ONLINE METHODS
Production and purification of linked transmembrane peptide complexes. Transmembrane peptides were produced as fusions to the carboxyl terminus of the 14-kilodalton trpEL sequence derived from the *Escherichia coli* tryptophan operon leader region, which directs fusion proteins to inclusion bodies and carries a carboxy-terminal unique methionine residue for cleavage mediated by cyanogen bromide. Peptide sequences fused to trpEL for this study were as follows: DAP12<sub>TM</sub> (33 amino acids), CSTSVPGVLAGIVGVDLTVLIA LAVYLFLGR; DAP12<sub>TM</sub> DKKDDKDK (40 amino acids), CSTSVPGVLAG IVYGDLTVVLTIALAVYLFGR; and DAP12<sub>TM</sub> NKG2C<sub>TM</sub> (63 amino acids), CSTSVPGVLAGIVGVDLTVLIALAVYLFGRGLTA EVGLGIIIIVATV2KLTVLPIFLEQN. In this study, predicted transmembrane domains are underlined; non-native sequences are in bold and served the following purposes: V indicates the position of a native methionine replaced with valine to avoid secondary cyanogen bromide cleavage of the fusion protein; DKKDDKDK was added to increase hydrophilicity and facilitate purification by high-performance liquid chromatography (HPLC); G was added to connect DAP12<sub>TM</sub> and NKG2C<sub>TM</sub> sequences through a short flexible linker. All transmembrane peptides were expressed as carboxy-terminal in-frame fusions to the trpEL sequence with an amino-terminal nine-histidine tag in the pMM-LR<sub>6</sub> vector (a gift from S.C. Blacklow). Transformed *E. coli* strain BL21 (DE3) cells were inoculated into 500 ml M9 minimal medium with Centrum multivitamins and stable isotope label(s) in 2.0-liter baffled flasks. Cultures were subsequently washed out with water before elution in 70% (vol/vol) formic acid. The covalently linked transmembrane trimer product was purified in a final HPLC step, as described above, and was identified by mass spectrometry and SDS-PAGE analysis.

NMR spectroscopy. The triple resonances experiments used for backbone assignment were all transverse relaxation optimized (tr), including three-dimensional tr-HNCA, tr-HN(CO)CA, tr-HN(CA)C and tr-HN(CC)C experiments were used for the collection of most distance restraints; because of the relatively large size of the protein-micelle complex, relaxation-optimized NOE-tr-HSQC yielded substantially better sensitivity than the regular NOE-tr-HSQC did, even for nondeuterated protein. The 15<sup>N</sup>-selected NOE-trHSQC experiments are three-dimensional 15<sup>N</sup>-selected NOE-tr-HSQC. With the residue-specific chemical shifts of backbone amide protons (1H<sup>-</sup>) and 15<sup>N</sup> nuclei, the 13<sup>N</sup>-selected NOE-tr-HSQC (mixing time, 80 ms) of samples containing uniform 15<sup>N</sup>, 13<sup>C</sup>-labeled protein and deuterated tetracetylp-chol-phosphocholine were used for correlation of the backbone amide and side-chain aliphatic and aromatic 1H resonances. The structured regions are almost all α-helical, as indicated by chemical shifts of 13<sup>C</sup>α and 13<sup>C</sup>β (where the superscripted α and β indicate carbon positions of amino acid side chains), analyzed with TALOS (for the prediction of polypeptide secondary structure from <sup>13</sup>C shifts) and the characteristic local NOE patterns of α-helix, and assignment of intra-residue and sequential NOEs in the 13<sup>N</sup>-selected NOE-trHSQC spectrum was straightforward. With the same approach, the assigned chemical shifts of aliphatic and amide protons were then used to assign the methyl 1H<sup>-</sup> and 13<sup>C</sup> resonances, which are mostly resolved in a two-dimensional 1H-13<sup>C</sup>-HSQC spectrum recorded with a constant-time 13<sup>C</sup> evolution of 56 ms (Supplementary Fig. 3). This was accomplished with three-dimensional 13<sup>N</sup>-selected NOE-tr-HSQC, recorded with 150 ms of mixing time and 56 ms of constant-time 13<sup>C</sup> evolution of the same samples with deuterated detergent. Specific stereo assignment of the γ-13C<sup>-</sup> of valine and β-13C<sup>-</sup> of leucine were obtained from a 10% 1<sup>3</sup>N-labeled protein sample by recording a 1H-13C-HSQC with 28 ms of constant-time 13<sup>C</sup> evolution as described<sup>31</sup>. For dimer and trimer samples in which the NMR readout strand could be labeled with 1<sup>3</sup>N and perdeuterated, a standard two-dimensional 13<sup>N</sup>-selected NOE-tr-HSQC was used for measurement of interstrand NOEs between backbone amide and side-chain aliphatic or aromatic protons (sample NOE trHSQC strips, Supplementary Fig. 2b). However, the single DAP12<sub>TM</sub> helix of the DAP12<sub>TM</sub>-DAP12<sub>TM</sub>-NKG2C<sub>TM</sub> trimer could not be perdeuterated; the best deuteration achievable at the methyl positions was ~75%. For detection of exclusively interhelical NOEs between DAP12<sub>TM</sub> backbone amide protons and DAP12<sub>TM</sub>-NKG2C<sub>TM</sub> side-chain methyl protons, the three-dimensional (1H-13C-HMQC)-NOE-tr(H-13C<sup>-</sup>-HSQC) experiment was used because this experiment selectively detects NOEs between protons attached to 13C and protons attached to 15N. In this experiment, the t<sub>1</sub>, t<sub>2</sub> and t<sub>3</sub> dimensions are labeled with 1H (methyl), 15N and 1H<sup>-</sup> frequency, respectively, and thus there are no diagonal peaks (sample NOE trHSQC strips, Supplementary Fig. 2d).

NMR data analysis. The NMRPipe software system<sup>32</sup> and CARA (computer-aided resonance assignment) software<sup>33</sup> were used for data processing and spectra analyses. TALOS<sup>30</sup> was used for the prediction of backbone dihedral angles from characteristic chemical shifts.

Structure calculation. The structure-determination program XPLOR-NIH<sup>14</sup> was used for structure calculation. A standard simulated annealing protocol<sup>15</sup> was run to satisfy all NMR-derived restraints. During the annealing run, the bath was cooled from 1,000 K to 20 K with a temperature step of 20 K, and 6.7 ps of Verlet dynamics at each temperature step, with a time step of 3 fs. The NOE restraints were enforced by flat-well harmonic potentials, with the force constant ramped from 25 to 50 kcal/mol Å<sup>2</sup> during annealing. Hydrogen bond restraints of 2 Å (O-H<sup>-</sup> and O-N, respectively) were enforced for helical regions (indicated by local NOEs and 13<sup>C</sup>α and 13<sup>C</sup>β chemical shift), with flat-well (±0.1 Å) harmonic potentials and a force constant ramped from 25 to 50 kcal/mol Å<sup>2</sup>. Also, for defined helical regions, backbone torsion angle restraints (δ = −60, ±8<sup>°</sup> and ±40) were applied, all with a flat-well (+10°).
harmonic potential with force constant ramped from 15 to 30 kcal/mol Å⁻². Other force constants used for NMR structure calculation were as follows (arrows indicate increasing force constant values over the given range): van der Waals force constant (K(q,q)) = 0.02 → 4.0 kcal/mol Å⁻², improper force constant (K(improper)) = 0.1 → 1.0 kcal/mol degree⁻² and bond angle force constant (K(bond angle)) = 0.4 → 1.0 kcal/mol degree⁻². For both dimer and trimer, a total of 75 structures were calculated with this protocol. From these structures, the 15 structures with lowest energy were chosen to represent the structural diversity of the NMR structures.

In vitro transcription, translation and assembly reactions. Full-length human DAP12, DAP10, NKG2C, NKG2D, Aε TCR, CD3ε, CD3δ and CD3γ and the mutant sequences were cloned into a modified pSP64 vector for in vitro translation with carboxy-terminal peptide affinity tags as described. In vitro transcription was done from linearized cDNA constructs using a T7 RiboMax Large Scale RNA Production kit and methyl-7-G cap analog (Promega), and capped, polyadenylated mRNAs were purified with the RNasy kit from Qiagen. Each 25-μl translation reaction contained 17.5 μl nuclease-treated rabbit reticulocyte lysate (Promega), 0.5 μl amino acid mixture without methionine or cysteine (Promega), 0.5 μl SUPERase-In RNase Inhibitor (Ambion), 1 μl each of 35S-labeled methionine and cysteine (Perkin Elmer), mRNA and 2.0 μl endoplasmic reticulum microsomes from a mouse hybridoma (IVD12) isolated as described. All in vitro translation and assembly reactions were done at 30 °C. An initial translation period of 20 min under reducing conditions was followed by a 1- to 2-hour assembly period. Reaction volumes were 25 or 50 μl as required for optimal signal.

Immunoprecipitation, electrophoretic analysis and densitometry. The following monoclonal antibodies to epitope tags were used for immunoprecipitation procedures: high-affinity, agarose-coupled anti-hemagglutinin (rat monoclonal antibody 3F10; Roche) and agarose-coupled anti-CD3ε (mouse monoclonal antibody UCH-T1; Santa Cruz). Translation and assembly reactions were stopped with 1 ml ice-cold 10 mM iodoacetamide in PBS, and microsomes were pelleted (10 min at 20,000g and 4 °C) and rinsed. Membrane pellets were extracted for 30 min at 4 °C with rotation in 400 μl immunoprecipitation buffer (0.5% (wt/vol) digitonin (Biosynth International), 10 mM iodoacetamide, 0.1% (wt/vol) BSA, 5 mg/ml leupeptin and 1 mM phenylmethyl sulfonyl fluoride in PBS). Lysates were precleared for 1 h with Sepharose 4 beads blocked with PBS-BSA, and immunoprecipitation was done for 2 h at 4 °C with rotation. Products were digested for 1 h at 37 °C with 500 U endoglycosidase H (New England Biolabs) in most experiments, then were separated by electrophoresis through 12% NuPAGE Bis-Tris gels (Invitrogen), transferred to polyvinylidene difluoride membranes and exposed to phosphorimager plates (GE Life Sciences). Gels were run under nonreducing conditions for all in vitro translation experiments (except Fig. 5d, right, which was run in reducing conditions). Densitometry was analyzed with the ImageQuant software package (Molecular Dynamics). Assembly efficiency was quantified as the ratio of receptor to DAP12 dimer (Fig. 2b, c), DAP12 dimer to DAP10 monomer (Fig. 2f), DAP10 dimer to DAP10 monomer (Fig. 5d, left), NKG2D to total DAP10 (Fig. 5d, right) or TCR to CD3ε (Fig. 5e,f) and are presented as the percentage of assembly compared with wild type (set as 100%).