CD229 (Ly9) is a cell surface glycoprotein of relative mass 120 kDa found on T and B lymphocytes, and it belongs to the CD150 receptor family. Like other receptors of this family, CD229 interacts with SAP/SH2D1a protein, mutation of which is responsible for the fatal X-linked lymphoproliferative disease. Receptors of the CD150 family function as costimulatory molecules, regulating cytokine production and cytotoxicity. Thus, their signaling and regulation in lymphocytes may be critical to an understanding of the pathogenesis of the X-linked lymphoproliferative disease. Here we show that CD229 interacts with the μ2 chain of the AP-2 adaptor complex that links transmembrane proteins to clathrin-coated pits. CD229 was the only member of the CD150 family associated with AP-2. We also show that the μ2 chain interacts with the Y470EK motif of CD229. The integrity of this site was necessary for CD229 internalization, but it was not involved in SAP recruitment. Moreover, CD229 binds to the AP-2 complex with B cell lines, and it is internalized rapidly from the cell surface on T cells after antibody ligation. In contrast, cross-linking of CD229 receptors with intact antibody inhibited CD229 internalization on B cells. However, when F(ab)'2 antibodies were used, CD229 internalization was similar on T and B cells, suggesting that Fcγ receptors control CD229 cell surface expression. Furthermore, CD229 was regulated by T cell receptor and B cell receptor signaling because coligation with antibodies against anti-CD3 and anti-IgM increased the rate of CD229 endocytosis. These data suggest that CD229 cell surface expression on lymphocytes surface is strongly and differentially regulated within the CD150 family members.

CD229 (also termed Ly9) is a cell surface glycoprotein of relative mass 120 kDa found on T and B cells (1). It is a member of the immunoglobulin superfamily, and it belongs to the CD150 family of receptors (2, 3). Six members of the CD150 family (CD150, CD229, CD84, CD244, Ntb-A, and Cs1) have one or more cytoplasmic tyrosine motifs with the consensus sequence Thr(Ile/Val)-Tyr(P)-X-Ile/Val). This motif functions as a docking site for the SAP3/SH2D1a protein (4–9). Mutations on the SAP gene, sh2d1a, are the responsible for the fatal X-linked lymphoproliferative disease after Epstein-Barr virus infection, familial hemophagocytic lymphohistiocytosis, and some cases of combined variable immunodeficiency (2, 4, 10–12). These disease phenotypes point to distinct roles for the six CD150 family/SAP signaling pathways in the control of T, B, and natural killer cell activation and homeostasis. Studies on SAP knock-out mice reveal that T cells have an impaired ability to differentiate into T helper 2 cells, resulting in increased resistance to T helper 2-mediated disease such as infection with Leishmania major. In addition, SAP−/− mice fail to resolve the lymphocytic choriomeningitis virus, showing an increased number of interferon-γ-producing cells in the spleen and liver (13) and a nearly complete absence of virus-specific long lived plasma cells and memory B cells (14). Altogether, these data indicate that SAP controls several distinct key T cell signal transduction pathways, which may be controlled differentially by the various CD150 receptors. It is crucial to elucidate the contribution of each member to these immune functions. Unlike other CD150 family members, 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 domains (15). Although the function of CD229 is unknown, other members of the subfamily such as CD84, CD244, and CD150 are known to activate lymphocytes (16–22), suggesting that the CD229 molecule is also involved in leukocyte activation. Recently, Ntb-A and Cs1 have also been shown to regulate cytotoxicity in natural killer cells (23, 24).

In this study, we have investigated the biochemical mechanism involved in CD229 signaling and intracellular trafficking by identifying proteins that interact with the CD229 cytoplasmic domain. Two clones encoding human AP-2 μ2 chain were isolated using CD229 as bait in a three-hybrid screen of a human B cell library. μ2, also termed AP50, is the 50-kDa subunit of the AP-2 complex. AP-2 is a key component of the endocytic machinery that links cargo membrane proteins to the clathrin lattice. It is a heterotrimer consisting of two large (100–115 kDa) α and β subunits or adaptins, one medium μ2 (50 kDa), and one small (17 kDa) σ2 subunit. The α-adaptin appears to contain the major membrane binding interface. The hinge domain of the β subunit binds to the clathrin heavy chain, providing a mechanism for the formation of polyhedral...

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The Cell Surface Expression of SAP-binding Receptor CD229 Is Regulated via Its Interaction with Clathrin-associated Adaptor Complex 2 (AP-2)*

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lattices. In addition to its structural role in coat assembly, AP-2 is also involved in the transport of proteins from the plasma membrane to the endosomal and lysosomal compartments. For effective cargo into coated pits, receptors should contain specific internalization signals, namely tyrosine-based motifs such as NPXY or YXX\(\Phi\) (where \(\Phi\) stands for any amino acid and \(\phi\) for a bulky hydrophobic residue), dileucine motifs, and acidic clusters (4–6). The structural analysis of the AP-2 core reveals that the binding site for YXX\(\Phi\) endocytic motifs is buried, indicating that a conformational change, probably triggered by phosphorylation in the disordered \(\mu_2\) linker, is necessary to allow YXX\(\Phi\) motif binding (27). Here, we demonstrate that the only member of the CD150 family which specifically binds \(\mu_2\) is CD229. We mapped the CD229-\(\mu_2\) interaction in Tyr\(^{470}\) of the CD229 cytoplasmic domain. Moreover, we show that TCR and BCR signaling regulates CD229 expression.

EXPERIMENTAL PROCEDURES

Cells and Reagents—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (In-vitrogen), 2 mM \(t\)-glutamine, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin. Jurkat and Daudi cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM \(t\)-glutamine, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin. Biotin, brefeldin A (BFA), and anti-mouse IgG peroxidase were obtained from Sigma. Monoclonal anti-\(\alpha\)-adaptin AP-2 (clone 100/2), also from Sigma, was used for immunoblotting. Rabbit anti-rat AP-2 (C-20), from Upstate Biotechnology (Lake Placid, NY), was used for immunoprecipitation and microscopy. Phosphotyrosine monoclonal antibody mixture horseradish peroxidase-conjugated was from Zymed Laboratories Inc. (San Francisco). Anti-GFP monoclonal was from Roche Applied Science. Anti-CD84 (clone CD84 1.1.1), generated at our laboratory (16), was used as a control IgG. Anti-human CD3 (clone 3Tb) was a gift from Dr. Lopez-Vilella (Hospital Clínic/HH11032). CD229 and mutated Fyn were inserted in the yeast strain CG1945, was confirmed by SDS-PAGE under nonreducing conditions and reducing conditions followed by silver staining.

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We mapped the CD229—\(\mu_2\) interaction in Tyr\(^{470}\) of the CD229 cytoplasmic domain. Moreover, we show that TCR and BCR signaling regulates CD229 expression.
FIG. 2. CD229 is the only member of the CD150 family which interacts with μ2. Yeast cotransfected with pBridge cytoplasmic tails of CD150 family members (CD150, CD244, CD229, CD84, NTB-A, and CS1) +/- Fyn and pGAD-SAP (A) or pACT2-μ2 (B) were analyzed for β-galactosidase activity. These data are the mean of duplicates and representative of three experiments.

antibody was detected using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Pierce).

Internalization Assay—Cells (1 × 10⁶/sample) were incubated with anti-human CD229 mAb (1 μg/ml = 1 × 10⁶) at 37 °C. At various time points (0–90 min), cells were chilled rapidly and acid stripped to remove cell surface-bound mAb as described elsewhere (28). Samples were washed twice in cold complete culture medium, and they were incubated for 25 min with biotinylated anti-CD229 followed by avidin-PE incubation (1). For recycling experiments, cells were incubated at 37 °C with anti-CD229 in the presence or absence of BFA at 10 μg/ml in complete culture medium for the indicated time periods. Samples were stripped and stained with biotin-conjugated anti-human CD229 followed by streptavidin-PE. For stimulation, cells were treated for 5 min with anti-CD3 or anti-IgM. Intact anti-CD229 or F(ab)₁ was added for an additional 30 min. Thereafter, cells were harvested, chilled, and acid stripped as described above. The samples were analyzed in a flow cytometer (FACS Caliber; BD Biosciences, Mountain View, CA) to detect PE (FL2) fluorescence (mean fluorescence intensity; MFI). The percentage of endocytosis was calculated using MFI values of PE as follows: 100 – [MFI at 37 °C/MFI at 4 °C] × 100.

Immunofluorescence Microscopy—COS-7 cells were cultured on coverslips, transfected with μ₂-EGFP cDNA, fixed, and permeabilized with –20 °C methanol for 15 min. After two washes, they were incubated for 30 min at room temperature with blocking buffer (phosphate-buffered saline containing 0.2% skim milk, 2% fetal bovine serum, 1% bovine serum albumin, 0.1 M glycine). Cells were then washed and incubated with 2 μg/ml rabbit anti-rat AP-2α-C for 1 h at 4 °C. After three washes, they were incubated with a biotinylated anti-rabbit (Biogenex, San Ramon, CA) at 4 °C for 30 min. After several additional washes, cells were incubated with streptavidin-Cy3 (Jackson Immunoresearch, West Grove, PA) at 4 °C for 20 min. Samples were washed and mounted in Fluoromount-G (Southern Biotechnology, Birmingham, AL). For endocytosis experiments, COS-7 cells were cultured on coverslips and cotransfected with human CD229 (WT or Y470F or Y470A mutants) with μ₂-EGFP cDNAs in duplicate. After 48 h they were labeled with 1 μg/ml biotinylated mAb CD229 at 4 °C for 30 min. After two washes with ice-cold phosphate-buffered saline, samples were incubated with streptavidin-Cy3 at 37 °C (to induce endocytosis) for 30 min. Cells were then washed and fixed in –20 °C methanol for 15 min. After washing them twice, cells were mounted and visualized as described above. Fluorescence images were obtained using a confocal microscope (TCS NT; Leica, Heidelberg, Germany).

RESULTS

CD229 Interacts with μ2 in a Yeast Three-hybrid Screen—The cytoplasmic tail of human CD229 was used as bait to screen a human B cell library. The CD229 cytoplasmic tail was cloned in the pBridge vector together with Fyn (7). The screening of ~2 × 10⁵ clones yielded two clones that encoded portions of human μ₂, the medium chain of clathrin-associated coated pit adaptor protein complex AP-2 (Fig. 1A). Because the μ₂ interaction with proteins is independent of phosphorylation, we assayed the binding by depleting the catalytic activity of Fyn. CD229 binding to μ₂ was similar in the presence and absence of kinase activity (Fig. 1B).

CD229 Is the Only Member of the CD150 Family of Receptors That Interacts with μ2—We assayed the interaction of μ₂ with all of the CD150 members using a semiquantitative β-galactosidase assay. All bound to SAP/H2D1α when Fyn was active, with the exception of CD150, which bound to SAP regardless of its phosphorylation status, as reported elsewhere (29) (Fig. 2A). Surprisingly, only CD229 was able to bind to μ₂ in conditions...
where Fyn was present or absent, whereas the rest of members failed to do so (Fig. 2B).

**CD229 Binds to μ2-EGFP Chimera and Integrates in AP-2 Endogenous Complex in COS-7 Cells**—To test the interaction CD229-μ2 in a mammal system, we made the μ2-EGFP construct and transiently transfected COS-7 cells alone or together with CD229. To determine whether CD229 and the μ2-EGFP chimera integrated in the endogenous AP-2 complex in COS-7 cells, we performed an immunoprecipitation with anti-μ2 chain of AP-2 (Fig. 3A). μ2-EGFP and CD229 coprecipitated with AP-2 endogenous complex (second and third lanes, respectively). Moreover, CD229-μ2-EGFP and AP-2 were in a complex (fourth lane). To test whether most μ2-EGFP was free or complexed with the endogenous AP-2, we performed μ2-EGFP transfection in COS-7 cells and immunochemical staining with anti-α chain AP-2. The majority of the μ2-EGFP protein colocalized with the endogenous AP-2 (Fig. 3B).

**Tyrosine 470 Is Required for CD229 Binding to μ2**—Analysis of the CD229 sequence revealed several putative motifs for μ2 binding (Y-X-X-φ) (where X stands for any amino acid and φ for a bulky hydrophobic residue) (Fig. 4A). A more restricted binding motif has been proposed for μ2:YPPφ, where P represents a polar amino acid (25). Following these criteria we found a suitable motif for the binding (Fig. 4A). The Y470F mutation abolished the interaction between CD229 and μ2 in a yeast system (Fig. 4B). COS cells were transfected with CD229 and μ2-EGFP-transfected COS cells. Cells were stained with biotinylated anti-CD229, and avidin-Cy3 was added at 37°C for 30 min, inducing endocytosis of the receptor. Cells were washed and mounted.

**Tyrosine 470 Is Essential for CD229 Endocytosis**—To determine the relevance of CD229-μ2 interaction for receptor internalization, we generated CD229 mutant constructs in which the Tyr470 residue was replaced by phenylalanine or alanine. We transiently transfected CD229 wild-type and mutants encoding constructs into COS-7 and measured their internalization after ligation with anti-CD229 mAb at 37°C. Thereafter, cells were stripped and stained with CD229 biotinylated anti-
body plus avidin-PE labeling, and samples were measured by flow cytometry (Fig. 5A). Within 30 min, 30–40% of wild-type CD229 surface receptors had been internalized. In contrast, internalization was completely abrogated, and CD229 surface expression even increased slightly, in CD229 mutants, which Tyr470 had been replaced by phenylalanine or alanine. We also transfected COS cells with the CD229WT and CD229Y470A mutants plus μ2-EGFP. After 24 h, cells were stained with biotinylated antibody at 4°C followed by avidin-Cy3 at 37°C (Fig. 5B). In cells transfected with CD229WT, the receptor was internalized and colocalized with the μ2-EGFP, whereas CD229Y470A and CD229Y470A internalization was impaired, and no colocalization with μ2 was found. Both results indicate that Tyr470 is needed for CD229 binding to μ2 and further endocytosis.

The Cytoplasmic Tail of CD229 Is Associated with the AP-2 Adaptor Complex in T and B Lymphocytes—Because CD229 is expressed only in cells of the immune system, we next determined whether the association of the AP-2 adaptor complex with CD229 occurred in lymphocytes. To this end, we performed coimmunoprecipitation experiments in two lymphocytic cell lines: Jurkat (T cell line) and Daudi (B cell line). Both cell lines were immunoprecipitated using anti-CD229 mAb and an anti-CD84 as a control mAb. Western blot was probed with anti-AP-2mAb (Fig. 6). A band representing AP-2α was found only in the lane in which CD229 was immunoprecipitated. Taken together, these data demonstrate that the AP-2 adaptor complex specifically interacts with the cytoplasmic tail of CD229 in T and B lymphocytes.

Anti-CD229 mAbs Trigger CD229 Internalization—Anti-CD229 mAbs induce the internalization of CD229 in COS cells after 30 min of mAb coligation. Because CD229 is expressed on lymphocytes and associates with the AP-2 complex, we assayed CD229 internalization in these cells. Endocytosis was assayed in the Jurkat T cell line and in the Daudi B cell line. CD229 was endocytosed rapidly in Jurkat cells, reaching a plateau within 15 min. The internalization of CD229 after incubation with intact anti-CD229 was comparable with that obtained with F(ab’)2 in Jurkat cells (Fig. 7A). In contrast, internalization of CD229 with intact mAb was impaired in B cells. However, when F(ab’)2 was used to coligate, the effect on CD229 internalization resembled that observed in T cells, indicating that CD229 endocytosis may be regulated through Fcγ receptors (Fig. 7B). In agreement with this observation, a reduction of intact antibody concentration used for ligation induced some CD229 endocytosis in B cells, indicating that a limiting mAb concentration elicits coligation with CD229 with minimal Fcγ receptor binding (data not shown). The internalization of CD229 after antibody coligation may represent a constitutive turnover rate of CD229 from the cell surface. To distinguish these two events (endocytosis versus turnover), we used cycloheximide and BFA, which block the expression of newly synthesized protein. BFA blockade inhibits the transport of proteins from the endoplasmic reticulum to the Golgi apparatus and also recycling of endocytosed proteins (30–32). Treatment with cycloheximide did not alter CD229 surface expression on T or B cells within the time period assayed for endocytosis (data not shown). Treatment with BFA for 2 h slightly reduced CD229 expression even after 60 min (20–25%) but did not significantly reduce the surface expression of CD229 in B cells (Fig. 7C). The transferrin receptor (CD71), which is rapidly recycled (33), was used as a control. After 15 min, CD71 surface expression was reduced more than 50% in BFA-treated cells (Fig. 7D). Altogether, these results indicate that CD229 expression on the cell surface is more stable on B than on T cells, and in both cases, the rate of turnover is lower than that for CD71.

Endocytosed CD229 Mainly Follows the Lysosomal Degradation Pathway—We next analyzed the events following CD229 ligation in lymphocytes. CD229, once internalized, may traffic to lysosomal vesicles for degradation or be recycled back to the cell surface. To distinguish these two events we assessed CD229 endocytosis in the presence or absence of BFA. If most CD229 were recycled back to the cell surface, the loss of CD229 after mAb ligation would be much greater in the presence of BFA. On the other hand, if most endocytosed CD229 were transferred to lysosomes, we would obtain identical results in
the presence or absence of BFA. CD229 surface levels decreased slightly on T cells induced by F(ab\')_2 anti-CD229 in the presence of BFA, suggesting that CD229 was partially recycled back to the cell surface (20–25%) but mostly trafficked to lysosomal degradation (Fig. 8A). In B cells, CD229 surface levels were similar in both cases. Thus, endocytosed CD229 follows mainly the lysosomal degradation pathway in these cells (Fig. 8B). Taken together, these results suggest that most CD229 is transported to the lysosomal compartment rather than recycled back to the cell surface.

CD229 Internalization Is Regulated after TCR or BCR Cross-linking—We next investigated whether antigen receptor cross-linking modulates CD229 internalization in T and B lymphocytes. When we assayed CD229 expression after TCR ligation at 30–60 min, the expression of CD229 did not vary significantly. In contrast, CD229 ligation did not affect CD3 expression in this period (data not shown). Unexpectedly, the coligation of CD229 and TCR enhanced CD229 internalization (Fig. 9A). Daudi cells were incubated with anti-IgM F(ab\')_2 and anti-CD229 for 30 min, and CD229 endocytosis was studied. CD229 internalization after IgM coligation was enhanced (Fig. 9B).

Taken together, these data suggest that most CD229 is transported to the lysosomal compartment rather than recycled back to the cell surface.

DISCUSSION

In this study, we have shown that CD229 binds to the \( \mu_2 \) chain of the AP-2 complex. It is well established that the \( \mu_2 \) subunit binds to the consensus sorting signal NPXY or YPP\( \phi \) (25). Although it has been suggested that \( \mu_2 \) interacts with dileucine motifs (34), peptides containing dileucine motifs bind only to the \( \beta \) subunit of AP1 and AP-2 complexes and not to the other subunits (35, 36). Analysis of CD229 revealed only one sequence that follows the YPP\( \phi \) consensus (Y\( ^{170} \)EK), which is conserved in mouse (37). Using site-directed mutagenesis, we mapped the \( \mu_2 \) interaction to CD229 in this motif. The single replacement of tyrosine by phenylalanine abrogates \( \mu_2 \) binding to CD229 in yeast. It has been proposed that the hydroxyl group of tyrosine contributes to a network of interactions with \( \mu_2 \) residues that form the hydrophobic pocket, explaining why phenylalanine does not efficiently replace tyrosine in YPP\( \phi \) signals (38, 39). As expected, the recruitment of SAP in the mutant is not altered because both molecules do not compete
CD229 but not that of any of the other members of CD150 family can interact with the AP-2 complex via its \(\mu_2\) chain. This intriguing difference suggests that the expression and internalization of CD229 are tightly and differentially regulated. The structure of CD229 differs from that of the rest of the CD150 family of receptors. It is the only member that has four extracellular Ig-like domains, and it has the longest cytoplasmic tail, comprising 180 amino acids, 16 threonines, 17 serines, and 8 tyrosines. Two unique tyrosine-based motifs (with the consensus amino acid sequence TV/I-Y) are critical for the binding to the SAP/SH2D1A protein and the Eaat-2 SH2 domain protein homolog to SAP in B cells.

Furthermore, CD229 can bind to endogenous AP-2 in COS cells, \(\mu_2\)-EGFP chimera can efficiently integrate in the endogenous AP-2 complex, and CD229 can co-preface with \(\mu_2\)-EGFP and the AP-2 endogenous complex. The complex is functional because CD229 internalization and CD229/\(\mu_2\)-EGFP colocalization were detected in COS cells after antibody-induced endocytosis. In agreement with our mapping data, CD229/\(\mu_2\) and CD229/\(\mu_4\) endocytosis was severely impaired. Although in our model a phenylalanine substitution abrogates the binding to \(\mu_2\), the FX\(\Phi\) motif can fit into the hydrophobic pocket of \(\mu_2\) but leads to a weaker interaction and slower endocytosis. Thus, Tyr470 is essential for CD229 endocytosis and may inhibit the internalization of this receptor after its phosphorylation in vivo. CD229 can be phosphorylated by Src-kinase Fyn in COS cells, and its possible interaction with a SH2 domain protein may further regulate the signaling and trafficking of the molecule, as proposed for CTLA-4.

Because CD229 is expressed in T and B lymphocytes, we have defined the mechanism of CD229 internalization and demonstrated that it can be regulated differentially in T and B cell lines. CD229 was endocytosed rapidly in response to antibody ligation in Jurkat T cells, whereas no internalization was observed in B cells. The same results were obtained in lymphocytes from peripheral blood. CD229 in B cells was inhibited by the Fc receptor coligation because Fab \(\gamma\) antibodies induce CD229 endocytosis in B cells. The CD229 uptake rate was similar using the intact and Fab \(\gamma\) antibodies in Jurkat cells lacking Fc receptors. In agreement with our results, the coligation of Fc receptors and BCR has been suggested to inhibit endocytosis by blocking the processing and presentation of the BCR-bound antigen (44). Using BFA and cycloheximide, we have determined that CD229, unlike transferrin receptor (CD71), exhibits a low constitutive rate of turnover, and therefore, the rapid internalization of CD229 represents ligand-induced endocytosis. After endocytosis, CD229 was segregated into two distinguishable pools, one that is shuttled to a nonrecycling degradation pathway and another that is recycled to the cell surface. The results suggest that 75–80% of CD229 in T cells become degraded via lysosomal compartments, and the remaining 20–25% of complexes are recycled to the cell surface.

In contrast, most CD229 is trafficked to the lysosomal degradation pathway in B cells.

Moreover, TCR and anti-IgM coligation enhances CD229 endocytosis, suggesting that the antigen receptor signaling regulates the availability of CD229 at the plasma membrane. Thus, endocytosis may represent a critical mechanism that modulates CD229 cell surface expression rapidly. After TCR activation, phosphatidylinositol 3-kinase activity and p38 MAPK become activated and catalyze the conversion of phosphatidylinositol 4,5-biphosphate to phosphatidylinositol 3,4,5-trisphosphate. The pleckstrin homology domain in the \(\alpha_2\) subunit of the AP-2 adaptor complex binds with high affinity to phosphatidylinositol 3,4,5-trisphosphate. This binding mediates two effects: localization of AP-2 in the membrane and increase in the affinity of \(\mu_2\) binding to endocytosis sorting signals (45, 46).

Because CD229 endocytosis is impaired when Fcy receptors are triggered and enhanced when the BCR is activated, this molecule may have a differential role in these different events. The ability to regulate CD229 internalization in T and B cells differentially may allow for different net effects of CD229 signaling which may be cell-specific. Recent studies indicate that CD229, unlike CD150 and CD84, inhibits interferon-\(\gamma\) secretion after TCR activation in T cells. TCR and BCR may increase the rate of CD229 endocytosis to prevent CD229 from delivering negative signals to the cell and Fcy receptor ligation retains the CD229 on the cell surface, thus favoring signaling through this receptor and the maintenance of an inhibitory response. The development of CD229 knockout mice may be a key step to define the physiological role of CD229.

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