7-H6 dimerizes in the manner of B7-2
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16 [30], 17B1.3 would block potential dimer formation, as evident from superposing B7-H6 in the
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18 17B1.3–B7-H6 complex onto the B7-2 dimer (**Fig. 3b**). Antibody binding to one monomer of the
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20 modeled B7-H6 dimer would result in major steric clashes with the other monomer. This could
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22 account for the inhibitory capacity of 17B1.3, if in fact B7-H6 dimerization is required for
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24 NKp30-mediated NK cell activation. To address this possibility, we used cellular assays to
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26 evaluate the functional effect of structure-based mutations designed to disrupt B7-H6
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28 dimerization. These experiments were carried out with full-length B7-H6 that included both
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30 transmembrane and cytoplasmic domains.

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33 Guided by our B7-2-based model of the B7-H6 dimer (**Fig. 3b**), we simultaneously
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35 mutated four residues in the predicted interface between IgV domains: Asp84Arg, Arg99Glu,
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37 Lys101Glu, and Ser102Arg (mut1). We reasoned that these drastic mutations, involving charge
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39 reversals and/or bulky side chains, should hinder, if not abolish, formation of potential B7-H6
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41 dimers at the cell surface. To validate the crystal structure, and as a control, we also mutated four
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43 B7-H6 residues in the interface with 17B1.3: Asn41Arg, Trp67Arg, Lys74Glu, and Glu115Arg
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45 (mut2).
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55 Both 17B1.3 mAb and NKp30-Fc bound to wild-type B7-H6 expressed on stably
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57 transfected BW cells, as did the anti-B7-H6 10E2.9 mAb [18] (**Fig. 4**). However, 17B1.3 mAb
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showed no binding to B7-H6 mut2 (**Fig. 4**), in agreement with the structure of the 17B1.3–B7-H6 complex (**Fig. 1a**). Binding of soluble NKp30-Fc was unaffected, as expected from the NKp30–B7-H6 structure where none of the four B7-H6 mut2 mutations are in the interface with the receptor (**Fig. 1b**) [9]. Also unaffected was binding of antibody 10E2.9, indicating that 10E2.9 and 17B1.3 recognize different epitopes on B7-H6. The IL-2-producing DO11.10 mouse T cell hybridoma expressing a chimeric receptor formed by the NKp30 extracellular domain fused to CD3 ζ (DOMSP30 cells) was used as a reporter cell system to evaluate B7-H6 signaling through NKp30 [7]. DOMSP30 reporter cells produced comparable amounts of IL-2 whether in the presence of BW cells expressing wild-type B7-H6 or B7-H6 mut2, showing that mutations which abolish 17B1.3 binding do not diminish NKp30-mediated cell activation (**Fig. 5**).

We next examined B7-H6 mut1, which contains four mutations designed to disrupt formation of putative B7-H6 dimers on the cell surface. Both 17B1.3 and NKp30-Fc bound to B7-H6 mut1 expressed on BW cells, whereas antibody 10E2.9 [17] did not (**Fig. 4**). Hence, 10E2.9 likely binds at or near the proposed interface between IgV domains in our B7-2-based model of the B7-H6 dimer (**Fig. 3b**). However, BW cells expressing B7-H6 mut1 were even more effective in stimulating IL-2 production by DOMSP30 reporter cells than BW cells expressing wild-type B7-H6 (**Fig. 5**). This result does not support our hypothesis that B7-H6 dimerization is involved in NKp30-mediated signaling, at least through the predicted IgV–IgV interface based on the B7-2 dimer (**Fig. 3b**). To further exclude B7-H6 dimerization, we conducted sedimentation equilibrium of soluble B7-H6 at relatively high initial loading concentrations of 4.55 and 2.33 mg/ml (corresponding to 164 and 83.9 μ M) at 25 °C and at three rotor speeds (18,000, 22,000 and 26,000 rpm). Global analysis of the equilibrium data collected

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4 at 295 nm revealed a monomer at all concentrations, suggesting a lower limit for a monomer–
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6 dimer dissociation constant of about 2.5 mM (data not shown).
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9 The 17B1.3–B7-H6 structure and accompanying functional analysis rule out prevention
10 of receptor–ligand binding or disruption of ligand dimerization as inhibitory mechanisms for
11 17B1.3. Another possible explanation is that antibody 17B1.3, being a large protein (~150 kDa
12 for the intact IgG), acts by sterically interfering with close cell–cell contacts at the NK cell (or
13 DOMSP30)–target cell interface, thereby blocking immunological synapse formation and NK
14 cell activation [34]. Even though 17B1.3 mAb does not prevent B7-H6 from binding NKp30 in
15 solution [18] or in the crystal (**Fig. 1b**), the situation may differ when B7-H6 and NKp30 are
16 attached to membranes, which confine protein movements to two dimensions.
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28 To assess this possibility, we measured NKp30-dependent activation of DOMSP30
29 reporter cells by P815 cells expressing B7-H6 in the presence or absence of 17B1.3 mAb in its
30 intact IgG (~150 kDa) or Fab (~50 kDa) form. Whereas both the IgG and Fab inhibited
31 activation, the far bulkier IgG was ~3-fold more effective (**Fig. 6**). This difference may be
32 understood in terms of our model of the ternary Fab 17B1.3–B7-H6–NKp30 complex (**Fig. 1b**),
33 in which the Fab, but not the IgG, could conceivably be accommodated in the NK cell–target cell
34 interface without complete disruption of NKp30–B7-H6 interactions. By contrast, the much
35 larger IgG molecule would impair NK cell–target cell apposition more effectively, thereby
36 indirectly preventing NKp30 from engaging B7-H6. Of note, we observed a similar ~3-fold
37 difference in effectiveness between the IgG and Fab forms of another anti-B7-H6 mAb, 4E5.5
38 (**Fig. 6**). Like 17B1.3, 4E5.5 inhibits NKp30-mediated NK cell activation without directly
39 blocking the binding of B7-H6 to NKp30 [18]. Since P815 cells express Fc receptors (FcR), it is
40 also possible that cross-linking of FcR and B7-H6 by 17B1.3 IgG may interfere with NKp30
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4 activation. However, this appears unlikely because an IgG molecule bound to FcR on the P815
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6 cell would have its Fab arms projecting away from B7-H6 molecules on the same P815 cell, such
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8 that the IgG molecule could not readily engage both FcR and B7-H6.
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10 11 12 13 14 **Conclusion**

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17 Antibodies have been shown to block signaling through cell surface receptors using a
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19 variety of mechanisms [19]. These include binding to the ligand-binding site of the receptor and
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21 preventing interaction with the ligand, binding to the receptor-binding site of the ligand and
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23 preventing interaction with the receptor, preventing dimerization of the receptor or ligand by
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25 binding to the dimerization site, and stabilizing an inactive conformation of the receptor. Our
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27 present findings add another likely category to this list: interference with cell–cell contacts in the
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29 case of cell surface receptors whose ligands are also cell surface proteins. This category may
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31 include a number of therapeutic antibodies already in the clinic or under development that block
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33 signal transmission by preventing receptor–ligand interactions at cell–cell interfaces.
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6 Institutes of Health.
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9 **Conflict of Interest:** E.V. and A.M. are co-founders of and shareholders in Innate-
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Figure Legends

Fig. 1. Structure of the Fab 17B1.3–B7-H6 complex and comparison with the NKp30–B7-H6 complex. (a) Ribbon diagram of the Fab 17B1.3–B7-H6 complex. V_LC_L, yellow; V_HC_H1, cyan; B7-H6, green. V_LCDR loops are labeled L1–L3; V_HCDR loops are labeled H1–H3. (b) Comparison of the Fab 17B1.3–B7-H6 and NKp30–B7-H6 complexes (Protein Data Bank accession code **3PV6**) [9]. The complexes were superposed through B7-H6. NKp30, magenta; NKp30-bound B7-H6, blue. Antibody 17B1.3 and NKp30 bind to spatially distinct, non-overlapping sites on the B7-H6 IgV domain. The NKp30–B7-H6 complex is oriented as if B7-H6 stands on the target cell surface at the bottom and NKp30 engages B7-H6 from an opposing NK cell above.

Fig. 2. Stereo view of the Fab 17B1.3–B7-H6 binding interface. The side chains of contacting residues are drawn in stick representation, with carbon atoms in green (B7-H6), yellow (V_L) or cyan (V_H), nitrogen atoms in blue, and oxygen atoms in red. Hydrogen bonds are drawn as dotted black lines.

Fig. 3. Models of putative B7-H6 dimers. (a) B7-1-based model of B7-H6 dimer with bound Fab 17B1.3 molecules (side view). The hypothetical B7-H6 dimer was constructed by superposing B7-H6 in the 17B1.3–B7-H6 structure onto the B7-1 dimer (**1I85**) [31] through the IgV domains. The C-termini of the B7-H6 monomers (cyan and yellow) are labeled. For clarity, only the IgV domains of the B7.1 dimer (red) are shown. 17B1.3 would not sterically interfere with formation

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4 of a B7-1-based B7-H6 dimer. (b) B7-2-based model of B7-H6 dimer with bound Fab 17B1.3
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6 molecules (side view). The hypothetical B7-H6 dimer was constructed by superposing B7-H6 in
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8 the 17B1.3–B7-H6 structure onto the B7-2 dimer (**1I8L**) [30] through the IgV domains. Only the
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10 IgV domains of the B7-2 dimer (red) are shown. Binding of 17B1.3 to one monomer (cyan) of
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12 the modeled B7-H6 dimer would result in major steric clashes with the other monomer (yellow).
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19 **Fig. 4.** Binding of 17B1.3, 10E2.9 mAbs and NKp30-Fc to BW cells expressing wild-type B7-
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21 H6 and mutants 1 and 2. BW cells were stained with 10E2.9 (upper panels), or 17B1.3 (middle
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23 panels) anti-B7-H6 mAbs or with NKp30-Fc (bottom panels) and analyzed by flow cytometry.
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25 mIgG1 and NKp44-Fc was respectively used as isotype control and Fc control. FACS profiles
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27 are representative of at least three experiments.
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33 **Fig. 5.** Activation of DOMSP30 reporter cells by BW cells expressing wild-type B7-H6 and
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35 mutants 1 and 2. DOMSP30, DOMSP46 reporter cells and parental DO11.10 cells were
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37 cocultured with the indicated cells for 24 hours. Cell activation was determined by evaluating IL-
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39 2 production in the coculture supernatant in a standard CTLL-2 survival assay. Data show mean
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41 plus standard deviation of one representative out of three independent experiments.
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48 **Fig. 6.** Activation of DOMSP30 cells by P815 cells expressing B7-H6 or B7-H1 without (Ø) or
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50 with 10 µg/ml of indicated mAb or Fab. Fab fragments were prepared by papain digestion of IgG
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52 17B1.3 and 4E5.5. Like 17B1.3, 4E5.5 is an inhibitory anti-B7-H6 mAb that does not block
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54 binding of B7-H6 to NKp30 [18]. Cell activation was determined by evaluating IL-2 production
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4 in the coculture supernatant in a standard CTLL-2 survival assay. Data show mean plus standard
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7 error of the mean of one representative out of two independent experiments.
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Table 1. Data collection and structure refinement statistics.

	Fab 17B1.3–B7-H6
<i>Data collection</i>	
Wavelength (Å)	1.502
Space group	$P2_12_12_1$
Unit cells (Å)	$a=89.6, b=138.0, c=171.4$
Resolution (Å) ^a	50–2.50 (2.59–2.50)
Observations	1,071,629
Unique reflections	74,515
Completeness (%) ^a	100 (99.8)
Mean $I/\sigma(I)$ ^a	22.2 (3.4)
R_{sym} (%) ^{a,b}	9.7 (55.9)
<i>Refinement</i>	
Resolution range (Å)	50–2.50
R_{work} (%) ^c	22.6
R_{free} (%) ^c	25.7
No. of protein atoms	9,890
No. of carbohydrate atoms	118
No. of water molecules	252
Average B -factors (Å ²)	
Protein	43.4
Carbohydrate	80.0
Water	44.2
r.m.s.d. from ideality	
Bond lengths (Å)	0.007
Bond angles (°)	1.39
Ramachandran statistics (%)	
Favorable	88.5
Allowed	10.8
Generous	0.4
Forbidden	0.2

^aValues in parentheses are statistics for the highest resolution shell.

^b $R_{\text{sym}} = \sum |I_j - \langle I \rangle| / \sum I_j$, where I_j is the intensity of an individual reflection and $\langle I \rangle$ is the average intensity of that reflection.

^c $R_{\text{work}} = \sum ||F_o| - |F_c|| / \sum |F_o|$, where F_c is the calculated structure factor. R_{free} is as for R_{work} but calculated for a randomly selected 5.0% of reflections not included in the refinement.

Table 2

Table 2. Interactions between Fab 17B1.3 and B7-H6.

Fab 17B1.3	B7-H6		
	Hydrogen bonds	Distance (Å)	Van der Waals contacts
V _L Tyr31	V _L Tyr31 O η –Glu75 O	2.87	Lys74, Glu75, Val76
V _L Tyr48			Phe72
V _L Leu49	V _L Leu49 O–Lys74 N ζ	2.83	Lys74
V _L Ser51	V _L Ser51 O γ –Lys74 N ζ	2.90	Lys74
V _L Asn52	V _L Asn52 O δ 1–Lys74 N ζ	2.90	Phe72, Lys74
V _H Thr28			Asn41
V _H Ser31	V _H Ser31 O γ –Gly111 O	3.29	Asn41, Gly111, Gln113
	V _H Ser31 O γ –Gln113 N ϵ 2	3.29	
V _H Tyr32	V _H Tyr32 O η –Glu115 O ϵ 2	2.83	Gln113, Glu115
V _H Asn33			Pro91
V _H Tyr52			Arg90, Pro91, Gly92, Pro110
V _H Leu101			Gln113, Glu115, Glu116
V _H Gly102			Trp67, Glu115
V _H Ala103			Trp67, Phe72
V _H Leu105			Phe89, Pro91
V _H Arg106	V _H Arg106 N η 1–Phe89 O	3.14	Phe89

Figure 1

Fig.1

Figure 2

Fig. 2

Figure 3

Fig. 3

(a)

(b)

Figure 4

Fig. 4

Figure 5

Fig. 5

Figure 6

Fig. 6