

# Identification of CD112R as a novel checkpoint for human T cells

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**T cell immunoglobulin and ITIM domain (TIGIT) and CD226 emerge as a novel T cell cosignaling pathway in which CD226 and TIGIT serve as costimulatory and coinhibitory receptors, respectively, for the ligands CD155 and CD112. In this study, we describe CD112R, a member of poliovirus receptor–like proteins, as a new coinhibitory receptor for human T cells. CD112R is preferentially expressed on T cells and inhibits T cell receptor–mediated signals. We further identify that CD112, widely expressed on antigen-presenting cells and tumor cells, is the ligand for CD112R with high affinity. CD112R competes with CD226 to bind to CD112. Disrupting the CD112R–CD112 interaction enhances human T cell response. Our experiments identify CD112R as a novel checkpoint for human T cells via interaction with CD112.**

T cell activation is orchestrated by the cosignaling network, which is involved in all stages of the T cell response (Croft, 2003; Zhu et al., 2011). The B7/CD28 family of Ig superfamily (IGSF) and several members of TNF receptor superfamily are the major groups of T cell cosignaling molecules (Chen and Flies, 2013). The importance of these cosignaling pathways has been emphasized in a variety of human diseases, including graft versus host disease, autoimmunity, infection, and cancer (Rosenblum et al., 2012; Yao et al., 2013; Drake et al., 2014).

Poliovirus receptor (PVR)–like proteins are a newly emerging group of IGSF with T cell cosignaling functions (Chan et al., 2012; Pauken and Wherry, 2014). This group of molecules share PVR signature motifs in the first Ig variable–like (IgV) domain and are originally known to mediate epithelial cell–cell contacts (Takai et al., 2008; Yu et al., 2009). The two ligands, CD155 (PVR/Necl-5) and CD112 (PVRL2/nectin-2), interact with CD226 (DNAM-1) to costimulate T cells, and they also inhibit T cell response through another coinhibitory receptor, T cell Ig and immunoreceptor tyrosine–based inhibitory motif (ITIM) domain (TIGIT; Yu et al., 2009). CD155 seems to be the predominant ligand in this ligand/receptor network because the interaction between CD112 and TIGIT is very weak (Yu et al., 2009). Adding to the complexity of this network, CD155, but not CD112, interacts with CD96, another PVR–like protein present on T cells and NK cells, though the function of this interaction is still unclear (Fuchs et al., 2004; Seth et al., 2007; Chan et

al., 2014). In addition to its intrinsic inhibitory function, TIGIT exerts its T cell inhibitory effects through ligating CD155 on DCs to increase IL-10 secretion or competes with the costimulatory receptor CD226 for ligand interaction (Yu et al., 2009; Lozano et al., 2012; Stengel et al., 2012). Although the molecular and functional relationship between CD226 and TIGIT is still unclear, this novel cosignaling pathway represents important immunomodulators of T cell responses, as well as valuable targets for future immunotherapy (Joller et al., 2011, 2014; Levin et al., 2011; Johnston et al., 2014; Zhang et al., 2014; Chauvin et al., 2015). In this study, we identified CD112R as a new coinhibitory receptor of the PVR family for human T cells.

## RESULTS AND DISCUSSION

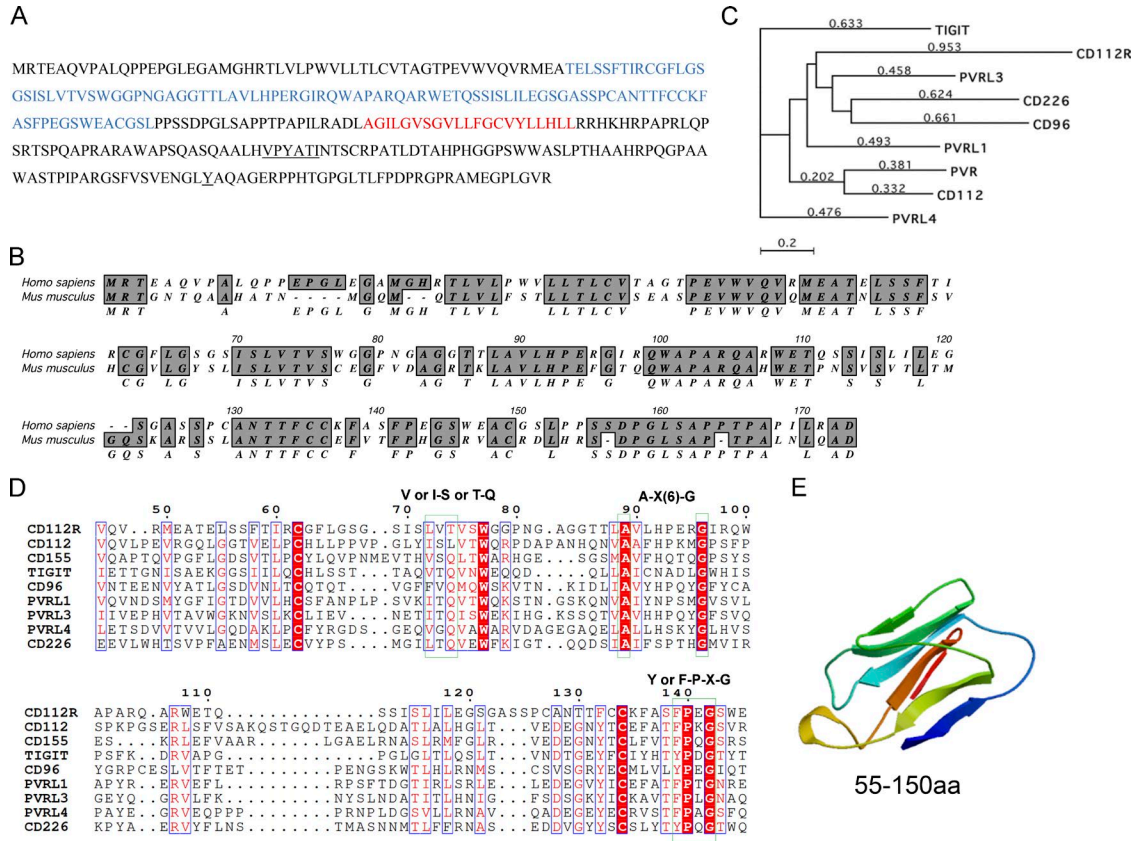
### Characterizing CD112R as a new receptor of the PVR family

We performed an extensive genome–wide search to look for genes that are both preferentially expressed on human T cells and encode transmembrane proteins with a single IgV extracellular domain. We discovered a candidate human gene previously named PVR–related Ig domain containing (PVR-IG; NCBI Nucleotide database accession no. BC073861). We renamed it as the receptor for CD112 (CD112R) to reflect its strong interaction with CD112 as described in this study. The CD112R gene encodes a putative single transmembrane protein, which is composed of a single extracellular IgV domain, one transmembrane domain, and a long intracellular domain (Fig. 1 A). Notably, the intracellular domain of human CD112R contains two tyrosine residues, one within an ITIM–like motif that is a potential docking site for phos-

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Abbreviations used: CHO, Chinese hamster ovary; IGSF, Ig superfamily; IgV, Ig variable–like; ITIM, immunoreceptor tyrosine–based inhibitory motif; MFI, median fluorescence intensity; PVR, poliovirus receptor; TIGIT, T cell Ig and ITIM domain; TT, tetanus toxoid.

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**Figure 1. Characterization of human CD112R protein.** (A) Protein sequence encoded by the human CD112R gene. Predicted extracellular IgV-like and transmembrane domains are highlighted in blue and red, respectively. Two tyrosines (Y233 and Y293) in the cytoplasmic domain are underlined with one within an ITIM-like motif underlined. (B) Alignment of the extracellular domains of human and mouse CD112R protein sequences using the MacVector 6.5 program. The shaded boxes refer to the shared amino acids among CD112R orthologues. (C) Guide tree analysis of human CD112R and the known PVR-like proteins via the Clustal W program in MacVector 6.5. (D) Multiple sequence alignment of the IgV domains of PVR-like proteins. Similar and identical residues among this group are shaded in red. The PVR signature motifs are outlined in green frames. Blue boxes mark conserved amino acids. (E) A predicted protein structure model of human CD112R IgV domain (55–150 aa) using human PVRL4 (Protein Data Bank accession no. 4JJH) as the template.

phatases (Billadeau and Leibson, 2002). The extracellular domain sequence of human and mouse CD112R have ~65.3% similarity (Fig. 1 B). Phylogenetic tree analysis of the first IgV of the PVR family reveals that CD112R is close to PVR-like proteins (Fig. 1 C). Alignment of the amino acid sequence indicates that the IgV domain of CD112R contains residues conserved among the PVR family (Fig. 1 D). These residues constitute at least three main motifs shared among the PVR family: Val, Ile–Ser, and Thr–Gln at position 72–74 aa of CD112R, Ala89–X6–Gly96, and Tyr139 or Phe139–Pro140–X–Gly142 (Yu et al., 2009). Using the first IgV domain of PVRL4 as a template, we constructed a structural model of CD112R. CD112R seems to adapt a V-set Ig fold consisting of a series of  $\beta$  sheets (Fig. 1 E).

Based on the mRNA expression data from BioGPS, the CD112R gene is preferentially transcribed in lymphocytes, including T lymphocytes and NK cells (unpublished data). Consistently, the CD112R gene is one of the genes heavily enriched in T cell subsets and NK cells (Benita et

al., 2010). We confirmed the CD112R expression in human immune cells by reverse transcript PCR (Fig. 2 A). Human DCs derived from monocytes did not express CD112R, whereas both NK and T cells contain a substantial amount of CD112R transcript. The expression of CD112R is further up-regulated in T cells upon activation. To further examine the expression of CD112R protein, we generated a mAb (clone 2H6) against human CD112R by immunizing mice with purified CD112R–Fc recombinant protein. The specificity of CD112R mAb was verified by its binding to CD112R transfectants by flow cytometry (Fig. 2 B) and Western blotting (not depicted). We found that CD112R is a monomer in cells because under reducing and nonreducing conditions, we detected CD112R protein at the expected size of a 36-kD monomer from lysates of CD112R transfectant (Fig. 2 C). B cells (CD19<sup>+</sup>), monocytes (CD14<sup>+</sup>), and neutrophils (CD66b<sup>+</sup>) freshly isolated from normal human blood do not express surface CD112R. A significant population of T (CD3<sup>+</sup>) and NK (CD56<sup>+</sup>) cells expresses low but detectable

surface CD112R, although the percentage of CD112R-expressing T cells varies in different donors (Fig. 2 D). Further analysis reveals that both CD16<sup>+</sup> and CD16<sup>-</sup> NK cell subsets express CD112R (Fig. 2 E). The majority of CD112R<sup>+</sup> T cells in the blood of healthy donors are CD8<sup>+</sup> T cells (Fig. 2 F). Phenotypic analysis indicates that they are mainly memory/effector T cells, as very few of CD112R-expressing cells are naive T cells (CD45RA<sup>+</sup>CCR7<sup>+</sup>; Fig. 2 G). CD4<sup>+</sup> T helper cells from fresh human blood do not express CD112R, but surface CD112R can be up-regulated upon stimulation (Fig. 2 H).

### Signal through CD112R inhibits TCR-mediated signal

We tested whether CD112R could serve as a T cell coreceptor to regulate T cell response. We examined whether the two tyrosines in the intracellular domain of CD112R (Fig. 1 A) can be phosphorylated to transduce a signal. We generated two CD112R mutants (Y293F and Y233F) via site-directed mutagenesis. HEK293T cells were transfected with WT or CD112R mutants and then treated with pervanadate. Interestingly, there was a significant amount of phosphorylated tyrosine signal of the CD112R protein, even without pervanadate treatment (Fig. 2 I). Pervanadate treatment further increased tyrosine phosphorylation of the CD112R protein, indicating that tyrosines in the CD112R intracellular domain are capable of being phosphorylated and therefore are able to mediate signal transduction. We also found that single mutation of tyrosine 233 to phenylalanine significantly reduced pervanadate-induced CD112R phosphorylation, whereas mutation at Y293 only had a little effect (Fig. 2 I). Because Y233 is within an ITIM-like motif, we further evaluated the potential interactions between CD112R and tyrosine phosphatases. We used the Molt4 cell line for this study, as this T cell leukemia cell line expresses a high level of CD112R (unpublished data). SHIP was strongly associated with CD112R in untreated Molt4 cells, and pervanadate treatment further increased this interaction (Fig. 2 J). SHP-1 and SHP-2 weakly associated with CD112R in untreated Molt4 cells, but these associations were enhanced greatly upon pervanadate treatment (Fig. 2 J). All of these results suggest that CD112R is able to recruit tyrosine phosphatases for signal transduction.

In addition, we investigated whether a signal through CD112R regulates TCR-mediated signals. We examined the NFAT pathway, which is strongly induced upon T cell activation and regulated by costimulatory signals (Chen and Flies, 2013). We used a Jurkat cell line (Jurkat-NFAT-Luc), which is stably transfected with a luciferase reporter under the control of the NFAT response element. By taking advantage of the well-known characteristics of an agonistic mouse CD28 mAb (clone 37.51), we constructed two chimeric molecules: the mCD28/hCD28 chimera composed of the extracellular domain of mouse CD28 and the transmembrane and intracellular domains of human CD28, and the mCD28/hCD112R chimera, which contains mouse CD28 extracellular domain and the transmembrane and intracellular domains of human

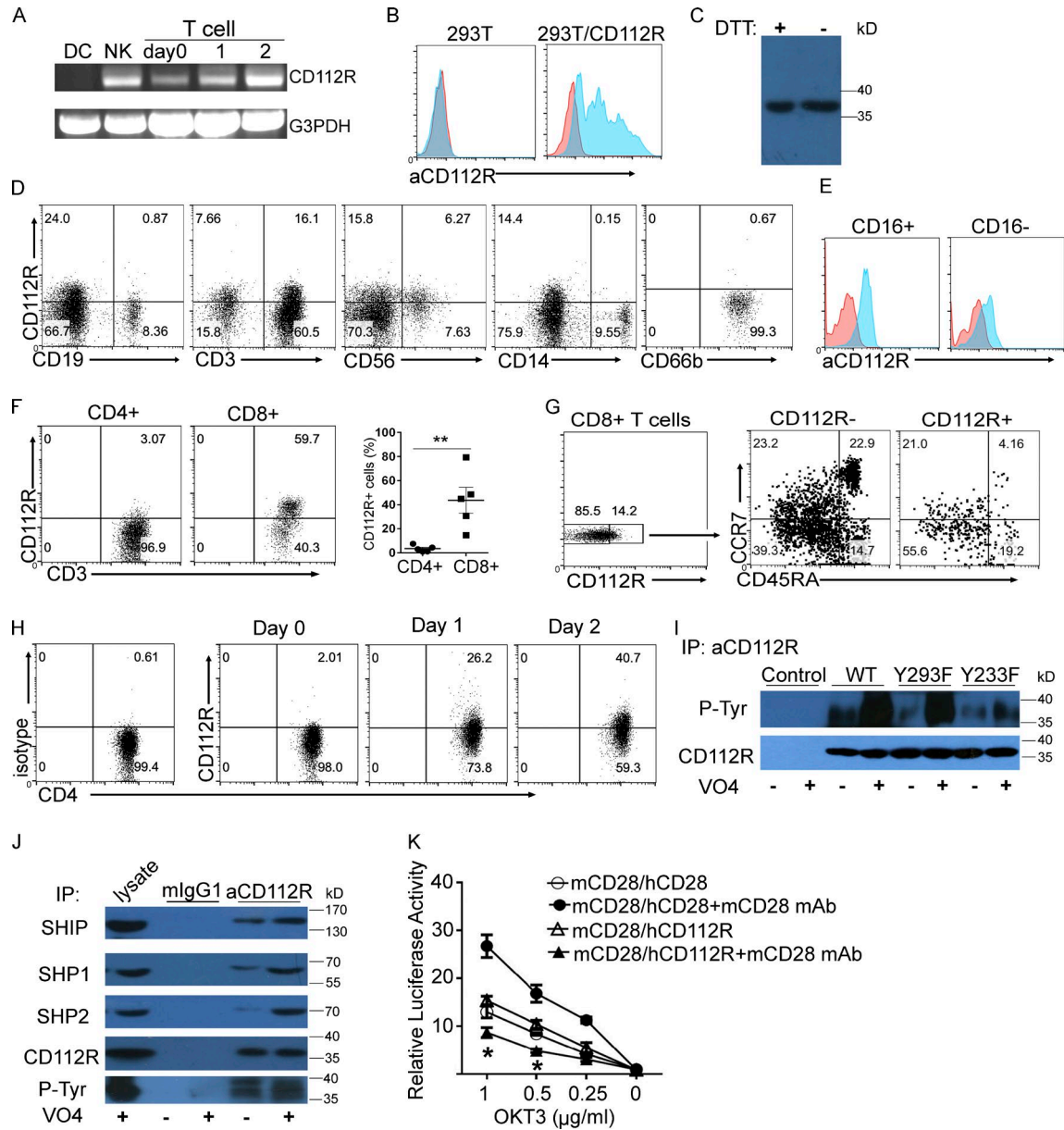
CD112R. Jurkat-NFAT-Luc cells were transfected with these two chimeric molecules, and cells expressing mouse CD28 were selected by flow cytometry sorting. We stimulated these two cell lines with human CD3 mAb (OKT3) together with control antibody or mouse CD28 mAb. The addition of the mouse CD28 mAb can cross-link the chimera, resulting in amplification of the intracellular signal of the chimeras. As expected, the addition of mCD28 mAb to mCD28/hCD28 transfectant amplified human CD28 signal and increased NFAT activity upon TCR stimulation. However, inclusion of mCD28 mAb in mCD28/hCD112R-expressing cells significantly inhibited the luciferase activation, suggesting that signal through CD112R inhibits TCR-mediated NFAT activation (Fig. 2 K). Thus, our results suggested that CD112R could be a new coinhibitory receptor that suppresses TCR signal.

### DCs and the majority of human cancer lines express a putative ligand for CD112R

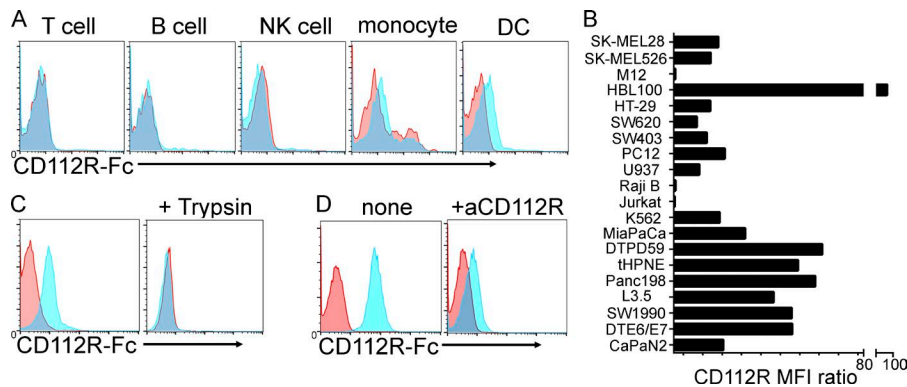
To identify the interacting partner for CD112R, we first looked for the presence of a potential ligand on human cells. We generated a CD112R-Fc fusion protein by cloning the extracellular domains of CD112R into an expression vector containing the constant region of mouse IgG2a. We stained immune cells from human peripheral blood with CD112R fusion protein for possible binding by flow cytometry. CD112R protein did not interact with T, B, or NK cells (Fig. 3 A). However, it had slight binding with human monocytes, suggesting the presence of a putative ligand for CD112R on human monocytes. Consistently, this interaction became more obvious when human monocyte-derived DCs were stained by CD112R protein (Fig. 3 A). We also stained human tumor cell lines with CD112R fusion protein for possible CD112R ligand. Virtually all of the adherent tumor cell lines had a strong binding signal to CD112R protein, suggesting a possible ligand on cancer cells for CD112R (Fig. 3 B). In contrast, most tumors of hematopoietic origin did not interact with CD112R. Our data implied that the putative ligand for CD112R on tumor cells could be a surface molecule mediating cellular adhesion. Another point of interest is that this potential ligand is sensitive to trypsin cleavage, as when we treated cancer cells with trypsin for a long period of time (>10 min), the tumor cells completely lost the CD112R-binding capacity (Fig. 3 C). CD112R protein bound to HEK293T cells strongly, and inclusion of a CD112R mAb (clone 2H6) blocked this interaction, further confirming the specificity of this interaction with CD112R protein (Fig. 3 D). These results suggest the presence of a putative surface ligand for CD112R on the majority of tumor cells and DCs.

### CD112 is the ligand for CD112R

The presence of a putative ligand for CD112R on cancer cells and its regulatory function on T cells led us on the path to identifying this specific ligand. Because CD112R is an Ig-containing protein, we predicted that the binding partner for CD112R should also be a member of IGSF. We tested



**Figure 2. CD112R expression in immune cells and its effect on TCR signal.** (A) Human CD112R transcript in human immune cells. RNAs were isolated from DCs, NK cells, and T cells stimulated by OKT3 plus CD28 mAb. The expression of CD112R was detected by PCR. G3PDH was used as a housekeeping gene. (B) HEK293T cells transduced with control or CD112R gene were stained with control (red) or CD112R mAb (clone 2H6; blue). (C) Cell lysate of HEK293T/CD112R transfectant was run in reducing (+DTT) and nonreducing (-) conditions and detected by CD112R mAb (clone 2H6). (D) Flow cytometry analysis of CD112R expression in human peripheral blood from healthy donors ( $n = 4$  donors) stained with indicated cell surface markers. (E) CD112R expression on different NK cell subsets: CD16<sup>+</sup> (CD56<sup>+</sup>CD16<sup>+</sup>) and CD16<sup>-</sup> (CD56<sup>+</sup>CD16<sup>-</sup>). The expression of CD112R (blue) in these two NK subsets is shown. (F) The CD112R expression on CD4<sup>+</sup>CD3<sup>+</sup> and CD8<sup>+</sup>CD3<sup>+</sup> T cell subsets. Graph (right) shows mean  $\pm$  SD frequencies of CD112R-expressing cells in each subset. (G) CD8<sup>+</sup> T cells were divided into two groups based on the expression of CD112R, and their expression of CD45RA and CCR7 was revealed. (H) Purified CD4<sup>+</sup> T cells were left unstimulated (day 0) or activated by anti-CD3/CD28 Dynabeads for different times, and the CD112R expression on T cells was detected by biotinylated CD112R mAb. (I) HEK293T cells were transiently transfected with WT or tyrosine mutants of CD112R. Cells were treated with or without pervanadate before analysis for tyrosine phosphorylation on CD112R. (J) Molt4 cell lysates were immunoprecipitated with CD112R mAb or mouse IgG1 (control) and blotted with different phosphatase mAbs as indicated. The presence of CD112R and tyrosine phosphorylation was demonstrated by immunoblotting with CD112R and phosphorylated tyrosine (P-Tyr) mAbs, respectively. Whole cell lysate serves as a detective control. (K) Jurkat-NFAT-Luc cells transfected with different chimeras as indicated were stimulated with OKT3 in the presence or absence of a mouse CD28 agonistic mAb. Data shows mean  $\pm$  SD of relative luciferase activity upon 4 h of stimulation. All data shown are representative of at least two independent experiments. IP, immunoprecipitation. F and K were analyzed by Student's *t* test; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .



**Figure 3. Expression of a putative ligand for CD112R.** (A) Immune cells in human blood and monocyte-derived DCs were stained with control (FLAG-Fc; red) or CD112R-Fc (blue) protein by flow cytometry. Immune cells were gated on individual lineage markers. T cell: CD3; B cell: CD19; NK cell: CD56<sup>+</sup>CD3<sup>-</sup>; monocyte: CD14; DC: CD11c. (B) Cultured human tumor cell lines were stained with control (FLAG-Fc) and CD112R protein, and the median fluorescence intensity (MFI) was determined by flow cytometry. CD112R MFI ratios (CD112R MFI/control MFI) are indicated. (C) SK-MEL28 melanoma line with or without trypsin treatment (10 min) was stained with control (FLAG-Fc; red) or CD112R-Fc (blue) protein by flow cytometry. (D) Blockade of CD112R binding by a CD112R mAb. HEK293T cells were stained with control (FLAG-Fc; red) or CD112R-Fc (blue) protein by flow cytometry with or without the presence of a CD112R mAb (clone 2H6). All data shown are representative of at least three independent experiments.

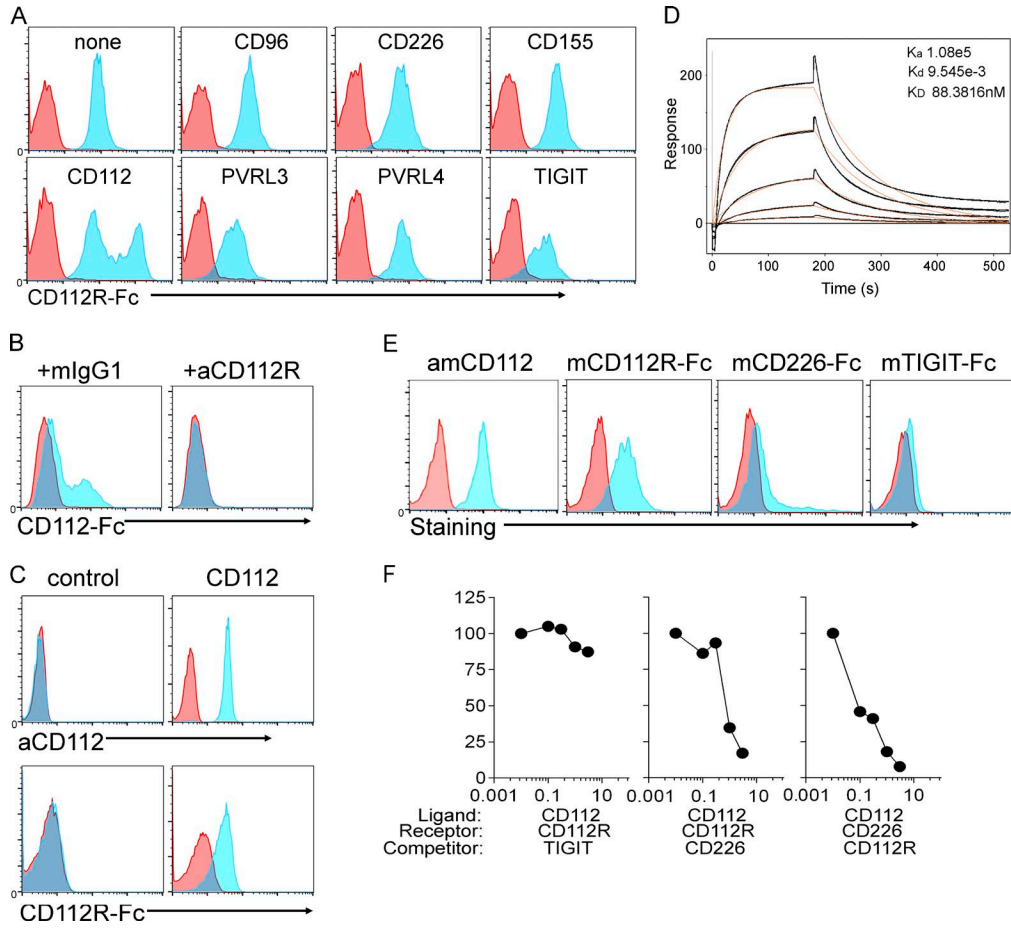
several groups of IGSF genes with known T cell modulatory functions, including the B7 family, butyrophilin-like molecules, T cell Ig mucin family, and PVR-like molecules (Zhu et al., 2011). We did not find any member from the B7, butyrophilin-like, or T cell Ig mucin family bound to CD112R protein (unpublished data). However, when PVR members were individually transduced into HEK293T cells, we detected an even stronger binding peak as CD112 (also called PVRL2/nectin-2) was transfected onto HEK293T cells (Fig. 4 A). This suggested that CD112 could be a binding partner for CD112R. We further verified this interaction by producing CD112-Fc fusion protein to stain CD112R-expressing cells. CD112 fusion protein bound to CD112R transfectant but not to control HEK293T cells. The inclusion of CD112R mAb was able to block this interaction, further demonstrating the specificity of this interaction (Fig. 4 B).

To determine whether CD112R directly interacts with CD112, we coated beads with CD112 or control protein. The presence of CD112 protein on coated beads was confirmed by CD112 mAb staining. CD112R protein bound to CD112-coated beads but not to control beads, demonstrating that CD112R directly interacts with CD112 (Fig. 4 C). Biacore measurement of this interaction revealed that the  $K_d$  of the CD112-CD112R interaction was 0.088  $\mu$ M (Fig. 4 D), which is much higher than the interaction between CD112 and CD226 ( $K_d = 8.97 \mu$ M or  $K_d = 0.31 \mu$ M) with similar measurement methods (Tahara-Hanaoka et al., 2006; Liu et al., 2012). The interaction between TIGIT and CD112 was too weak to determine the affinity by our Biacore experiments. Therefore, our results support that CD112R is a new receptor for CD112 with higher affinity than CD226.

We also verified that the CD112R-CD112 interaction is conserved in mice. We transduced RMA-S lymphoma cells with mouse CD112 gene to generate a stable cell line expressing surface mouse CD112 (RMA-S/mCD112; Fig. 4 E, left). We stained these cells with several mouse PVR-like receptor fusion proteins for binding. As shown in Fig. 4 E, mouse CD112R fusion protein bound strongly to RMA-S/mCD112 cells, but not to mock RMA-S transfectant. Mouse CD226 fusion protein weakly interacted with RMA-S/mCD112 transfectant, whereas the interaction of TIGIT protein with RMA-S/mCD112 cells was negligible (Fig. 4 E). Collectively, our studies suggest that CD112R is the receptor for CD112 with the highest affinity both in human and mouse.

#### Competitive analysis between CD112R and other PVR member interactions

We investigated whether CD226 and TIGIT, two known receptors for CD112, compete with CD112R for binding. We coated CD112 protein on beads and stained with CD112R protein in the presence of different concentrations of TIGIT or CD226 protein. Inclusion of TIGIT had little effect on disrupting this interaction, whereas CD226 was a good inhibitor of the CD112-CD112R binding (Fig. 4 F). This result is consistent with the relatively higher affinity between the CD112-CD226 pair than the CD112-TIGIT interaction (Yu et al., 2009; Martinet and Smyth, 2015). However, when CD112R was used as a competitor, the CD112-CD226 interaction was significantly inhibited even in a relatively low concentration. Thus, our competition studies indicate that CD112R and CD226 share a common binding site on CD112. This conclusion was further supported by our studies that a CD112



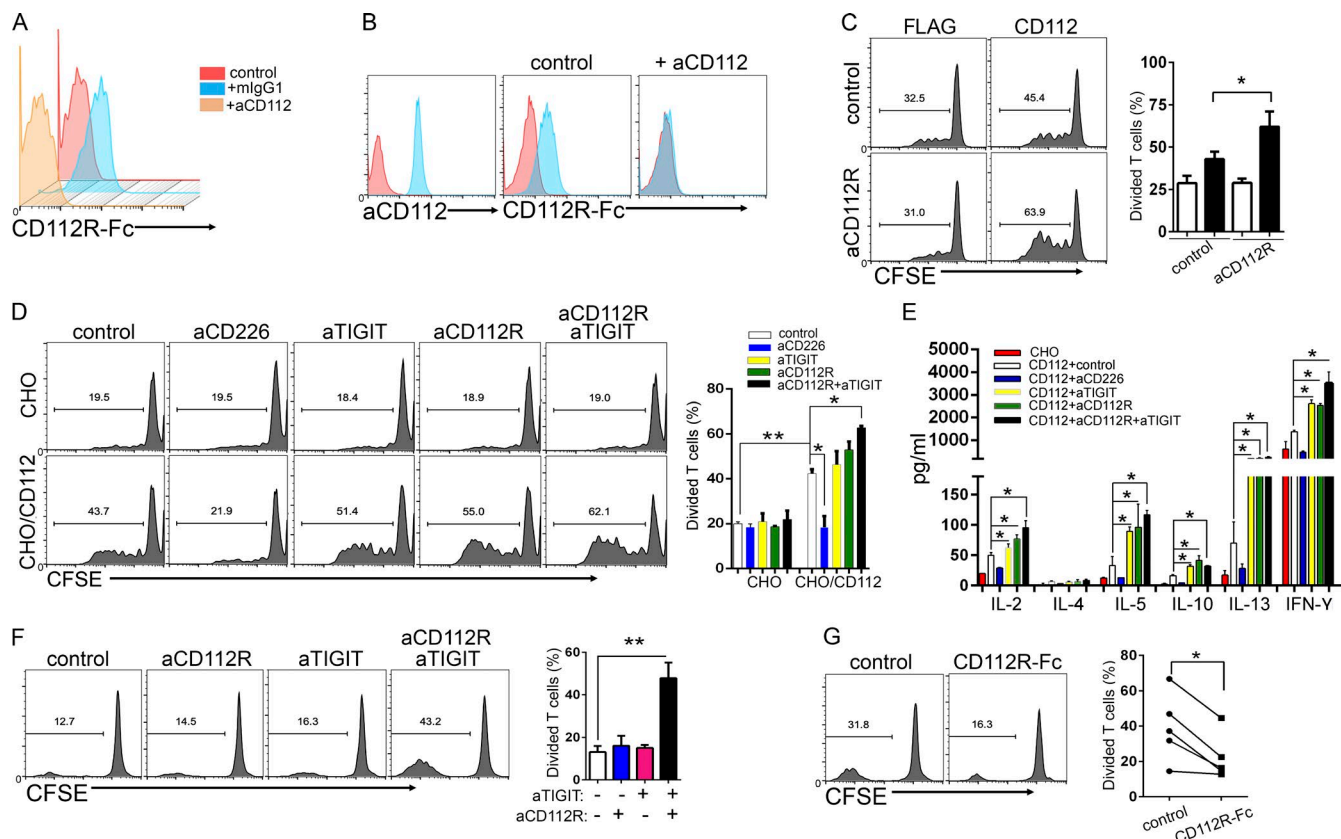
**Figure 4. Identification of CD112 as a ligand for CD112R.** (A) HEK293T cells were transiently transfected with different PVR-like gene plasmids as indicated and stained with control (FLAG-Fc; red) or CD112R-Fc (blue) protein. (B) HEK293T cells transfected with CD112R gene were incubated with anti-CD112R mAb (clone 2H6) or control mlgG1 as indicated before being stained with CD112-Fc (blue) or control FLAG-Fc (red). (C) Beads coated with CD112 (right) or control protein (left) were stained with CD112 mAb (blue) or isotype control (red) to confirm the presence of CD112 on beads. Beads were also incubated with CD112R fusion protein (blue) or control (red) for direct interaction. (D) Biacore 3000 analysis of CD112R binding to CD112. The surface plasmon resonance sensorgrams were recorded with threefold serial dilutions starting at the highest concentration of 333 nM. The fitting curves are in orange. (E) RMA-S/mCD112 (blue) or control RMA-S (red) cells were stained for binding by mCD112 mAb or mCD112R, mCD226, and mTIGIT fusion protein, respectively. (F) Competitive binding assay for CD112 among CD112R, CD226, and TIGIT proteins. Beads coated with CD112 were stained by CD112R-Fc protein in the presence of different concentrations of TIGIT or CD226 protein, whereas beads coated with CD112 were stained by CD226-Fc in the presence of different concentrations of CD112R protein. All data shown are representative of at least two independent experiments.

mAb (clone TX31) blocked the binding of CD112 to both CD112R and CD226 (Fig. 5 A and not depicted).

**CD112 mediates the CD112R binding to DCs and tumor cells**

Our initial studies revealed that CD112R protein binds to many cell types, including DCs and human cancer cells. Here, we further investigated whether CD112 was the surface molecule responsible for the CD112R interaction. Human T, B, or NK cells do not express any detectable CD112 protein, whereas monocytes express a significant level of surface CD112. Human monocyte-derived DCs express high levels of CD112, and this expression can be further up-regulated by TLR agonists (unpublished data). Therefore, the expression profile of CD112 on immune cells is consistent with the

pattern of CD112R binding (Fig. 3 A). The CD112R interaction with DCs was completely blocked when DCs were preincubated with a CD112-blocking mAb (clone TX31), implying that CD112 on DCs mediated the CD112R interaction (Fig. 5 A). We also found that CD112 expression on tumor cells correlates with that of CD112R protein binding. The majority of adherent tumor cells constitutively expressed a high level of CD112, whereas most tumor cells of hematopoietic origin were CD112 negative (not depicted). To directly confirm that CD112 is the ligand mediating the interaction, we preincubated tumor cells with CD112-blocking mAb and stained them with CD112R protein. As the representative result in Fig. 5 B shows, inclusion of a CD112-blocking mAb completely eliminates the CD112R binding to the human



**Figure 5. CD112 binds to CD112R to inhibit T cell response.** (A) Human monocyte-derived DCs stimulated with LPS overnight were preincubated with mlgG1 or CD112 mAb (clone TX31) and then stained for CD112R protein binding. DCs stained with control FLAG protein are shown in red. Data shown are representative of three different experiments ( $n = 3$  donors). (B) Human pancreatic cell line PANC198 was stained with isotype control (red) or CD112 mAb (blue) for CD112 expression (left). Cells were preincubated with control mlgG1 or CD112 mAb (clone TX31) before being stained by control (FLAG-Fc; red) or CD112R fusion protein (blue). Data shown are representative of two independent experiments. (C) Purified human T cells were CFSE labeled and stimulated with OKT3 together with plate-coated CD112-Fc or control protein (FLAG-Fc). Control (mouse IgG1) or CD112R mAb was added during cell culture. Cells were gated on CD8<sup>+</sup> T cells, and their division was analyzed based on the dilution of CFSE. The CFSE-diluted cells indicated were counted as divided T cells. Data shown are representative of at least three independent experiments ( $n > 3$  donors). (D and E) CFSE-labeled CD4<sup>+</sup> T cells were cultured with mytomycin-treated CHO stimulators expressing CD112 or control CHO stimulator cells. Antibodies as indicated were added from the beginning of culture. After 5 d of culture, cell division was analyzed based on the dilution of CFSE (D). (E) IL-2 (day 2) and other cytokines (day 5) in the supernatant were measured by a human T helper cytokine panel. Data are representative of three independent experiments. (F) Purified human T cells were labeled with CFSE and were co-cultured with autologous DCs in the presence of TT. Control (mouse IgG1), CD112R mAb, or TIGIT mAb was included at the beginning of the culture. The proliferation of TT-specific CD4<sup>+</sup> T cells was determined by CFSE dilution of the human CD3 and CD4 double-positive cells. Data are representative of three independent experiments ( $n = 3$  donors). (G) In the same culture condition as in F, CD112R-Fc or control protein (FLAG-Fc) was included at the beginning to examine the effect on TT-specific T cell response.  $n = 5$ . \*,  $P < 0.05$  using two-way ANOVA. All bar graphs in C–F represent the mean  $\pm$  SD results; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (Student's *t* test). The numbers in the histograms in C, D, F, and G refer to the percentages of divided T cells.

pancreatic cancer cell line PANC198. In all of the tumor cell lines ( $n > 8$ ) we tested so far, preincubating tumor cells with the CD112-blocking mAb completely prevented CD112R fusion protein from binding (not depicted). Furthermore, PVR-like proteins are known to mediate heterointeractions among members (Takai et al., 2008; Martinet and Smyth, 2015). The presence of high-affinity ligand CD112 on the majority of cell types could have hidden any possible weak binding between CD112R and other PVR members. We coated beads with individual PVR-like proteins and stained for CD112R protein binding. No PVR-like protein except

CD112 was able to interact with CD112R protein (unpublished data). Collectively, our studies support that CD112 is the main ligand, if not the only one, that mediates the interaction of CD112R with DCs and tumor cells.

#### CD112 interacts with CD112R to suppress T cell response

To test the potential function of the CD112–CD112R interaction on T cell response, we labeled purified human T cells with CFSE and stimulated them with plate-coated CD112-Fc in the presence of human CD3 mAb (Fig. 5 C). Immobilized CD112-Fc modestly increased human T cell

division, as revealed by dilution of CFSE dye. This costimulatory effect of CD112 on T cell response could be mediated through CD226, a known T cell costimulatory receptor for CD112 (Shibuya et al., 2003; Tahara-Hanaoka et al., 2004). Inclusion of a CD112R-neutralizing mAb (clone 2H6; Fig. 4 B) further enhanced the costimulatory effect of CD112 (Fig. 5 C), indicating that CD112 interacts with CD112R to inhibit T cell proliferation.

Similarly, when T cells were stimulated by cellular-based CD112 (a Chinese hamster ovary [CHO] cell stimulator), we saw a significant increase in CD4<sup>+</sup> T cell division (Fig. 5 D). CD226 is the costimulatory receptor responsible because inclusion of a CD226-blocking mAb completely eliminated the effect. Inclusion of either a TIGIT- or CD112R-blocking mAb slightly promoted this expansion, whereas the combination of these two antibodies significantly enhanced T cell proliferation (Fig. 5 D). As a result, the combinatory blockade of CD112R and TIGIT significantly promoted the secretion of cytokines, including IL-2, IL-5, IL-10, IL-13, and IFN- $\gamma$  (Fig. 5 E). Similarly, the combinatory blockade of CD112R and TIGIT significantly promoted the expansion of CD8<sup>+</sup> T cells (not depicted). When we cultured naive CD4<sup>+</sup> human T cells with CHO stimulator to further look at the potential effect of CD112R on CD4<sup>+</sup> T helper cell differentiation, the combination of CD112R and TIGIT mAbs was able to increase the frequency of IFN- $\gamma$ - and IL-17-producing T cells (not depicted).

To further evaluate the function of endogenous CD112-CD112R interaction on the T cell response, we examined the effect of this pathway in an antigen-specific T cell response. Purified human T cells were labeled with CFSE and cultured with autologous monocyte-derived DCs in the presence of tetanus toxoid (TT). The inclusion of CD112R- or TIGIT-blocking mAb alone had a minor effect on TT-specific T cell response. However, the combination of CD112R and TIGIT mAbs was able to significantly augment T cell proliferation (Fig. 5 F), demonstrating a synergistic effect of these two inhibitory receptors on T cell response. But the addition of CD112R-Fc fusion protein modestly inhibited T cell proliferation in the same model (Fig. 5 G), further confirming an overall positive effect of CD112 on T cells (Fig. 5 C).

Collectively, our results suggest that CD112R is a new coinhibitory receptor for CD112. Therefore, previous studies about CD112 function should be reevaluated in the context of CD112R. The molecular and functional relationships among the receptors for CD112, including CD112R, CD226, and TIGIT, have yet to be fully explored.

## MATERIALS AND METHODS

**Cloning and bioinformatics analysis of CD112R.** Human CD112R (also called PVRIG; NCBI Nucleotide database accession no. BC073861) cDNA was cloned from human thymus tissue cDNAs (Takara Bio Inc.) by PCR. The full-length coding region was further put into a pcDNA3.1(-) expression vector by restricted enzyme digestion. Mouse

CD112R gene (Gm36869; NCBI Gene ID 102640920) was identified by searching for CD112R orthologue (HomoloGene; National Center for Biotechnology Information). Mouse CD112R cDNA (NCBI Reference Sequence accession no. XM\_011240964.1) was synthesized from GenScript and cloned onto a pcDNA3.1(-) expression vector.

The domain structure of human CD112R was analyzed by the SMART interface (<http://smart.embl-heidelberg.de/>). CD112 orthologous proteins were identified and collected from the NCBI HomoloGene database. Sequence alignments of the extracellular domains of human CD112R and other PVR-like proteins were analyzed via the Clustal W program in MacVector 6.5 (MacVector, Inc.). PVRL4 (Protein Data Bank accession no. 4JJH) was selected as the template for comparative protein structure modeling. The structural model of the IgV domain of CD112R was constructed with the multiple mapping method server using the optimal combination of two alignment methods, MUSCLE (European Bioinformatics Institute) and HHalign (Max Planck Institute).

**Fusion proteins and antibodies.** The extracellular domains of CD112R and other PVR-like molecules were cloned and fused into a pMIgV expression vector containing the constant region of mouse IgG2a. Fusion proteins were expressed by transiently transfecting the freestyle HEK293F cells using the polyethylenimine transfection method, and fusion proteins were purified for supernatant using a protein A-Sepharose column according to the manufacturer's instructions (GE Healthcare).

Mouse anti-human CD112R (clone 2H6; IgG1) was generated from a hybridoma derived from the fusion of SP2 myeloma with B cells from a mouse immunized with human CD112R-Fc. Hybridoma was adapted and cultured in Hybridoma-serum-free media (Life Technologies). Antibodies in supernatant were purified by HiTrap protein G affinity column (GE Healthcare). LEAF purified mouse IgG1 (clone MG1-45) and functional grade human CD112 mAb clone TX31 were purchased from BioLegend. Functional grade human TIGIT mAb (clone MBSA43) was purchased from eBioscience. Human CD226 mAb (clone DX11) was purchased from Abcam. All other antibodies used in flow cytometry were purchased from BD, eBioscience, R&D Systems, or BioLegend.

**Immunoblotting.** Mutants for the two tyrosines (Y233 and Y293) in human CD112R intracellular domain were made by changing respective tyrosine to phenylalanine. Assays for pervanadate-induced tyrosine phosphorylation were performed as previously described (Zhu et al., 2013). In brief, HEK293T cells transfected with individual plasmid were incubated with pervanadate for 10 min before lysis. Cell lysates were immunoprecipitated with CD112R mAb (clone 2H6) and protein G magnetic beads (Invitrogen). After SDS-PAGE, blots were analyzed for phosphotyrosine using 4G10 (EMD Millipore) or CD112R mAb (clone 2H6).



Molt4 cell, a T cell leukemia cell line expressing CD112R, was used to analyze the association of CD112R with possible phosphatases. In brief, Molt4 cells were incubated with pervanadate before being lysed in radioimmuno-precipitation assay buffer. Cell lysate was immunoprecipitated with anti-CD112R (clone 2H6). The possible associated phosphatases were detected by the following antibodies: anti-SHP-1 (Santa Cruz Biotechnology, Inc.), anti-SHP-2 (Santa Cruz Biotechnology, Inc.), and anti-SHIP (Santa Cruz Biotechnology, Inc.).

**Biacore assay.** All biosensor experiments were run on a Biacore 3000 instrument (GE Healthcare). PBS with 0.005% P20 buffer (Gibco) was used as the running buffer for both the immobilization and kinetics experiments. Amine-coupling chemistry was used to immobilize protein FLAG, CD112, and CD155 to a CM5 sensor chip surface at 25°C. Kinetic experiments were carried out with threefold serial dilutions of CD112R: 4, 12, 36, 111, and 333 nM. All samples were diluted in PBS buffer and were injected for 3 min across the surface at a flow rate of 20  $\mu$ l/min, and the dissociation of analyte from the surface-bound ligands was monitored for 5 min. All analyte concentrations were performed in duplicate. Buffer blanks were used to double reference the obtained kinetic data. Raw sensogram data were processed and fit using the Scrubber software package (version 2.0b; Biological Software).

**Jurkat-NFAT-Luc activation assay.** The mCD28/hCD28 and mCD28/hCD112R chimeras were generated by PCR and cloned into a pcDNA3.1(-) expression vector. We transduced chimera genes into Jurkat cells stably expressing a luciferase reporter under the control of NFAT response element (Jurkat-NFAT-Luc; Promega). Transfectants were selected with Zeocin and further enriched by flow cytometry sorting. Transfected Jurkat cells were stimulated with coated human CD3 mAb (OKT3) for 4 h with or without mouse CD28 mAb (clone 37.51). The presence of mouse CD28 mAb acts as an agonist to amplify signals transduced by the intracellular domain of the chimeras. After stimulation, cells were lysed with the ONE-Glo Luciferase Assay System (Promega) and measured for luminescent signal instantly.

**T cell proliferation assay.** Human blood from healthy donors was obtained from the Bonfils Blood Center in Denver, CO. OKT3 mAb (anti-human CD3) was precoated in the 96-well plates at the indicated concentrations. CD112-Fc or control protein FLAG-Fc at 5  $\mu$ g/ml was also immobilized in the wells. Human T cells were negatively selected and purified by a human pan-T cell selection kit or naive human CD4 T cell selection kit (Miltenyi Biotec). T cells were CFSE labeled, added into each well at  $2.5\text{--}3 \times 10^5$  per well, and cultured for 3 d. Cells were collected and stained with cell surface markers before flow cytometry analysis.

For cellular-based T cell activation assay, CFSE-labeled T cells were stimulated with stimulator cells (CHO cells ex-

pressing membrane-bound anti-CD3 mAb fragments; Leitner et al., 2010). Stimulator cells expressing human CD112 and control stimulator cells were established by transfection and followed with flow cytometry sorting. Stimulator cells were treated with mitomycin C before being co-cultured with CFSE-labeled human T cells at the ratio of 1:5. Control (mouse IgG1) or blocking mAbs against different PVR-like proteins were added at the beginning of the culture. T cell proliferation was assessed by CFSE dilution after 5-d culture. IL-2 (day 2) and other cytokines (day 5) in supernatant were measured by a human T helper cytokine panel (LEGENDplex; BioLegend). For intracellular cytokine production, cultured T cells were restimulated with PMA<sup>+</sup> ionomycin for 4 h to detect intracellular cytokines.

**TT-specific human T cell response.** For in vitro TT stimulation, autologous DCs were co-cultured with CFSE-labeled purified human T cells at different ratios in the presence of 50 ng/ml TT (List Biological Laboratories) for 10–14 d. Antibodies or fusion proteins were added from the beginning of culture. Cell division of human CD4<sup>+</sup> T cells was examined by FACS for CFSE dilution as described previously (Zhu et al., 2013).

**Statistical analysis.** Student's *t* test was used for statistical analysis, and *p*-values reflect comparison with the control sample. *P*-values <0.05 were considered statistically significant. The error bars in figures represent SD.

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