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An Essential Role of Sialylated *O*-Linked Sugar Chains in the Recognition of Mouse CD99 by Paired Ig-Like Type 2 Receptor (PILR)[1]

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Abstract

The paired Ig-like type 2 receptor (PILR), which comprises both inhibitory and activating isoforms, is well conserved among most mammalians. The inhibitory PILRα possesses an ITIM in its cytoplasmic domain, whereas the activating PILRβ does not have an ITIM but transduces activating signals by associating with the ITAM-bearing DAP12 adapter molecule. Both mouse PILRα and PILRβ recognize mouse CD99, which is broadly expressed on various cells, including lymphocytes, and is involved in the regulation of immune responses. We herein report that sialylated *O*-linked sugar chains on CD99 are essential for the recognition by PILR. Mutations of one of two *O*-glycosylation sites on CD99 significantly reduced recognition of CD99 by the activating PILRβ, whereas recognition by the inhibitory PILRα was not affected. In contrast, mutations of both *O*-glycosylation sites on CD99 completely abrogated the recognition by both PILRα and PILRβ. PILR did not recognize CD99 treated with neuraminidase, and CD99 expressed on cells transfected with core 2 β-1,6-*N*-acetylglucosaminyltransferase was not recognized by PILR. NK cells expressing endogenous activating PILRβ receptors mediated cytotoxicity against cells expressing wild-type CD99 but not cells expressing mutant CD99 that lacked *O*-glycosylation sites. These findings indicate that sialylated *O*-linked sugar structures on CD99 play an important role in the recognition of PILR.

Several families of NK cell receptors, such as human killer cell Ig-like receptor (KIR)[4] and mouse Ly49, consist of activating and inhibitory receptors. Inhibitory receptors possess ITIM in their cytoplasmic domains and deliver inhibitory signals upon recognition of their ligands.

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[4]Abbreviations used in this paper: KIR, killer cell Ig-like receptor; β3Gal-T, β1,3-galactosyltransferase; C2GnT, core 2 β-1,6-*N*-acetylglucosaminyltransferase; GalNAc, *N*-acetylgalactosamine; GalNAc-α-*O*-benzyl, benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; PILR, paired Ig-like type 2 receptor; ppGaNTases, UDP-*N*-acetylgalactosamine: polypeptide *N*-acetyl-galactosaminyltransferases; Siglec, sialic acid-binding Ig-like lectin; ST3GalI, β-galactoside α-2,3-sialyltransferase 1.

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In contrast, the highly related activating receptors in these families do not possess ITIM but deliver activating signals by associating with ITAM-bearing adapter molecules such as DAP12 (1). Many of the inhibitory receptors on NK cells recognize self-Ags such as MHC class I, whereas their paired activating receptors typically show weak affinity for MHC class I, but in some cases recognize viral products (2–5). These activating and inhibitory receptors play an important role in the regulation of NK cell activation (1,6–8).

In our studies to identify activating NK cell receptors, we have previously cloned the activating paired Ig-like type 2 receptor (PILR)β from a cDNA library of mouse NK cells as a molecule that associates with DAP12 (9). The PILR family is conserved among most mammalians (10) and consists of the activating PILRβ isoform and the inhibitory PILRα isoform, both of which have highly homologous extracellular domains (9,11,12). PILR is expressed on mouse NK cells, macrophages, and dendritic cells and regulates the function of these cell populations (9,11,12). Furthermore, we found that both PILRα and PILRβ recognize mouse CD99 (9). Although the affinity of activating PILRβ for CD99 appears to be significantly lower when compared with the affinity of the inhibitory PILRα for CD99, NK cells expressing PILRβ mediated significant cytotoxicity against CD99-expressing cells (9). Therefore, the activating PILRβ seems to be involved in target cell recognition by NK cells. Furthermore, analysis using an anti-CD99 mAb indicated that CD99 is highly expressed on activated T cells (13). Because PILRα and PILRβ are expressed on dendritic cells (9,12), interaction between CD99 and PILR might be involved in the regulation of dendritic cell function by T cells.

From our analysis of the recognition of mouse CD99 by PILR, we found that CD99 expressed on certain cell lines is not recognized by PILR. This suggested that posttranscriptional modification of CD99 might be involved in the recognition by PILR. We herein report that sialylated *O*-glycans on CD99 are required for the recognition of PILR. Furthermore, expression of the core 2 branching enzyme, core 2 β-1,6-*N*-acetylglucosaminyltransferase (C2GnT), completely abrogated the recognition of CD99 by PILR. NK cells showed cytotoxicity against cells expressing wild-type CD99, but not cells expressing a mutant CD99 that lacks *O*-glycosylation. These data indicated that specific *O*-glycan structures on CD99 are involved in its recognition by PILR.

Materials and Methods

Cells and transfectants

Mouse CD99 (GenBank accession number: NM_025584) was transfected into various cell lines by using the pMx-IRES-GFP retrovirus expression vector, and GFP-positive cells were purified by using a FACSAria (BD Biosciences). Mouse C2GnT (GenBank accession number: U19265) was also transfected into CD99 or mock transfectants by using the pMx-IRES-human-CD8α retrovirus vector. These transfectants were stained with PE-conjugated anti-human CD8α mAb (eBioscience), and the stained cells were purified by using a FACSAria.

Anti-CD99 mAb

Wistar rats (Japan SLC) were immunized with mouse CD99-Ig fusion protein using TiterMax Gold (TiterMax) as an adjuvant. Two weeks after immunization, lymph node cells were fused with SP2/0, and a clone that recognized mouse CD99-transfected Ba/F3 cells was obtained (clone 5-2). The mAb produced in culture supernatant was purified by protein G affinity chromatography (GE Healthcare).

Ig fusion proteins

The cDNA-encoding extracellular domain of mouse PILRα (GenBank accession number: NM_153510) was inserted into the *Xho*I cloning site of a modified pME18S expression vector

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that contained a mouse CD150 leader segment and the Fc segment of human IgG1 (9). The cDNA-encoding mouse PILRβ (GenBank accession number: NM_133209) was also inserted into a similar expression vector that has an additional IgA tail piece sequence at the C terminus of the Fc segment (9,14). These plasmids were transfected into COS-7 cells by using 293fectin (Invitrogen). After 72 h, the culture supernatants were collected, and the amounts of Ig-fusion protein were determined by standard ELISA. The amount of Ig fusion protein used for staining was 5 μg/ml.

Flow cytometry

Cells were incubated for 30 min on ice with a saturating concentration of mouse PILR-human IgG1 Fc fusion protein or rat anti-mouse CD99 mAb (5-2), followed by PE-conjugated anti-human IgG or anti-rat IgG Ab (Jackson ImmunoResearch). Splenocytes prepared from C57BL/6 mice (Japan SLC) were stimulated with anti-CD3 mAb (1 μg/ml) or anti-mouse IgM F(ab′)$_2$ Ab (10 μg/ml, Jackson ImmunoResearch) for 48 h. Freshly isolated splenocytes and activated splenocytes were stained with PE-conjugated anti-CD4, PE-conjugated anti-CD8α, and PE-conjugated anti-B220 mAbs in combination with biotinylated PILRα-Ig or PILRβ-Ig fusion protein or anti-CD99 mAb. Biotinylated proteins were detected by using APC-conjugated streptavidin (Jackson ImmunoResearch). Stained cells were analyzed by using a FACSCalibur (BD Biosciences). Data were analyzed by CellQuest Pro software (BD Biosciences).

Treatment of cells with glycosylation inhibitor or neuraminidase

CD99-transfected Ba/F3 or mock-transfected Ba/F3 cells were cultured in the presence of various concentrations of benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (GalNAc-α-*O*-benzyl) (Sigma-Aldrich) for 48 h. CD99-transfected Ba/F3 and mock-transfected Ba/F3 cells were incubated with neuraminidase from *Arthrobacter ureafaciens* (1 U/ml, Roche) at 37°C for 3 h. Thereafter, cells were analyzed by flow cytometry or by Western blotting.

Western blot analysis

Cells were washed in cold PBS and disrupted with lysis buffer (20 mM Tris, 150 mM NaCl (pH 7.5)) containing 1% Brij 98 (Sigma-Aldrich). The total cell lysates were separated by using 5–20% SDS-PAGE gels (ATTO) under reducing condition, and proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore). Membranes were blotted with rat anti-mouse CD99 mAb (5-2) or PILRα-Ig fusion protein (5 μg/ml), followed by incubation with HRP-labeled anti-rat IgG or anti-human IgG Ab (Amersham Biosciences), respectively. All the blots were developed by using an ECL kit (Pierce Biotechnology), and chemiluminescence was detected with a LAS-1000 instrument (Fujifilm).

Surface biotinylation and immunoprecipitation

Cell surface biotinylation was performed as described previously (15). Briefly, cells were washed with ice-cold PBS and then were incubated with sulfo-NHS-LC-biotin (Pierce Biotechnology) at 100 μg/ml for 15 min at room temperature. Thereafter, biotinylated cells were washed with ice-cold PBS and were disrupted with lysis buffer. CD99 was immunoprecipitated by protein G-coupled Sepharose preincubated with anti-mouse CD99 mAb (5-2) The precipitates were separated on 5–20% SDS-PAGE gels under reducing condition and were transferred onto polyvinylidene difluoride membranes. Biotinylated proteins were detected by using peroxidase-conjugated streptavidin (Sigma-Aldrich).

Generation of CD99 mutants

cDNA for mouse CD99 in which all of the serine and threonine residues were mutated to alanines (CD99M) were generated by oligonucleotide synthesis. The mutated residues were

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reverted to the original serine or threonine residues by point mutation. The CD99W-T45A, CD99W-T50A, and CD99W-T45A-T50A proteins were generated by mutating threonine residues at position 45 and/or 50 of wild-type CD99 to alanine.

NK cell-mediated cytotoxicity assay

Mouse NK cells were purified from splenocytes of C57BL/6 mice as described previously (15). Briefly, surface Ig^+, $CD4^+$, and $CD8^+$ splenocytes were removed by mAb-coated magnetic beads (Qiagen), and DX5-positive NK cells were purified by using a MACS purification system (Miltenyi Biotec). Purified NK cells were expanded in the presence of 2500 U/ml IL-2 for 7 days. Cytotoxic assays were performed by using PKH2 fluorescence dye-labeling methods (Sigma-Aldrich), as described previously (16).

Results

Posttranscriptional modification of CD99 affects the recognition by PILR

In previous studies, we have demonstrated that both mouse PILRα and PILRβ recognize mouse CD99 expressed on Ba/F3 cells, a mouse pro-B cell line (9). As shown in Fig. 1*A*, PILRα-Ig and PILRβ-Ig fusion proteins bind to CD99-transfected Ba/F3 cells but not to mock transfectants. However, both PILRα-Ig and PILRβ-Ig bound minimally to WEHI-231 (a mouse B cell line) or IIA1.6 (a FcγRII-negative mutant of the A20 mouse B cell line) transfected with CD99 (Fig. 1*A*). To analyze the cell-surface expression of CD99 on WEHI-231 or IIA1.6 cells, we generated a mAb that specifically recognizes mouse CD99. Anti-CD99 mAb, 5–2, recognized CD99-transfected Ba/F3 cells but not mock-transfected Ba/F3 cells (Fig. 1*B*). Anti-CD99 mAb also recognized both WEHI-231 and IIA1.6 cells transfected with CD99 but not mock transfectants (Fig. 1*B*). These data suggested that certain posttranscriptional modifications, such as glycosylation, might be involved in the recognition of CD99 by PILR. To test our hypothesis, CD99 transfectants were biotinylated and CD99 was immunoprecipitated from lysates using anti-CD99 mAb. As shown in Fig. 1*C*, CD99 precipitated from Ba/F3 cells showed a molecular mass of ~25 kDa, whereas CD99 precipitated from WEHI-231 showed heterogeneous molecular weights of 25 and 23 kDa. Additionally, CD99 precipitated from IIA1.6 migrated at ~20 kDa. These different molecular weights of CD99 expressed on these cell lines might reflect differences in the glycosylation pattern of CD99. Indeed, both human and mouse CD99 have been reported to be *O*-glyco-sylated type I transmembrane proteins (17,18). In contrast, human and mouse CD99 do not possess possible *N*-glycosylation sites. These data suggested that *O*-glycosylation of CD99 might affect the recognition by PILR.

Involvement of *O*-glycosylation on CD99 in the recognition by PILR

GalNAc-α-*O*-benzyl is a sugar analog that specifically inhibits *O*-glycosylation through its ability to compete with GalNAc-*O*-Ser/ Thr, which is a substrate for β1,3-galactosyltransferase (β3Gal-T), which generates core 1 structures of *O*-glycans (19). Therefore, membrane proteins on cells treated with GalNAc-α-*O*-benzyl are poorly *O*-glycosylated. To analyze whether *O*-glycosylation on CD99 is involved in the recognition by PILR, parental Ba/F3 cells or CD99-transfected Ba/F3 cells were treated with GalNAc-α-*O*-benzyl at different concentrations. Viability of cells was not affected by GalNAc-α-*O*-benzyl treatment (data not shown). As shown in Fig. 2*A*, treatment of CD99 transfectants with GalNAc-α-*O*-benzyl resulted in a dose-dependent inhibition of binding by both PILRα-Ig and PILRβ-Ig fusion proteins. In contrast, cell-surface expression of CD99 itself was not affected by the treatment with GalNAc-α-*O*-benzyl when the expression was analyzed by using an anti-CD99 mAb. We then analyzed the molecular mass of CD99 on the cells treated with GalNAc-α-*O*-benzyl by Western blotting. Although the molecular mass of CD99 expressed on non-treated cells was 25 kDa, the molecular mass of CD99 expressed on GalNAc-α-*O*-benzyl-treated cells was significantly

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decreased (Fig. 2*B*). These data suggested that *O*-glycosylation on CD99 plays an important role in the recognition of CD99 by PILR.

Identification of *O*-glycosylation sites on CD99 involved in the recognition by PILR

We analyzed the glycosylation sites of mouse CD99 that are responsible for the recognition by PILR. *O*-glycosylation is generated on serine or threonine residues (20). When CD99 was analyzed by using the NetOGlyc 3.1 algorithm (www.cbs.dtu.dk/services/NetOGlyc/) (21), four serine or threonine residues were predicted to be possible *O*-glycosylation sites (Fig. 3*A*). Single-point mutations of any of these possible *O*-glycosylation sites did not abrogate the recognition of CD99 by PILRα-Ig when they were analyzed by transient transfection to 293T cells (data not shown). We then considered the possibility that there may be additional *O*-glycosylation sites on CD99 not predicted by this computer algorithm. Therefore, we generated CD99 in which all the serine and threonine residues were mutated to alanine (CD99M). We transfected the CD99M and CD99W into Ba/F3 cells and stained the transfectants with PILR-Ig fusion protein. Both PILRα-Ig and PILRβ-Ig recognized the wild-type CD99 but not CD99M. In contrast, the CD99M was recognized by anti-CD99 mAb, demonstrating that *O*-linked glycosylation of CD99 is not required for cell-surface expression of this protein (Fig. 3*B*). These data also indicated that *O*-glycosylation on CD99 might be required for the recognition by PILR.

To identify the glycosylation sites exactly, we generated a series of CD99M in which individual mutated alanines were reverted to the original serine or threonine residues and transfected the CD99M revertants into Ba/F3 cells (Fig. 3*B*). The transfectants were stained with PILRα-Ig, PILRβ-Ig, and anti-mouse CD99 mAb. When a CD99M in which amino acid residue 45 was reverted to threonine (CD99M-A45T) was transfected into Ba/F3 cells, the CD99M-A45T-transfected cells were recognized by both PILRα-Ig and PILRβ-Ig. In contrast, a CD99M in which amino acid residue 50 was reverted to threonine (CD99M-A50T) was recognized by PILRα-Ig but not by PILRβ-Ig. There was no significant difference in CD99 expression between these different mutants, as determined by staining with an anti-CD99 mAb. Other CD99M revertants were stained with anti-CD99 mAb but not with PILR-Ig. There was no significant difference in the staining intensity with anti-CD99 mAb among these revertants (data not shown). These data suggested that threonines at amino acid residues 45 and 50 are *O*-glycosylation sites involved in the recognition by PILR, although residue 50 was less important in the recognition by PILRβ-Ig than was residue 45. However, because all serine and threonine residues were mutated in CD99M, the overall structure of the CD99 protein might be altered by these extensive mutations. Therefore, to minimize the effect of the mutations, we generated a mutant CD99 in which only threonines at amino acid residues 45 and/or 50 were mutated to alanine from the wild-type CD99 (CD99W-T45A, CD99W-T50A, and CD99W-T45A-T50A). These mutants were also expressed on the cell surface at the same level as wild-type CD99. Ba/F3 cells transfected with CD99W-T45A or CD99W-T50A were recognized by PILRα-Ig. However, binding of PILRβ-Ig was severely impaired by these single-point mutations. In particular, the mutation at residue 45 affected the PILRβ-Ig binding more efficiently than did the mutation at residue 50. In contrast, the CD99W-T45A-T50A was not recognized by either PILRα-Ig or PILRβ-Ig (Fig. 3*B*). Because a single-point mutation of threonine residue at position 45 or 50 did not abrogate the recognition of CD99 by PILRα-Ig, *O*-glycosylation at threo-nine at amino acid residue 45 or 50 seems to be enough for the recognition by PILRα, whereas *O*-glycosylation at both threonines is required for the efficient recognition by PILRβ.

We then analyzed the molecular weights of these CD99 mutants by SDS-PAGE. A series of mutant CD99 were transiently transfected into 293T cells, and cell lysates of these transfectants were analyzed by Western blot analysis using anti-CD99 mAb (Fig. 3*C*). Although wild-type

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CD99 showed a molecular mass of 25 kDa, CD99M in which all serines and threonines were mutated to alanines showed a molecular mass of 17 kDa, which is almost the same as the predicted molecular mass of the CD99 polypeptide. CD99M revertants, in which amino acid residue 45 or 50 was reverted to threonine (CD99M-A45T or CD99M-A50T), showed larger molecular weights than CD99M, although the molecular weights were different between them. In contrast, molecular weights of other revertants were 17 kDa and were the same as CD99M (Fig. 3*C*). Furthermore, the molecular mass of CD99W-T45A-T50A, in which only two threonines at amino acid residues 45 and 50 of wild-type CD99 were mutated to alanine, was also 17 kDa. These data suggest that only the two threonine residues at positions 45 and 50 in CD99 are *O*-glycosylated, although several threonine and serine residues were predicted to be possible *O*-glycosylation sites by the NetOGlyc 3.1 algorithm.

Role of sialic acids on the recognition of CD99 by PILR

When the amino acid homology of PILR to other proteins was analyzed, 4 –10% of amino acids of PILR are identical with the sialic acid-binding Ig-like lectin (Siglec) family of proteins, which recognizes sialic acids on glycans (10,22). Interestingly, an arginine residue that is essential for sialic acid recognition by Siglecs is conserved in both PILRα and PILRβ at amino acid residue 133 (23–25). We addressed whether sialic acid is involved in the recognition of CD99 by PILR. Ba/F3 cells transfected with wild-type CD99 or CD99W-T45A-T50A were treated with neuraminidase and stained with PILR-Ig or anti-CD99 mAb. As shown in Fig. 4*A*, neuraminidase treatment completely abrogated the recognition of CD99 by PILRα-Ig and PILRβ-Ig, but not by anti-CD99 mAb. We then analyzed the molecular mass of CD99 on Ba/F3 cells treated with neuraminidase by Western blot to determine the effect of the enzyme treatment. The molecular mass of wild-type CD99 on neuraminidase-treated cells was dramatically decreased and was similar to CD99W-T45A-T50A. In contrast, the molecular mass of CD99W-T45A-T50A was not affected by the neuraminidase treatment (Fig. 4*B*). These data indicated that CD99 is heavily sialylated and that *O*-linked sugar chains modified with sialic acids are required for the recognition of CD99 by PILR.

Modification of *O*-glycans on CD99 by core 2 branching enzyme inhibits the recognition by PILR

Core 1 and core 2 structures are the main components of *O*-glycans. The core 2 *O*-glycan branch is generated by C2GnT, and there are several isozymes of C2GnT (26,27). Additionally, E-selectin and P-selectin recognize several ligands specifically modified by C2GnT (28,29). We addressed whether C2GnT is involved in the ligand recognition by PILR by introducing C2GnT into CD99-transfected Ba/F3 cells or mock-transfected Ba/F3 cells (Fig. 5*A*). Interestingly, neither PILRα-Ig nor PILRβ-Ig bound to Ba/F3 cells cotransfected with C2GnT and CD99, whereas PILRα-Ig and PILRβ-Ig bound well to Ba/F3 cells transfected with CD99 alone. There was no difference in expression levels of CD99 between these transfectants when they were analyzed by anti-CD99 mAb. Similar results were obtained when CD99 was cotransfected with C2GnT into 293T cells (data not shown). Western blot analysis of CD99 indicated that there was no significant difference in the molecular mass of CD99 on transfectants in the presence or absence of C2GnT (Fig. 5*B*). These data suggested that the core 2 branch in *O*-glycans on CD99 inhibits the recognition by PILR, although C2GnT does not increase the total amount of glycans on CD99. To exclude the possibility that C2GnT affected the recognition of CD99 by PILR indirectly, CD99 was transfected into Ba/F3 cells in the presence or absence of C2GnT, and binding of PILR-Ig to CD99 was analyzed by SDS-PAGE. As shown in Fig. 5*B*, CD99 immunoprecipitated from CD99-transfected parental Ba/F3 cells were blotted with PILRα-Ig, whereas the CD99 precipitated from C2GnT-transfected Ba/F3 cells was not blotted with PILRα-Ig. These data suggest that PILR directly recognizes specific *O*-glycan structures on CD99 that lack core 2 glycans.

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We then addressed whether CD99 recognition by PILR is regulated by intrinsic C2GnT. C2GnT is expressed in B cells and is involved in the generation of the B220 epitope on CD45 (28). Because CD99 is expressed on T cells upon activation (13), we compared recognition of CD99 expressed on activated T cells and B cells by PILRα-Ig or PILRβ-Ig (Fig. 6). Naive $CD4^+$ T cells do not express CD99 and are not recognized by PILRα-Ig and PILRβ-Ig. When $CD4^+$ T cells were activated, they expressed CD99 and were recognized by PILRα-Ig, but not by PILRβ-Ig. In contrast, naive B cells expressed CD99 but were not recognized by PILRα-Ig and PILRβ-Ig. Although CD99 expression on B cells was slightly up-regulated after stimulation, activated B cells were also not recognized by PILRα-Ig and PILRβ-Ig. These data suggested that expression of intrinsic C2GnT in B cells regulates recognition of CD99 by PILR, as observed on C2GnT-transfected cells. Interestingly, naive $CD8^+$ T cells were well recognized by PILRα-Ig, but not by PILRβ-Ig, although naive $CD8^+$ T cells do not express CD99. This indicates that naive $CD8^+$ T cells express unknown ligands for PILRα. Because activated $CD4^+$ T cells that express CD99 were recognized by PILRα-Ig, but not by PILRβ-Ig, recognition of CD99 by PILRα and PILRβ is regulated differently in distinct cell types.

Involvement of *O*-glycan on CD99 in the cytotoxicity mediated by NK cells

Previously, we have shown that DAP12-associated PILRβ triggers NK cell-mediated cytotoxicity against CD99-expressing cells (9). Because PILRβ does not recognize CD99W-T45A-T50A that lacks two *O*-glycosylation sites in CD99 (Fig. 3*B*), we analyzed cytotoxicity of IL-2-expanded mouse NK cells against Ba/F3 cells transfected with wild-type CD99, CD99W-T45A, CD99W-T50A, or CD99W-T45A-T50A (Fig. 7). NK cells showed significant cytotoxicity against Ba/F3 cells transfected with wild-type CD99, but not against parental Ba/F3 cells. NK cells did not kill Ba/F3 cells transfected with CD99W-T45A-T50A and CD99W-T45A that are not recognized by PILRβ-Ig. NK cells showed only weak cytotoxicity against Ba/F3 cells transfected with CD99W-T50A that was partially recognized by PILRβ-Ig (Fig. 3*B*). The expression levels of these mutant CD99 proteins on Ba/F3 cells were the same as that of wild-type CD99 (Fig. 3*B*). These data indicated that full *O*-glycosylation of CD99 is required for optimal PILRβ-mediated target cell recognition by NK cells.

Discussion

In the present study, we have demonstrated that mouse CD99 is an *O*-glycosylated protein and that the *O*-glycosylation on CD99 is essential for the recognition by both the inhibitory PILRα and activating PILRβ. Furthermore, we have identified two threonine residues on CD99 that are *O*-glycosylated, and these *O*-glycosylation sites are required for the recognition by PILR. Neuraminidase treatment of CD99-expressing cells abrogated the recognition of CD99 by PILR. Additionally, expression of core 2 branching enzyme, C2GnT, significantly reduced the recognition of CD99 by PILR. These data suggested that certain sialylated carbohydrate structures are involved in the recognition of CD99 by PILR. PILR belongs to a member of the Ig superfamily and has a weak homology to the Siglec family of proteins that recognize sialic acids attached to the end of glycans, although the chromosomal location of PILR is different from the Siglec family (10,12). Furthermore, an arginine residue, which is conserved among Siglecs and is essential for the recognition of sialic acid by Siglecs, is also conserved in PILR. Indeed, point mutation of the arginine residue abrogated recognition of CD99 by PILR-Ig fusion protein (J. Wang and H. Arase, unpublished observation). Therefore, the arginine residue on PILR seems to be involved in the recognition of sialic acids on CD99, and PILR might recognize the ligand in a similar way with Siglec family proteins.

Glycans on cell-surface molecules are involved not only in the direct interaction with other molecules but also in the structure of the proteins (30). Therefore, PILR might recognize a three-dimensional structure of *O*-glycosylated CD99 rather than directly recognizing

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carbohydrates. However, the glycosylated PILR ligand was still recognized by the PILRα-Ig fusion protein after boiling and treatment with SDS, a treatment that disrupts the three-dimensional structure of proteins (Fig. 5*B*). This suggested that PILRα directly recognizes specific *O*-glycans on CD99 rather than the specific structure of *O*-glycosylated CD99. In contrast, PILRβ-Ig did not bind to the denatured CD99 (J. Wang and H. Arase, unpublished observation). Additionally, a single-point mutation at threonine residues significantly affected the recognition of PILRβ but not PILRα. Therefore, PILRβ might recognize CD99 in a structure-dependent manner. Alternatively, the low affinity of PILRβ to CD99 compared with PILRα might be responsible for the difference.

Glycans usually have complicated structures, and it is not easy to determine *O*-glycan structures precisely. Although we have not yet elucidated the structure of *O*-glycans on CD99 that is responsible for the recognition by PILR, our data provide some information on the specificity of PILR. In mammals, *N*-acetylgalactosamine (GalNAc) is first transferred to serine or threonine residues by a family of UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferases (ppGaNTases) (26). Thereafter, galactose is added to the GalNAcα1 →Ser/Thr by β3Gal-T, which generates a core 1 structure, Galβ1 →3GalNAcα1 →Ser/Thr (31). GalNAc-α-*O*-benzyl is a sugar analog for GalNAcα1 →Ser/Thr and functions as a putative substrate for β3Gal-T, which inhibits the addition of core 1 structure on GalNAcα1 →Ser/Thr (19). Because CD99 transfectants incubated with GalNAc-α-*O*-benzyl were not recognized by PILR, core 1 structure seems to be required for the recognition of CD99 by PILR. The core 1 structure is a substrate for both β-galactoside α-2,3-sialyltransferase 1 (ST3GalI) and C2GnT. ST3GalI transfers sialic acids onto the core 1 structure to generate *N*-acetylneuraminic acid (NeuAc)α2 →3Galβ1 →3GalNAcα1 →Ser/Thr. In contrast, C2GnT transfers *N*-acetylglucosamine (GlcNAc) to the core 1 structure to generate Galβ1 →3 (GlcNAcβ1 →6) GalNAcα1 →Ser/Thr. Because these enzymes function competitively (32), NeuAcα2 →3Galβ1 →3GalNAcα1 →Ser/Thr is mainly generated when ST3GalI activity is dominant. In contrast, when C2GnT activity is dominant, Galβ1 →3(GlcNAcβ1 →6) GalNAcα1 →Ser/Thr is mainly generated. Because recognition of CD99 by PILR was inhibited in the presence of C2GnT or by the treatment with neuraminidase, PILR might recognize certain glycan structures related to NeuAcα2 →3Galβ1 →3GalNAcα1 →Ser/Thr. However, parental Ba/F3 or 293T cells were not recognized by PILR, although the NeuAcα2 →3Galβ1 →3GalNAcα1 →Ser/Thr structure itself must be present on various cell-surface proteins other than CD99. Additionally, neuraminidase treatment significantly reduced the molecular mass of CD99, suggesting that CD99 expressed on Ba/F3 cells is polysialylated similar to the neural cell adhesion molecule (33). Taken together, PILR might recognize a certain glycan structure specific to CD99 or might recognize both certain peptide sequences and glycan structures on CD99.

Expression levels of C2GnT vary among cell types (34,35). Therefore, we considered the possibility that the amount of C2GnT in WEHI-231 and IIA1.6 cells might be higher than that in Ba/F3 cells, possibly explaining why CD99 expressed on WEHI-231 and IIA1.6 cells is not recognized by PILR. Although there was no relationship between the expression levels of C2GnT transcripts and recognition by PILR among these cell lines (data not shown), both WEHI-231 and IIA1.6 are of B cell origin and express the B220 epitope on CD45, which requires C2GnT (28). This suggested that both B cell lines express functional C2GnT activity. Indeed, activated T cells, but not B cells, were recognized by PILR, although both express CD99 at the same level. Selectin family members, such as P-selectin and L-selectin, generally require core 2 *O*-glycans for ligand recognition (28). Additionally, galectin-1 requires core 2 *O*-glycans for the binding to CD43 or CD45 (34,35). In contrast, PILR specifically recognizes CD99 not modified by C2GnT. Because it has not been reported that C2GnT affects ligand recognition by Siglec family members, the ligand-binding specificity of PILR seems to be different from that of the Siglec family.

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In summary, our data demonstrate that mouse CD99 is *O*-glycosylated and that PILR specifically recognizes mouse CD99 in an *O*-glycan-dependent manner. The inhibitory PILRα is highly expressed on most $CD14^+$ monocytes in humans and $CD11b^+$ granulocytes and dendritic cells in mice (Ref. 12 and unpublished observation). Additionally, activated mouse T cells express CD99 (13), which is recognized by PILRα-Ig (Fig. 6). Human T cells are also recognized by human PILRα-Ig (J. Wang and H. Arase, unpublished observation). Therefore, PILR-mediated recognition of ligands modified with appropriate *O*-glycans might play an important role in the regulation of immune responses both in humans and mice. We have previously shown that mouse CMV possesses a ligand for inhibitory Ly49 receptors on NK cells to down-regulate immune responses (4). Therefore, certain pathogens might alter the glycosylation of cellular ligands that are recognized by the inhibitory PILR to evade an immune response. Furthermore, it has been suggested that NK cells might recognize certain carbohydrate structures on target cells (36). Indeed, NK cells express various lectin-like molecules such as Ly49 and NKR-P1. However, these lectin-like molecules expressed on NK cells seem to recognize specific protein structures rather than carbohydrate structures (4,5, 37,38); therefore, the importance of carbohydrate recognition by NK cell receptors has remained unclear. In our present studies, NK cells failed to recognize targets expressing CD99 that mutated to lack two *O*-linked glycosylation sites. Therefore, the activating PILRβ on NK cells might be involved in the recognition of carbohydrates on certain targets. Further functional analyses of CD99 by the inhibitory PILRα or activating PILRβ may reveal novel regulatory mechanisms of the immune system that are dependent on carbohydrate recognition.

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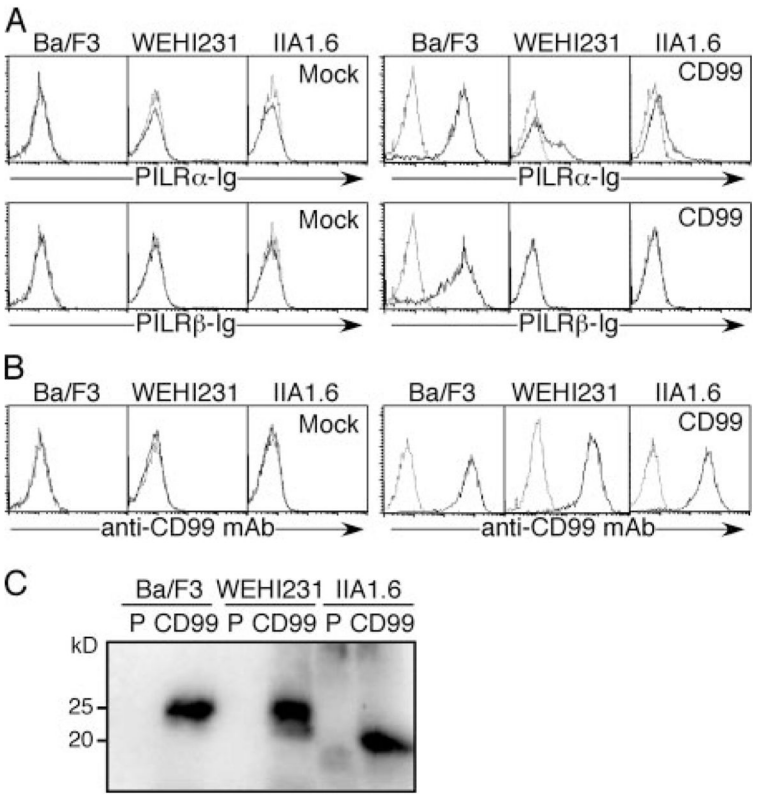

FIGURE 1.

Posttranscriptional modification of CD99 affects the recognition by PILR. *A*, Mouse CD99- or mock-transfected Ba/F3, WEHI-231, and IIA1.6 cells stained with control Ig (dotted line), PILRα-Ig (solid line) or PILRβ-Ig (solid line). *B*, Mouse CD99- or mock-transfected Ba/F3, WEHI-231, and IIA1.6 cells stained with anti-CD99 mAb (5–2, solid line) or control mAb (dotted line). *C*, Western blot analysis of CD99 expressed on several cell lines. CD99 was transfected into Ba/F3, WEHI-231, and IIA1.6 cells, and cell-surface molecules were biotinylated. CD99 was precipitated from the lysates of CD99-transfected cells (CD99) or parental cells (P) by using anti-CD99 mAb. The precipitates were analyzed by SDS-PAGE, and biotinylated proteins were detected by using peroxidase-conjugated streptavidin.

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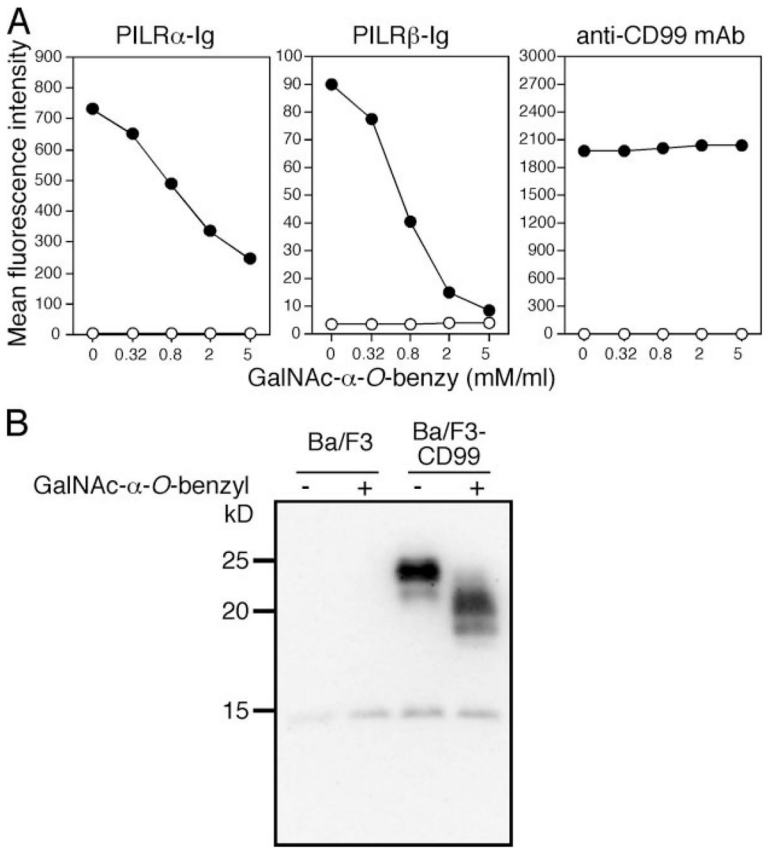

FIGURE 2.

Involvement of *O*-glycosylation of CD99 in the recognition by PILR. *A*, Effect of GalNAc-α-*O*-benzyl on the recognition of CD99 by PILR. CD99- or mock-transfected Ba/F3 cells were incubated with GalNAc-α-*O*-benzyl at the indicated concentrations for 2 days and were stained with PILR-Ig or anti-CD99 mAb. Mean fluorescence intensities of the stained cells are shown. *B*, Western blot analysis of CD99 expressed on Ba/F3 cells treated with GalNAc-α-*O*-benzyl. CD99- or mock-transfected Ba/F3 cells were cultured in the presence (+) or absence (−) of GalNAc-α-*O*-benzyl (5 mM) for 2 days, and cell lysates were analyzed by SDS-PAGE. CD99 was detected by Western blotting using anti-CD99 mAb.

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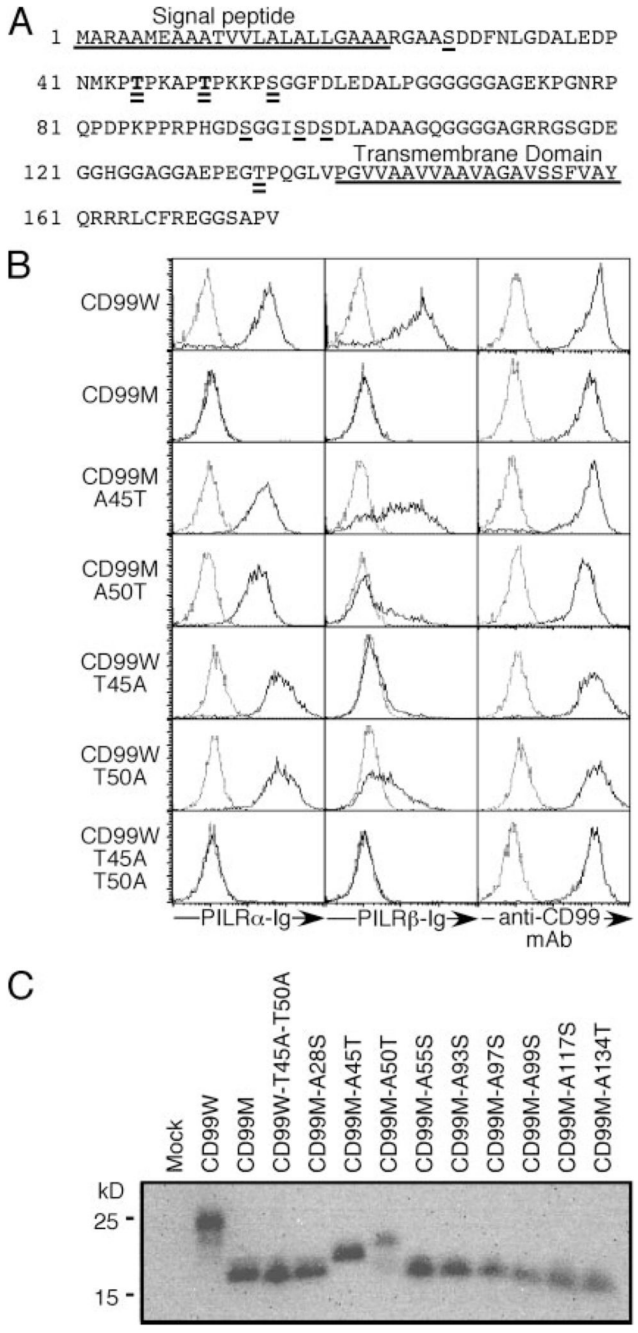

FIGURE 3.

Identification of *O*-glycosylation sites on CD99. *A*, Possible *O*-glycosylation sites were predicted by using an *O*-glycosylation prediction program (www.cbs.dtu.dk/services/netoglyc). Serine and threonine residues that were predicted to be possible *O*-linked glycosylation sites are double underlined. Serine and threonine residues that were not predicted to be *O*-glycosylation sites are underlined. Threonines at 45 and 50 residues are indicated as boldface characters. *B*, Ba/F3 cells were transfected with various CD99 mutants and the transfectants were stained with PILRα-Ig (solid line), PILRβ-Ig (solid line), or control Ig (dotted line). Expression of CD99 was analyzed by staining with anti-CD99 mAb (solid line) or control mAb (dotted line). *C*, Western blot analysis of various CD99 mutants. Lysates

of 293T cells transfected with wild-type CD99 and CD99 mutants were separated by SDS-PAGE and were blotted with anti-CD99 mAb.

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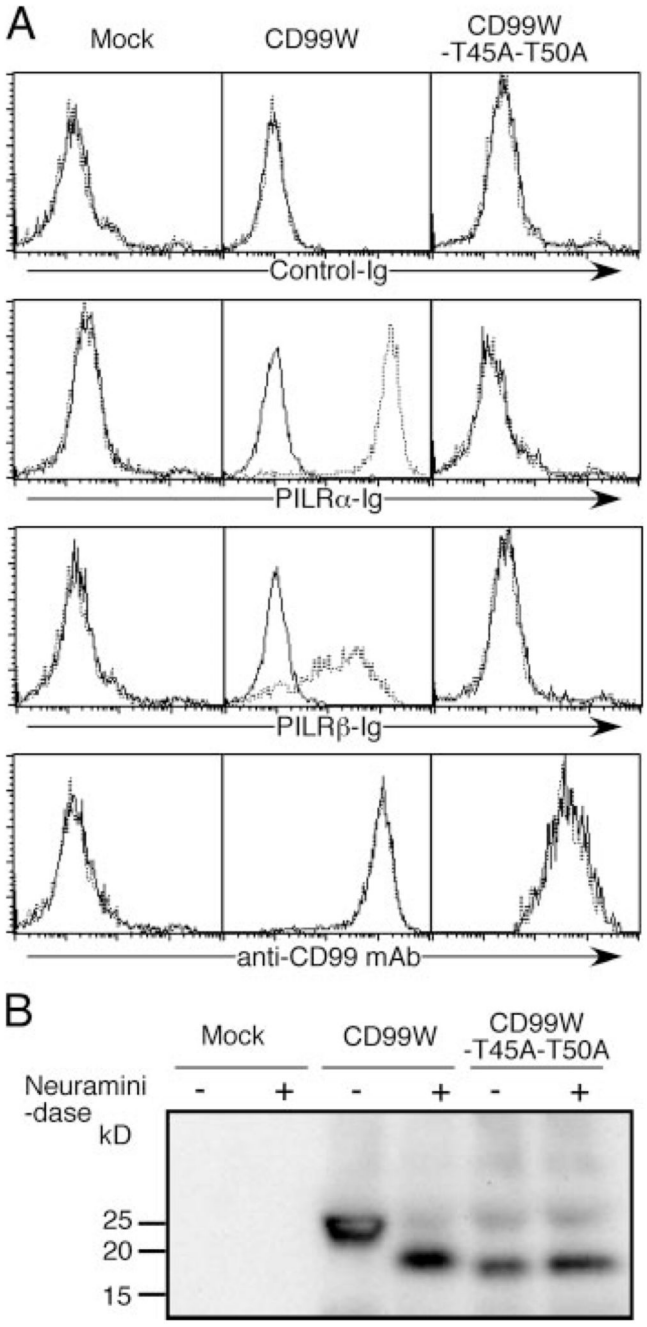

FIGURE 4.

Sialic acid is involved in the recognition of CD99 by PILR. *A*, Mock-, CD99-, or CD99W-T45A-T50A-transfected Ba/F3 cells were incubated in the presence (solid line) or absence (dotted line) of neuraminidase and were stained with PILRα-Ig, PILRβ-Ig, or control-Ig fusion protein. Expression of CD99 was analyzed by staining with anti-CD99 mAb. *B*, Western blot analysis of neuraminidase-treated cells. Mock-, CD99-, or CD99W-T45A-T50A-transfected Ba/F3 cells were incubated in the presence (+) or absence (−) with neuraminidase. Cell-surface molecules were biotinylated, and lysates of cells were precipitated with anti-CD99 mAb. The precipitates were separated by SDS-PAGE, and biotinylated proteins were detected by using peroxidase-conjugated streptavidin.

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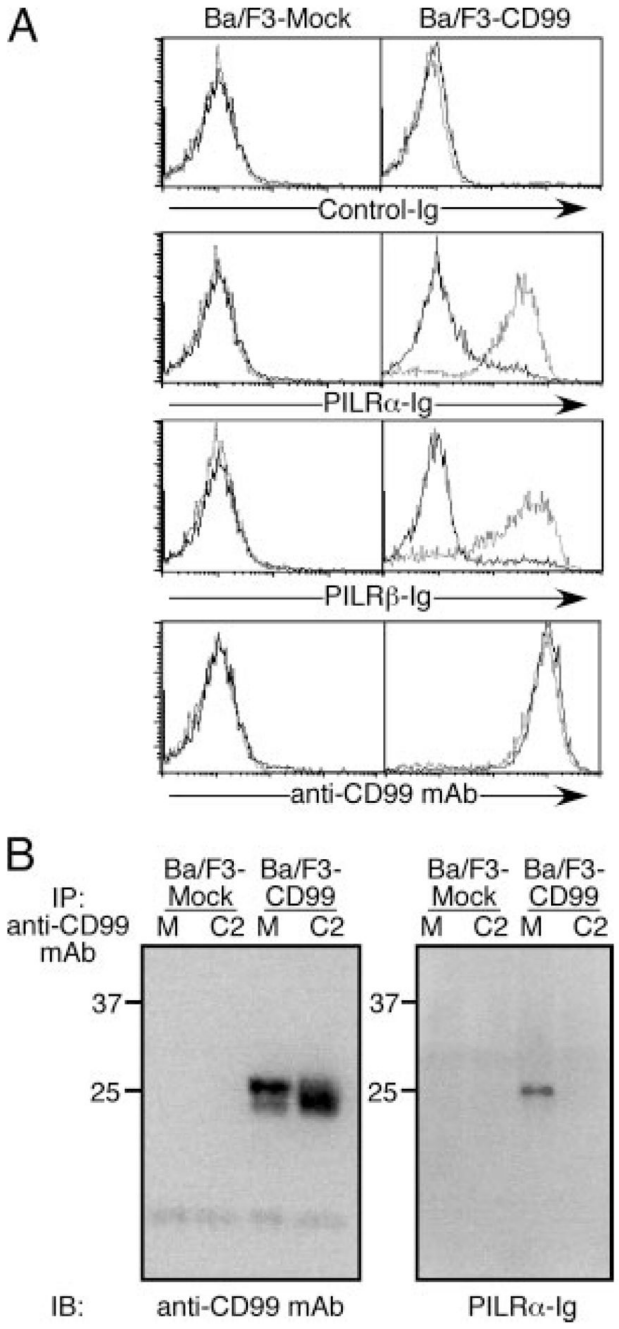

FIGURE 5.

Expression of core 2-branching enzyme abrogates PILR recognition. *A*, Mock- or CD99-transfected Ba/F3 cells were further transfected with core 2-branching enzyme (solid line) or control vector (dotted line) and were stained with control-Ig, PILRα-Ig, PILRβ-Ig, or anti-CD99 mAb. *B*, Western blot analysis of CD99 modified with core 2-branching enzyme. CD99 was precipitated from cell lysates of mock- or CD99-transfected Ba/F3 cells that were further transfected with core 2-branching enzyme (C2) or control vector (M) by anti-CD99 mAb. The precipitants were blotted with PILR-Ig (*right*) or anti-CD99 mAb (*left*), respectively.

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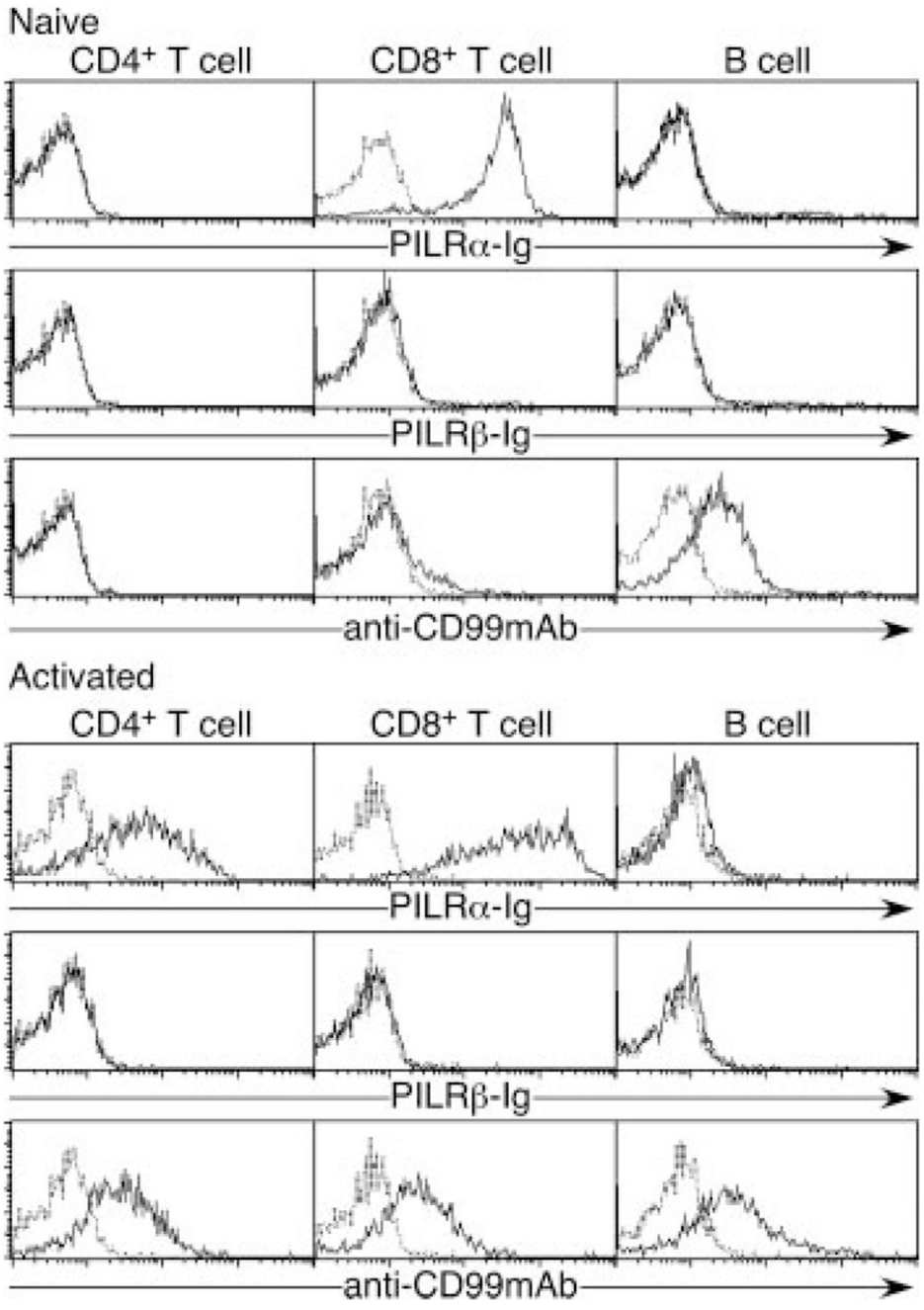

FIGURE 6.

Recognition of activated $CD4^+$ T cells, but not B cells, by PILRα. Freshly isolated splenocytes or splenocytes activated with anti-CD3 mAb or anti-IgM Ab were stained with PE-conjugated anti-CD4, PE-conjugated anti-CD8, or PE-conjugated anti-B220 mAb in combination with biotinlylated PILRα-Ig, PILRβ-Ig, or anti-CD99 mAb, followed by APC-conjugated streptavidin (solid line). Cells were also stained with control-Ig or control mAb (dotted line). Staining patterns of PILRα-Ig, PILRβ-Ig, and anti-CD99 mAb on $CD4^+$, $CD8^+$, or $B220^+$ cells are shown.

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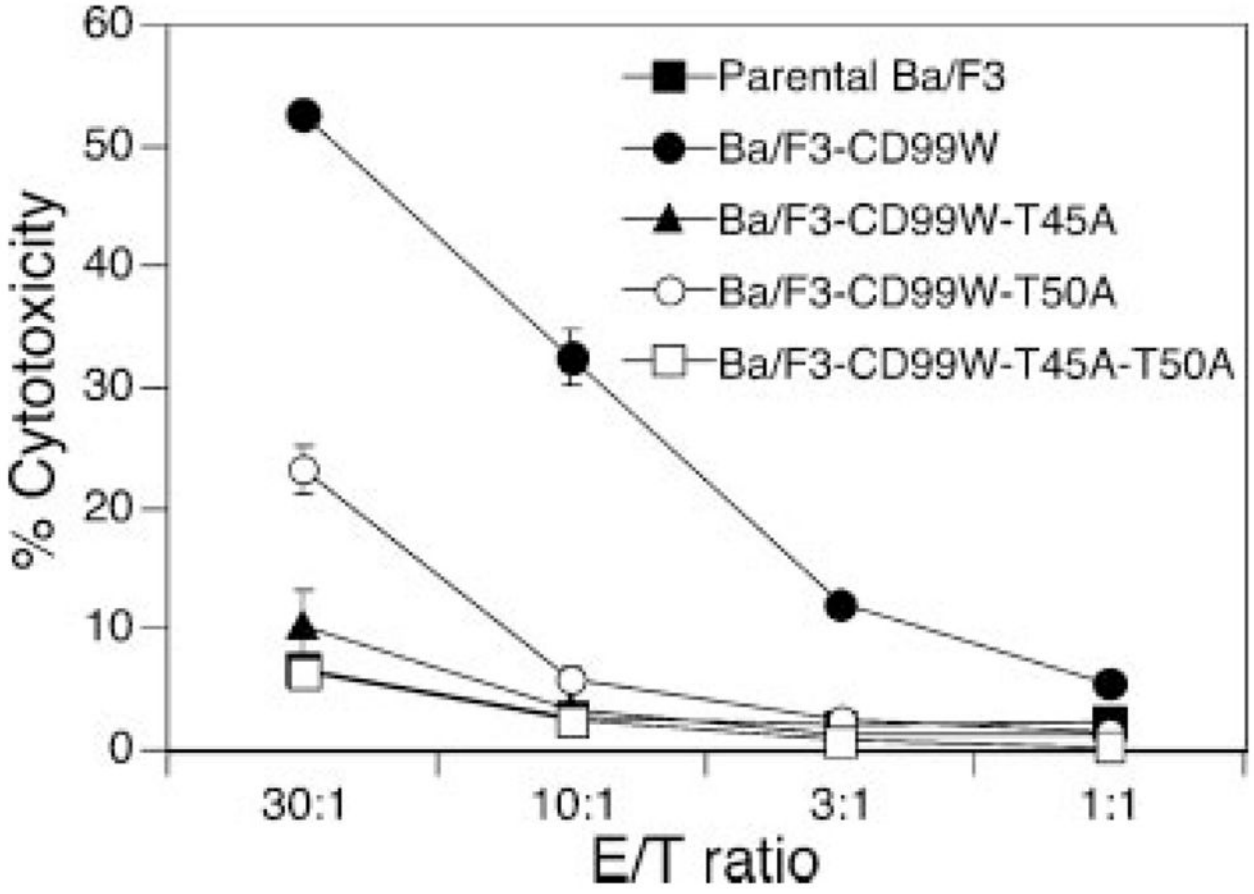

FIGURE 7.

Cytotoxicity of NK cells against Ba/F3 cells transfected with wild-type CD99 or mutant CD99. Cytotoxicity of IL-2-expanded NK cells against wild-type CD99-transfected Ba/F3 cells (●), CD99W-T45A-T50A-transfected Ba/F3 cells (□), CD99W-T45A (▲), CD99W-T50A (○), and parental Ba/F3 cells (■) is shown. Means (±SD) of triplicate assays are shown. Data are representative of three independent experiments.