Supporting Information

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SI Text

Generation of Fusion Proteins. The coding sequence of TIGIT was cloned as a fusion to the Fc fragment of IgG1 to generate the recombinant Fc-fused protein. The C-terminal Gln of TIGIT at amino acid 139 position of the TIGIT was fused to the heavy chain hinge of a deglycosyltaed Fc (N297A), followed by the CH2 and CH3 regions. The ORF of the recombinant protein was codon optimized for high-level expression in mammalian cells. The optimized DNA sequence was synthetically synthesized by GeneArt with the addition of flanking DNA sequences corresponding to EcoRI and NotI restriction sites at the 5′ and 3′ ends of the DNA fragment, respectively. The expression vector was constructed by double digestion of the optimized DNA fragment with EcoRI and NotI, followed by its ligation into pIRESpuro3 (Clontech Laboratories, Inc.). For the production of DNAM-1-Ig fusion protein, the sequence encoding the extracellular portion of DNAM-1 was amplified by PCR using the 5′ primer CCGATATCGCCGCCACCATGGATTATCCTACTTTACTTTTG (including EcoRV restriction site) and the 3′ primer CGGATCCACAAAGAGGGTATATTGGTTAT (including BamHI restriction site). This PCR-generated fragment was cloned into the expression vector containing the mutated Fc portion of human IgG1 (Fc mut pIRESpuro3).

The resulting constructs were transfected into HEK-293T cells by using the FuGENE 6 Transfection Reagent (Roche Diagnostics). After 48 h, transfected cells were subjected to antibiotic selection with 5 μg/mL puromycin (Sigma–Aldrich). Stable pools were analyzed for protein secretion by SDS/PAGE. Supernatants were collected and purified on a Poros 20 protein G column in the High Pressure Perfusion Chromatography Station, BioCAD (PerSeptive Biosystems).

Generation of Transfectants. For the generation of 721.221 cells expressing PVR, the PVR cDNA was amplified by PCR using the 5′ primer CCCAAGCTTGCCGCCACCATGGCCGCCGCGTGGC (including HindIII restriction site) and the 3′ primer GGTCTAGATCACCTTGTGCCCTCTGTCTG (including XbaI restriction site). For the generation of 721.221 cells expressing PVRL2, the PVRL2 cDNA was amplified by PCR by using the 5′ primer CCCAAGCTTGCCGCCACCATGGCCCGGGCCGCTGCC (including HindIII restriction site) and the 3′ primer CGGGCCCTCACACATACATGGCCCGGGA (including ApaI restriction site). For the generation of 721.221 cells expressing PVRL3, the PVRL3 cDNA was amplified by PCR using the 5′ primer TATAAGCTTGCCACCATGGCGCGGACCCTGCGG (including HindIII restriction site) and the 3′ primer GGTCTAGACTAAACATACCACTCCCTCCTG (including XbaI restriction site). The PVR, PVRL2 and PVRL3 constructs were cloned into pcDNA3 vector (Invitrogen Life Technologies) and was stably expressed in 721.221 cells.

For the generation of BW cells expressing the chimeric TIGIT-ζ protein, the extracellular portion of the human TIGIT protein was amplified by PCR from cDNA isolated from peripheral blood lymphocytes using the 5′ primer CGAATTCGCCGCCACCATGCGCTGGTGTCTCCTCC (including EcoRI restriction site) and the 3′ primer TCCATCTAGCAAGTAGCAGAGTGGAATCTGGAACCTGGCACC (including the first twenty one nucleotides of the mouse ζ-chain transmembrane portion). The mouse ζ-chain was amplified by PCR using the 5′ primer GGTGCCAGGTTCCAGATTCCACTCTGCTACTTGCTAGATGGAA (including last twenty one nucleotides of human TIGIT extracellular portion) and the 3′ primer CCGAATTCTTAGCGAGGGGCCAGGGTCTG (including XbaI restriction site). The two amplified fragments were mixed, and PCR was performed with the 5′ EcoRI primer and the 3′ XbaI primer for generation of the TIGIT-ζ construct. The TIGIT-ζ construct was cloned into pcDNA3 expression vector (Invitrogen Life Technologies) and was stably expressed in BW cells.

For the generation of YTS NK tumor line expressing TIGIT fused to an HA tag the signal peptide of TIGIT was amplified by PCR using the 5′ primer CGGATCCGCCGCCACCATGCGCTGGTGTCTCCTCC (including BamHI restriction site) and the 3′ primer AGCGTAATCTGGAACATCGTATGGGTAGGGAGCCTGCCTCAGCCC (including the twenty seven nucleotides coding for the HA tag). TIGIT was amplified by PCR using the 5′ primer TACCCATACGATGTTCCAGATTACGCTCTCGCCTCAGGAATGATGACA (including the 27 nucleotides coding for the HA tag) and the 3′ primer CCTGTACACTAACCAGTCTCTGTGAAGAAG (including Bsp1407 restriction site). The two amplified fragments were mixed, and PCR was performed with the 5′ BamHI primer and the 3′ Bsp1407 primer for the generation of the TIGIT-HA construct.

For generation of the TIGIT protein mutated in Y231A, we used the TIGIT-HA construct as a template and amplified by PCR the 3′ end of the gene by using an internal 5′ primer GTCCTGAGTGCCAGAAGCCT bearing the mutation and the gene-specific 3′-edge primer (including the Bsp1407 restriction site). The PCR product was used as a 3′ primer together with the gene-specific 5′-edge primer (including the BamHI restriction site) for the generation of the mutated full-gene cDNA.

The TIGIT-HA and the two mutated constructs were then cloned into the lentiviral vector SIN18-pRLL-hEFIαp-EGFP-WRPE (1), instead of the GFP coding sequence. Lentiviral viruses were produced by transient three-plasmid transfection as described (2). These viruses were used to transduce YTS cells in the presence of polybrene (6 μg/mL). All transfectants were periodically monitored for expression by staining with the appropriate mAb.

ELISA. Ninety-six-well dishes were coated with 25 ng of protein per well in 100 mM sodium carbonate buffer, washed and blocked with 5% milk in PBS. 50 L of hybridoma culture supernatant was applied for 1 h at room temperature. Plates were washed with sodium carbonate buffer and then incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma). Plates were washed and incubated with 100 L of 1 mg/mL *p*-nitrophenyl phosphate disodium (PNPP) (Sigma). Positives clones were further confirmed by staining of BW/TIGIT-ζ and YTS/TIGIT-HA.

Flow Cytometry. Cells were stained with either mAbs or Ig fusion proteins as previously described (1).

BW Assay. Fifty thousand BW or BW-transfected cells were incubated in RPMI medium (Sigma–Aldrich) plus 10% FCS with 50,000 irradiated (3,000 rad) target cells as indicated in the figure for 48 h at 37 °C and 5% CO_2. Supernatants were collected, and the level of IL-2 was quantified by using anti-IL-2 mAb and standard ELISA (BD PharMingen).

1. Xu K, Ma H, McCown TJ, Verma IM, Kafri T (2001) Generation of a stable cell line producing high-titer self-inactivating lentiviral vectors. *Mol Ther* 3(1):97–104.

2. Kafri T, Blomer U, Peterson DA, Gage FH, Verma IM (1997) Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nat Genet* 17(3):314–317.

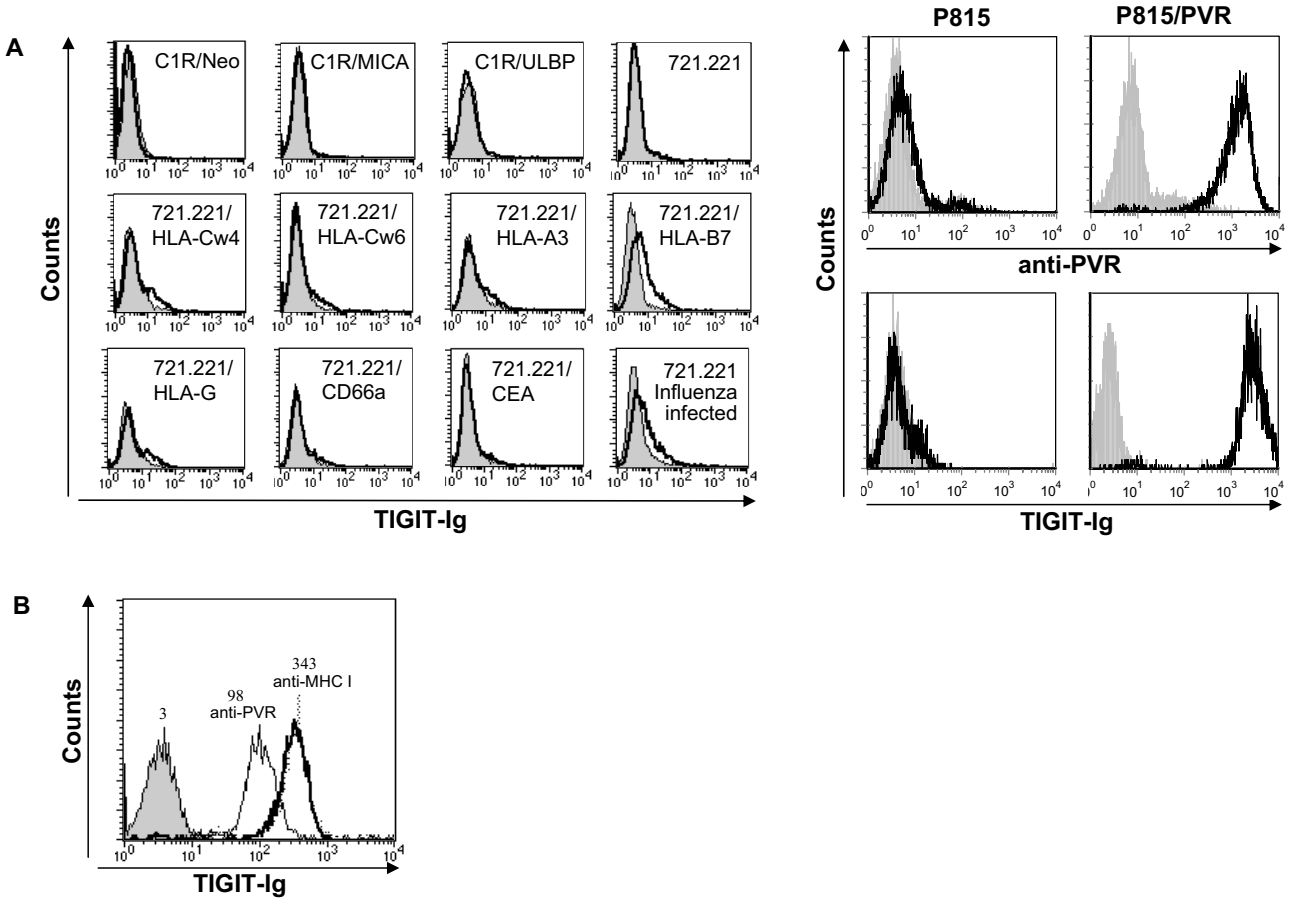

Fig. S1. TIGIT-Ig binds to PVR (CD155). (*A*) The TIGIT-Ig fusion protein, composed of the extracellular domain of the TIGIT receptor fused to the Fc portion of human IgG1, was used for staining several transfectants expressing various ligands for different inhibitory and activating NK receptors or infected with influenza (black lines). TIGIT-Ig bound neither to the inhibitory ligands HLA-Cw4/Cw6/A3/B7/G, CD66a (CEACAM1) and CEA nor to the activating ligands MICA, ULBP1 and hemagglutinin (cell infected with influenza). A very strong binding of TIGIT-Ig was observed to P815 cells expressing PVR, also stained with anti-PVR mAb. Gray filled histograms are the background staining with the secondary phycoerythrin- or fluorescein-conjugated antibody only. (*B*) Flow cytometry analysis of the colon carcinoma cell line RKO (chosen for its strong binding to TIGIT-Ig, see Fig. S2) stained with TIGIT-Ig fusion protein either alone (black empty histogram), or after blocking with mAb against PVR (gray empty histogram), or with a control Ab against MHC I (dashed empty histogram). The filled gray histogram is the background staining with the secondary phycoerythrin-conjugated antibody only. Numbers indicate median fluorescence intensity (MFI).

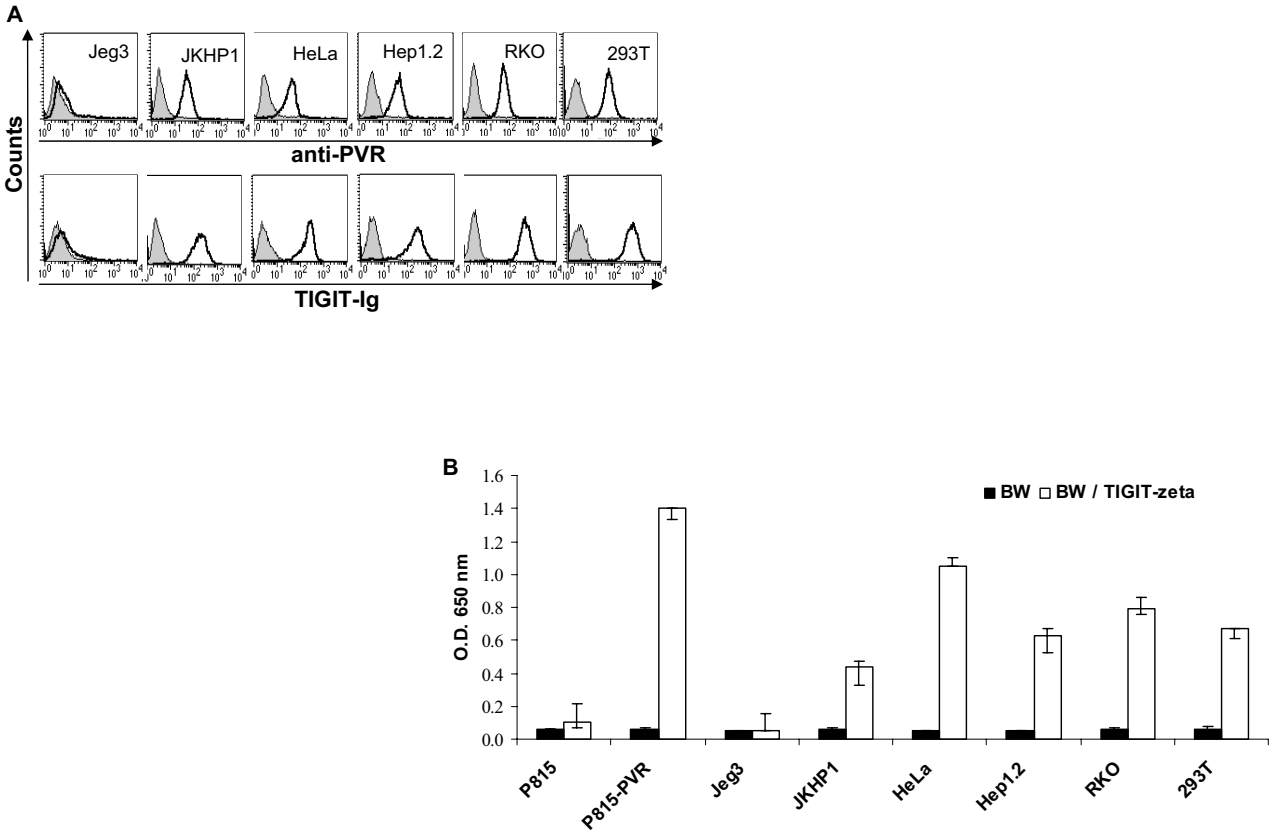

Fig. S2. TIGIT activation by PVR-expressing cells. (*A*) A perfect correlation was observed between the expression of PVR and TIGIT-Ig binding. Flow cytometry analysis of PVR expression by using specific mAb (*Upper*) or TIGIT-Ig fusion protein binding (*Lower*) of the indicated cell lines (black lines). Gray filled histograms for anti-PVR staining is the background staining with the secondary fluorescein-conjugated antibody only. Gray filled histograms for TIGIT-Ig staining is the background staining with the secondary phycoerythrin-conjugated antibody only. (*B*) To test the functional relevance of TIGIT-PVR interaction, the extracellular portion of TIGIT was cloned in frame with the transmembrane and cytoplasmic portions of a mouse ζ-chain. The TIGIT-ζ construct was expressed in BW cells (BW/ TIGIT-ζ). Engagement of the TIGIT receptor on those cells would elicit the secretion of murine IL-2 (mIL-2). The BW parental cells and the BW/ TIGIT-ζ cells were coincubated for 48 h with P815, P815/PVR or the various cell lines. Mouse IL-2 secretion was measured by ELISA. Significant amounts of mIL-2 were detected in the supernatant of BW/ TIGIT-ζ cells coincubated with P815/PVR and all other cell lines expressing PVR.

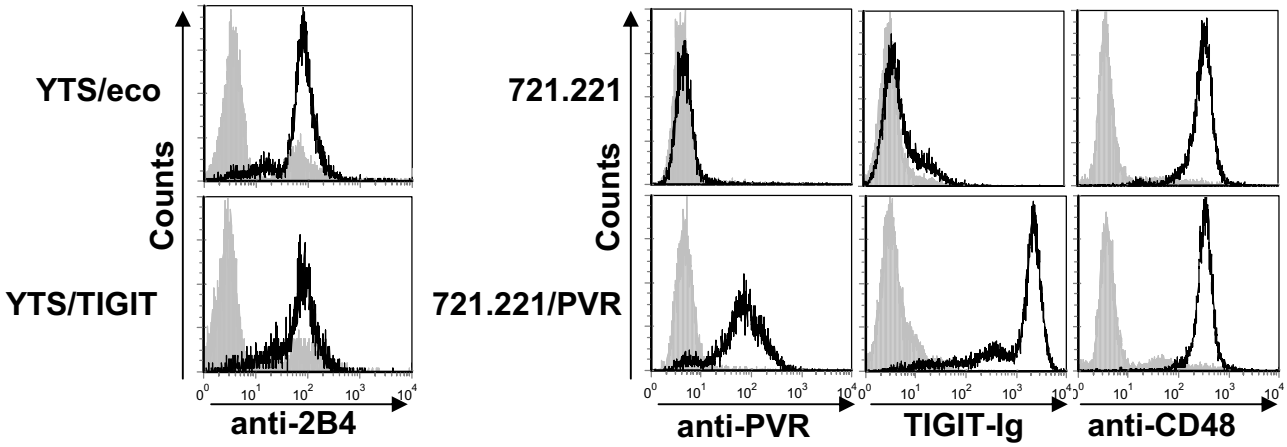

Fig. S3. Characterization of YTS and 721.221 transfectants. Because the killing of 721.221 cells by YTS cells is mediated mainly through the interaction between the activating 2B4 receptor on YTS cells and its ligand, CD48, on the target cells, it is important to demonstrate that 2B4 is present in equal levels on the parental YTS/eco (*Upper Left*) and on YTS/TIGIT cells (*Lower Left*). We also expressed PVR in 721.221 cells and demonstrated that it is indeed recognized by the anti-PVR mAb and by the TIGIT-Ig fusion protein (*Right*). We further showed that CD48 is present in equal levels on all 721.221 cells (*Right*). Gray filled histograms, background staining with the secondary fluorescein- or phycoerythrin-conjugated antibody only.

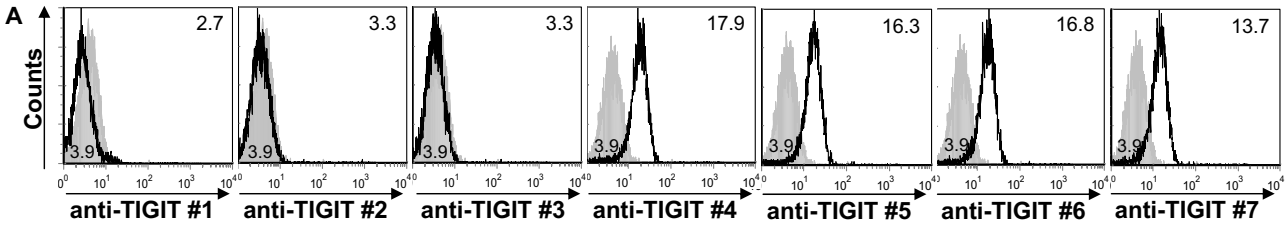

Fig. S4. Binding of the various anti-TIGIT mAbs to primary NK cells. FACS analysis of IL-2 activated bulk NK cell cultures stained with seven different antibodies directed against TIGIT. Gray shaded histograms, background staining with the secondary fluorescein-conjugated antibody only. Mouse antibodies #4-7, which recognized TIGIT in the highest affinity (Fig. 1*A*), also recognized TIGIT on IL-2-activated bulk NK cells.

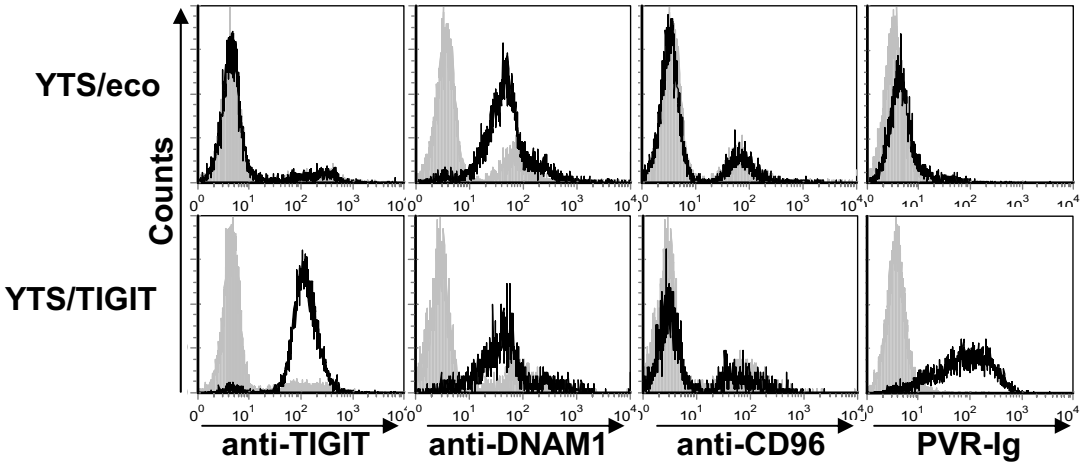

Fig. S5. DNAM1 on YTS cells cannot interact with PVR. Flow cytometry analysis of YTS/eco (*Upper*) and YTS/TIGIT (*Lower*) stained with the indicated mAbs and PVR-Ig fusion protein (black lines). Gray filled histograms are background staining with the secondary fluorescein- or phycoerythrin-conjugated antibody only. Notably although YTS cells express DNAM-1 the PVR-Ig, a fusion protein that is composed from the extracellular domain of PVR and the Fc portion of a human IgG, demonstrated no binding, whereas YTS/TIGIT is recognized by PVR-Ig.