Supplementary Table S1. Primer sequences used for generating cDNA encoding WT LAG-3 (LAG-3), WT CD4 (CD4), their mutants and LAG-3/CD4 fusion proteins.

Gene	Forward Primer	Reverse Primer
LAG-3	5′-CCGCTCGAGATGTGGGAGGCTCAGTT-3′	5′-ATAAGAATGCGGCCGCTCAGAGCTGCTCCGGCTC-3′
LAG-3ΔCY[1]	5′-CCGCTCGAGATGTGGGAGGCTCAGTT-3′	5′-ATAAGAATGCGGCCGCTCATCTTCTCCAAAGGTGA-3′
LAG-3ΔEP[2]	5′-CCGCTCGAGATGTGGGAGGCTCAGTT-3′	5′-ATAAGAATGCGGCCGCTCATTGCTCCAGCTCCTCTATCT-3′
LAG-3S484A[3]	5′-CCAAGACGATTTGCTGCCTTAGAGCAA-3′	5′-TTGCTCTAAGGCAGCAAATCGTCTTGG-3′
LAG-3S497A[4]	5′-CCGCAGGCTCAGGCCAAGATAGAGGAG-3′	5′-CTCCTCTATCTTGGCCTGAGCCTGCGG-3′
LAG-3S484, 497A[5]	5′-TTAGAGCAAGGGATTCACCCTCCGCAG -3′	5′-CTGCGGAGGGTGAATCCCTTGCTCTAA-3′
CD4	5′-CCGCTCGAGATGAACCGGGGAGTCCCTT-3′	5′-ATAAGAATGCGGCCGCTCAAATGGGGCTACATGTCTT-3′
CD4ΔCY	5′-CCGCTCGAGATGAACCGGGGAGTCCCTT-3′	5′-ATAAGAATGCGGCCGCTCAGCGCCTTCGGTGCCGGC-3′
LAG-3-CD4[6]	5′-GGCTTTCACCTTTGGTGTGTCAGGTGCC-3′	5′-GGCACCTGACACACCAAAGGTGAAAGCC-3′
CD4-LAG-3[7]	5′-CTAGGCATCTTCTTCAGAAGACAGTGGCGA-3′	5′-TCGCCACTGTCTTCTGAAGAAGATGCCTAG-3′
LAG-3-EGFP	5′-CCGCTCGAGATGTGGGAGGCTCAGTT-3′	5′-TCCCCCGGGGGAGCTGCTCCGGCTCGGGCT-3′
LAG-3ΔCY-EGFP	5′-CCGCTCGAGATGTGGGAGGCTCAGTT-3′	5′-TCCCCCGGGGTCTTCTCCAAAGGTGAAAGC-3′
LAG-3ΔEP-EGFP	5′-CCGCTCGAGATGTGGGAGGCTCAGTT-3′	5′-TCCCCCGGGGTTGCTCCAGCTCCTCTATCTT-3′
CD4-EGFP	5′-CCGCTCGAGATGAACCGGGGAGTCCCTT-3′	5′-TCCCCCGGGGAATGGGGCTACATGTCTTCTG-3′
CD4ΔCY-EGFP	5′-CCGCTCGAGATGAACCGGGGAGTCCCTT-3′	5′-TCCCCCGGGGGCGCCTTCGGTGCCGGCA-3′
LAG-3-CD4-EGFP	5′-GGCTTTCACCTTTGGTGTGTCAGGTGCC-3′	5′-GGCACCTGACACACCAAAGGTGAAAGCC-3′
CD4-LAG-3-EGFP	5′-CTAGGCATCTTCTTCAGAAGACAGTGGCGA-3′	5′-TCGCCACTGTCTTCTGAAGAAGATGCCTAG-3′
LAG-3S484A-EGFP	5′-CCAAGACGATTTGCTGCCTTAGAGCAA-3′	5′-TTGCTCTAAGGCAGCAAATCGTCTTGG-3′
LAG-3S497A-EGFP	5′-CCGCAGGCTCAGGCCAAGATAGAGGAG-3′	5′-CTCCTCTATCTTGGCCTGAGCCTGCGG-3′
LAG-3S484, 497A-EGFP	5′-TTAGAGCAAGGGATTCACCCTCCGCAG -3′	5′-CTGCGGAGGGTGAATCCCTTGCTCTAA-3′

[1]ΔCY, cytoplasmic deletion mutant; [2]ΔEP, EP motif (Glu-Pro repetitive sequence in CY) deletion mutant; [3]S484A indicates that Ser^{484} residue changes to Ala^{484}; [4]S497A indicates that Ser^{497} residue changes to Ala^{497}; [5]S484, 497A indicates that both Ser^{484} residue and Ser^{497} residue change to Ala^{484} and Ala^{497}; [6]LAG-3-CD4 indicates that a fusion protein has an extracellular domain and transmembrane of LAG-3 combined with the cytoplasmic domain of CD4; [7]CD4-LAG-3 indicates a fusion protein has an extracellular domain and transmembrane of CD4 combined with the cytoplasmic domain of LAG-3.

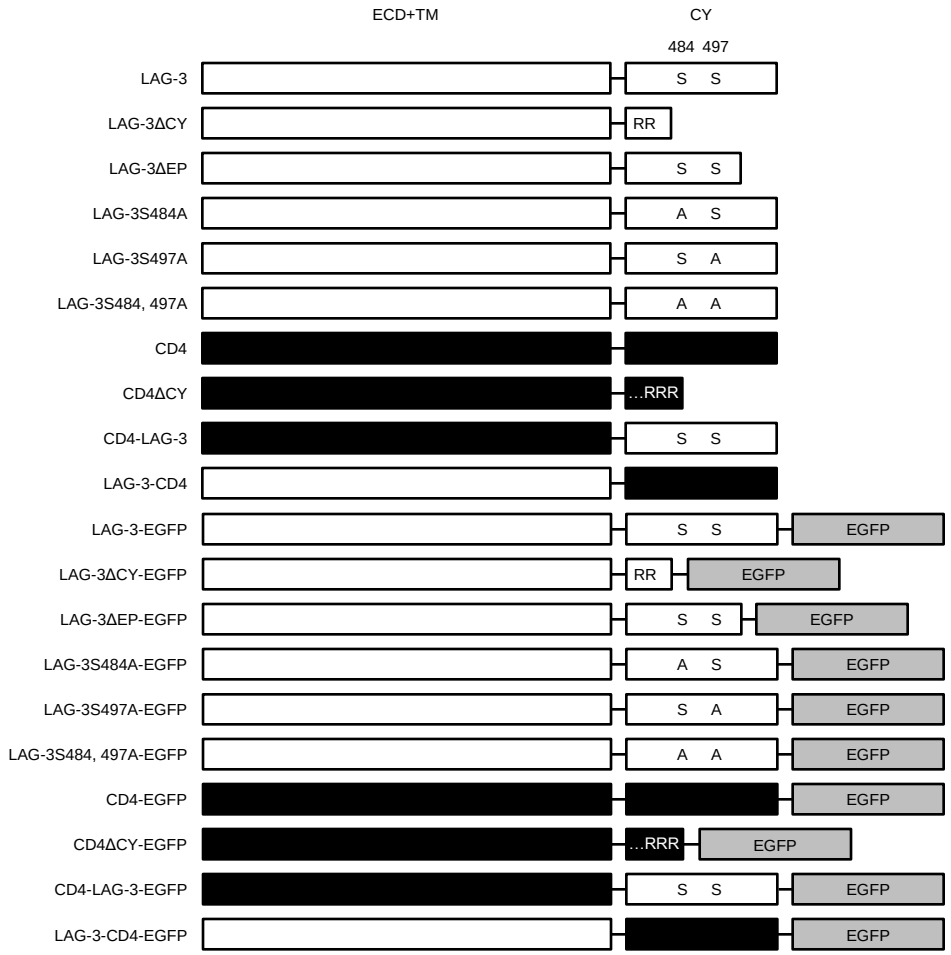

Figure S1. Schematic diagram of WT LAG-3 (LAG-3), WT CD4 (CD4), their mutants and LAG-3/CD4 fusion proteins. White box indicates LAG-3 domain, black box indicates CD4 domain, and grey box indicates EGFP domain. “S” indicates serine residue, “A” indicates alanine residue, and “R” indicates arginine residue. Numbers (484 and 497) indicate the position of amino acid residues in the LAG-3 coding region. ECD indicates extracellular domain, TM indicates transmembrane region, and CY indicates cytoplasmic domain.

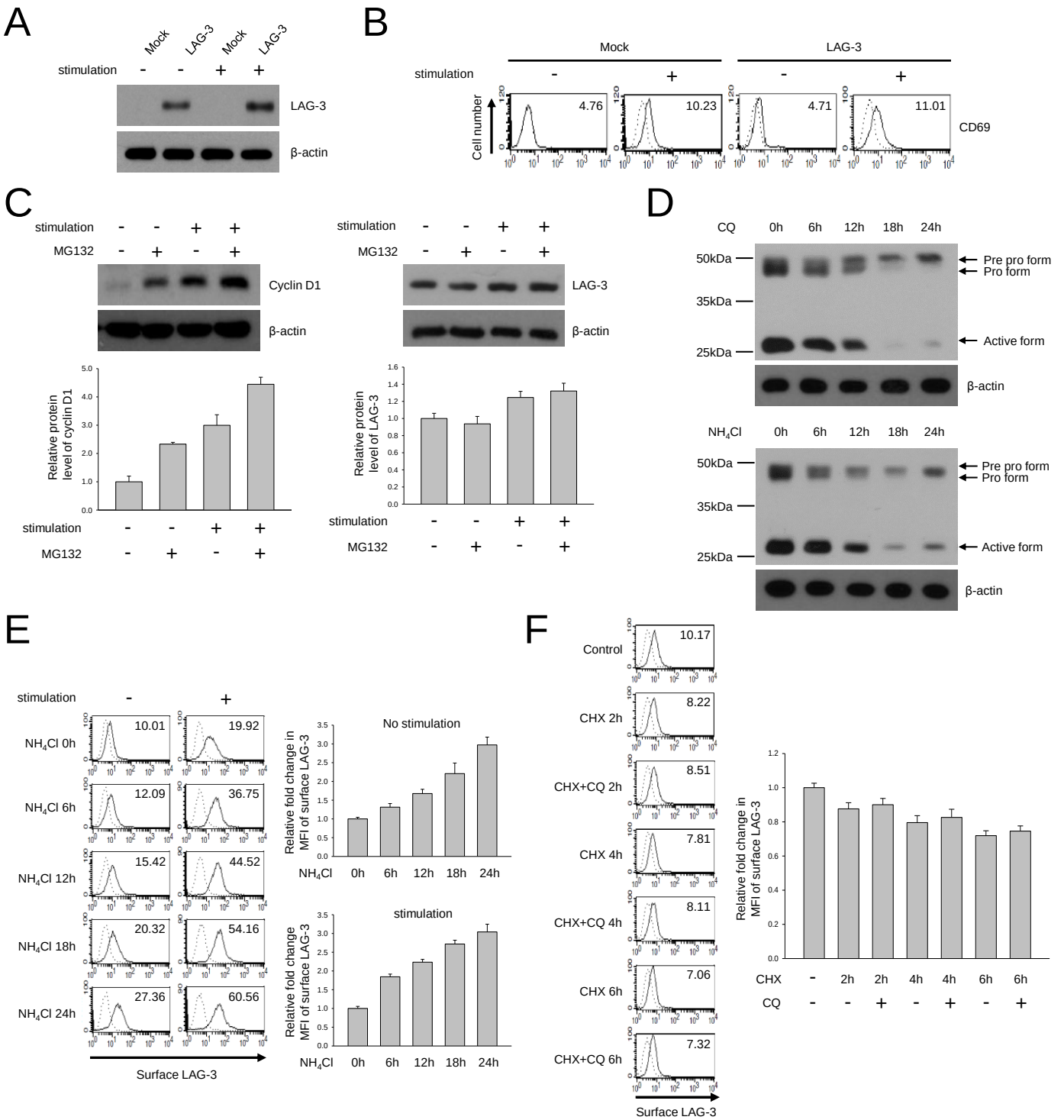

Figure S2. (A) Total LAG-3 expression in LAG-3 expressing Jurkat cells. Empty vector (mock) or LAG-3 expressing Jurkat cells were stimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 24 h. Before or after stimulation, cell lysates were immunoblotted with anti-human LAG-3 Ab and anti-β-actin Ab. **(B) Surface induction of CD69 on Jurkat cells upon activation.** Empty vector transfectants (mock) and human LAG-3 transfectants (LAG-3) were stimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 24 h. After stimulation, the cells were stained with PE-conjugated anti-human CD69 Ab and analyzed by flow cytometry. Dotted lines show the staining result with the isotype control, and thin lines show the staining result with the anti-human CD69 antibody. **(C) Cyclin D1 expression is largely dependent on the activity of the proteasome complex, but the expression of LAG-3 is not dependent on the activity of the proteasome complex.** LAG-3 overexpressing Jurkat cells were treated with 20 μM of MG132 for 4 h. After treatment with MG132, western blot analyses were performed to analyze the expression of cyclin D1 (*left panel*) and LAG-3 (*right panel*). The inhibitory effect of MG132 on the expression of cyclin D1 and LAG-3 was quantified and indicated in each panel. **(D) Chloroquine (CQ) and ammonium chloride (NH_4Cl) inhibit the activity of cathepsin D, one of lysosomal enzymes.** LAG-3 expressing Jurkat cells were treated with 100 μM of CQ (*upper panel*) or 10 mM of ammonium chloride (*lower panel*) for different time points and monitored the expression pattern of cathepsin D by western blot analyses. **(E) Lysosomal inhibition by ammonium chloride (NH_4Cl) increases the cell surface expression of LAG-3.** LAG-3 expressing Jurkat cells were stimulated in the presence or absence of NH_4Cl (10 mM) for different time points. After stimulation, cells were stained with PE-conjugated anti-human LAG-3 Ab. Representative histograms are shown in the *left panels*. Dotted line, background staining of isotype control Ab; thin line, specific staining of LAG-3. Each number in the histograms indicates MFI. Relative fold changes in MFI of LAG-3 surface expression are shown in the *right panels*. **(F) Surface expression of LAG-3 in the presence of cycloheximide (CHX) and chloroquine (CQ).** LAG-3 expressing Jurkat cells were treated with CHX (20 μg/ml) in the presence or absence of CQ (100 μM). After stimulation, cells were stained with PE-conjugated anti-human LAG-3 Ab. Representative histograms are shown in the *left panels*. Dotted line, background staining of isotype control Ab; thin line, specific staining of LAG-3. Each number in the histograms indicates MFI. Relative fold changes in MFI of LAG-3 surface expression are shown in the *right panels*.

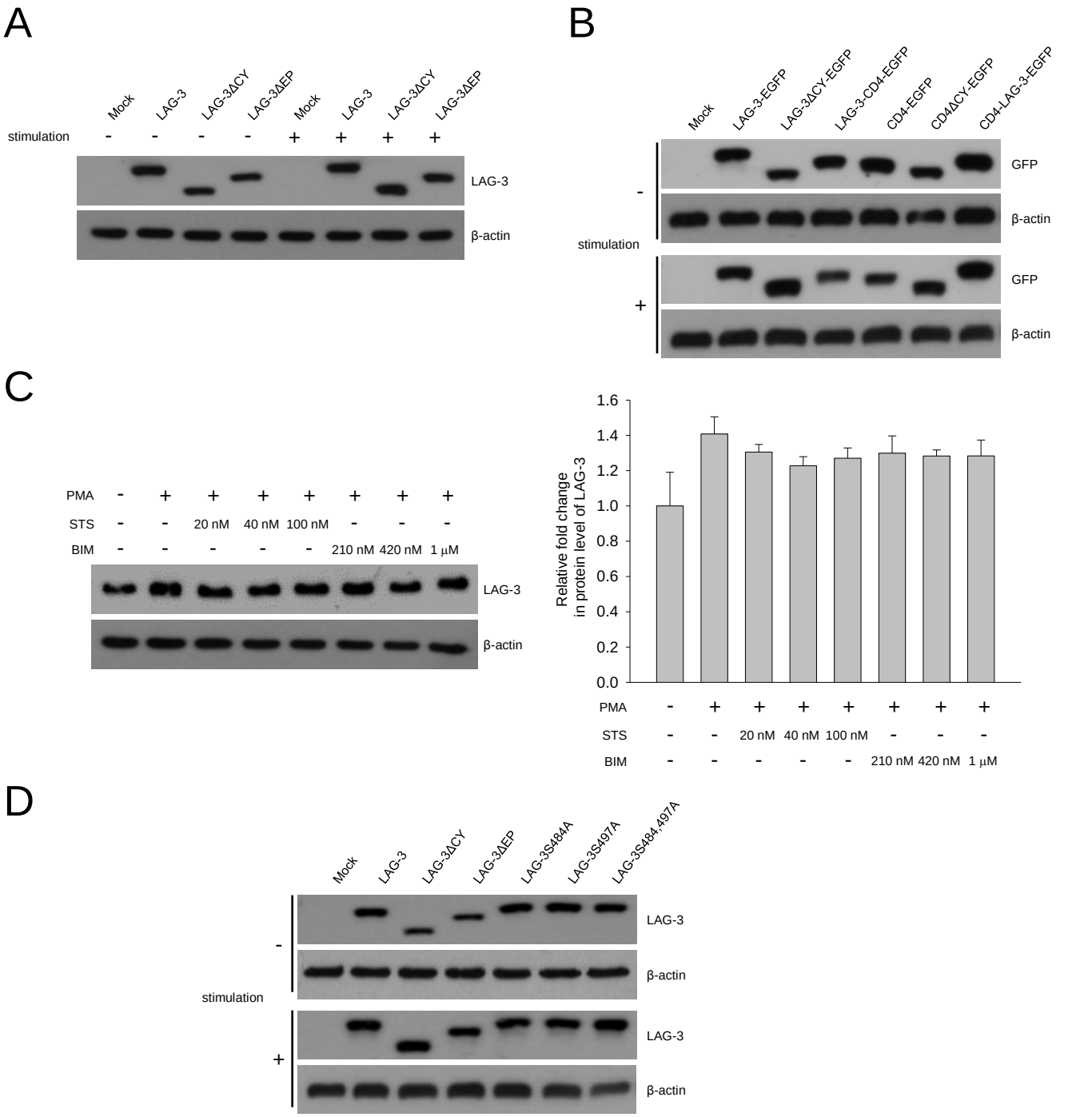

Figure S3. (A) The expression levels of LAG-3 deletion mutants were comparable to that of wildtype (WT) LAG-3 in Jurkat cells. Empty vector (mock) expressing Jurkat cells, WT LAG-3 (LAG-3) expressing Jurkat cells, LAG-3ΔCY expressing Jurkat cells and LAG-3ΔEP expressing Jurkat cells were stimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 24 h. Before or after stimulation, cell lysates were immunoblotted with anti-human LAG-3 Ab and anti-β-actin Ab. Data are representative of three independent experiments. **(B) The expression levels of LAG-3/CD4-EGFP fusion proteins in Jurkat cells.** Jurkat cells bearing the empty vector or cells expressing the indicated fusion proteins were stimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 24 h. Before or after stimulation, cell lysates were immunoblotted with anti-GFP Ab and anti-β-actin Ab. Data are representative of three independent experiments. **(C) The level of total LAG-3 expression was not changed after treatment of various concentrations of staurosporine (STS) and Bisindolylmaleimide I (BIM).** LAG-3 expressing Jurkat cells were stimulated with PMA (10 ng/ml) in various concentrations of the PKC inhibitors, STS or BIM. After stimulation, cell lysates were immunoblotted with anti-human LAG-3 Ab and anti-β-actin Ab. Data are representative of three independent experiments. **(D) The expression levels of several amino acid substitution mutants of LAG-3 were comparable to that of wildtype (WT) LAG-3 in Jurkat cells.** Jurkat cells bearing the empty vector or cells expressing the indicated proteins were stimulated with PMA (10 ng/ml) for 24 h. Before or after stimulation, cell lysates were immunoblotted with anti-human LAG-3 Ab and anti-β-actin Ab. Data are representative of three independent experiments.