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Journal

The Journal of Immunology, 193(2)

ISSN

0022-1767

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Publication Date

2014-07-15

DOI

10.4049/jimmunol.1400556

Peer reviewed

THE JOURNAL OF IMMUNOLOGY

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J Immunol 2014; 193:610-616; Prepublished online 16 June 2014;
doi: 10.4049/jimmunol.1400556
http://www.jimmunol.org/content/193/2/610

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Human NKG2E Is Expressed and Forms an Intracytoplasmic Complex with CD94 and DAP12

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The NKG2 family of NK receptors includes activating and inhibitory members. With the exception of the homodimer-forming NKG2D, NKG2 receptors recognize the nonclassical MHC class I molecule HLA-E, and they can be subdivided into two groups: those that associate with and signal through DAP12 to activate cells, and those that contain an ITIM motif to promote inhibition. The function of NKG2 family member NKG2E is unclear in humans, and its surface expression has never been conclusively established, largely because there is no Ab that binds specifically to NKG2E. Seeking to determine a role for this molecule, we chose to investigate its expression and ability to form complexes with intracellular signaling molecules. We found that NKG2E was capable of associating with CD94 and DAP12 but that the complex was retained intracellularly at the endoplasmic reticulum instead of being expressed on cell surfaces, and that this localization was dependent on a sequence of hydrophobic amino acids in the extracellular domain of NKG2E. Because this particular sequence has emerged and been conserved selectively among higher order primates evolutionarily, this observation raises the intriguing possibility that NKG2E may function as an intracellular protein. ***The Journal of Immunology*****, 2014, 193: 610–616.**

Natural killer receptors have a wide range of effects and are expressed by both NK cells (1, 2) and cytotoxic $CD8^+$ T cells (3, 4). They are involved in protection against invading pathogens (5, 6), and dysregulation of certain NK receptors has been implicated in autoimmunity (3, 7). Consequently, a delicate balance has to be maintained between the activating and inhibitory receptors present.

One such group is the NKG2 family, which is comprised of seven receptors, known as NKG2A, -B, -C, -D, -E, -F, and -H (8). With the exception of the more distantly related and homodimer-forming NKG2D (9) and the orphan receptor NKG2F (10), NKG2 family proteins assemble into heterodimers with CD94, a C-type lectin-like molecule that allows for interaction of the complex with HLA-E (11, 12). CD94/NKG2 heterodimers are expressed on most NK cells, and the inhibitory family members are expressed by many subsets of $CD8^+$ T cells. Although CD94 is associated with all NKG2 signaling complexes save NKG2D and NKG2F, activating NKG2 receptors also require the binding of the adaptor molecule DAP12, also known as KARAP (13), for cell surface expression and signaling (14). DAP12 contains an ITAM motif, allowing for activation of NK cells by the NKG2C/CD94/DAP12 complex through the Syk and ZAP70 kinases (14). In contrast, NKG2A contains an ITIM motif in its intracellular (IC) portion, allowing it to directly activate the SHP1/2 phosphatases (15) and obviating the need for an additional adaptor molecule (16). Meanwhile, NKG2F associates with DAP12 but not CD94, and thus fails to be expressed on cell surfaces (10).

Whereas NKG2A and NKG2C have been linked to autoimmune diseases such as celiac disease (17) and rheumatoid arthritis (18), as well as chronic viral infections (19), there are few data available for NKG2E. Crucially, NKG2E and NKG2C have identical transmembrane (TM) sequences (see Fig. 1C), suggesting that DAP12 will associate with both with equal efficiency (20). Furthermore, NKG2E was reported to successfully bind to HLA-E/peptide complexes (21). Finally, NKG2E has been shown to have activating properties in response to Qa-1 (22) and to be important for defense against viral infections in mice (23). Consequently, NKG2E is often cited in conjunction with NKG2C as being a de facto activating receptor.

However, there are no data that directly verify NKG2E surface expression in humans, and the association of NKG2E with DAP12 has never been demonstrated. Additionally, mouse NKG2E is unrelated to the human gene and it lacks a large portion of the extracellular (EC) domain that we dubbed the ECII region. Unfortunately, investigations into the function human (h)NKG2E are hampered by the lack of a specific Ab, as those currently available also bind to hNKG2C and hNKG2A with similar affinity (Supplemental Fig. 1A). Despite our best efforts, we were also

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Received for publication February 28, 2014. Accepted for publication May 13, 2014.

This work was supported by National Institutes of Health Grants R01 DK067180 and R01 DK058727 (to B.J.) and AI068029 (to L.L.L.), as well as by the Digestive Disease Research Core Center at the University of Chicago. L.L.L. also receives support as an American Cancer Society Professor.

G.A.O., F.T., J.S., B.M., and C.C. performed experiments; G.A.O. provided input into the conceptual development and execution of the studies; J.-C.G. and L.B.B. performed the genetic analysis; G.A.O. and B.S. analyzed the results and created the figures; L.L.L. provided constructs and cell lines; L.B.B. and L.L.L. participated in discussion and review of the manuscript; B.S. and B.J. wrote the manuscript; and B.J. devised the research and supervised all investigations.

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The online version of this article contains supplemental material.

Abbreviations used in this article: dN/dS, ratio of the rate of nonsynonymous to synonymous nucleotide mutations; EC, extracellular; EGFP, enhanced GFP; ER, endoplasmic reticulum; h, human; IC, intracellular; IRES, internal ribosome entry site; PDI, protein disulfide isomerase; PM, partial mutant; TM, transmembrane.

unable to generate an Ab of adequate specificity. Consequently, it has yet to be determined whether NKG2E can be expressed at a protein level in human NK or T cells and, if so, whether it is able to function as a receptor. It has been previously noted that NKG2E transcripts are increased in NKG2A$^-$ CTLs that have undergone NK reprogramming in patients with celiac disease, but whether and how this may contribute to pathogenesis are unknown (17, 24).

We sought to address this gap in the literature by investigating NKG2E at a protein level, using FLAG and MYC tagging in absence of a specific Ab, as previously described (14). Intriguingly, we found that although NKG2E is translated, unlike NKG2C it fails to be expressed on cell surfaces efficiently and is instead retained intracellularly. Furthermore, we observed that this retention is dependent on the ECII portion of the EC domain. Finally, we demonstrate that NKG2E is assembled into a multiprotein complex with CD94 and DAP12.

Materials and Methods

Cell culture

The 293T cell line is a human renal epithelial cell line and was cultured in RPMI 1640 medium with 10% FBS and antibiotics. The Ba/F3 cell line is a murine B cell line and was cultured in DMEM with 10% FBS, 1% L-glutamine, and 1% streptomycin/penicillin.

Transfection

Human CD94, NKG2C, NKG2E, and DAP12 were cloned for mammalian expression into pcDNA4/TO (Invitrogen), papuroFLAG (containing N-terminal FLAG tag), pcDNA-myc (Invitrogen, containing C-terminal MYC tag), pIRES–enhanced GFP (EGFP) (Clontech), and SX vectors by standard cloning techniques. 293T cells were transfected with plasmids using Lipofectamine 2000 reagent (Invitrogen) after reaching ~80% confluency. The standard transfection protocol provided by the manufacturer was used. After transfection, cells were cultured for 24–30 h before staining for flow cytometry. Ba/F3 cells were transfected with the Amaxa Nucleofector using Nucleofection solution V and program T-20. Cells were cultured for 24–30 h after transfection before staining for flow cytometry. Ba/F3 cells expressing CD94/DAP12 and CD94/NKG2C-IRES-EGFP/DAP12 were previously described (14). For the generation of stable transfectants of CD94/NKG2E-IRES-EGFP/DAP12, Ba/F3 cells were transfected with Amaxa and sorted for expression of EGFP, then cultured in medium with selective antibiotics.

Reagents for flow cytometry

PE- and allophycocyanin-conjugated anti-CD94 were purchased from BD Biosciences. Unconjugated anti-MYC Ab was purchased from Covance. Unconjugated anti-NKG2E Ab was purchased from Abnova. Fluorochrome-conjugated anti-FLAG Ab was purchased from Sigma-Aldrich. Anti-DAP12 (14) and HLA-E tetramers loaded with HLA-G leader peptide (11) were received as gifts. Unconjugated F(ab′)$_2$ goat anti-mouse IgG2a isotype control was purchased from SouthernBiotech.

Reagents for confocal microscopy

Anti-human CD94 mouse IgG2a (Beckman Coulter), anti– protein disulfide isomerase (PDI) rabbit (Abcam), and anti–pan-cadherin mouse IgG1 (Abcam) were used as the primary Abs for confocal microscopy. The secondary Abs used were goat anti-mouse IgG2a-FITC, donkey anti-rabbit Cy5, and goat anti-mouse IgG1-Cy5 and were from Jackson ImmunoResearch Laboratories.

Reagents for immunoprecipitation and Western blot

Anti-hCD94 (Beckman Coulter) and anti–hNKG2-N-18 (Santa Cruz Biotechnology) were used for immunoprecipitation. Abs used for blotting were anti–hNKG2-N-18 and mouse anti-hDAP12. The associated secondary Abs were HRP-conjugated donkey anti-goat and goat anti-mouse Abs (from Jackson ImmunoResearch Laboratories).

Flow cytometry analysis

For surface staining, cells were incubated with fluorochrome-conjugated Abs according to standard protocols. Unconjugated Abs were revealed with appropriate fluorochrome-conjugated F(ab) goat anti-mouse IgG isotype. For IC staining, cells were fixed and permeabilized using Cytofix/Cytoperm solution (BD Biosciences). Fluorescence was analyzed on a four-color FACSCanto or FACSCalibur with quadrants set to score as negative >99% of control Ig-stained cells.

Confocal microscopy

293T cells were grown on coverslips overnight, followed by transfection with CD94, DAP12, and NKG2C or NKG2E using Lipofectamine 2000 (Invitrogen). Cells were fixed with 3.7% paraformaldehyde and stained with Abs for receptor complex and subcellular localization markers for 1 h at room temperature. Abs were chosen to have different species of origin or IgG isotype specificity when of mouse origin. Coverslips were washed in PBS, mounted with ProLong antishade reagent (Invitrogen), and analyzed by confocal microscopy using a TCS SP2 AOBS confocal microscope and workstation (Leica). Samples were obtained with sequential acquisition of different wavelengths and a ×63 (oil) objective lens with a numerical aperture of 1.4.

Western blot and immunoprecipitation

293T cells were transfected with human CD94, DAP12, and NKG2C or NKG2E. Ba/F3 cell lines were transfected with Nucleofector solution V (Amaxa), sorted for high EGFP expression, and selected by antibiotics. Cells were cultured, harvested, and lysed. In brief, cells were lysed in buffer containing freshly added protease and phosphatase inhibitors (50 mM Tris HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 10 mM iodoacetamide, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitor mixture tablets). For immunoprecipitation, lysates in 0.5% Triton X-100 were incubated with anti-hCD94 or anti–hNKG2-N-18, specific for NKG2C and NKG2E but not for NKG2A. Equal amounts of protein were subjected to SDS-PAGE under reducing conditions in 12% gels, transferred to nitrocellulose membranes (Bio-Rad Laboratories), and probed with goat anti-hNKG2 and mouse anti-hDAP12, followed by HRP-conjugated donkey anti-goat and goat anti-mouse Abs. To control for loading differences, blots were probed for total DAP12 and NKG2. Binding of secondary Abs was visualized using an ECL kit (GE Healthcare).

Generation of reciprocal chimeras and mutants

NKG2C and NKG2E chimeras were created by recursive PCR as previously described (Current Protocols, John Wiley & Sons). In brief, primers spanning different regions of NKG2E and NKG2C were used to create DNA fragments, which were reciprocally hybridized and the final products amplified. In the NKG2E/C chimera the last 17 aa of NKG2E were replaced with the last 9 aa of NKG2C, whereas in the NKG2C/E chimera the reverse was performed. NKG2E mutants were created by standard PCR mutagenesis using custom primers. Chimeras and mutants were cloned into pcDNA-myc and pIRES-EGFP. Final constructs were confirmed by DNA sequencing.

Phylogenetic analysis of the NKG2C and NKG2E family members

The alignment of NKG2C and NKG2E among primates and two rodents (mouse and rat). was done using the software MUSCLE (25, 26) and visualized using ClustalX (27). The phylogenetic tree based on protein sequence alignment was inferred by RAxML 7.2.8 under an LG+F+Gamma4 model of sequence evolution (empirical amino acid frequencies and four discrete γ categories). Bootstrap values were based on 100 replicates. The ratio of the rate of nonsynonymous to synonymous nucleotide mutations (dN/dS) was calculated using the previously described method of Li et al. (28).

Results

NKG2E is translated and associates with CD94 and DAP12, but does not translocate to the cell surface

To assess first whether NKG2E could form a complex with CD94 and DAP12, we transfected the human renal epithelial 293T cell line and the murine Ba/F3 B cell line previously used to study CD94 with CD94, DAP12, and either NKG2E- or NKG2C-expressing plasmids, with both NKG2 members tagged with C-terminal MYC (14). We then stained for surface MYC and found that although NKG2C was present on the cell surface as expected, NKG2E-transfected cells expressed minimal NKG2E extracellularly (Fig. 1A, *left panels*). The presence of both proteins after transfection and lack of NKG2 expression in untransfected controls

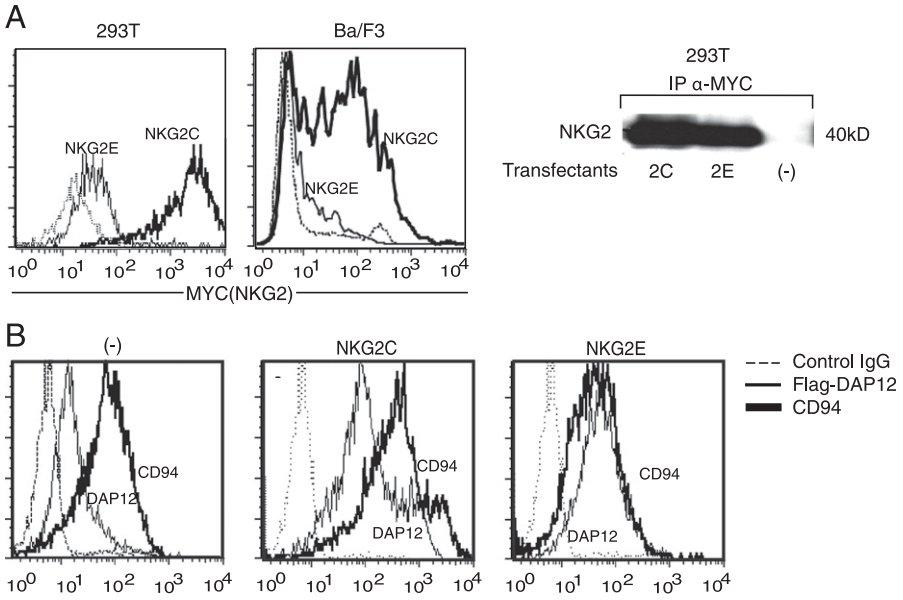

FIGURE 1. NKG2E is translated and associates with CD94, but it fails to be expressed on cell surfaces. (**A**) NKG2C but not NKG2E is expressed on cell surfaces. 293T (*left*) and Ba/F3 (*middle*) cells were transfected with MYC-tagged NKG2C or NKG2E in conjunction with DAP12 and CD94. Cells were surface stained using anti-MYC mAb and analyzed by flow cytometry. Protein expression was verified by Western blot (*right*). Data are representative of at least three independent experiments. (**B**) CD94 and DAP12 are expressed on cell surfaces when transfected with NKG2C but not NKG2E. Ba/F3 cells were transfected with CD94 and FLAG-tagged DAP12 alone (*left*) or additionally with NKG2C (*middle*) or NKG2E (*right*) and analyzed for CD94 and DAP12 surface expression by flow cytometry using anti-CD94 or anti-FLAG mAb. Data are representative of at least three independent experiments.

were confirmed by immunoblotting (Fig. 1A, *right panel*). To verify that the lack of NKG2E surface expression was not caused by an unanticipated side effect of tagging with MYC, we transfected Ba/F3 cells with N-terminal FLAG-tagged DAP12, CD94, and NKG2E or NKG2C cloned into bicistronic IRES-EGFP. EGFP$^+$ cells were then surface stained using anti-CD94 and anti-FLAG Abs. We found that NKG2C transfection led to major upregulation of CD94 and DAP12 on the surface compared with cells transfected with CD94 and DAP12 alone, whereas the presence of NKG2E failed to induce surface expression (Fig. 1B). Overall, these data demonstrate that NKG2E transcripts are translated within cells, but NKG2E proteins fail to be expressed on cell surfaces in the presence of CD94 and DAP12.

NKG2E forms an IC complex with CD94 and DAP12

Given the finding that NKG2E is not present on cell surfaces, we were intrigued by the possibility that NKG2E might form IC complexes with CD94 and DAP12. We first sought to determine whether NKG2E is capable of retaining CD94 within the IC compartment. To study a potential effect of NKG2E on surface CD94 expression, we transfected 293T cells with CD94 alone, CD94 with DAP12, or CD94, DAP12, and NKG2E or NKG2C. Cells were then stained in two steps, first with anti-CD94 conjugated to allophycocyanin for surface detection, then with anti–CD94-PE for IC detection after fixation and permeabilization. We found that there was a basal level of surface CD94 expression that was unaffected by the presence of DAP12, and that coexpression of NKG2C caused a significant increase in CD94 extracellularly (Fig. 2A). In contrast, transfection with NKG2E resulted in substantial downregulation of surface CD94 compared with cells transfected with CD94 alone or CD94 in conjunction with DAP12, suggesting that NKG2E not only fails to be expressed on cell surfaces, but that it additionally is capable of preventing surface CD94 expression.

To further elucidate the subcellular compartmentalization of CD94 in the presence of NKG2E, we performed confocal microscopy on 293T cells transfected with CD94, DAP12, and NKG2E or NKG2C. Cells were stained for CD94 (green) and PDI or pan-cadherin (both shown in red). PDI exclusively stains the ER, whereas both the ER and plasma membrane stain positively by pan-cadherin. We found that when CD94 was transfected with DAP12 and NKG2C, it colocalized with cadherin but not PDI, suggesting an association with the plasma membrane (Fig. 2B). In contrast, CD94 colocalized with both cadherin and PDI when transfected with NKG2E, indicating an association with the ER rather than the plasma membrane (Fig. 2B).

DAP12 is required for the surface expression of NKG2C (2) forming associations via interactions between TM residues (20). Because the TM domain of NKG2E is identical to that of NKG2C (Fig. 2C), we wondered whether NKG2E was capable of forming a complex with DAP12 and CD94 despite its inability to be expressed on cell surfaces. To that end, we transfected 293T cells with various combinations of CD94, DAP12, and NKG2E or NKG2C. We then performed coimmunoprecipitation experiments, using an anti-CD94 Ab to pull down the protein complex and subsequently probing using anti-DAP12. As expected, we were able to confirm that CD94 formed a complex with DAP12 when cotransfected with NKG2C. Intriguingly, we also noted that NKG2E had a similar capacity to form complexes with DAP12 and CD94 despite its inability to localize to cell surfaces (Fig. 2D).

To verify our findings, we also tested the association between NKG2, CD94, and DAP12 in Ba/F3 cells. After transfection with CD94, DAP12, and/or NKG2E or NKG2C, we again used coimmunoprecipitation to analyze protein associations. When an anti-CD94 Ab was used to pull down the complex, we found that roughly the same amount of NKG2E bound CD94 as did NKG2C (Fig. 2E, *top row*), despite lower levels of expression overall (Fig. 2E, *second row*). Additionally, we used an anti-NKG2 Ab to pull down and probed using an anti-DAP12 Ab, and we found that NKG2E was able to associate with DAP12 (Fig. 2E, *third row*). Total DAP12 levels are shown as a loading control (Fig. 2E, *bottom row*). Finally, NKG2E levels were increased upon incubation with the proteasomal inhibitor MG132, and we noted a corresponding rise in DAP12 in complex with NKG2E (Fig. 2F).

Overall, these data demonstrate that NKG2E forms an IC complex with CD94 and DAP12, even though it fails to reach the cell surface.

Hydrophobic residues in the EC portion of NKG2E are responsible for its IC retention

We next sought to determine which portion of NKG2E was responsible for its failure to be expressed on cell surfaces. Protein trafficking to membranes is dictated by the presence of specific recognition sequences within the TM, IC, or EC segments. When

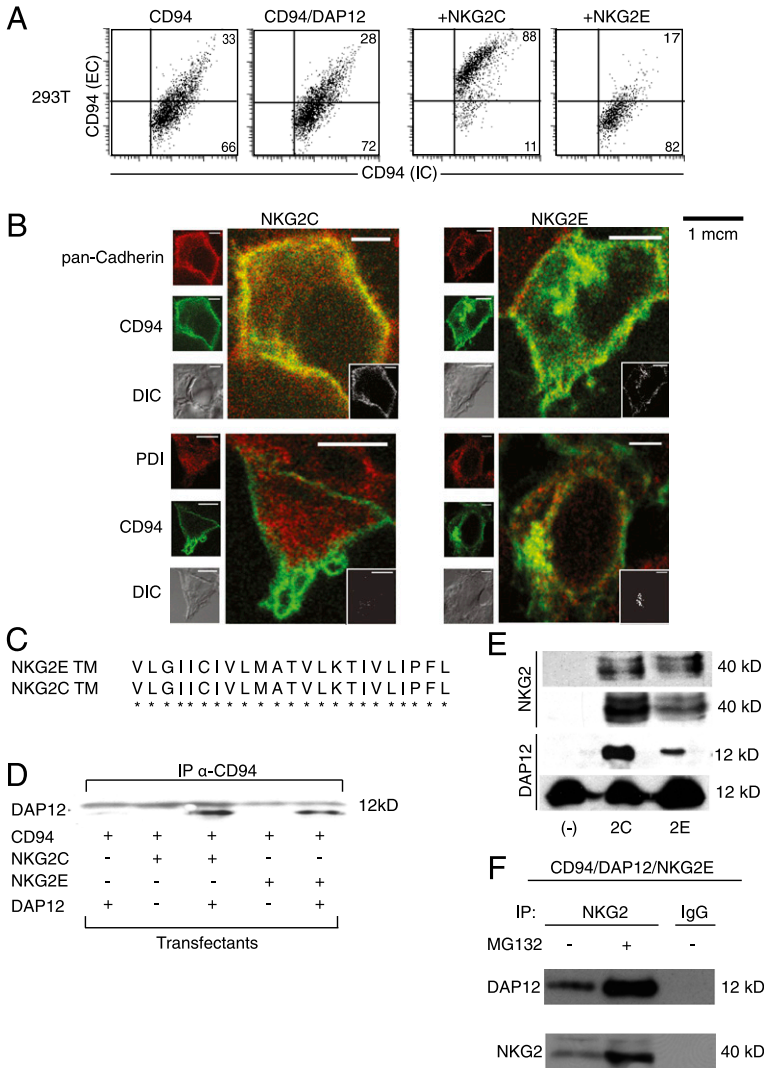

FIGURE 2. NKG2E forms an IC complex with DAP12 and CD94 and is trafficked to the ER but not the plasma membrane. (**A**) 293T cells were transfected with CD94 alone (*first panel*), CD94 and DAP12 (*second panel*), CD94/DAP12/NKG2C (*third panel*), or CD94/DAP12/NKG2E (*fourth panel*). Cells were stained for surface expression of CD94 and were permeabilized and stained for IC CD94. Data are representative of at least three independent experiments. (**B**) 293T cells were transfected with CD94, DAP12, and either NKG2C (*left panels*) or NKG2E (*right panels*) and assessed by confocal microscopy. CD94 is shown in green whereas differential interference contrast (DIC) is shown in gray for all samples. Pan-cadherin is shown in red in the *top two panels*, whereas PDI is shown in red on the *bottom two panels*. Images are representative of at least three independent experiments. Scale bars, 1 μm. (**C**) NKG2C and NKG2E have identical TM domains, suggesting that NKG2E can associate with CD94. (**D**) DAP12 associates with CD94 in the presence of NKG2C or NKG2E in 293T cells. 293T cells were transfected as in (B). Coimmunoprecipitation was performed on lysates using an anti-CD94 mAb to pull down the NKG2/CD94/DAP12 complex, which was then probed using an anti-DAP12 mAb. Data are representative of at least three independent experiments. (**E**) NKG2E forms a complex with DAP12 and CD94 in Ba/F3 cells. Ba/F3 cells were transfected as in (B). Cell lysates were immunoprecipitated with anti-CD94 and probed with anti-NKG2 (*top row*). Lysates were also immunoprecipitated with anti-NKG2 and probed with anti-DAP12 (*third row*). Total lysates were blotted with anti-NKG2 (*second row*) and anti-DAP12 (*bottom row*) as loading controls. Data are representative of at least three independent experiments. (**F**) Proteasomal inhibitor MG132 increases expression of CD94/NKG2E/DAP12 complexes. Ba/F3 cells were transfected with CD94, DAP12, and NKG2E and were then treated with MG132 or with a vehicle control. Lysates were subsequently immunoprecipitated using an anti-NKG2 mAb or an IgG control. The resulting complex was probed using an anti-DAP12 mAb (*top row*) or an anti-NKG2 mAb (*bottom row*). Data are representative of at least three independent experiments.

comparing the sequences of NKG2E and NKG2C, we found that most of the nonhomologous amino acid differences were localized to the end of the EC C terminus in a region we dubbed the ECII segment (Fig. 3A). Furthermore, we noted that the ECII region of NKG2E contained a great deal of hydrophobic residues (Fig. 3A), which have previously been implicated in the retention of proteins in the ER (29).

To test whether the ECII region of NKG2E was responsible for its lack of expression on cell surfaces, we generated three pairs of chimeric molecules by exchanging the IC, ECI, or ECII segments of NKG2E and NKG2C (Fig. 3A and data not shown). We noted no change in receptor expression patterns between chimeras generated by switching either the IC or ECI sequences upon transfection into 293T cells along with CD94 and DAP12 (data not shown). However, when the ECII domain of NKG2E was replaced with that of NKG2C (NKG2E-C), we saw robust up-regulation of surface expression compared with native NKG2E, as detected by tetramers of HLA-E loaded with HLA-G leader peptide (Fig. 3B) and by an anti-NKG2E Ab (Supplemental Fig. 1B). Conversely, cells expressing NKG2C containing the ECII domain of NKG2E (NKG2C-E) were only weakly stained by the tetramer (Fig. 3B). Lysates were probed by Western blot to verify chimera protein expression (data not shown).

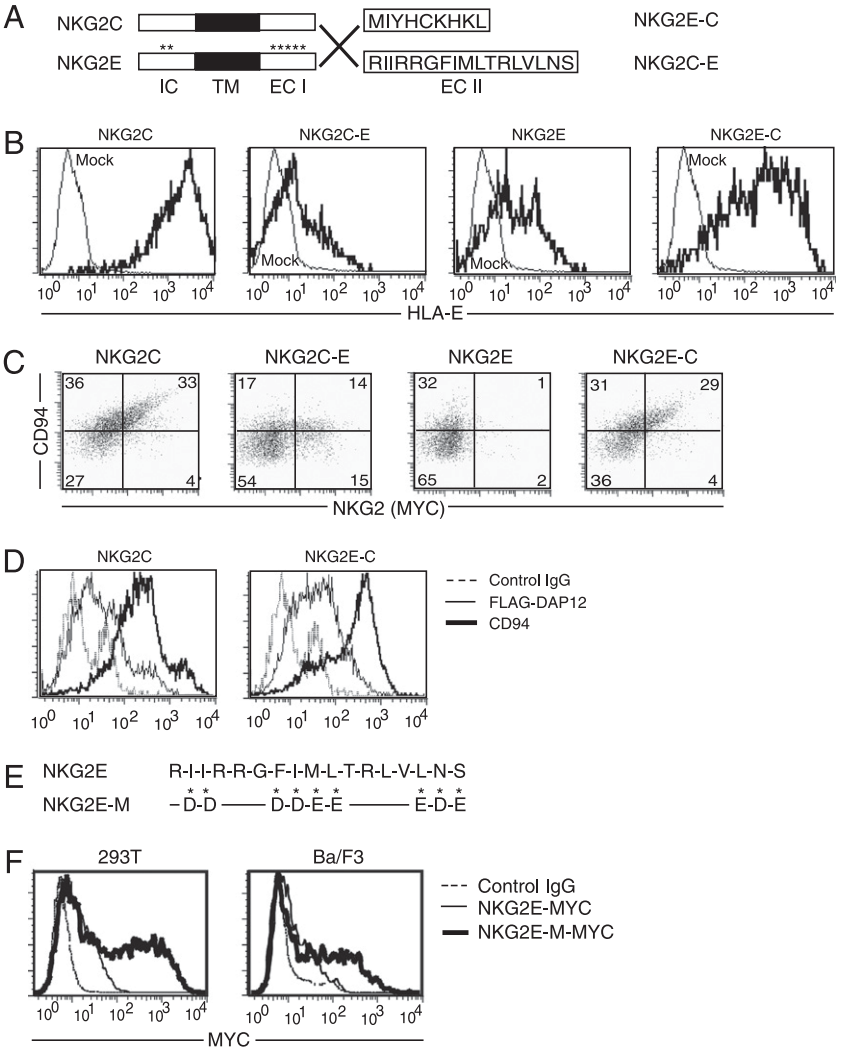

FIGURE 3. Hydrophobic residues in the EC portion of NKG2E cause it to be retained intracellularly. (**A**) Generation of chimeras is shown schematically. The final 17 aa of NKG2E were used to replace the final 9 aa of NKG2C to create the NKG2C-E chimera, whereas the reverse was performed to create the NKG2E-C fusion protein. (**B**) Evaluation of chimera function by flow cytometry. 293T cells were transfected with the indicated MYC-tagged fusion or control NKG2 in addition to CD94 and DAP12 and were evaluated for their ability to recognize HLA-E tetramers loaded with HLA-G leader peptide. Data are representative of at least three separate experiments. (**C**) Evaluation of chimera surface expression by flow cytometry. Ba/F3 cells were transfected as in (B), then stained with anti-CD94 and anti-MYC mAbs. Data are representative of at least three independent experiments. (**D**) Surface expression of DAP12 and CD94 was assessed to verify complex formation and translocation. Ba/F3 cells were transfected with untagged NKG2C or NKG2E-C, FLAG-tagged DAP12, and CD94 and then analyzed by flow cytometry using anti-FLAG and anti-CD94 mAb. Data are representative of at least three independent experiments. (**E**) Schematic of the mutations generated in the ECII domain of NKG2E. Hydrophobic residues were replaced with either aspartic or glutamic acid according to whichever more closely approximated the size of the replaced residue. (**F**) Replacement of all three stretches of hydrophobic residues resulted in surface expression of NKG2E. MYC-tagged mutant or control NKG2E was transfected into 293T (*left*) and Ba/F3 (*right*) cells in conjunction with DAP12 and CD94, and surface expression was evaluated by flow cytometry using an anti-MYC Ab. Data are representative of at least three experiments.

We then sought to verify this expression pattern by using Ba/F3 cells, which better approximate physiological systems. Cells were transfected with CD94, DAP12, and MYC-tagged NKG2E, NKG2C, or one of our chimeras and then stained for CD94 and MYC expression. The inability of NKG2E to be expressed on cell surfaces was even more pronounced than in 293T cell line (Fig. 3C). We again noted that the surface association of CD94 and NKG2C was strongly reduced upon replacement of its ECII domain, whereas NKG2E gained the ability to coexpress with CD94 at cell surfaces upon receipt of the ECII domain of NKG2C (Fig. 3C). To further confirm that the ECII domain was critical for cellular localization and that our data were not an artifact from MYC tagging, we transfected Ba/F3 cells with CD94, NKG2E-C, or NKG2C, as well as with N-terminal FLAG-tagged DAP12. Staining for CD94 revealed that its expression was highly upregulated in conjunction with NKG2E-C (Fig. 3D). Furthermore, DAP12 surface levels were increased when transfected with NKG2E possessing the NKG2C ECII domain (Fig. 3D).

Upon further examination of the ECII region of NKG2E, we found a large number of hydrophobic residues (9 of 17) assembled into three sequences (Fig. 3E). Suspecting that one or more of these sequences might be responsible for the IC retention of NKG2E, we generated substitutions of each hydrophobic sequence for negatively charged aspartic or glutamic acids, choosing either D or E to best approximate the size of the native residue replaced. In total, we created three partial mutants and one full mutant: NKG2E partial mutant 1 (NKG2E-PM1), I225I226 to D225D226; NKG2E-PM2, F230I231M232L233 to D230D231E232E233; NKG2E-PM3, L236N237S238 to E236D237E238; and NKG2E-M, which has all the hydrophobic residues replaced (Supplemental Fig. 2A). We noted that whereas NKG2E-PM1 and NKG2E-PM3 were not efficiently expressed on cell surfaces after transfection with DAP12 and CD94, NKG2E-PM2 expression was increased roughly 10-fold, with surface expression of the NKG2E-M mutant lacking all hydrophobic sequences having increased a further 10-fold (Supplemental Fig. 2B). We transfected NKG2E-M into both Ba/F3 and 293T cells, noting that compared with native NKG2E, NKG2E-M was expressed at significantly higher levels on the surface when transfected with DAP12 and CD94 (Fig. 3F).

Taken together, these data indicate that the hydrophobic residues of the ECII region are responsible for its IC retention.

NKG2E is present only in higher order primates and is strongly conserved

In the mouse, NKG2C and NKG2E are very closely related proteins with an overall homology of 91% in their amino acid sequences (22) and no differences in their ECII domains. This observation is in accordance with previous studies showing that, in mice, these two molecules serve identical functions (22). In contrast, the ECII domains of the human NKG2C and NKG2E are markedly divergent and, as our study shows, such divergence leads to major functional differences between these two molecules. Interestingly, phylogenetic analysis revealed that only *Pan troglodytes* (chim-

panzees) and *Pongo abelii* (orangutans) have a clearly homologous version of human NKG2E, as defined by the presence of the unique ECII domain (Fig. 4A). This places the appearance of the human-like NKG2E at only ~16 million years ago (Fig. 4B). Since its appearance, however, the distinctive amino acid sequence of NKG2E and particularly the ECII domain appear to be strongly conserved. To formally test whether NKG2E is evolving under strong selective constraints, we calculated dN/dS ratios using both the entire NKG2E sequences as well as restricting the analyses to the ECII region. We found that dN/dS ratios for the entire NKG2E were significantly <1 in all pairwise comparisons (humans versus chimpanzees, NKG2E dN/dS = 0.15, $p = 4.98 \times 10^{-6}$; humans versus orangutans, NKG2E dN/dS = 0.44, $p = 0.03$; chimpanzees versus orangutans, NKG2E dN/dS = 0.45, $p = 9.12 \times 10^{-3}$). When focusing uniquely on the ECII region a similar trend was observed. Between humans and orangutans we found a dN/dS of 0.27 (lower than that observed for the entire sequence). This value was nonsignificant ($p > 0.05$), which could be expected given the short length of the sequence (only 51 bp). Between humans and chimpanzees, the nucleotide sequence coding the ECII region is 100% identical between the two species, which is again compatible with the notion that the ECII region is probably evolving under strong selective constraints after the divergence between humans and chimpanzees. Finally, we also noted that all ECII hydrophobic sites that we showed to be important for the regulation of surface expression of NKG2E (Supplemental Fig. 2) are 100% conserved across the three species. Collectively, these analyses provide statistical support that NKG2E has been evolutionary conserved since its recent emergence in the Hominidae, which indicates that NKG2E plays an important and nonredundant role in host immunity.

Discussion

The exact role of NKG2E has remained poorly defined, largely due to technical limitations imposed by the lack of a specific Ab. Using tagged NKG2E and DAP12 as previously described, we

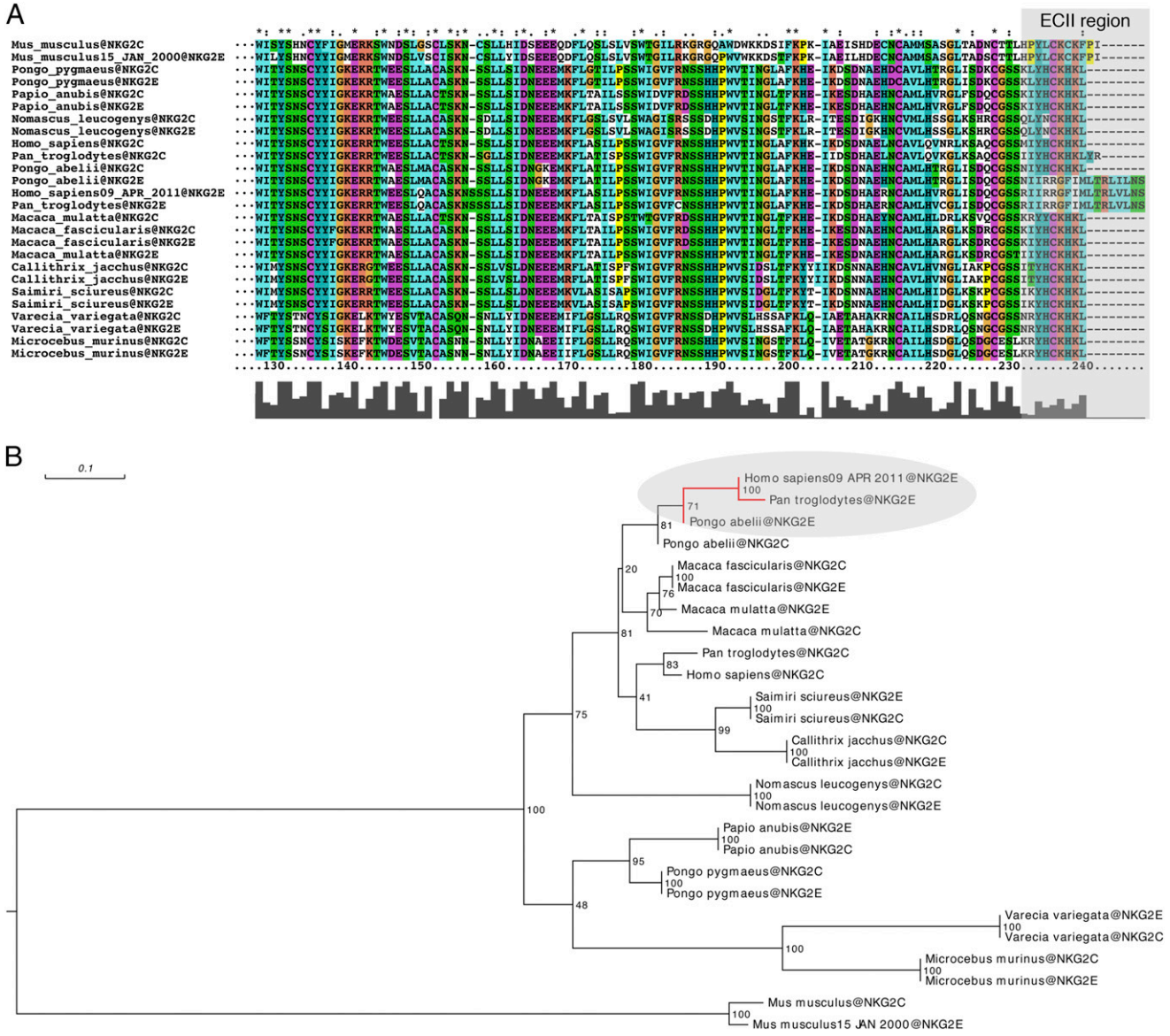

FIGURE 4. Phylogenetic analysis of the NKG2C-E family members. (**A**) Multiple sequence alignment of the protein sequence of NKG2C and NKG2E among primates and two rodents (mouse and rat). The alignment was done using the software MUSCLE and visualization using ClustalX. (**B**) Phylogenetic tree based on protein sequence alignment. The tree was inferred by RaxML 7.2.8 under an LG+F+Gamma4 model of sequence evolution (empirical amino acid frequencies and four discrete γ categories). The values indicated correspond to bootstrap support values based on 100 replicates.

found that NKG2E is translated and forms an IC complex with CD94 and DAP12.

DAP12 is a critical adaptor molecule required for surface expression and signaling through not only NKG2C and the activating members of the NKG2 family, but also through other activating NK receptors such as NKp44 (30) and the non-ITIM–bearing killer cell inhibitory receptors (31). Consequently, it plays a key role in the activation of inflammatory and killing pathways by NK cells (1, 2, 6) and pathological cytotoxic T cells that have acquired an NK-like phenotype in the context of chronic viral infections (19) and autoimmune disorders such as celiac disease (17). Our experiments suggest that NKG2E associates with DAP12, suggesting two possible functions for NKG2E: it may play a role in the recognition of IC molecules, or it may act in a regulatory capacity by binding to adaptor molecules and preventing their association with other activating receptors on the cell surface. NKG2E was shown to have a higher affinity for HLA-E than did the corresponding NKG2C complex (21). Given that NK cells themselves express HLA-E, it is possible that the role of the CD94/NKG2E/DAP12 receptor in NK cells is to recognize IC HLA-E complexed to peptides that direct trafficking to the ER rather than the cell surface. Alternatively, soluble HLA-E could be taken up by NK cells and subsequently recognized by IC CD94/NKG2E/DAP12 complexes. Such a mechanism has been implicated in NK cell activation by HLA-G recognized by IC KIRD2L4 (32). However, the mechanism by which these complexes would be targeted to the ER remains unknown.

Another intriguing possibility is that NKG2E could potentially function as a regulatory molecule that limits destructive inflammatory responses by reducing free levels of DAP12 that could bind other inflammatory receptors. We did note that the proteasome inhibitor MG132 increased expression of DAP12 and NKG2E. We also have data suggesting that association with NKG2E results in increased ubiquitination of DAP12, but these studies were limited by the fact that we had no recourse but to transfect nonlimiting amounts of NKG2C and NKG2E, because there is no Ab that can differentiate between the two (Supplemental Fig. 1B). Furthermore, we were unable to show that NKG2E impacted CD94/NKG2C-mediated activation of NK cells (data not shown).

Even though the specific role of NKG2E remains to be determined, our study demonstrates that NKG2E is functional in that it can retain CD94 in the IC compartment and form a complex with DAP12. Furthermore, our study reveals that NKG2E acts as an IC protein and that this characteristic is linked to the hydrophobic residues present in its ECII domain. Importantly, phylogenetic analysis suggests that NKG2E has evolved relative recently, is present only in *P. troglodytes* (chimpanzees) and *P. abelii* (orangutans), and that the ECII region that is responsible for its IC retention is highly conserved, suggesting that the ECII region may play an important role in higher primates. Further studies may uncover whether NKG2E signaling represents a key IC activation pathway or, alternatively, the molecule may play a novel regulatory role in restricting the availability of binding partners for other activating receptors.

Disclosures

The authors have no financial conflicts of interest.

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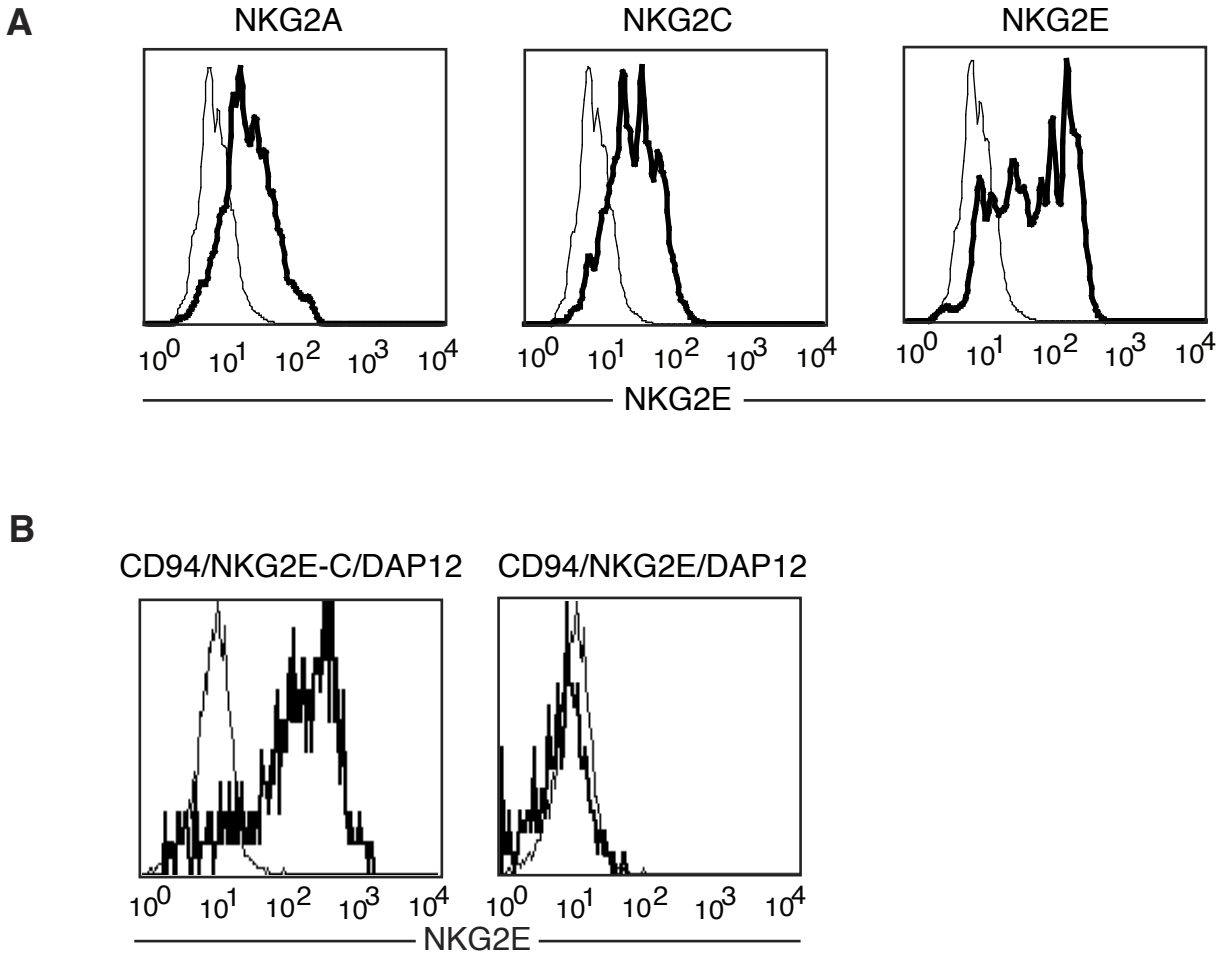
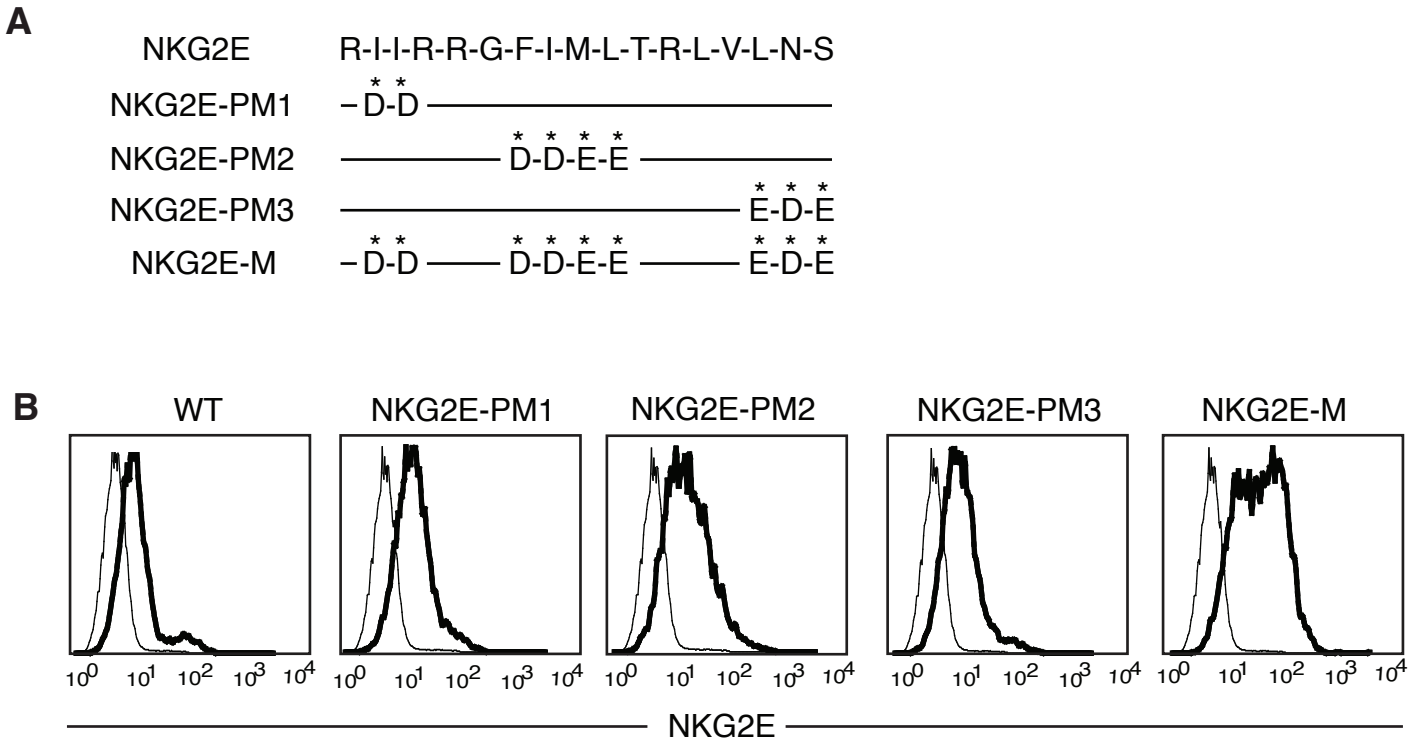


Supplemental Figure 1: (A) Antibodies purportedly specific for NKG2E display cross reactivity towards NKG2A and NKG2C as well. 293T cells were transfected with NKG2A, NKG2C, or NKG2E, then assessed for intracellular NKG2E expression. **(B)** Chimeric NKG2E-C goes to the cell surface and is recognized by an NKG2E-specific antibody. Ba/F3 cells were transfected with CD94, DAP12, and NKG2E or NKG2E-C.

Figure S1



Supplemental Figure 2: (A) Schematic of the partial mutations of hydrophobic residues generated in the ECII domain of NKG2E. **(B)** Partial mutation of the ECII region results in only mild upregulation of NKG2E on the cell surface. 293T cells were transfected with a mutant NKG2E in conjunction with CD94 and DAP12 and assessed for surface expression of NKG2E using an anti-NKG2 family antibody. Mutation of either the first or third hydrophobic sequence (NKG2E-PM1 and NKG2E-PM3, respectively) does not result in increased surface expression. NKG2E-PM2 surface expression is increased roughly 10 fold. Robust upregulation is only observed when the NKG2E-M mutant is used, which has all three hydrophobic sequences replaced.

Figure S2