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CD229 (Ly9) Lymphocyte Cell Surface Receptor Interacts Homophilically through Its N-Terminal Domain and Relocalizes to the Immunological Synapse[1]

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CD229 is a member of the CD150 family of the Ig superfamily expressed on T and B cells. Receptors of this family regulate cytokine production and cytotoxicity of lymphocytes and NK cells. The cytoplasmic tail of CD229 binds to SAP, a protein that is defective in X-linked lymphoproliferative syndrome. To identify the CD229 ligand, we generated a soluble Ig fusion protein containing the two N-terminal extracellular domains of human CD229 (CD229-Ig). CD229-Ig bound to CD229-transfected cells, whereas no binding was detected on cells expressing other CD150 family receptors, showing that CD229 binds homophilically. Both human and mouse CD229 interacted with itself. Domain deletion mutants showed that the N-terminal Ig-domain mediates homophilic adhesion. CD229-CD229 binding was severely compromised when the charged amino acids E27 and E29 on the predicted B-C loop and R89 on the F-G loop of the N-terminal domain were mutated to alanine. In contrast, one mutation, *R44A,* enhanced the homophilic interaction. Confocal microscopy image analysis revealed relocalization of CD229 to the contact area of T and B cells during Ag-dependent immune synapse formation. Thus, CD229 is its own ligand and participates in the immunological synapse. *The Journal of Immunology,* **2005, 174: 7033–7042.**

Interactions between cell surface molecules, expressed by various hemopoietic cell types, regulate immune responses. A considerable number of these cell surface molecules belong to the Ig superfamily (IgSF),[4] which has evolved as a large but diverse group of genes encoding proteins involved in bimolecular recognition (1). The extracellular domains of IgSF proteins are involved in multiple specific cell-cell interactions, both homophilic and heterophilic. These interactions trigger intracellular signals that determine numerous cellular functions including activation, differentiation, proliferation, and acquisition of effector function and/or tolerance induction (2). Even though most of these cell surface receptors lack intrinsic enzymatic activity, they recruit intracellular kinases, phosphatases, and adapter proteins in response to engagement by their ligands or counterreceptors (3).

CD229, previously known as Ly9, is expressed in T and B lymphocytes, and thymocytes (4–7). It belongs to the CD150 family, a group of structurally related leukocyte cell surface receptors of the IgSF. Members of this family of immunoreceptors are involved in the functional regulation of several immune cell types, including helper and cytotoxic T lymphocytes, NK cells, and macrophages (8–11). The CD150 family includes a number of cell surface receptors, CD229, CD48, CD84, CD150 (SLAM), CD244 (2B4), NTB-A (Ly108), CS1 (novel Ly9; CRACC), CD84-H1, and BLAME (BCM-like) (9). These molecules are type I transmembrane glycoproteins that contain a cytoplasmic tail, with the exception of CD48, which has a GPI anchor to the membrane. The extracellular ectodomain of this group of proteins comprises N-terminal V-set Ig domains lacking the canonical disulfide bond and C-terminal C2-set Ig domains, which are characterized by similar patterns of conserved cysteines. Although the extracellular domains of CD2 and CD58 share some of these characteristics (12, 13), the homology between these two leukocyte receptors and members of the CD150 family is low (<15% identity) (14). Unlike other members of the CD150 family, CD229 has four rather than two extracellular Ig domains. Domains 1 and 3 are similar, as are domains 2 and 4, suggesting that CD229 arose from a progenitor with two Ig domains (5). The cytoplasmic domain of CD229, similar to several members of the CD150 family, contains two copies of the tyrosine-based motif (T-I/V-Y-x-x-V/I) (6). This unique motif is a docking site for SH2-binding phosphatases and the adapter molecules SAP and EAT-2 (15, 16). It has recently been shown that SAP is required for CD229 tyrosine phosphorylation in T lymphocytes (17). The *SH2D1A* gene, which encodes SAP, is deleted or mutated in patients with X-linked lymphoproliferative disease (18, 19), an unusual immunodeficiency characterized by uncontrolled polyclonal expansion of both T and B cells after an EBV infection, resulting in fatal mononucleosis. Some other clinical manifestations of this disease include B cell lymphoma and dysgammaglobulinemia (20). The lethal immunodeficiency observed in X-linked lymphoproliferative disease patients points to a critical role of the CD150 family of SAP-binding cell surface receptors in the regulation of the immune system (9). Recently,

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[4] Abbreviations used in this paper: IgSF, Ig superfamily; wt, wild type; CMAC, chloromethyl derivative of aminocoumarin; SEE, staphylococcal enterotoxin E.

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CD229 was shown to associate with the μ-chain of the AP-2 adaptor complex, which links transmembrane molecules with clathrin-coated pits (21). TCR and CD229 coligation enhances CD229 endocytosis, suggesting that Ag receptor signaling regulates the availability of CD229 at the plasma membrane. CD229 is the only member of the CD150 family that binds to the AP-2 complex.

Through their extracellular domain, the CD150 immunoreceptors interact with specific ligands. One common feature of this subset of molecules is that they interact with members of the same family; CD150, CD84, CS1, and NTB-A all participate in homophilic interactions, whereas CD244 (2B4) is a receptor for CD48 (22–26). The counterreceptor of CD229 is currently unknown.

During Ag recognition, T cells undergo substantial membrane and cytoskeletal rearrangements that lead to the formation of the immunological synapse (27, 28). The TCR, accessory cell surface molecules, and intracellular signaling molecules that are incorporated into and around the contact zone between T cells and APCs all contribute to the modulation of the functional state of the synapse. The immunological synapse appears to be essential to establish the persistent signaling necessary for full activation and is implicated in the control of the quality and extent of biological T cell responses. Recently, it has been reported that CD244 (2B4), which is a member of the CD150 family, redistributes to the central zone of the cytotoxic NK immune synapse (29). However, the behavior of CD229 and other members of the family during immunological synapse formation remains to be elucidated.

Here we show that CD229 interacts homophilically through its N-terminal V-like domain and identify the amino acid residues essential for binding. We also show that CD229 localizes to the contact site between T cells and Ag-presenting B cells during Ag-dependent immune synapse formation.

Materials and Methods

Cells and reagents

COS-7 cells, the lymphoblastoid B cell line Raji, and the murine myeloma cell line NS-1 were obtained from the American Type Culture Collection. The Armenian hamster kidney cell line was obtained in our laboratory by transfecting a primary culture of kidney cells with SV40. The Jurkat-derived human cell line Vβ8$^+$ J77cl20 was kindly provided by A. Alcover and has been described previously (30). Cell lines were cultured in complete RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin, and L-glutamine (Life Technologies). The following mAbs were produced in our laboratory: anti-human CD84 (clone CD84.1.21) (23), anti-human CD229 (clones HLy9.1.84, HLy9.1.38, HLy9.1.25, HLy9.1.77) (6) and anti-human CD150 (clone SLAM 4) (31). The anti-CD244 mAb (clone 69-IgG1) was generated by fusing myeloma cell line NS_1 with the spleen cells of a BALB/c mouse immunized three times with the human CD244-Ig fusion protein (decribed bellow). The mAbs anti-murine CD229 (clone Ly9ab3) and anti-murine CD84 (clone mCD84.7) were generated by fusing NS_1 with spleen cells obtained from Armenian hamsters that were immunized three times with the Armenian hamster kidney cell line transiently transfected with murine full-length Ly9 cDNA or the Ig fusion protein of murine CD84 (mCD84-Ig), respectively. Hybridomas were subcloned at least three times. mAbs were purified using the Affi-Gel Protein A MAPS II kit (Bio-Rad) from concentrated supernatants obtained from the culture of hybridomas in INTEGRA CL 1000 flasks (Integra Biosciences).

Anti-human CD48 mAb (clone 156.4H9) and LFA-1 (clone 68-5A5) were a gift from R. Vilella (Hospital Clínic, Barcelona, Spain), and NTB-A mAb (clone MA127) was a gift from A. Moretta (32). The following mAbs were obtained from BD Pharmingen: anti-human CD3-FITC; CD45-FITC; CD2-PE; CD58-PE; biotin anti-mouse Igκ; and TNP. Biotin-conjugated anti-human IgG, streptavidin-Cy3 and anti-mouse-Cy3 were purchased from Jackson ImmunoResearch Laboratories and streptavidin-PE was from Southern Biotechnology Associates. Anti-mouse IgG labeled with HRP was obtained from Sigma-Aldrich.

Chimeric CD229 molecules and splice variants

Two human/mouse CD229 cDNAs chimeric molecules were produced. Chimeric molecule hDI/II mDIII/IIV was made by joining the cDNA that encodes domains I and II of human CD229 with domains III and IV of murine CD229, whereas the chimeric molecule hDI/DIII/DIV mDII was constructed using domains I, III, and IV of human CD229 and domain II of murine CD229. The chimera hDI/II mDIII/IV was constructed with two fragments generated by PCR. The first PCR product was generated using human CD229 cDNA as template and the HLy9 wild-type (wt) forward oligonucleotide (Table I) and overlapping reverse oligonucleotide 5′-TCT TCT GGA GGC TC/C TGG ATC TGT ACA GAA C-3′. The second PCR product was generated using the murine (C57BL/6) CD229 cDNA as template and overlapping forward oligonucleotide 5′-GTT CTG TAC AGA TCC AG/G AGC CTC CAG AAG A-3′ and reverse oligonucleotide 5′-TTC TCC AAA TCC TCA CCC CG-3′. Each PCR product was used for a recombinant PCR containing the primers HLy9 wt and reverse primer of murine CD229.

Chimeric molecule hDI/DIII/DIV mDII was constructed using three products generated by PCR. The first PCR product, corresponding to the first domain of human CD229, was amplified using human CD229 cDNA as template and HLy9 wt forward oligonucleotide (Table I) and overlapping reverse oligonucleotide 5′-GGC TTC TGG AGC TTC TCA TAG ACG AAA AGG GTG-3′. The second PCR product, corresponding to the second domain of murine CD229, was generated using murine (C57BL/6) CD229 cDNA as template and the overlapping forward oligonucleotide 5′-CAC CCT GTT CGT CTA TGA GAA GCT CCA GAA GCC-3′ and overlapping reverse oligonucleotide 5′-GTT CCT CCT CTG GAG GCT CCT GTG CAG AAT TGC CA-3′. The third PCR product was amplified using human CD229 cDNA as template and the overlapping forward oligonucleotide 5′-CTG GCA ATT CTG CAC AGG AGC CTC CAG AGG A-3′, and the HLy9 wt reverse oligonuclotide (Table I). PCR products were used for a recombinant PCR to anneal overlapping ends. The human/mouse chimeric molecules were cloned into pcDNA3.1/V5/His-TOPO expression vector (Invitrogen) fully sequenced and transfected in COS cells. As control, murine CD229 full-length cDNA was transfected in COS cells and tested with the anti-human CD229 mAbs to rule out possible cross-reactivity of these mAbs with murine CD229.

A CD229 isoform that lacks the first Ig domain (CD229 ΔDI) was isolated from the thymus by RT-PCR and subcloned in PCIneo expression vector. The reactivity of CD229 mAbs (HLy9.1.25, HLy9.1.38, HLy.1.77 and HLy9.1.84) with COS cells transfected with the chimeric molecules, and the splicing variant CD229 ΔDI cDNAs was analyzed using flow cytometry.

Construction of the fusion proteins

The human CD229-Ig (CD229-Ig), human CD244-Ig (CD244-Ig), murine CD84-Ig (mCD84-Ig), and murine CD229-Ig (mCD229-Ig) fusion proteins

Table I. *CD229 primers for site-directed mutagenesis*

Mutation	Sense Primer (5′–3′)	Antisense Primer (5′–3′)
HLy9wt	gctagcatgagtcagctgcaaatattctct	gcagcagctgcttttcctttcaggtgaa
D25A	ctcagtagccacagagattg	tcaatctctgtggctactga
E27A	gtatacacagcgattgagaac	gttctcaatcgctgtgtctac
E29A	cacagagattgcgaacgtcat	atgacgttcgcaatctctgtg
K37A	gattggtcccgcaaatgctct	agagcatttgcgggaccaatc
L40A	caaaaatgctgctggctttcgc	gcgaaagcagcagcatttttg
R44A	tcgcagctcccaaagaaaatg	catttttctttgggagctgcga
K46A	cgtcccgcagaaaatgtaacc	ggtttacatttctgcgggacg
E47A	cgtcccaaagcaaatgtaacc	ggttacatttgctttgggacg
R89A	gcccagataaaccaagcgaatt	aattcgcttggtttatctgggc

containing the CD33 leader peptide and the Fc region of human IgG1 were obtained by inserting the sequences corresponding to the first and second extracellular Ig domains into the mammalian expression vector signal pIg-Tail (R&D Systems). The oligonucleotides used were as follows: human CD229 forward oligonucleotide, 5′-AGG AAG ATC TAA AGG ACT CAG CCC CAA CAG TGG T-3′, and reverse oligonucleotide, 5′-AGG AAG ATC TAC TTA CCT GTT CTG GAG GCT CCT GGA TCT GTA C-3′; human CD244 forward oligonucleotide, 5′-AGG GGG ATC CAT GCC AGG GTT CAG CTG ACC A-3′, and reverse oligonucleotide, 5′-ACC AGG ATC CAC TTA CCT GTG AAT TCC TGA TGG GCA TTC TGA C- 3′; murine CD84 forward oligonucleotide, 5′-TCG AGG ATC CAA AAG ATG CAG ACC CGG TGG T-3′, and reverse oligonucleotide, 5′-TAA CGG ATC CAC TTA CCT GTA TG AAG CTT GGA GTG TCT G-3′; murine CD229 forward oligonucleotide, 5′-GTT CAG ATC TAA AGG AAA CAC CTC CAA CAG TGA T-3′, and reverse oligonucleotide, 5′-GTC AAG ATC TAC TTA CCT GTT CTG GAG GCT CCT GTG CAG AAT TGC CA-3′. Human CD84-Ig was obtained in our laboratory (23). The constructs corresponding the Ig fusion proteins were subcloned in the expression vector pCI-neo (Promega) and NS-1 stable transfectants were obtained by electroporation and selection with 1.2 mg/ml geneticin (G418) (Life Technologies). Eight million cells were electroporated (280 V, 950 μF) with 8 μg of linearized DNA using the Gene Pulser II Apparatus (Bio-Rad). The transfected cells were plated in flat-bottom 96-well tissue culture plates (Corning) by limiting dilution, and clones producing high amounts of fusion protein were cultured in INTEGRA CL 1000 flasks (Integra Biosciences). The supernatants containing the fusion proteins were purified as described for the mAbs.

Site-directed mutagenesis

Site-directed mutagenesis of single residues was performed using a set of sense and antisense primers containing the appropriate mutation (Table I). wt CD229 cDNA was used as template. The first PCR used either the sense primer 5′-AGT TCT GAA AAT AGA TCA TCA TGG-3′ plus an antisense primer containing the mismatched base or a sense primer that contained the mismatched base plus the antisense primer 5′-AGC TGC TTT TCC TTT CAG GTG-3′ to create two PCR products that overlapped within the region spanned by the mutagenized sense and antisense primers. The reactions were conducted as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 2 min, with a final extension period of 72°C for 6 min. Aliquots of 3 μl of a 1/25 dilution of each PCR product were used as templates for a recombinant PCR containing the primers of the full-length CD229. The conditions of the PCR were the same as before. PCR products were cloned into the pcDNA3.1/V5/His-TOPO expression vector (Invitrogen). After confirming mutated clones by automatic sequencing, each cDNA mutant was transfected into COS-7 cells.

COS transfection

One million COS-7 cells were transiently transfected with 3 μg of cDNA corresponding to human CD229 or the CD229 mutants, CD229 chimeras, CD229 splice variant (CD229 ΔDI), CD84, CD150, CD244, CD48, CD2, CD58, and NTB-A using 100 μl of Nucleofector Solution V according to the manufacturer's protocol (AMAXA Biosystems). The cells were analyzed 24 h after transfection either by flow cytometry or by immunocytochemistry.

Immunocytochemistry

Transiently transfected COS-7 cells were cultured on glass coverslips in 24-well tissue culture plates (Corning). After 48 h, cells were fixed with 4% paraformaldehyde at 4°C and then blocked with 3% BSA (Sigma-Aldrich). Cells were labeled with HLy9.1.25, HLy9.1.38, and HLy9.1.77 mAbs and CD229-Ig fusion protein for 1 h at room temperature. After a washing in PBS, samples were incubated with Cy3-conjugated anti-mouse IgG or biotin-conjugated anti-human IgG (Jackson ImmunoResearch Laboratories). Samples were washed twice in PBS and mounted in Fluoromount-G (Southern Biotechnology). Fluorescence images were obtained using a confocal spectral microscope (TCS SL; Leica).

Flow cytometry

Flow cytometry was conducted with transfected cells stained with anti-human mAbs, followed by biotinylated anti-mouse κ L chain, plus streptavidin-PE (BD Pharmingen). Ig fusion protein staining was performed using 2 μg of Ig fusion protein followed by incubation with biotin-conjugated anti-human IgG (Jackson ImmunoResearch Laboratories) and then streptavidin-PE. The stained cells were analyzed using a FACSCalibur (BD Biosciences Immunocytometry Systems) equipped with CellQuest software. Ten thousand cells were counted for each sample.

Molecular modeling of the CD229 N-terminal domain

We used the alignment mode of SWISS-MODEL, an automated modeling process based on a user-defined target-template alignment (33). We obtained a first model of 78 residues (corresponding to residues 13–90 of the N-terminal domain of CD229) from the alignment between our target and CD58 and a second model of 42 residues (corresponding to residues 63–104 of the N-terminal domain of CD229) from the alignment between our target and CD2. The final model presented here is 90 residues long and was constructed using the three-dimensional alignment CE method with the first and second models (34). Our final model shows the archetypical fold of V Ig variable domains characterized by nine β strands forming two antiparallel β sheets. The first β strand was not modeled.

Immunological synapse formation and immunofluorescence microscopy

To distinguish B (Raji) from T (Jurkat Vβ8$^+$ J77cl20) lymphocytes, Raji cells were loaded with the fluorescent cell tracker chloromethyl derivative of aminocoumarin (CMAC; 10 μM) or 5-chloromethylfluorescein diacetate (3 μM) (Molecular Probes). B cells were incubated for 20 min at 37°C with 2 μg/ml staphylococcal enterotoxin (SEE) (Toxin Technology). Jurkat cells were mixed with Raji cells (proportion, 1:1) and incubated for 15 min at 37°C. Cells were plated onto poly-L-lysine-coated slides (50 μg/ml; Sigma), incubated for 20 min at 37°C, and fixed in 4% formaldehyde. For immunofluorescence assays, samples were blocked with PBS containing 2% BSA, and Fc receptors were blocked with 15% of heat-inactivated rabbit serum (Life Technologies). Cells were labeled with mAbs followed by Cy3-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories). For visualization of immunological synapse formation, confocal images were acquired using a Leica TCS SL laser scanning confocal spectral microscope (Leica Microsystems Heidelberg) with argon and helium-neon lasers attached to a Leica DMIRE2 inverted microscope. All images were obtained using a ×63 oil immersion objective lens (NA 1.32) equipped with phase contrast optics. Due to the small size of the cells, electronic zoom was necessary for higher magnification and better image resolution. The images of CMAC-labeled Raji cells were obtained using a mercury lamp HBO 50W UV filter as light source after each acquisition of fluorescence confocal and phase contrast images. Image assembly and treatment were performed using the Leica Confocal Image Processing Software.

B cell line Raji was negatively selected for CD229 expression using three sequential pannings. Briefly, polystyrene bacteriological petri dishes (100 × 15 mm) were coated overnight at 4°C with of HLy9.1.84 mAb at 10 μg/ml in PBS. The dishes were washed twice with PBS and blocked with PBS containing 0.2% BSA (Sigma-Aldrich) for 1 h at room temperature. The dishes were washed once with PBS, and then 10×10^6 Raji cells were added and incubated for 40 min at 4°C. Nonadherent Raji cells were collected. This procedure was repeated three times. The collected Raji cells expressed almost undetectable CD229 levels (Raji CD229$^-$). These cells were used to perform immunological synapse formation studies. Contacts of Jurkat cells with Raji CD229$^-$ cells that expressed detectable levels of CD229 in the confocal images were excluded from the analysis.

Statistical analysis

The Sigma Stat statistical package (Jandel Scientific Software) was used in the analysis of the results. All results are reported as mean ± SEM. Statistical significance was set at $p < 0.05$. ANOVA and Student's t test with Bonferroni's correction were used.

Results

CD229 interacts homophilically

To identify the ligand of CD229, we assessed binding of a CD229-Ig fusion protein to COS-7 cells transiently transfected with CD2, CD58, or members of the CD150 family. Binding was detected only with CD229. CD2-, CD58-, CD84-, CD150-, CD48-, CD244-, and NTB-A transfected cells were all negative (Fig. 1). The specific binding of CD244-Ig to CD48 was used as a positive control (Fig. 1). CD229 homophilic binding was also observed in mice (Fig. 2*A*). Moreover, the CD229-CD229 homophilic interaction is not species restricted because human CD229 can bind to mouse CD229 and vice versa (Fig. 2*B*). This is in contrast to CD84 homophilic binding, which is species restricted, because human CD84 does not bind to murine CD84, although murine CD84 can bind to itself (Fig. 2*A*).

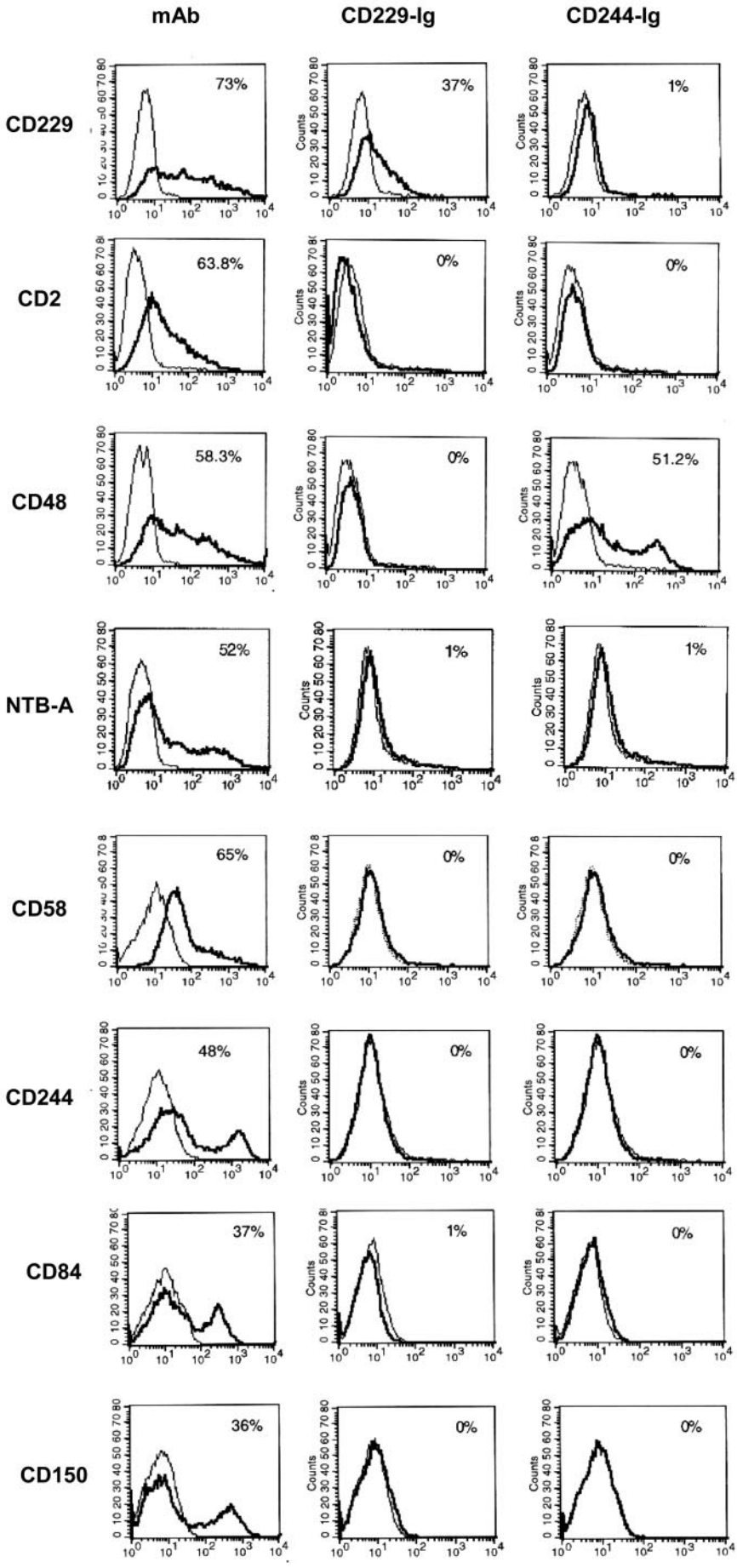

FIGURE 1. CD229 exhibits homotypic binding. COS-7 cells transfected with human CD229, CD2, CD48, NTB-A, CD58, CD244, CD84, and CD150 cDNA were labeled with CD229-Ig or CD244-Ig fusion proteins. Protein expression was detected using specific Abs (*left column*). Transfected cells were assayed for binding of CD229-Ig or CD244-Ig (*middle* and *right columns*). Immunostaining controls: isotype controls (*left column*) and Ctl-Ig (murine CD84-Ig; *middle* and *right columns*) are represented with dotted lines. The percentage of positive cells is indicated in each histogram.

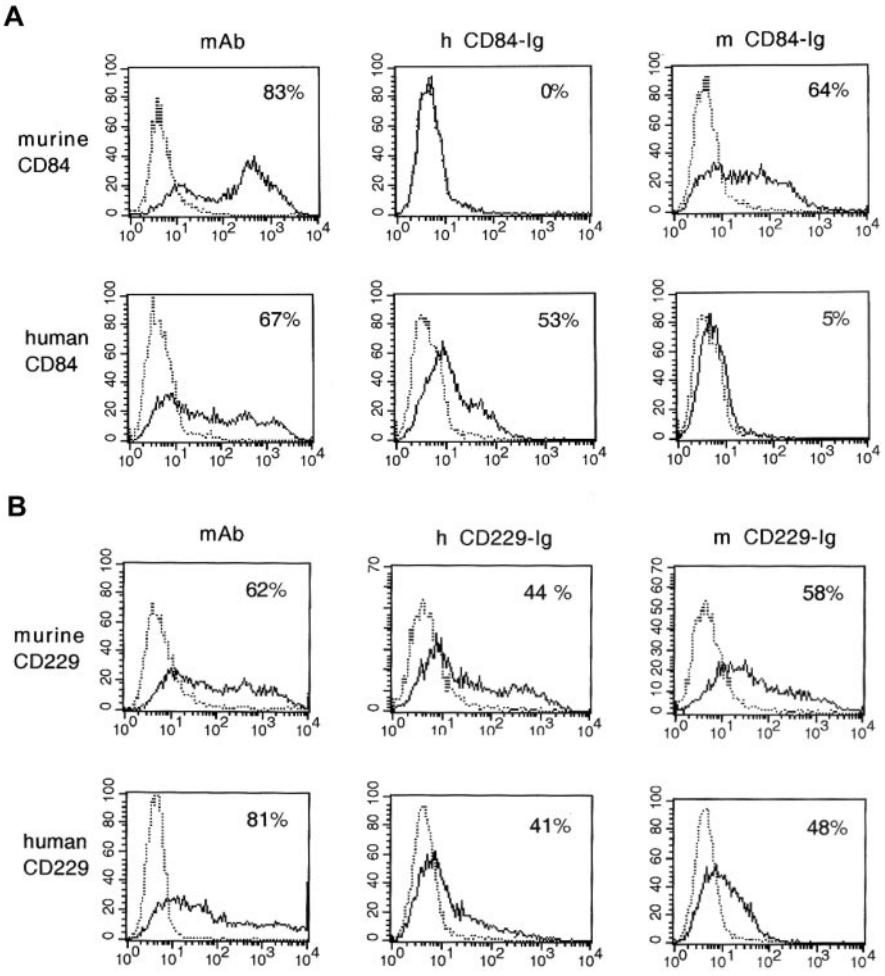

FIGURE 2. Human and murine CD229 receptors interact homophilically. *A,* COS-7 cells transfected with full-length murine CD84 (*upper panel*) or full-length human CD84 (*lower panel*) were labeled with specific Abs mCD84.7 and CD84.1.21 (*left column*). Human CD84-Ig (hCD84-Ig) or murine CD84-Ig (mCD84-Ig) binding was assayed (*middle* and *right columns*). *B,* COS-7 cells transfected with full-length murine CD229 (*upper panel*) or the full-length human CD229 (*lower panel*) were labeled with specific Abs Ly9ab3 and HLy9.1.25 (*left column*). Human CD229-Ig (hCD229-Ig) or murine CD229-Ig (mCD229-Ig) binding was assayed (*middle* and *right columns*). Immunostaining controls: isotype controls (*left column*) and Ctl-Ig (CD244-Ig; *middle* and *right columns*) are represented with dotted lines. The percentage of positive cells is indicated in each histogram.

Domain mapping of CD229 mAbs

To identify the Ig-like domains that are involved in the homophilic interaction of CD229, domain mapping was performed for the four mAbs previously produced by our laboratory (6). mAb binding to COS cells transfected with CD229 human/mouse chimeras indicated that HLy9.1.25, HLy9.1.38, and HLy9.1.77 were directed against Ig domain I, whereas HLy9.1.84 was reactive with Ig domain II (Table II). Moreover, only mAb HLy9.1.84 was able to bind with COS cells transfected with a CD229 isoform that lacks the first Ig domain (CD229 ΔDI), further demonstrating that this mAb recognizes the second Ig domain. Cross-blocking studies showed that the mAbs recognized three epitopes: HLy9.1.25 and HLy9.1.38 recognize epitope A; HLy9.1.77 recognize epitope B; and HLy9.1.84 recognize epitope C (Table III).

The N-terminal domain of CD229 mediates homophilic interaction

CD229-Ig binding was tested with CD229 ΔDI. This isoform was expressed on the cell surface of transfected COS cells as shown by positive staining with mAb HLy9.1.84. Deletion of the first V domain completely abolished binding of CD229-Ig, indicating that the direct and exclusive interaction between two opposing V domains is crucial for the homophilic CD229 interaction (Fig. 3).

Furthermore, monovalent Fab of mAb HLy9.1.77, which maps to the first Ig domains and reacts with epitope B, were able to partially block (60%) the binding of CD229-Fc to wt CD229-transfected cells. In contrast, Abs recognizing epitope A, located in domain I, and mAb recognizing epitope C, located within domain

Table II. *Mapping the interaction domain of CD229 mAbs*[a]

mAbs	CD229 hDI/DII mDIII/DIV	CD229 hDI/ DIII/DIV mDII	CD229 ΔDI	mAb Interaction Domain[b]
HLy9.1.25	+	+	−	DI
HLy9.1.38	+	+	−	DI
HLy9.1.77	+	+	−	DI
HLy9.1.84	+	−	+	DII

[a] COS cells were transiently transfected with chimeric human (h)/mouse (m) constructs or the isoform lacking the first V-like Ig domain (CD229 ΔDI) described in *Materials and Methods.* CD229 mAbs were assayed by flow cytometry: +, Detection of protein expression, −, nondetection of protein expression.

[b] Domain recognized by the mAb.

Table III. *Ability of test mAbs to block the binding of labeled mAbs to CD229–300.19 cells*[a]

	Labeled mAbs			
Test mAbs	HLy9.1.25	HLy9.1.38	HLy9.1.77	HLy9.1.84
HLy9.1.25	+++	++	−	−
HLy9.1.38	+++	+++	−	−
HLy9.1.77	−	+	+++	−
HLy9.1.84	−	−	−	+++

[a] Symbols represent the relative ability of test mAbs to block the binding of the indicated labeled mAbs to CD229–300.19 cells. Symbols indicate the percentage decrease in the number of fluorescence-positive cells: −, <20% of positive cells; +, 20–50% of positive cells; ++, 50–80% of positive cells; +++, >80% of positive cells.

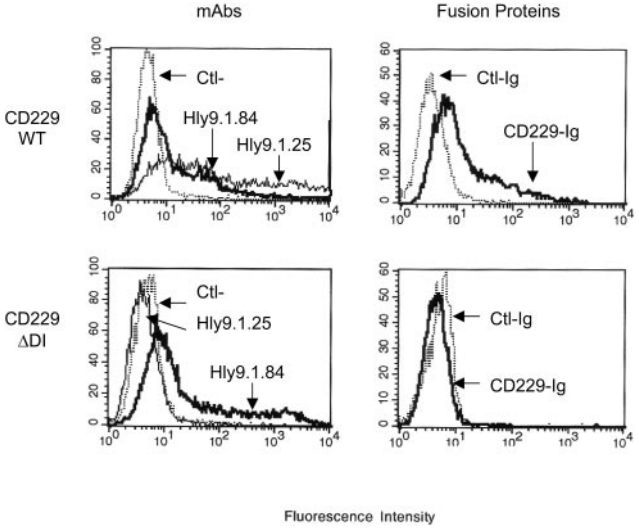

FIGURE 3. Homophilic binding of CD229 is mediated by the first extracellular domain. COS-7 cells transiently transfected with cDNA encoding full-length CD229 (*upper panel*) or the thymus CD229 isoform that lacks the first Ig domain (CD229 ΔD1), which contains DII/DIII and DIV (*lower panel*), were stained with HLy9.1.25 or HLy9.1.84 (*right column*). CD229-Ig binding (*left column*). Immunostaining controls: isotype controls (*left column*) and Ctl-Ig (murine CD84-Ig; *right column*) are represented with dotted lines.

II, did not significantly block CD229-Ig binding to CD229-transfected COS cells (data not shown). These data demonstrate that the first Ig domain mediates homophilic binding of CD229.

Identification of the key amino acid residues on the GFCC′C″ face involved in CD229 homophilic interaction

Surface-exposed charged amino acids have been proposed to be involved in the intermolecular interaction of the N-terminal Ig domains of CD2 and its counterreceptor CD58 (35, 36). To identify key amino acid residues of CD229 that contribute to homophilic binding, a number of point mutations were generated by site-directed mutagenesis. Several charged amino acids that are conserved between human and murine CD229, located at the GFCC′C″ face of the N-terminal Ig domain, were mutated to alanine. Binding of the CD229-Ig fusion protein was tested in COS cells transfected with wt and mutated CD229. COS cells expressing mutant CD229 molecules at levels comparable with wt were analyzed by flow cytometry and immunocytochemistry (Table IV and Fig. 4). To assess whether any of these mutations resulted in alterations of their tertiary structure that could affect their adhesion to a CD229 ligand, we tested their immunoreactivity with three mAbs, HLy9.1.25, HLy9.1.38, and HLy9.1.77, that recognize epitopes located within the first Ig-like domain. All of the mutants tested reacted similarly with these mAbs, suggesting no significant alteration of the general tertiary structure (Fig. 4*B*). Our results show that mutation of E27 and E29 on the predicted B-C loop and R89 on the F-G loop of the N-terminal domain all cause a disruption of the binding by >80%, whereas the rest of the mutants had no significant effect on the binding activity of CD229 (Table IV and Fig. 4*A*). Surprisingly, one mutation, *R44A,* caused a significant increase in binding to CD229-Ig (Table IV and Fig. 4).

CD229 redistributes to the contact area of T-B cell conjugates in an Ag-dependent manner

Here we used confocal microscopy to examine the localization of CD229 during Ag-dependent immune synapse formation. Superantigen activation of Vβ8$^+$ Jurkat cells has been described to trigger molecular polarization and segregation, forming a canonical immunological synapse (27). Jurkat T cells activated with the bacterial superantigen SEE-pulsed Raji cells formed immunological synapses similar to those described previously in mouse T cells activated with peptide Ag-bearing B cells. Both Jurkat and Raji cells expressed comparable levels of CD229. CD229 moved to the interface of the T-B cell conjugate after Ag triggering (Fig. 5*B*). CD229 was re-localized in 13.2% of the T-B cell conjugates analyzed in the absence of SEE. However, after incubation with SEE, the number of conjugates in which CD229 relocalized to the T-B cell contact area increased to a 38.0%, similar to the relocalization levels of CD3 (Fig. 5 and Table V). In contrast, CD45 was homogeneously distributed around the cell surface (Fig. 5 and Table V). The relocalization of CD229 was observed on both the Jurkat T cell and the Raji APC (Table VI). Moreover, the relocalization of CD229 was significantly affected by the presence of CD229 on APCs, because we observed that CD229 relocalization was significantly reduced when CD229 was not present on APCs in both the presence and the absence of SEE. In contrast, no significant differences in the relocalization of CD3 were observed (Fig. 6). To further analyze the localization of CD229 at the immunological synapse, colocalization studies were performed. We observed that the compact clusters of CD3 were embedded in large accumulations of CD229, similar to those observed with the integrin LFA-1 (Fig. 5*A*). Together, these data show that CD229 localizes to the immunological synapse.

Table IV. *Site-directed mutagenesis dissection of the homophilic adhesion*

CD229[a]	Location on First Ig-like Domain[b]	Relative CD229 Expression[c]	% Binding[d]
wt		1.0	100
D25A	BC loop	1.7	96
E27A	BC loop	0.9	21
E29A	BC loop	1.5	4
K37A	CC′ loop	1.1	73
R44A	C′C″ loop	1.47	204
K46A	C″	0.7	51
E47A	C″	0.99	69
R89A	FG loop	0.95	0

[a] COS cells transiently transfected with wt CD229 and mutant CD229 cDNAs.
[b] Strand assignment based on the modeling of CD229 described in *Materials and Methods.*
[c] Expression of wt and mutant CD229 proteins labeled with HLy91.25 mAb were measured by flow cytometry. Fluorescence intensities of wild-type or mutant CD229 vs wt CD229 are shown.
[d] Positive cells of wt and mutant CD229 proteins assayed with CD229-Ig were measured by flow cytometry. Values are percent of positive cells of wt or mutant CD229 vs wt CD229.

Discussion

In this study, we have demonstrated that CD229 is a self-ligand, interacting through its N-terminal V-like domain, in which we have identified three residues critical for the homophilic interaction. We observed that a soluble CD229-Fc fusion protein binds to CD229-transfected cells, but not to cells transfected with the cDNA of other members of the CD150 family or the related cell surface molecules CD2 and CD58. Our data are consistent with the observation that the only known ligands of the CD150 family of cell surface receptors are other members of the same family. With the exception of CD48, which binds to CD244, ligand interactions within this family have been shown to be homophilic (CD84-CD84, CD150-CD150, CS1-CS1, and NTB-A-NTB-A) (9, 25, 26). Here we have shown that both human and murine CD229 can

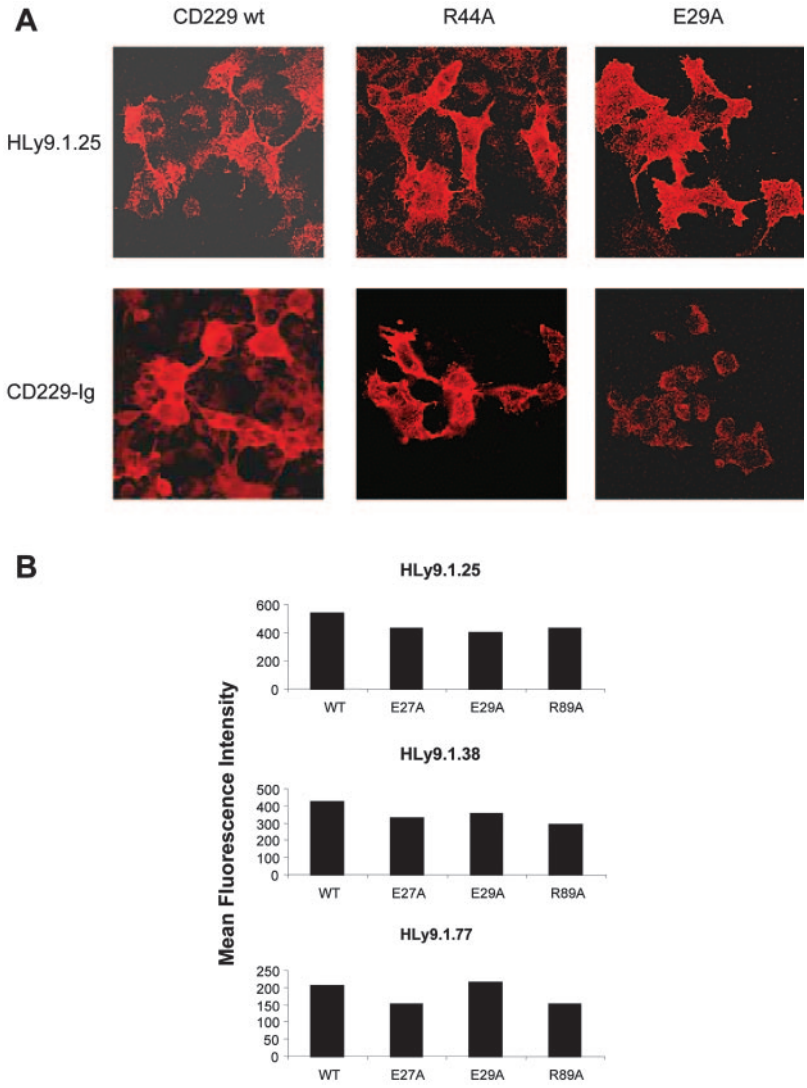

FIGURE 4. Identification of residues involved in homophilic interaction of CD229. *A,* COS-7 cells transiently transfected with full-length CD229, *E29A,* or *R44A* cDNA were stained with HLy9.1.25 mAb followed by Cy3-conjugated anti-mouse Ig (*upper panel*). The transfected cells were assayed with CD229-Ig followed by biotinylated anti-human Fc Ab and avidin-Cy3 (*lower panel*). *B,* COS-7 cells transiently transfected with full-length CD229, *E27A, E29A,* and *R89A* cDNAs were stained with HLy9.1.25, HLy9.1.38, and HLy9.1.77 mAbs followed by biotinylated anti- κ and streptavidin-PE. Mean fluorescence intensities are shown.

bind to itself, indicating that the homophilic interaction of this molecule occurs in different species. Moreover, the CD229-CD229 homophilic interaction is not species restricted, because human CD229 also bound to murine CD229 and vice versa. This is in contrast to our results with CD84 and to previous reports for other members of the CD150 family where their interaction has been shown to be species specific (23, 37). These data indicate that residues involved in the homophilic interaction of CD229 may be preserved between these two species.

Our structure-function analysis, using domain deletion mutants, revealed that the homophilic interaction of CD229 is mediated by the membrane distal N-terminal Ig domain. If additional Ig domains were directly involved in this interaction, cells expressing a CD229 that only lacks the N-terminal domain would be expected to display a residual capacity to bind to CD229-Fc. However, soluble ligand binding was completely abolished upon deletion of the N-terminal V domain. Moreover, single amino acid point mutation of residues located in the first Ig-like domain abolished the CD229-CD229 interaction. N-terminal domains are generally important for the interaction of cell-adhesion molecules that belong to the IgSF (38).

When homology can be demonstrated with well-characterized proteins, many properties of the three-dimensional structure can be predicted. The model building analysis of the N-terminal domain of CD229 described here used the known structure of CD2 and CD58 as a guide (35). The basic structure of the V-like N-terminal domain of CD229 is a predicted tertiary fold of a stacked pair of β-pleated sheets. There are 9 β strands, with strands A, B, E, and D lying in one sheet and strands C, C′, C″, F, and G lying antiparallel in the other (Fig. 7). Site-directed mutagenesis in CD2 and CD58 has shown that charged residues located on the GFCC′C″ face are critical for their interaction. The structural analysis of the ligand-binding domains of CD2 and CD58 confirmed that the binding is dominated by electrostatic contacts between binding surfaces exhibiting considerable electrostatic complementarity. Our results show that exposed residues on the GFCC′C″ face of the N-terminal domain also play a crucial role in homophilic interaction of CD229. Single mutations of the negatively charged residues E27 and E29 located in the predicted B-C loop, and the positively charged residue R89 on the F-G loop, abolished the CD229-CD229 interaction (Fig. 6). On the basis of mAb binding profiles, the presence of gross structural perturbation or misfolding, as a consequence of specific mutations was highly unlikely (Fig. 4*B*). Thus, we speculate that the negatively charged residues (E27 and E29) may interact with the positively charged residue (R89) in the counterreceptor interacting interface between the N-terminal adhesion domains of CD229. However, a crystal structure of CD229 binding to itself will be needed to confirm the actual way in which these residues interact at the molecular level.

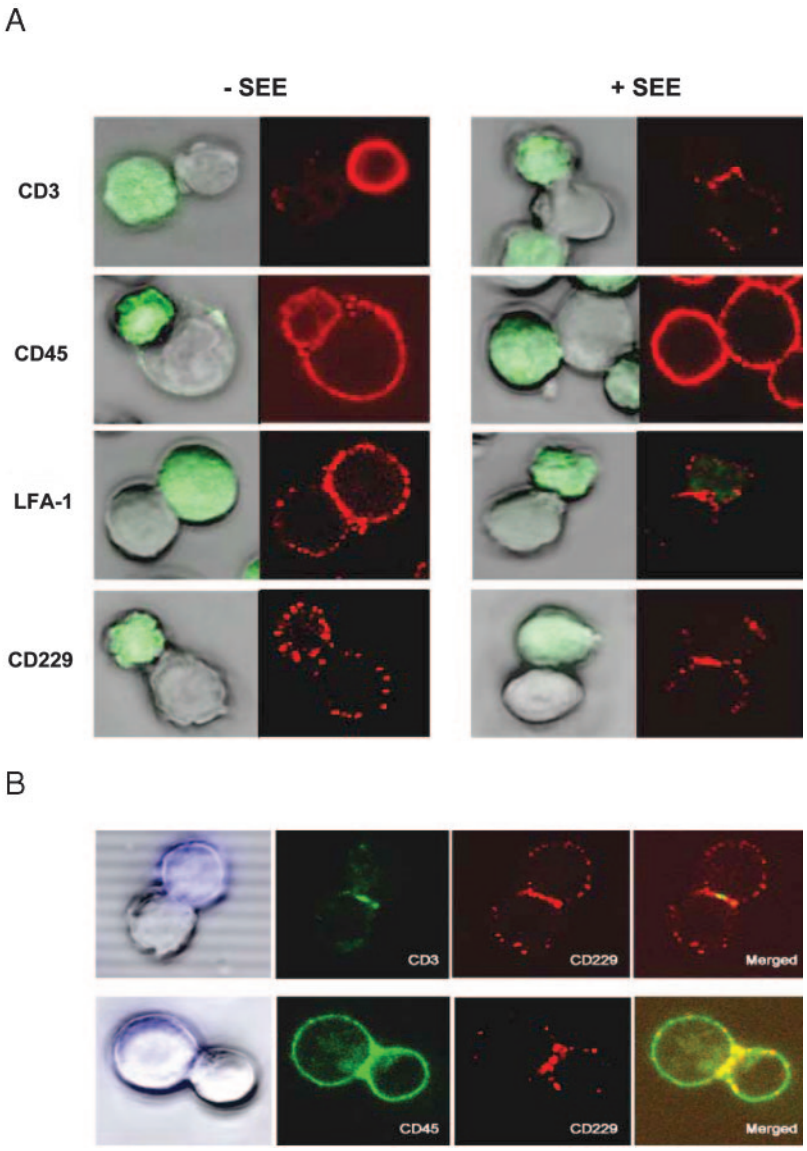

FIGURE 5. CD229 localizes to the immune synapse. *A,* Jurkat-Raji conjugates incubated for 10 min in the presence or absence of SEE were allowed to adhere to poly-L-lysine, fixed, and stained (red) for CD3, CD45, LFA-1, and CD229. Phase contrast images merged with green fluorescence from 5-chloromethylfluorescein diacetate-loaded Raji cells are shown. *B,* Jurkat-Raji conjugates incubated for 10 min in the presence of SEE and double-stained for CD229 (red) and CD3 or CD45 (green). Phase contrast images merged with blue fluorescence from CMAC-loaded Raji cells are shown.

As suggested for CD2 and CD58, we hypothesize that CD229 molecules expressed on the surface of adjacent cells establish head-to-head contacts. Of interest in this context is the observation that dynamic binding between CD2 and CD58 counterreceptors on opposing cells optimizes immune recognition through stabilization of T cell and APC cell contact (35).

Surprisingly, mutation of residue R44 located in the C′C″ loop enhanced the binding of CD229 to itself, a phenomenon that has been reported for other ligand-receptor pairs (39). This observation may aid the design of fusion proteins or small peptides that could be used to alter CD229 function in a therapeutic context.

Binding of the CD229-Ig could be observed only in transfected cells overexpressing CD229, indicating that its affinity for itself is weak. Nevertheless, CD229 binding is very specific, because it bonds only to itself and not to any other member of the CD150 family. Low affinity interactions are a common feature of homophilic receptors of the Ig superfamily including CD150 (K_d 200 μM). These interactions are characterized by remarkably fast on/off rates that foster rapid and extensive exchanges between partners on opposing cell surfaces. This is thought to allow cells to rapidly form and break contacts, conferring plasticity at the cell-cell contact areas or enabling the rapid surveillance of target membranes (38, 40). Whereas the affinity of adhesion molecules often

Table V. *Frequency of relocalization at the Raji-Jurkat IS*[a]

Conjugates	− SEE	+ SEE
CD3	1.1 ± 1.1	47.4 ± 7.2
CD45	8 ± 2.8	9.1 ± 0.4
CD229	13.2 ± 1.0	38.0 ± 1.8

[a] Quantification (%) of cell conjugates in which CD3, CD45, and CD229 were relocalized at the T cell-APC contact area in the presence or absence of SEE. At least 100 conjugates from three independent experiments were analyzed. Results correspond to the arithmetic mean ± SD.

Table VI. *Fold induction of fluorescence intensity at the IS*[a]

	CD229 (n = 10)	CD3 (n = 5)	CD45 (n = 5)
IS vs T cell	4.2 ± 2.0	13.1 ± 4.6	0.9 ± 0.2
IS vs APC	6.4 ± 3.0		0.9 ± 0.3

[a] Fluorescence intensity in the IS with respect to the rest of the T cell or APC membrane fluorescence. n is the number of T cell-APC contacts analyzed from two different experiments. Values are arithmetic means ± SD. IS, Immune synapse.

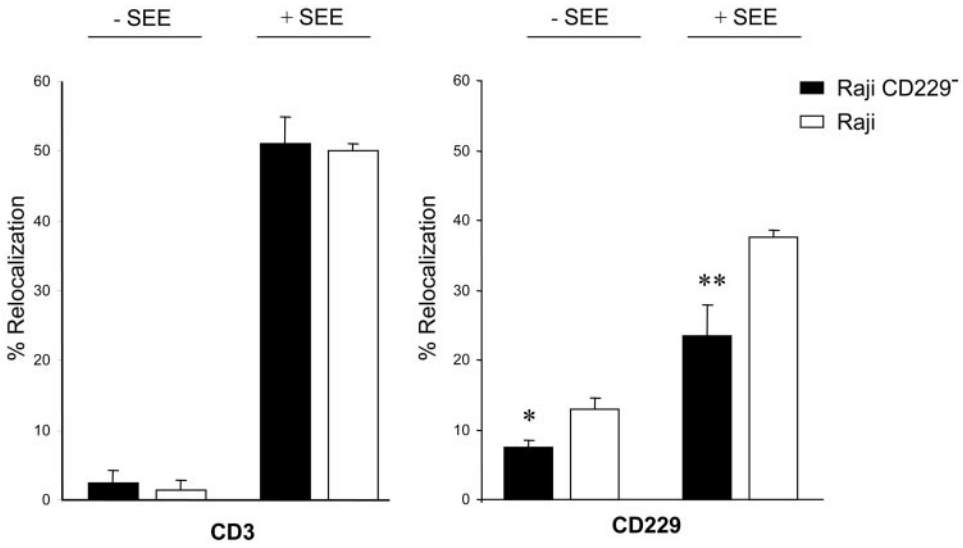

FIGURE 6. CD229 relocalization is affected by the presence of CD229 on APCs. Quantification of CD229 and CD3 relocalization to the T-B contact area of Jurkat-Raji or Jurkat-Raji CD229$^-$ conjugates in the presence or absence of SEE. More than 100 contacts were analyzed in three independent experiments. Histograms represent the arithmetic mean ± SD. *, $p < 0.05$ compared with Jurkat-Raji with Jurkat-Raji CD229$^-$ conjugates not treated with SEE. **, $p < 0.05$ compared with Jurkat-Raji with Jurkat-Raji CD229$^-$ conjugates treated with SEE.

reflects a specific conformation of the extracellular binding domain, avidity modulation involves changes in the cellular distribution, which leads to clusters of molecules and thereby specifically increases the number of available receptors at the site of cell-cell interaction. This avidity can be further enhanced by the participation of other adhesion molecules at the contact site, such as the integrins (41). Thus, the lateral receptor mobility and accumulation of multiple interface bonds will be required for the development of appreciable adhesion.

To explore the functional relevance of CD229 homophilic binding, we analyzed its localization during immunological synapse formation. Immune receptors and components of their signaling cascades are spatially organized, and this spatial organization plays a central role in the initiation and regulation of signaling (42). The synapse is established and maintained to a large extent by the interaction of glycoproteins expressed on the surface of adjacent cells. Homophilic interactions of cell surface proteins typically result in their accumulation at sites of cell-cell contact. These molecules not only function as adhesion molecules but also translate biochemical information. By doing so, they regulate the initiation and prolongation of signaling triggered by ligand engagement of the immune cell signaling receptors. The ability to segregate receptors provides a mechanism for the compartmentalization of signaling components within the membrane, concentrating certain components in the contact area and excluding others with negative regulatory activity. Our results show that CD229 redistributes to the contact area established between T cells and enterotoxin E (SEE)-pulsed Ag-presenting B cells. The redistribution could be observed in both the T and the B cell. Moreover, the presence of CD229 on the B cell affected the relocalization of CD229 on the T cell, indicating a role of CD229 homophilic interaction in its cellular distribution (Fig. 6). However, CD229 homophilic interaction was not required for CD3 relocalization to the immunological synapse. Interestingly, the accumulation of CD229 was more evident at the periphery of the contact region, surrounding the compact clusters of CD3 (Fig. 5*B*). The molecular segregation of CD229 may be explained by the four-Ig domain structure of this cell surface molecule. It has been observed that cell surface molecules of the Ig superfamily that are present in the central synapse contain just two Ig domains and that this may be important to ensure the right distance for interactions between two cells. This is also the case for the CD150 family member CD244 (2B4) (29). Consistent with this notion, the interaction of CD2 with an elongated form of CD48 significantly inhibits immune recognition (43). Recent studies in our laboratory indicate that CD229 inhibits rather than induce T cell activation and cytokine production (44). Thus, we hypothesize that CD229 is excluded from the central synapse to prevent it from delivering negative signals. More studies including dynamic redistribution analysis of CD229 will be required to elucidate its contribution to the modulation of the functional state of the immunological synapse.

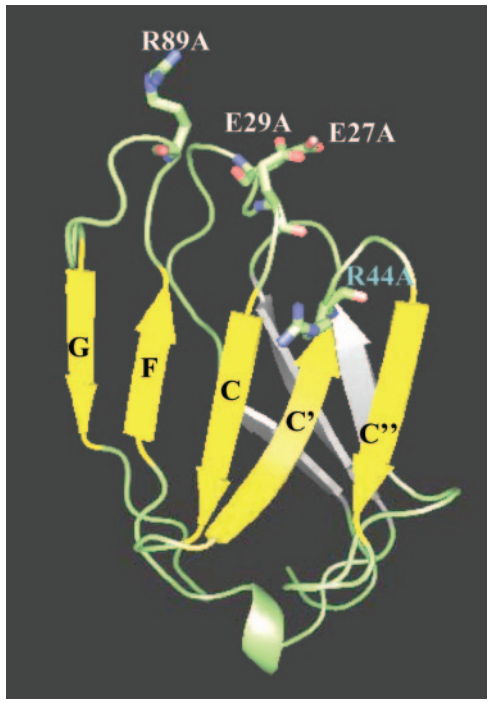

FIGURE 7. Molecular model of the N-terminal IgV set domain of human CD229. Ribbon diagram of the N-terminal domain of CD229 showing the predicted homophilic interface. The β strands GFCC′C″ are labeled according to the standard convention. The face GFCC′C″ is gold, and the ABED face is gray. The mutated amino acids that strongly modify the interaction are indicated on the model according to the one-letter amino acid codes. Substituted amino acids that abolish adhesion are labeled in white, whereas residue 44, which strongly enhances adhesion, is labeled in light blue. Negatively changed atoms are labeled in red, and positively charged atoms are labeled in blue.

Immunological synapse occurring during cognate interactions between B and helper T cells, within the B cell follicles of secondary lymphoid organs, are required for humoral immune responses to many Ags (45). Expression of SAP by $CD4^+$ T cells is essential for T cells to provide B cell help and establish long term humoral immunity (46). The fact that CD229 is a SAP-associated homophilic receptor expressed on both T and B cells and that it localizes at the immune synapse suggests that this molecule has a role in the regulation of T-B interactions. Further evidence supporting the importance of CD229 in regulating T cell help for B cells is the recent finding that CD229 is preferentially expressed by effector T follicular helper cells (47).

The identification of CD229 as a homophilic receptor and characterization of the binding site is an important step in advancing our understanding of how this interaction can regulate the immune response.

Disclosures

The authors have no financial conflict of interest.

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