ONLINE METHODS

Production and purification of linked transmembrane peptide complexes. Transmembrane peptides were produced as fusions to the carboxyl terminus of the 14-kilodalton trpLE 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 trpLE for this study were as follows: $DAP12_{TM}$ (33 amino acids), CSTVSPGVLAGIV**V**GDLVLTVLIALAVYFLGRL; $DAP12_{TM}$ **DKDKDKDK** (40 amino acids), CSTVSPGVLAGIV**V**GDLVLTVLIALAVYFLGR**DKDKDKDK**; and $DAP12_{TM}$-$NKG2C_{TM}$ (63 amino acids), CSTVSPGVLAGIV**V**GDLVLTVLIALAVYFLGRL**G**TAEVLGIISIVLVATVLKTIVLIPFLEQN. In these, 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; **DKDKDKDK** was added to increase hydrophilicity and facilitate purification by high-performance liquid chromatography (HPLC); **G** was added to connect $DAP12_{TM}$ and $NKG2C_{TM}$ sequences through a short flexible linker. All transmembrane peptides were expressed as carboxy-terminal in-frame fusions to the trpLE sequence with an amino-terminal nine-histidine tag in the pMM-LR6 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 grown at 37 °C to an absorbance of ~0.6 at 600 nm and were cooled for 1 h to 18 °C before overnight induction at 18 °C with 100 μM isopropyl β-D-thiogalactopyranoside. Full deuteration of transmembrane peptides required growth in D_2O with deuterated glucose (Cambridge Isotope Laboratories). Inclusion bodies were extracted from sonicated *E. coli* cell suspensions with 6 M guanidine HCl, 50 mM Tris, pH 8.0, 200 mM NaCl, 1% (vol/vol) Triton X-100 and 5 mM β-mercaptoethanol. For creation of the $DAP12_{TM}$ homodimer, the cleared inclusion-body lysate was batch-bound overnight to His-Select nickel affinity gel (Sigma-Aldrich) and was washed with a urea solution (8 M) containing 5 mM β-mercaptoethanol. The column was then washed with a urea solution containing 20 μM $CuSO_4$ and 2 mM oxidized glutathione, which was subsequently washed out with water before elution in 70% (vol/vol) formic acid. Digest with cyanogen bromide in 70% (vol/vol) formic acid (1 h under argon; 0.1 g/ml) liberated the $DAP12_{TM}$ peptides from the trpLE fusion partners. The digest was dialyzed in water, lyophilized and loaded onto a ZORBAX SB-C3 column (Agilent) in 100% (vol/vol) formic acid. Fragments were separated on a gradient of 40% acetonitrile (0.1% (vol/vol) trifluoroacetic acid) to 75% (vol/vol) isopropanol–25% (vol/vol) acetonitrile (0.1% (vol/vol) trifluoroacetic acid), and the peak representing the pure disulfide-linked $DAP12_{TM}$ peptide dimer was identified by matrix-assisted laser desorption–ionization–time of flight mass spectrometry and SDS-PAGE analysis. A similar strategy was used to generate the heterotrimeric peptide complex: trpLE-$DAP12_{TM}$ and trpLE-$DAP12_{TM}$-$NKG2C_{TM}$ fusion proteins from separate cultures were combined and crosslinked on the nickel-affinity matrix as described above. An extra ZORBAX SB-C3 HPLC separation step was then done to isolate the disulfide-linked fusion heterodimer before treatment with cyanogen bromide 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.

For dimeric and trimeric mixed-label ^{15}N-NOE spectroscopy (NOESY) samples (dimer and trimer), inclusion-body lysates from two separate *E. coli* expression and labeling cultures were mixed such that the nondeuterated, ^{13}C-labeled fusion was in two- to fourfold excess relative to the ^{15}N-, ^{2}H-labeled fusion, and this mixture was subjected to the nickel-column purification and oxidative crosslinking protocol described above.

NMR sample preparation. NMR samples were prepared by codissolution of 1–2 mg lyophilized peptides with approximately 30 mg (protonated or perdeuterated) tetradecylphosphocholine detergent (FOS-Choline; Anantrace) in hexafluoroisopropanol and drying of the solution to a thin film under a nitrogen stream. Thin films were redissolved in 8 ml of a urea solution (8 M) containing approximately 2.4 mg (protonated or deuterated) SDS detergent (Cambridge Isotope Laboratories) and were dialyzed overnight in 20 mM sodium phosphate buffer, pH 6.8, for removal of the denaturant. Refolded samples were concentrated to 300 μl and loaded into Shigemi microcells (Sigma) with 5% (vol/vol) D_2O. Final detergent concentrations were 250 mM FOS-Choline 14 and 25 mM SDS.

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-HNCACB and tr-HN(CO)CACB[28,29]. These experiments used dimer and trimer samples labeled with ^{15}N, ^{13}C or and 85% ^{2}H at a ^{1}H frequency of 600 MHz at 30 °C. Three-dimensional ^{15}N-selected NOESY–tr-HSQC experiments were used for the collection of most distance restraints; because of the relatively large size of the protein-micelle complex, relaxation-optimized NOESY–tr-HSQC yielded substantially better sensitivity than the regular NOESY-HSQC did, even for nondeuterated protein. The ^{13}C-selected NOESY experiments are three-dimensional ^{13}C-selected NOESY-HSQC. With the residue-specific chemical shifts of backbone amide protons ($^{1}H^{N}$) and ^{15}N nuclei, the ^{15}N-selected NOESY–tr-HSQC (mixing time, 80 ms) of samples containing uniform ^{15}N-, ^{13}C-labeled protein and deuterated tetradecylphosphocholine were used for correlation of the backbone amide and side-chain aliphatic and aromatic ^{1}H resonances. The structured regions are almost all α-helical, as indicated by chemical shifts of $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ (where the superscripted α and β indicate carbon positions of amino acid side chains), analyzed with TALOS software (for the prediction of polypeptide secondary structure from ^{13}C shifts)[30] and the characteristic local NOE patterns of α-helix, and assignment of intra-residue and sequential NOEs in the ^{15}N-selected NOESY spectrum was straightforward. With the same approach, the assigned chemical shifts of aliphatic and amide protons were then used to assign the methyl ^{1}H and ^{13}C resonances, which are mostly resolved in a two-deimensional ^{1}H-^{13}C HSQC spectrum recorded with a constant-time ^{13}C evolution of 56 ms (**Supplementary Fig. 3**). This was accomplished with three-dimensional ^{13}C-selected NOESY-HSQC, recorded with 150 ms of mixing time and 56 ms of constant-time ^{13}C evolution of the same samples with deuterated detergent. Specific stereo assignment of the γ-^{13}C of valine and δ-^{13}C of leucine were obtained from a 10% ^{13}C-labeled protein sample by recording a ^{1}H-^{13}C HSQC with 28 ms of constant-time ^{13}C evolution as described[31]. For dimer and trimer samples in which the NMR readout strand could be labeled with ^{15}N and perdeuterated, a standard three-dimensional ^{15}N-selected NOESY–tr-HSQC was used for measurement of interstrand NOEs between backbone amide and side-chain aliphatic or aromatic protons (sample NOESY strips, **Supplementary Fig. 2b**). However, the single $DAP12_{TM}$ helix of the $DAP12_{TM}$-$DAP12_{TM}$-$NKG2C_{TM}$ trimer could not be perdeuterated; the best deuteration achievable at the methyl positions was ~75%. For detection of exclusively interhelical NOEs between $DAP12_{TM}$ backbone amide protons and $DAP12_{TM}$-$NKG2C_{TM}$ side-chain methyl protons, the three-dimensional (^{1}H-^{13}C HMQC)-NOESY-(^{1}H-^{15}N tr-HSQC) experiment was used because this experiment selectively detects NOEs between protons attached to ^{13}C and protons attached to ^{15}N. In this experiment, the t_1, t_2 and t_3 dimensions are labeled with ^{1}H (methyl), ^{15}N and $^{1}H^{N}$ frequency, respectively, and thus there are no diagonal peaks (sample NOESY strips, **Supplementary Fig. 2d**).

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

Structure calculation. The structure-determination program XPLOR-NIH[34] was used for structure calculation. A standard simulated annealing protocol[35] 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 $Å^{-2}$ during annealing. Hydrogen bond restraints of 2 and 3 Å (O-H^{N} and O-N, respectively) were enforced for helical regions (indicated by local NOEs and $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ chemical shift), with flat-well (±0.1 Å) harmonic potentials and a force constant ramped from 25 to 50 kcal/mol $Å^{-2}$. Also, for defined helical regions, backbone torsion angle restraints (ϕ = −60, &mm.psi; = −40) were applied, all with a flat-well (±10°)

doi:10.1038/ni.1943

harmonic potential with force constant ramped from 15 to 30 kcal/mol rad^{-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_{vdw}) = 0.02 → 4.0 kcal/mol Å^{-2}, improper force constant (K_{impr}) = 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, A6 TCRα, A6 TCRβ, CD3γ, CD3δ and CD3ε and the mutant sequences were cloned into a modified pSP64 vector for *in vitro* translation with carboxy-terminal peptide affinity tags as described[3]. *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 RNeasy 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 ^{35}S-labeled methionine and cysteine (Perkin Elmer), mRNA and 2.0 µl endoplasmic reticulum microsomes from a mouse hybridoma (IVD12) isolated as described[3]. 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 with addition of oxidized glutathione to 4 mM. 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 20,000*g* 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 DAP12 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%).

28. Kay, L.E., Ikura, M., Tschudin, R. & Bax, A. Three-dimensional triple-resonance NMR spectroscopy of isotopically enriched proteins. *J. Magn. Reson.* **89**, 496–514 (1990).
29. Salzmann, M., Wider, G., Pervushin, K. & Wuthrich, K. Improved sensitivity and coherence selection for [15N,1H]-TROSY elements in triple resonance experiments. *J. Biomol. NMR* **15**, 181–184 (1999).
30. Cornilescu, G., Delaglio, F. & Bax, A. Protein backbone angle restraints from searching a database for chemical shift and sequence homology. *J. Biomol. NMR* **13**, 289–302 (1999).
31. Szyperski, T., Neri, D., Leiting, B., Otting, G. & Wuthrich, K. Support of 1H NMR assignments in proteins by biosynthetically directed fractional 13C-labeling. *J. Biomol. NMR* **2**, 323–334 (1992).
32. Delaglio, F. *et al.* NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* **6**, 277–293 (1995).
33. Keller, R. *The Computer Aided Resonance Assignment Tutorial* (Cantina Verlag, Goldau, Switzerland, 2004).
34. Schwieters, C.D., Kuszewski, J.J., Tjandra, N. & Clore, G.M. The Xplor-NIH NMR molecular structure determination package. *J. Magn. Reson.* **160**, 65–73 (2003).
35. Nilges, M., Clore, G.M. & Gronenborn, A.M. Determination of three-dimensional structures of proteins from interproton distance data by dynamical simulated annealing from a random array of atoms. Circumventing problems associated with folding. *FEBS Lett* **239**, 129–136 (1988).