Structure of the human NK cell NKR-P1:LLT1 receptor:ligand complex reveals clustering in the immune synapse

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Signaling by the human C-type lectin-like receptor, natural killer (NK) cell inhibitory receptor NKR-P1, has a critical role in many immune-related diseases and cancer. C-type lectin-like receptors have weak affinities to their ligands; therefore, setting up a comprehensive model of NKR-P1-LLT1 interactions that considers the natural state of the receptor on the cell surface is necessary to understand its functions. Here we report the crystal structures of the NKR-P1 and NKR-P1:LLT1 complexes, which provides evidence that NKR-P1 forms homodimers in an unexpected arrangement to enable LLT1 binding in two modes, bridging two LLT1 molecules. These interaction clusters are suggestive of an inhibitory immune synapse. By observing the formation of these clusters in solution using SEC-SAXS analysis, by dSTORM super-resolution microscopy on the cell surface, and by following their role in receptor signaling with freshly isolated NK cells, we show that only the ligation of both LLT1 binding interfaces leads to effective NKR-P1 inhibitory signaling. In summary, our findings collectively support a model of NKR-P1:LLT1 clustering, which allows the interacting proteins to overcome weak ligand-receptor affinity and to trigger signal transduction upon cellular contact in the immune synapse.

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Natural killer (NK) cells are innate immune lymphocytes equipped with a wide range of activating and inhibitory surface receptors, allowing them to sensitively recognize and kill malignant, infected, or other transformed cells through “missing-” and “induced-self” mechanisms and through antibody-dependent cell-mediated cytotoxicity (ADCC). In addition, NK cells also contribute to the initiation and development of the adaptive immune response, secreting several classes of cytokines, especially proinflammatory IFN-γ. Interestingly, recent findings show that NK cells can even maintain a form of immunological memory, thus further highlighting the principal roles NK cells play in immunity, particularly through their receptors.
NK receptors comprise two structurally divergent classes: the families of immunoglobulin-like receptors and C-type lectin-like receptors (CTLRs). CTLRs are encoded within the natural killer gene complex (NKC, human chromosome 12), and, unlike C-type lectins, CTLRs neither bind calcium ions nor engage carbohydrate ligands. Instead, CTLRs are known to interact with protein ligands. For example, receptors such as Ly49, CD94/NKG2, or NuMA appear to recognize MHC class-I-like molecules, whereas receptors of the NKR-P1 subfamily recognize structurally highly related Clr/Oc1i CTLRs. These are encoded by CLEC2 genes and are genetically tightly linked to the NKR-P1-coding KLR genes. This unique CTRL:CTR interaction system is involved in both non-MHC missing-self and induced-self recognition. Several inhibitory and activating NKR-P1 receptors have been described in mice and rats; however, the human receptor NKR-P1 (CD161, KLRB1 gene) remains since 1994 the only human orthologue described so far. Nevertheless, based on structural and functional homology to NKR-P1, the human activating CTLR:ligand pairs Nkp65:KACL (KLRF1:CLEC2A) and Nkp80:AICL (KLRF1:CLEC2B) have been proposed as the activating counterparts of human NKR-P1.

Human NKR-P1 (CD161) was first reported as a marker of NK cells, in which NKR-P1 acts as an inhibitory receptor up-regulated by IL-12. However, NKR-P1 is also expressed by natural killer T (NKT) cells, mucosa-associated invariant T (MAIT) cells, and other subsets of T lymphocytes, wherein NKR-P1 acts as a co-stimulatory receptor, increasing IFN-γ secretion. NKR-P1 is even detected in immature CD16+ CD56 NK cells and in precursors of Th17 and MAIT cells in the umbilical cord blood. Recently, NKR-P1 was identified in glioma-infiltrating T cells, having an inhibitory, immunosuppressive role in T cell-mediated killing of glioma cells. In addition, NKR-P1 promotes transendothelial migration to immunologically privileged niches upon interaction with its endogenous ligand, lectin-like transmembrane protein 1 (LTL1).

LTI (gene CLEC2D) is primarily expressed on activated monocytes and B cells. In these cells, LTI helps to maintain NK cell self-tolerance. However, LTI can induce its expression on NK and T cells. Furthermore, LTI is up-regulated on glioblastoma, prostate and triple-negative breast cancer, and B cell non-Hodgkin's lymphoma. In addition, LTI contributes to immune evasion by dampening NK cell cytotoxicity. Interestingly, increased numbers of CD16+ TH2 cells have been detected in glioma tumors. Conversely, the functions of NKR-P1 receptors on IL-17-producing regulatory T cells, on subsets of Tc17 cells, and on all TH17 cells are particularly relevant because these cells have been implicated in several autoimmune diseases (Crohn's disease, multiple sclerosis, rheumatoid arthritis, and psoriasis). Therefore, the analysis of NKR-P1 receptors and ligands such as LTI is essential to gain a deeper insight into the structure-function relationships underlying both physiological and pathogenic processes in the immune system.

Human NKR-P1 and LTI are type II transmembrane glycoproteins with similar protein topology: an N-terminal cytoplasmic signaling domain, a transmembrane helix, a flexible stalk region, and a C-terminal C-type lectin-like domain (CTLD). Moreover, both NKR-P1 and LTI were shown to form disulfide homodimers, likely involved in their stalk regions. However, the structure of Nkp65:KACL complex is the only one among all complexes of the human C-type lectin-like (CTL) receptor:ligand subfamily that has been solved so far. A subsequent study further showed that the interaction between Nkp65 and KACL is protein-based and independent of glycosylation. Based on these data, a model of the NKR-P1:LTI complex was subsequently proposed, and key interaction residues were identified through surface plasmon resonance (SPR) analysis of NKR-P1 and LTI mutants, highlighting the fast kinetics of this interaction. Furthermore, the structures of related mouse NKR-P1B ectodomain complexes with murine cytomegalovirus (MCMV) immunoevasin protein ml2, or with its cognate ligand CIRb, have been recently reported. Previously, we reported the first structure of LLT1 forming a non-covalent dimer regardless of glycosylation and following the conserved dimerization mode of CLEC2-encoded ligands observed for CD69 and Clec47. Notwithstanding, no comprehensive model of the CTLR:ligand complexes’ dimer:dimer interaction, corresponding to the natural state of these proteins when expressed on the cell surface, is available yet. Here, we investigate the structure of human NKR-P1 and examine the effects of NKR-P1 dimerization on LLT1 binding. We present the crystal structure of NKR-P1 in complex with LLT1, explain the previous in-solution interaction observations, and show a novel assembly of the complex utilizing two different non-symmetric binding sites on LLT1. Our results explain how the human NKR-P1 receptor overcomes its weak affinity for LLT1 by ligand binding-induced cross-linking and clustering and elucidate the mode of signal transduction of this receptor within the NK cell immune synapse, thereby providing a good model for the future description of related homologous low-affinity complexes.

**Results**

**Structure of the human NKR-P1 ectodomain**

Two crystal structures of the human NKR-P1 ectodomain were solved: the structure of glycosylated NKR-P1 possessing uniform Asn-GlcNAc-Man9,Gal-N glyco and of deglycosylated NKR-P1 with N-glycans cleaved off after the first GlcNAc residue (NKR-P1 deglyco); statistical data on all structures are outlined in Table 1. NKR-P1 in both crystal structures follows the general fold characteristic of a CTL domain—two α-helices (α1 and α2) and two antiparallel β-sheets with the conserved hydrophobic WIG motif within the domain core (Figs. 1a and 2a). The two β-sheets are formed by β-strands β0, β1, β1’ and β3, and β2, β2’, β3, and β4, respectively (assignment according to Zelensky and Greedy), also used to describe other related CTL structures. In addition, three intramolecular disulfide bonds stabilize the domain: Cys94-Cys105, Cys122-Cys210, and Cys189-Cys202.

The human NKR-P1 homodimer is similar to the murine dectin-1 homodimer

The asymmetric unit of NKR-P1 glyco contains two monomers, whereas the asymmetric unit of NKR-P1 deglyco contains eight NKR-P1 monomers. All these monomers are arranged into very similar homodimers, with pairwise RMSD on Cα atoms up to 0.5 Å (Fig. 2b). However, these homodimers have an unexpected configuration: they do not follow the usual dimerization mode observed for CTLDs of the CLEC2 ligands such as CD69 or LLT1 with helix α2 at the dimerization interface. Instead, the dimerization interface of NKR-P1 is formed by helix α1, as in the murine C-type lectin-like pattern recognition receptor dectin-1 (https://doi.org/10.2210/pdb2CLS/pdb), with which human NKR-P1 shares only 32% sequence identity of the CTLD (Fig. 2c). The RMSD of the Cα atoms between NKR-P1 and dectin-1 dimers is 3.7 Å in the overlapping region (matching 196 from the total 250 residues of the NKR-P1 dimer). The structurally distinct region mainly covers helices α2, whose positions differ up to 7 Å between NKR-P1 and dectin-1. We also observed a highly similar arrangement with helix α1-centered dimerization interface in the structure of a covalent disulfide dimer of rat NKR-P1B receptor ectodomain (https://doi.org/10.2210/pdb67D/pdb) with 1.4 Å RMSD of the Cα atoms between these two dimers (Supplementary Fig. 1a). On the contrary, the non-classical dimer of mouse NKR-P1 (https://doi.org/10.2210/pdb66D/pdb) has an entirely different overall arrangement (Supplementary Fig. 2a).

The dimerization interface of the NKR-P1 homodimer consists of six protein–protein and several water-mediated hydrogen bonds (Supplementary Table 1), a peptide bond interaction via delocalized electrons (Lys126-Glu127), and a small hydrophobic core comprising Leu19, Ala120, and Ile68 from both chains (Fig. 3). The contact surface area is ca. 500 Å². Compared to the helix α2-centered LLT1 dimer (7–12 hydrogen bonds, stronger hydrophobic core, 500–800 Å²...
Glycosylation of human NKR-P1 affects its dimerization

The NKR-P1 ectodomain contains three potential N-glycosylation sites at residues Asn116, Asn157, and Asn169 (Fig. 1a). Glycosylation at Asn157. This suggests that although the glycosylation of the wild-type NKR-P1 ectodomain is purely monomeric50. Thus, glyco participation in dimerization and, therefore, its ability to form multimeric complexes is limited. Conversely, in NKR-P1 glyco, the first GlcNAc unit at Asn169 is well defined in electron density maps of both chains, A and B, of the NKR-P1 dimer, while no electron density was found for glycosylation at Asn157. This suggests that although the glycosylation on either Asn116 or Asn157 can contribute to the dimer formation, dimerization might be impaired by steric hindrance if both N-glycans are present simultaneously. To test this hypothesis, we expressed NKR-P1 SI59A mutant, thus abrogating glycosylation on Asn157. The overall fold of the mutant is comparable to the wild-type NKR-P1, as assessed by CD spectroscopy (Supplementary Fig. 3a). Indeed, when analyzed by analytical ultracentrifugation, the mutant protein exhibited substantial levels of oligomeric species (Supplementary Fig. 3b), whereas the wild-type NKR-P1 ectodomain is purely monomeric63. Thus, glycosylation heterogeneity may affect the propensity of human NKR-P1 to dimerize and, therefore, its ability to form multimeric complexes with LLT1.

Structure of the human NKR-P1:LLT1 complex

The crystal structure of the NKR-P1:LLT1 complex is formed by deglycosylated NKR-P1 and LLT1 ectodomains. The asymmetric unit of the crystal contains a complex of dimeric NKR-P1 with dimeric LLT1 and an extra dimer of NKR-P1 (Fig. 4a). These NKR-P1 dimers have the same helix α1-centered dimerization interface as the structures of the
unbound NKR-P1 dimers described above (Fig. 2b). The LLT1 dimer retains the expected α2-centered dimerization mode (Fig. 2c), identical to that previously described in unbound LLT1 structures.

LLT1 has clearly identifiable GlcNAc units at residues Asn95 and Asn147. The NKR-P1 glycosylation observed in the electron density of the complex matches the glycosylation identified in the NKR-P1_deglyco structure.

The LLT1 homodimer engages its partner bivalently, i.e., one dimer interacts with two NKR-P1 dimers related by crystallographic symmetry: each LLT1 monomer binds to a different subunit of a distinct NKR-P1 homodimer (Fig. 4a). There is no apparent induced fit of the binding partners – the RMSD of Cα atoms between the non-interacting and the interacting NKR-P1 dimers (NKR-P1_glyco and the complex) is 0.5 Å, and that of LLT1 (https://doi.org/10.2210/pdb4QKI/pdb) is 0.7 Å. Moreover, the N-linked glycosylation chains do not directly contribute to the interaction.

LLT1 engages NKR-P1 in two distinct interaction modes. NKR-P1 and LLT1 establish two types of contact in this structure – the primary (LLT1 chain B:NKR-P1 chain D) and the secondary (LLT1 chain A:NKR-P1 symmetry-related chain C) interaction modes. The primary interaction mode matches well the structure of the homologous human NKp65:KACL complex (https://doi.org/10.2210/pdb4I0P/pdb) – the RMSD of Cα atoms of the two complexes is 1.3 Å (one chain of the receptor and one chain of the ligand in each case, Fig. 4b, lower left). Similarly, in the structure of mouse NKR-P1B complexed with MCMV immunoevasin m12, the observed interaction interface matches the primary mode in the present structure of the human NKR-P1 complex, although the area covered by the m12 protein is considerably larger (Supplementary Fig. 1b, c). The recently described structure of the mouse NKR-P1B:Clrf complex (https://doi.org/10.2210/pdb6E7D/pdb) also showed an interaction interface common for both NKR-P1 receptors (Supplementary Fig. 2b, c). However, the receptor:ligand arrangement observed in the second interaction mode of the NKR-P1:LLT1 complex is unique and differs from all known homologous complexes in the ligand orientation (Fig. 4b, lower right, and Supplementary Fig. 2b).
loops and by β3 and β4 strands (Fig. 4c, d), creating a flat surface for interaction with LLT1. By contrast, in LLT1, the primary and secondary interaction interfaces are substantially different, albeit sharing a small number of residues. While the loops L0', L0, L3, L5, L6, and strands β3 and β4 form the primary interaction patch of LLT1 (Fig. 4c), residues of the loops L2 and L5, strand β2' and helix α2 are involved in the second interaction interface (Fig. 4d). Both interaction modes place the membrane-proximal parts of the receptor and the ligand on opposite sides of the complex, creating a plausible model for interaction between two neighboring cells.

The primary interaction mode is established through nine direct and several water-mediated hydrogen bonds, in addition to two charge-supported and π-π stacking (Tyr201-Arg175) interactions, with a total contact surface area of ca. 800 Å² (Supplementary Table 1). The four strongest bonds occur between the NKR-P1 residues Asp147, Ser199, and Arg181 and the LLT1 residues Arg153, Lys169, and Asn120, respectively (Fig. 4c, d).

Besides interaction with LLT1, NKR-P1 dimers bound in primary and secondary modes also have non-negligible mutual accessory contact (NKR-P1 chain D and NKR-P1 chain C in symmetry-related position, C_sym). The interface area has 440 Å² and is established through seven direct hydrogen bonds (Supplementary Table 1). Four residues are towering above the interface: Asn143 and Arg146 of chain D and Asn174 and Asn176 of chain C_sym (none of the asparagine residues is a glycosylation site). The interface also has several water-mediated contacts.

**NKR-P1:LLT1 complex formation in solution**

To characterize the size and shape of the NKR-P1:LLT1 complex in solution, we have performed analytical ultracentrifugation (AUC), microscale thermophoresis (MST), and small-angle X-ray scattering coupled to size-exclusion chromatography (SEC-SAXS) experiments.
The acquired AUC data are concentration-dependent and reflect the dynamic nature of this interacting system, similar to the results from the SEC-SAXS measurements. The free human NKR-P1 ectodomain is monomeric, with a sedimentation coefficient $s_{20,w}$ of 2.15 corresponding to an estimated molecular mass of 18 kDa, matching the expected value of 17.5 kDa closely. The LLT1 ectodomain forms a stable non-covalent dimer (2.9 S) that does not dissociate into monomers, except in very low concentration, as previously characterized.

When increasing the loading concentration of the NKR-P1:LLT1 equimolar mixture, the sedimentation coefficient of the complex increases as well, reaching an $s_{20,w}$ value of 3.7 S at the highest analyzed concentration (Supplementary Fig. 3c). This value, when corrected for non-ideality caused by the high protein loading concentration used (18 mg/ml), corresponds to an estimated zero-protein-concentration sedimentation coefficient $s^0_{20,w}$ value of 4.5 S, and a moderately elongated particle with approximate dimensions of 10–15 × 5–4 × 5–4 nm. That compares well with the 8–10 × 5–6 × 4–5 nm dimensions expected for the possible NKR-P1:LLT1 interaction assemblies observed in the complex’s crystal structure, i.e., monomer:dimer:monomer or dimer:dimer (note that while N-glycan chains are not present in the deglycosylated complex’s structure, they were present during all our analyses in solution).

**Fig. 3 | Glycosylation affects the dimerization interface of human NKR-P1.**
a Dimerization interface of human NKR-P1. Subunits of human NKR-P1 are shown as Cα-trace (blue and cyan), and the dimer contact residues are shown as sticks with carbon atoms colored in light blue (blue subunit) and orange (cyan subunit); for clarity, only the residues of the blue subunit are labeled. The first GlcNAc unit N-linked to Asn116 and the carbohydrate chain N-linked to Asn169, observable in the NKR-P1_glyco structure, are shown with carbon atoms colored yellow and green, respectively. b Top view of the dimerization interface. The NKR-P1 subunits surfaces are colored blue and cyan. The GlcNAc units bound to Asn116 are shown as sticks with carbon atoms in yellow. Contact residues between the GlcNAc bound to chain A, and the chain B, are shown in yellow, whereas contact residues between the GlcNAc bound to chain B, and the chain A, are shown in purple. Hydrogen bonds are shown as green-dashed lines with a detailed view on the right-hand side. c Mixed glycosylation states at the dimer interface in the NKR-P1_deglyco structure. The GlcNAc unit N-linked to Asn157 of chain A is modeled with an occupancy of 0.5, while the second GlcNAc unit present at Asn166 of chain B is not modeled. Contours of 2mFo−DFc (2.8σ, cyan) and mFo−DFc (1σ, green) electron density maps are shown. d Small hydrophobic core in the central part of the NKR-P1 dimerization interface (subunits colored as in (a)). The central residues are shown as spheres with carbon atoms in yellow. The carbon atoms of Ile168 residues (whose mutation decreases the ability of NKR-P1 to bind LLTI) are shown in orange.
Secondary interaction mode is involved in NKR-P1:LLT1 binding in solution

To understand whether the secondary interaction mode observed within the crystal structure is also utilized in the solution, we designed an N120R, R153E, K169A triple mutant of LLT1 (LLT1SIM), thus abolishing the three strongest contacts in the LLT1 secondary interaction interface (Fig. 4d). The LLT1SIM mutant was expressed and purified in the same way as the wild-type LLT1 and displayed a comparable CD spectrum (Supplementary Fig. 3a). NKR-P1:LLT1SIM equimolar mixture concentration series reached an $s_{20,w}$ value of 3.3 S (corresponding to an estimated $s^2_{20,w}$ value of 3.9 S), clearly showing the formation of the complex of a smaller size compared to the wild-type NKR-P1:LLT1 mixture, possibly a monomer:dimer assembly (Supplementary Fig. 3d). By integrating the whole continuous size distribution c(s) curves and plotting the resulting weight-average S values against the LLT1 proteins’ concentrations used, binding isotherms were constructed for both dilution series and firstly best-fit to the simplest hetero-association binding model $A + B \iff AB$, where $A$ is the LLT1 dimer and $B$ is the NKR-P1 monomer (Supplementary Fig. 4a). LLT1SIM showed about three-fold weaker overall affinity than the wild-type LLT1, which was further corroborated by independent MST analysis using fluorescently labeled NKR-P1 titrated with LLT1 or LLT1SIM (Supplementary Fig. 4b). To analyze the difference between wild-type LLT1 and LLT1SIM concerning the binding of the second NKR-P1 monomer, we also best-fit the data using the $A + B \iff AB + B \iff BAB$ model (Supplementary Fig. 4c). While for the wild-type LLT1 this improved the fit and provided two different $K_D$ values, possibly corresponding to primary and secondary interaction modes, for LLT1SIM, the fitted $K_D$ values...
remained unchanged, disproving the binding of the second NKR-P1 monomer. Similarly, when the AUC and MST data were globally fitted together with the AUC parameters fixed at the previously best-fit values, the LLT1SIM MST data could be fitted equally well with both AB and BAB models. In contrast, the fit is poor for the AB model in the case of wild-type LLT1 (Supplementary Fig. 4d). Taken together, our data show that the secondary interaction interface is not just a crystal contact but contributes significantly to the overall affinity of the NKR-P1:LLT1 interaction.

SEC-SAXS analysis confirms NKR-P1:LLT1 higher-order complex formation

Observing assemblies of even higher order is unlikely in the AUC sedimentation velocity experiment, given the fast kinetics of this interaction, the long time-scale of the experiment, and the fact that protein concentration steadily decreases along the sedimentation boundary. However, the NKR-P1:LLT1 complex’s crystal structure suggests a chain-like arrangement of receptor:ligand dimers. To determine if such assemblies exist in the solution, we performed a SEC-SAXS analysis of the NKR-P1:LLT1 equimolar mixture at a 15 mg/ml loading concentration. Two peaks of absorbance at 280 nm, corresponding to two distinct peaks, for each collected SAXS frame, the radius of gyration was calculated using AUTORG (blue circles). For further analysis, six intervals of the SAXS data were selected and separately merged (frames 355–367, 368–378, 379–388, 389–399, 431–440, 481–491; denoted as columns with diagonal hatching). SAXS scattering curve (a), Kratky plot (b), and pair distance distribution function (c) for the intervals 389–399 and 481–491 are shown in the inset for data quality assessment of the merged data. All six merged data intervals were then individually analyzed by OLGOMER (Supplementary Data 1 and Supplementary Fig. 5). Diffraction data from the SEC-SAXS experiment have been deposited (https://doi.org/10.17632/268ww2m4j3.1)81.

Fig. 5 | SEC-SAXS analysis of NKR-P1:LLT1 shows higher-order complex formation. Overlay of the size-exclusion chromatography profile (black line) and SAXS scattering signal (red line) for the NKR-P1:LLT1 equimolar mixture at a 15 mg/ml loading concentration. Both signals show two distinct peaks. For each collected SAXS frame, the radius of gyration was calculated using AUTORG (blue circles). For further analysis, six intervals of the SAXS data were selected and separately merged (frames 355–367, 368–378, 379–388, 389–399, 431–440, 481–491; denoted as columns with diagonal hatching). SAXS scattering curve (a), Kratky plot (b), and pair distance distribution function (c) for the intervals 389–399 and 481–491 are shown in the inset for data quality assessment of the merged data. All six merged data intervals were then individually analyzed by OLGOMER (Supplementary Data 1 and Supplementary Fig. 5). Diffraction data from the SEC-SAXS experiment have been deposited (https://doi.org/10.17632/268ww2m4j3.1)81.

plateaus in the main peak suggest a dynamic balance between complex formation and dissociation (Fig. 5). As a result, the merged scattering curves could not be fitted well to scattering curves simulated from any single structure of the NKR-P1:LLT1 complex or its components. Therefore, we constructed models of assemblies of the NKR-P1:LLT1 complex in varying lengths (number of protein chains), considering all possible permutations of the order of the molecules using only primary, only secondary, or both interaction modes in an alternating fashion. The resultant library of all these models (in total, 49 different structures) was then used by the OLGOMER software to best-fit the sampled data intervals (Supplementary Fig. 5) as well as the individual SAXS experimental curves (Supplementary Data 1). By this approach, the sampled SAXS data fit reasonably well to the superimposed calculated scattering curves of groups of NKR-P1:LLT1 complex structures of higher stoichiometry ($\chi^2$ 1.29–3.47; note that complete glycosylation was not modeled, but it contributed to the scattering; Supplementary Fig. 5). Following the continuously decreasing radius of gyration calculated from the SAXS data, the stoichiometry of the best-fit structures of the NKR-P1:LLT1 complex also constantly decreases with the retention volume. The SAXS scattering curve of the first peak is best-fit with two to three LLT1 dimers interacting in a chain-like oligomer with two to three NKR-P1 dimers, the curve of the second peak to one LLT1 dimer interacting with one NKR-P1 dimer in primary or secondary mode. Notably, OLGOMER generally favored assemblies that combine primary and secondary interaction modes against models with the primary or secondary modes alone.
LLT1 ligation induces NKR-P1 receptor clustering

To answer the biological relevance of these higher-order complexes in the context of a live cell, we performed single-molecule localization microscopy studies with an NKR-P1-expressing cell line and quantified the surface distribution of NKR-P1 with respect to LLT1 or LLT1\textsuperscript{sim} binding. Full-length NKR-P1 transfectants generated using the piggyBac system were induced to express the receptor in a limited time to allow single-molecule localization microscopy. Cells were then incubated in the presence or absence of soluble LLT1 or LLT1\textsuperscript{sim}, fixed, and labeled with anti-NKR-P1 AlexaFluor\textsuperscript{®} 647 mAb. Direct stochastic optical reconstruction microscopy (dSTORM) images were acquired, and Voronoi tessellation cluster analysis was used to assess the effect of soluble LLT1 on the nanoscale organization of NKR-P1 on the cell surface (Fig. 6a). In the absence of LLT1, we detected clusters of fluorescent events with an average area of 1870 ± 777 nm\textsuperscript{2} (Fig. 6b) and an average diameter of 41 ± 7 nm (Fig. 6c), mostly clusters of events with a diameter ranging from 10 to 40 nm (Fig. 6d), indicative of AlexaFluor\textsuperscript{®} 647 doubly labeled NKR-P1 homodimers (ca. 6 nm + 10 nm linking error per each mAb ±15 nm of localization precision; please note that the apparent size of fluorescent clusters does not directly correspond to the size of receptor clusters). The addition of soluble LLT1 significantly increased the observed cluster of events diameters (41 ± 7.5 pm; p < 0.0001, Fig. 6c, d), the area (2713 ± 1180 nm\textsuperscript{2}; p < 0.0001, Fig. 6b), and the number of events detected in the clusters (38.8 ± 12.3; p < 0.0001, Fig. 6e), whereas the density of events in clusters remained unchanged, as expected (Fig. 6f). Furthermore, the effect of LLT1\textsuperscript{sim} addition on NKR-P1 nanoscale organization was statistically indistinguishable from the negative control while being significantly different from the effect of LLT1 addition. Based on the relative comparison of these three distinct receptor states (free, LLT1-bound, and LLT1\textsuperscript{sim}-bound), we can assume that the secondary interaction interface in LLT1 is necessary to form nanoscale NKR-P1 clusters upon interaction. No significant difference in the total density of events per inner cell surface (evaluated area) was found (Fig. 6g), pointing to stable expression levels of NKR-P1 throughout the measurement. Consequently, the size of NKR-P1 nanoclusters increases (beyond one homodimer unit) not because of differences in NKR-P1 expression levels but because LLT1 cross-links two or more NKR-P1 homodimers.

LLT1 secondary interaction mode is required for NKR-P1 inhibitory signaling

Next, we analyzed the effect of soluble LLT1 or LLT1\textsuperscript{sim} on the inhibitory potential of native NKR-P1 expressed on the surface of freshly isolated NK cells in an NK cell-mediated cytotoxicity assay thus investigating further the influence of NKR-P1 ligand binding-induced cross-linking on cellular signalization. NK cells isolated from the blood of three different healthy donors were activated with IL-2 and mixed with the K562 target cells in a 4:1 effector:target ratio and incubated for 4 h with PBS buffer as a negative control or with the soluble LLT1 or LLT1\textsuperscript{sim} (both in two different concentrations). In the absence of NKR-P1 ligand, K562 cells are well-susceptible to NK cell-mediated lysis (Fig. 7; PBS control, less than 10% live K562 cells). As expected, the lysis was substantially blocked in the presence of soluble LLT1. By contrast, the LLT1\textsuperscript{sim} variant with a mutated secondary interaction interface could not block NK cell-mediated cytotoxicity. As observed by dSTORM on the cell surface (Fig. 6), LLT1\textsuperscript{sim} does not efficiently cross-link NKR-P1. This mutant also fails to signalize via the NKR-P1 inhibitory receptor pathway. Therefore, based on both microscopy and cytotoxicity assay data, we conclude that NKR-P1 cross-linking triggered by LLT1 ligation is biologically relevant and indispensable for cellular signalization and requires interaction in both primary and secondary interaction modes.

Discussion

Currently, there are only two similar CTL:CTL NK cell receptorligand complexes with known 3D structure: human NKp65:KACL\textsuperscript{37} and mouse NKR-P1B:Clrb\textsuperscript{42}. These homologous complexes are topologically similar to the primary interaction mode of the NKR-P1:LLT1 complex (Fig. 4b). Sequence identity of the extracellular part of NKR-P1 with NKp65 is 33% and with murine NKR-P1 42%. In comparison, sequence identity of the extracellular portion of LLT1 with KACL is 49% and with murine Clrb 51%. The structure of the human NKp65:KACL complex comprises two monomeric Nkp65 units interacting separately and symmetrically with a dimeric KACL ligand. Crystal structure of the murine NKR-P1:Clrb complex shows two Clrb dimers interacting with one NKR-P1 dimer placed between them (each Clrb dimer interacts with one of the two NKR-P1 chains). The arrangement of the murine complex is similar to the herein presented NKR-P1:LLT1 complex’s structure, apart from the fact that the murine complex is symmetric while we observe two distinct binding interfaces, the primary and the secondary interaction mode of LLT1.

Despite different oligomeric forms of NKR-P1:LLT1, NKR-P1:Clrb, and NKp65:KACL complexes, the primary receptor:ligand interaction is similar in all three cases at the level of monomer:monomer superposition – the receptor:ligand pair of monomers overlaps basically along the whole chain. The most structurally conserved regions are generally sequentially conserved β-sheets in the core of the proteins. Supplementary Fig. 6a shows in red the fragments with conserved 3D position, amino acid type, and length of at least three amino acids. Such criteria are fulfilled by these fragments: in ligands KCFYFS (in human LLT1 residues 85–90), NWT (95–97), WIGL (132–135), WKW (143–145), and WICSK (152–156), and in receptors – WIGL (in human NKR-P1 residues 153–156) and ICQ (209–211). Therefore, it seems that for their interaction, the mutual orientation of the receptor and ligand scaffold is more important than the actual interaction interface.

We noted only three amino acid interaction pairs conserved in the interaction interface in at least two of the three homologous complexes. They are gathered around residues Tyr165-Thyr171-Phe148, Arg175-Arg181-Arg188, and Tyr177-Thyr183-Phe160 of the ligand (Supplementary Table 2 and Supplementary Fig. 6a). The arginine residues form equivalent hydrogen bonds in Clrb and KACL cases. In LLT1, the arginine assumes a different conformation and forms an intramolecular hydrogen bond with Asn133. The primary interaction mode in NKR-P1:LLT1 mainly relies on main chain contacts that enable fast kon/koff kinetics, thus corroborating the previously published SPR-based findings\textsuperscript{90,91} and our AUC analysis. Hence, a topologically similar complex is formed in all three cases, although the underlying intermolecular recognition mechanism is semi-independent of the actual fold and amino acid composition (Supplementary Fig. 6b). However, the secondary interaction mode is unique to the NKR-P1:LLT1 complex and is not found in the other related complexes.

Y. Li and coworkers reported that the orientation of Nkp65 bound to its ligand precludes the putative α2-centered dimerization of Nkp65\textsuperscript{92}. Similarly, a hypothetical Nkp65 α-centered dimer is also implausible based on steric hindrance and the lack of stabilizing interactions. This observation contrasts with the α-centered dimerization of NKR-P1 present in both its unbound and complexed crystal structure. Interestingly, the single-nucleotide polymorphism (SNP) c.503 T > C of the human KLRB1 gene, causing the substitution of isoleucine 168 for threonine in the NKR-P1 CTLD, was reported to have a 37% frequency of the Thr168 allele\textsuperscript{95}. The authors showed that the Thr168 isoform of NKR-P1 has a lower affinity to LLT1 and a weaker inhibitory effect on NK cells. They proposed that Ile168 forms a part of the interaction interface between NKR-P1 and LLT1, directly affecting NKR-P1 recognition by NKR-P1. However, the structure of the NKR-P1 homodimer shows that Ile168 is found at the dimerization interface rather than at the membrane-distal interaction interface – more specifically, in a small hydrophobic pocket within the dimerization interface.
Fig. 6 | Soluble LLT1 affects NKR-P1 distribution on the cell surface. NKR-P1 stable transfectants were incubated in the presence or absence of soluble LLT1 or LLT1SIM, and the cell surface distribution of NKR-P1 was monitored by super-resolution microscopy. a Representative brightfield (BF) and dSTORM images of full-length NKR-P1 HEK293 stable transfectants on PLL-coated slides incubated without (black) or with LLT1 (red) or LLT1SIM (green), fixed and stained with AlexaFluor® 647-labeled anti-NKR-P1 mAb; scale bars represent 5 µm. The 10 µm² regions (red boxes in dSTORM images) are magnified and shown with corresponding clusters of fluorescent events (CoE) maps and binary maps; scale bars represent 1 µm. b–g Analysis of full-length NKR-P1 distribution relative changes induced by the presence of its LLT1 or LLT1SIM soluble ligands: average cluster of events area (b), average cluster of events diameters (c), size distributions of cluster of events diameters overlaid with Poisson distribution functions (d), average events per cluster of events (e), density of events detected per cluster of events (f) and total density of the detected events (g). In b, c and e, f, each plotted point represents the mean value obtained from the analysis of the total inner surface of a single cell. The box-plot center represents the overall mean value, the bounds of the box represent the interquartile range, and the whiskers represent ±SD. Data are from n = 45 NKR-P1 control cells and n = 41 or n = 47 NKR-P1 cells incubated with LLT1 or LLT1SIM in seven or four independent experiments, respectively. One-way ANOVA with Bonferroni correction, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns. not significant. Source data are provided as a Source Data file.
interface (Fig. 3d). Therefore, we propose that substituting the nonpolar isoleucine residue with polar threonine caused by c.503 T > C SNP indirectly affects the binding affinity because this substitution destabilizes the α1-centered NKR-P1 homodimer. Glycosylation often significantly impacts receptor homooligomerization, as recently evidenced for, e.g., NK cell activation receptor NKP30. NKR-P1 homo-dimerization is also regulated by its glycans; specifically, the glycans present on Asn16 and Asn57 (Fig. 3b). Core glycan chains present at these residues contribute partially to the α1-centered dimerization interface, but at the same time, the glycans clash together. As a result, the stability of the α1-centered NKR-P1 homodimer is improved by abrogating N-glycosylation on Asn157, leaving only the Asn16 glycan at the dimer interface (Supplementary Fig. 3b). Interestingly, a c.470 A > G SNP causing N157S mutation is also listed in the human genome variation database. However, the clinical significance of N157S mutation was not yet investigated, although it could significantly affect NKR-P1 signalization via stabilizing its ligand-bound state. The oligomeric state of the receptor might thus modulate the overall NKR-P1:LLT1 binding affinity. The Nkp65:KACL complex stands out for its high affinity (Kd ~0.67 nM)—ca. 3000× stronger than that of Nkp80:ACL (Kd ~2.3 μM) and 70,000–130,000× than that of NKR-P1:LLT1 (Kd ~48 μM); this study 90 μM). Due to the exceptionally high Nkp65:KACL binding affinity, any putative ancestral α1-centered dimerization interface may have been lost in Nkp65. In contrast, the NKR-P1 and Nkp80 receptors may have evolved to compensate for their low affinity to their ligands by utilizing the α1-centered dimerization and enabling an increased avidity effect.

The SPR analysis of single-residue mutants of both binding partners, performed by J. Kamishikiryo and updated by S. Kita and their coworkers based on the published LLT1 structure, identified several key residues of NKR-P1 and LLT1 essential for their interaction and proposed several pairs of interacting residues (Supplementary Table 3). The mutated residues that had detrimental or moderate adverse effects on binding in these SPR studies are mainly found in the primary interaction interface (Fig. 1 and Supplementary Table 3) – in LLT1: Tyr165, Asp167, Lys169, Arg175, Arg180, and Lys181, and in NKR-P1: Arg181, Asp183, Glu186, Tyr198, Tyr201, and Glu205. However, the proposed interaction pairs do not always agree with the mutual orientation of both proteins observed in the present crystal structure of the NKR-P1:LLT1 complex. For example, the suggested LLT1:NKR-P1 pairs Tyr177/Tyr198 and Arg175/Glu200 correspond well with our observed interaction pairs LLT1:Tyr177/Or/NKR-P1:Ser199 O and LLT1:Arg175/NKR-P1:Glu200:OE2, respectively. On the other hand, the proposed pairing of Glu179 from LLT1 loop L6 with Ser193 and Thr195 from NKR-P1 loop LS only resembles the crystal structure contacts of Glu179 with Arg181 (loop L3) and Tyr198 (strand β4), located near NKR-P1 loop LS. In contrast to the SPR studies, we observed no contact between Tyr165 and Phe152, although LLT1:Tyr165 is used in the primary interface, and Phe152 is close to the NKR-P1 L0 interaction region. Lastly, we cannot confirm the suggested direct bond between LLT1:lys169 and NKR-P1:Glu205. Although these residues are close to each other in the primary mode (the closest distance of 4.3 Å), they clearly do not form a pivotal bond in the interaction interface. Furthermore, the Lys169 side chain is rather flexible, as suggested by the low quality of its electron density map in the primary mode, in contrast to all other nearby side chains with well-defined positions.

Because the secondary binding mode observed in the NKR-P1:LLT1 structure involves a different region of LLT1 than the region used in the primary binding mode, the orientation of LLT1 and NKR-P1 in this binding mode does not match the interaction pairs proposed in the SPR studies. Notwithstanding, the NKR-P1 interaction interface is very similar in primary and secondary modes; therefore, some of the previously proposed NKR-P1 interaction residues are also involved in the secondary mode (Arg181, Asp183, Tyr198, and Tyr201). Interestingly, residue LLY169 establishes several contacts with NKR-P1 (Arg181, Ser199, and Glu200) in secondary interaction mode. However, the previously proposed pair Lys169/Glu205 was not observed in this mode either, and these residues are even farther apart – ca.11 Å. The presence of Lys169 in both primary and secondary interaction interfaces of LLT1 suggests that this residue plays an important role in the overall complex formation. Accordingly, the previously reported LLT1 mutation Lys169Glu would lead to a co-localization of several negative side chains in the secondary interface (Glu200 and Asp183 of NKR-P1, and mutated Glu169 of LLT1), thus indicating that the disruption of the NKR-P1:LLT1 interaction observed in the previous SPR experiments most likely resulted from the weakening of the secondary rather than the primary interface. J. Kamishikiryo subsequently restored binding by introducing the Glu205Lys mutation in NKR-P1. Based on our structure, this effect would be explained by strengthening the primary interface. Thus, the previously published SPR-based interaction data largely agree with the interaction modes observed between NKR-P1 and LLT1 in the present crystal structure, although some previously suggested interaction pairs were misassigned and are not present in either interaction interface.

Several authors have previously suggested an avidity effect of multimerization upon the interaction that compensates for the low affinity of the NKR-P1:LLT1 complex. Interestingly, in the present structure of this complex, we indeed observe the formation of a chain of repeating NKR-P1 and LLT1 homodimers. This pseudo-linear multimer has a zigzag shape in which the membrane-proximal parts of the two proteins are on opposite sides (Fig. 8), and it is structurally based on the alternating helix α1/α2-centered NKR-P1/LLT1 homodimers (the chain-forming effect) and the simultaneous involvement of both primary and secondary interaction modes (steric effect). On the contrary, an artificially constructed multimeric model of NKR-P1:LLT1 engaged in just the primary mode shows a chain of homodimers with a non-linear, almost helical conformation (Supplementary Fig. 6c). Furthermore, the NKR-P1 stalk regions sterically clash, and the LLT1 stalk regions are exposed outside the complex core in many different directions. Thus, such a multimer is unlikely compatible with cell
membrane anchoring and formation within the immune synapse. NKR-P1:LLT1 engaged in just the secondary mode would also form a helical multimer with both receptor and ligand stalk regions pointing in many different directions outside the complex core (Supplementary Fig. 6d). At the same time, when bound to LLT1 dimer in both primary and secondary interaction mode, the neighboring NKR-P1 molecules create a mutual accessory contact of not an insignificant energetic contribution to the stability of the whole assembly (Fig. 4a, marked with a black star). This accessory contact encompasses a similar number of hydrogen bonds and size as the secondary binding interface between NKR-P1 and LLT1 itself (Supplementary Fig. 6e), and it could thus be considered an additional stabilizing element favoring a combination of the primary and secondary binding modes rather than a single one of them.
To determine whether such zipper-like oligomers of NKR-P1:LLT1 occur not only in crystal and to some extent in solution (Fig. 5 and Supplementary Fig. 5) but also on the cell surface, we used single-molecule localization microscopy to examine full-length NKR-P1 transfectants labeled with anti-NKR-P1 AlexaFluor® 647 mAb in the presence or absence of soluble LLT1 and LLT1SM (Fig. 6). We observed a significant increase in the NKR-P1 cluster of fluorescent events size and area upon adding LLT1, but not its secondary interaction mode mutant, LLT1SM. Although the experimental setup with one binding partner embedded in the membrane and the other being presented as soluble does not fully describe the biological reality of intercellular contact, we assume that limiting the interaction to a two-dimensional space between membranes of two adjacent cells in the case of full-length disulfide proteins would only strengthen it. Our experiment aimed to test the hypothesis of the NKR-P1 receptor’s ability to form clusters (cross-linked oligomers) in the context of the cell membrane upon engaging the dimeric species of LLT1. This experimental setup allowed us to normalize the concentration of LLT1 throughout the measurement while ensuring the presence of primarily dimeric LLT1 in the solution. Additionally, our SEC-SAXS data were best-fit with multimer chains in alternating primary and secondary interaction modes (Supplementary Fig. 5), even though the whole library of all possible models, including those composed purely of primary or secondary mode, were included in the analysis. Acceptable χ² values were obtained primarily for multimer models containing the alternating arrangement. Taken together, we conclude that combining both interaction modes is necessary for a biologically plausible multimeric interaction, as further supported by the NK cell-mediated cytotoxicity assay results (Fig. 7).

Albeit such functional multimerization of NK CTLRs has been mostly overlooked, the formation of similar nanoclusters is well described for interaction between the immunoglobulin family of KIRs and MHC class I glycoproteins or interaction between KRR2DL1 and NK2CD5. Moreover, a periodic zipper-like network of interacting dimers was reported in the crystal structures of the co-stimulatory immunocomplexes B7-1:CTLA-4 and B7-2:CTLA-4. Interestingly, B7-1 is expressed on the cell surface in a dynamic equilibrium between monomers and non-covalent dimers. Upon interaction with the co-stimulatory receptor CD28, B7-1 forms an interaction network composed of B7-1 and CD28 homodimers. The uncoupling of this interaction is facilitated by B7-1 dissociation to monomers, whereas insertion of B7-1 obligate dimer leads to prolonged, abnormal signaling between antigen-presenting cells and T-cells.

Although as full-length proteins, both LLT1 and NKR-P1 form covalent disulfide homodimers on the cell surface, to our knowledge, the dimeric state of their CTL ectodomains has not yet been assessed in live cells. Conversely, the formation of the helix α2-centered non-covalent homodimer of soluble LLT1 ectodomain has been previously characterized, and likely occurs within the full-length protein as well. The human NKR-P1 helix α1-centered dimer uses fewer intermolecular contacts, has a smaller contact surface area than helix α2-centered dimeric CTLRs, and is thus less stable. Nevertheless, its formation would expectedly increase in the context of the full-length NKR-P1 receptor disulfide homodimer. At the same time, the length of the NKR-P1 stalk region (25 amino acids) confers enough flexibility for CTLD monomer/dimer equilibrium within the disulfide homodimer of the full-length receptor itself, which would be then further regulated by polymorphism and glycosylation heterogeneity at the NKR-P1 dimerization interface (Fig. 8c, bottom left). Thus, similarly to the B7-1:CD28 system, an equilibrium between the monomeric and dimeric states of CTL ectodomains, modifying and fine-tuning the ability of NKR-P1 to form stable higher-order complexes with LLT1, may consequently regulate the strength and signaling of the NKR-P1:LLT1 system, while cross-linking of NKR-P1 by LLT1 within the immune synapse may provide enough avidity for stable signal transduction by this low-affinity interaction complex.

In conclusion, the presented data show that the crystal structure of the NKR-P1:LLT1 complex constitutes a novel way of C-type lectin-like NK cell receptor/glind multimerization on the cell surface that explains how ligand binding overcomes low affinity through receptor cross-linking within the immune synapse.

Methods

Protein expression and purification

Stabilized H176C form of the soluble LLT1 ectodomain (Gln72-Val191) was transiently expressed in HEK293S GnTI cells, as previously described. The N120R, R153E, K169A secondary interaction mode mutant LLT1SM was cloned and produced in the same way. The C-type lectin-like domain of human NKR-P1 was produced similarly in stably transfected HEK293S GnTI cells. Briefly, the expression construct corresponding to the extracellular CTL domain of NKR-P1 (Gly90-Ser225) was subcloned into the pPINGGTneo plasmid (kindly provided by Prof. Ray Owens, University of Oxford), flanked by the N-terminal secretion leader and by the C-terminal His-tag (with the ETG and the KHHHHHH at the N- and C-termini of the secreted protein, respectively). Suspension culture of HEK293S GnTI cells was transferred with a 1:3 (w/w) mixture of the expression plasmid and 25-kDa linear polyethyleneimine. The stably transfected cell pool was selected on 200 ng/ml G418 within three weeks. The secreted proteins were purified from the harvested media by two-step chromatography - an IMAC was performed on a Talon column (GE Healthcare), followed by SEC on a Superdex 200 10/300 GL (GE Healthcare) in 10 mM HEPES pH 7.5 with 150 mM NaCl and 10 mM Na2HPO4. For dsSTORM microscopy and in vitro NK cell assays (see below), the buffer was exchanged for a tissue culture grade PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4). For deglycosylation, GST-fused Endo F1 was added in a 1:100 weight ratio to proteins in SEC buffer with 50 mM citrate pH 5.5 and incubated for 2 h at 37 °C. The deglycosylated proteins were then purified by batch affinity chromatography on Glutathione Sepharose 4B resin (GE Healthcare) followed by SEC, as described above.

Crystallization

NKR-P1 glycosylated (structure NKR-P1_glyco) – Soluble human NKR-P1 ectodomain at 20 mg/ml in SEC buffer was crystallized using the sitting drop vapor diffusion method. Drops (100 nl of reservoir solution and 100 nl of protein solution) were set up using a Cartesian Honeybee 961 robot (Genomic Solutions) at 294 K. The reservoir consisted of 20% (v/v) ethylene glycol, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4. For deglycosylation, GST-fused Endo F1 was added in a 1:100 weight ratio to proteins in SEC buffer with 50 mM citrate pH 5.5 and incubated for 2 h at 37 °C. The deglycosylated proteins were then purified by batch affinity chromatography on Glutathione Sepharose 4B resin (GE Healthcare) followed by SEC, as described above.
X-ray diffraction data collection, processing, and structure solution

All diffraction data were collected at the Diamond Light Source (Harwell, UK) at beamline I03 using a wavelength of 0.9762 Å and a PILATUS3 6M detector. The crystal-detector distance was set to 340 mm, exposure time per image was 0.02 s, oscillation width was 0.1°, and the temperature was 100 K. 7200 images were collected for each dataset. In the case of the NKR-P1:LLTI complex, only 5000 images were finally used for data processing. All diffraction images were indexed and integrated using the XDS package49, scaled using AIMLESS50, and 5% of randomly selected reflections were utilized as an Rfree set. The phase problem was solved by molecular replacement using NKR-P1 glyco: in program BALBES51 using the structure of the human NK cell receptor KLRG1 bound to E-cadherin (https://doi.org/10.2210/pdb3F7T/pdb); NKR-P1_deglyco: 6 chains found in PHASER52 using murine NKR-PIA (https://doi.org/10.2210/pdb3T3A/pdb)53 were completed with 2 chains found in MOLREP54; NKR-P1:LLTI: 4 chains found in BALBES as NKR-P1 chains (using the structure of murine dectin-1, https://doi.org/10.2210/pdb2BPD/pdb)48 were completed with two more chains in MOLREP, and all 6 chains were manually reinterpretated as 4 NKR-P1 chains and as 2 LLTI chains. Refinement was performed using REFMAC55 and manual editing in COOT56. The last cycle of refinement was completed using all reflections. The final data processing and structure parameters are outlined in Table 1.

Crystal structure quality assessment

NKR-P1_glyco - The structure, comprising one dimer of the glycosylated human NKR-P1 CTLD, is overall well defined in the electron density map, corresponding to the high resolution of the structure (1.8 Å). Glycosylation at the dimerization interface does not show the overlapping features observed in the structures of deglycosylated NKR-P1 (below). GlcNAc was modeled on Asn116 with full occupancy in both chains, whereas glycosylation at Asn157 was not observed in the electron density map. All modeled glycosylation chains (GlcNAc3Man9, at A/Asn169, GlcNAc3Man9 at B/Asn169, and GlcNAc at residues Asn116 in both chains) are well localized in the electron density map.

NKR-P1_deglyco - The asymmetric unit comprises four dimers of the human NKR-P1 CTLD deglycosylated after the first GlcNAc unit. The length of the localized part of the protein chain varies from the shortest chains A, F, and G, with modeled residues Leu91–Leu214, to the longest chain, with residues Gly90–Arg218. GlcNAc at residue Asn169 is well localized, while GlcNAc units bound to Asn116 and Asn157 at the dimerization interface are present in alternative and overlapping positions; residue Asn116 also shows alternative conformers. GlcNAc units attached to Asn157 and Asn116 were modeled with 0.5 occupancies, and only the most distinct units from each overlapping pair were modeled (Table 1).

NKR-P1:LLTI - The asymmetric unit contains two dimers of the human NKR-P1 CTLD and one dimer of the LLTI CTLD. The structure has a well-defined electron density map, and all protein chains can be unambiguously assigned. The most distinct difference peaks correspond to non-interpretable small ligands. LLTI has well-localized GlcNAc units at residues Asn95 and Asn147. Localized GlcNAc units of NKR-P1 are the same as those identified in the NKR-P1_deglyco structure (previous paragraph).

CD spectroscopy

Circular dichroism (CD) spectra of wild-type and S159A NKR-P1 and wild-type and SIM LLTI were recorded using a Chirascan Plus CD spectropolarimeter with ProData Chirascan software (Applied Photophysics) and a 0.1 cm pathlength quartz cell. Spectra were recorded over the wavelength range of 195–260 nm in steps of 1 nm at room temperature. The sample concentrations were 0.2 mg/ml and 0.3 mg/ml for NKR-P1 and LLTI protein samples, respectively, in 10 mM HEPES, 150 mM NaCl pH 7.5 sample buffer. The CD signal was expressed as ellipticity, and the resulting spectra were buffer subtracted. Secondary structure composition was analyzed using the CDNN 2.1 software (Applied Photophysics).

Analytical ultracentrifugation

NKR-P1:LLTI complex formation and dimerization of NKR-P1 S159A mutant were analyzed in an analytical ultracentrifuge ProteomeLab XL-1 (Beckman Coulter)57. For the sedimentation velocity experiment, samples of glycosylated NKR-P1, LLTI, and their equimolar mixtures with increasing concentrations in SEC buffer were spun in the An-50 Ti rotor (Beckman Coulter) at 48,000 rpm at 20 °C, and 150 scans with 0.003 cm spatial resolution were recorded in 5 min steps using absorbance optics. The centrifuge was operated using ProteomeLab software (Beckman Coulter). Buffer density and protein partial specific volumes were estimated in SEDNTERP (http://www.jphi.ac.uk/) . Data were analyzed with SEDFIT58 using the continuous (s) distribution model. Binding isotherms (and figures illustrating AUC data) were prepared in GUSSI59 and then best-fit in SEDPHAT60 using hetero-association models A + B ⇔ AB or A + B ⇔ AB + B + ABB, where A is the LLTI dimer and B is NKR-P1 monomer, respectively. Only Kd and sedimentation coefficients of AB or ABB were floated in the fit; the other parameters were kept constant at known values.

Microscale thermophoresis

For the microscale thermophoresis (MST) measurements of NKR-P1:LLTI and NKR-P1:LLTISIM interactions, the NKR-P1 was fluorescently labeled with Atto 488 NHS-ester (Merck) at pH 6.5; the excess label was removed by size exclusion chromatography. Fluorescently labeled 100 nM NKR-P1 was mixed with dilution series of LLTI or LLTISIM and analyzed in standard capillaries in an NT.15 Monolith using the Mo.Control software (NanoTemper), 30% LED excitation power, and 60% MST power. Raw data were analyzed in PALMIST61; the exported isotherms were best-fit in SEDPHAT60 and figures prepared in GUSSI59.

Small-angle X-ray scattering

SEC-SAXS data for the NKR-P1:LLTI complex were collected at the Diamond Light Source (Didcot, UK) at beamline 21 using an Agilent 1200 HPLC system with 2.4 ml Superdex 200 column (GE Healthcare), a Pilatus P3-2M detector, 12.4 kV radiation, and 4.014 m sample-to-detector distance. The human NKR-P1 and LLTI ectodomains with GlcNAc3Man9 glycans diluted in 10 mM HEPES, 150 mM NaCl, 10 mM NaF, pH 7.5 were mixed at a 1:1 molar ratio. The data were collected at 293 K for buffer and protein samples at a 15 mg/ml loading concentration. The data in selected intervals (frames 355–367, 368–379, 380–399, 390–401, 412–421) were solvent-subtracted in SCATTER (developed by Robert Rambo at the Diamond Light Source, https://www.bioisis.net/tutorials/9) and then in the intervals separately merged and characterized using the ATSAS62 package. As proof of the data quality, the scattering plot, Kratyk plot, and pair distance distribution function P(r) are shown in Fig. 5 for intervals 389–399 (a sample data range from the first peak) and 481–491 (a sample data range from the second peak). The scattering and Guinier plots for all the data ranges are shown in Supplementary Fig. 5. Agreement between the SAXS data and our 3D crystal structure of the NKR-P1:LLTI complex was evaluated using OLGOMER63. Library of NKR-P1 and LLTI structural models representing monomers, dimers, and their interacting multimers in various permutations of primary and secondary interaction modes was generated in PyMOL from the herein solved crystal structures by applying symmetry operations (in total, 49 different models). The OLGOMER algorithm was then let to select their combinations to best-fit all individual scattering curves and curves resulting from the six selected merged data intervals (Supplementary Data 1 and Supplementary Fig. 5).
Super-resolution dSTORM microscopy

Full-length NKR-P1 stable transfectants were generated in HEK293S GnT1 cell line using the piggyBac transposon-based system with doxycycline-inducible protein expression. Microscopy samples were prepared from cells treated with 5 ng/ml doxycycline overnight. Cells were washed with PBS and incubated for 1 h at 37 °C with 8.4 mg/ml LTL1 or LTL1™ in PBS—PBS alone. After incubation, cells were allowed to settle on the surface of PLL-coated glass slides for 25 min at 37 °C. Cells were then fixed with 4% PFA and 0.2% GA for 10 min at room temperature and washed three times with PBS. For antibody staining, cells were first blocked with 5% BSA for 30 min and stained with Alexa Fluor® 647 anti-human CD161 antibody (clone HP-3G10; BioLegend) at 10 ng/ml in blocking solution for 60 min. Samples were washed five times with PBS before post-fixation with 4% PFA and 0.2% GA for 10 min. Finally, cells were treated with 15 mM NaN3 and washed twice with PBS. dSTORM images were acquired with Elyra PS.1 using Zen Black edition software (Carl Zeiss). Fiducial markers (FluoSpheres 880 nm; Thermo Fisher) were diluted in PBS and allowed to settle on the sample for 1 h (the final dilution of the bead stock was 100,000×). Before each measurement, the buffer was exchanged for glucose oxidase/catalase/MEA-based imaging buffer, and the sample was sealed with cover glass and silicon to prevent buffer oxidation. For each cell, 2 × 10^3 raw images were acquired using the high NA STORM illumination mode with an exposure time of 15 ms, using 100% of 642 nm laser power and 100×, 1.46 numerical aperture, oil immersion objective. We used the same batch and concentration of the antibody to ensure consistent labeling within the single measurements for all experiments. Moreover, we always measured conditions with and without the soluble ligand in the same batch. Cells were then incubated with 100,000×) (https://doi.org/10.17632/268ww2m4j31) and deposited there under the accession numbers of https://doi.org/10.2210/pdb5MGR/pdb (NKR-P1_glyco), https://doi.org/10.2210/pdb5MGS/pdb (NKR-P1_deglyco), and https://doi.org/10.2210/pdb5MTG/pdb (NKR-P1:LLT1). Links to the other PDB entries in this article are https://doi.org/10.2210/pdb62BDP/pdb, https://doi.org/10.2210/pdbB2CL8/pdb, https://doi.org/10.2210/pdb5YF7/pdb, https://doi.org/10.2210/pdb3T3A/pdb, https://doi.org/10.2210/pdb40OP/pdb, https://doi.org/10.2210/pdb4Q6K/pdb, https://doi.org/10.2210/pdb5J2S/pdb, and https://doi.org/10.2210/pdb6E7D/pdb. Diffraction data have been deposited in the SBGrid Data Bank under the codes 778 (NKR-P1_glyco; https://doi.org/10.15785/SBGRID/778); 779 (NKR-P1_deglyco, https://doi.org/10.15785/SBGRID/779) and 780 (NKR-P1:LLT1; https://doi.org/10.15785/SBGRID/780). Diffraction data from the SEC-SAXS experiment have been deposited to Mendeley Data (https://doi.org/10.17632/268ww2m4j31). The total output of the OLIGOMER analysis of the SEC-SAXS data is available as Supplementary Data 1. Source data are provided with this paper.

Super-resolution image data analysis

Super-resolution dSTORM images were reconstructed from raw image sequences with the ThunderSTORM plug-in77 for the ImageJ processing software. Sub-pixel localization of molecules was performed by fitting an integrated Gaussian point-spread function models using the maximum likelihood estimation fitting method. Reconstructed dSTORM images were corrected for drift using fiducial markers and for multiple localizations from a single source by merging events within 20 nm appearing in subsequent frames (with an off-gap of 5 frames). Voronoi tessellation cluster analysis was performed in ClusterVisu™ on a whole inner cell surface (cell edges and other concentrated membrane regions were excluded from analysis). A threshold value of 250 was chosen by comparing the distribution of Voronoi polygon surface values between localizations in the experimental areas and randomized regions of the same density of events. Clusters containing less than two fluorescent events were discarded from the data set. Multiple means were compared with one-way analysis of variance (ANOVA) with Bonferroni correction in OriginPro 2018. Graphs show mean values, and error bars represent the S.D. Values of p > 0.05 are indicated as not significant; statistically significant R values are indicated with asterisks (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

NK cell-mediated cytotoxicity assay

The influence of LTL1 or LTL1™ on inhibition of the cytotoxic activity of primary NK cells was assessed by flow cytometry. Buffy coats for human NK cell isolation were purchased from the Institute of Hematology and Blood Transfusion (IHB, Prague, Czech Republic) as material for research use. The IHB arranged the consent of the donors. Primary NK cells were isolated by negative selection using an NK cell isolation kit (Miltenyi Biotec) according to the manufacturer’s protocol. Purified NK cells were cultured overnight at 1 × 10^6 cells/ml in RPMI 1640 supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 80 ng/ml IL-2 (Sigma–Aldrich) for their activation. K562 target cells were stained with CellTrace Violet Proliferation Kit (Thermo Fisher). Following the staining, 1 × 10^6 target cells were mixed with activated NK cells in a 40:1 (E:T) ratio, and LTL1 or LTL1™ proteins were added, keeping the final reaction volume at 20 µl (6.5 µl of K562 and 6.5 µl of NK cell suspensions and 7 µl of the concentrated protein stock solutions in PBS, to a final protein concentration of 50 or 250 µM). After 4 h of incubation, the cell mixture was centrifuged at 300 × g and stained with 1 µg/ml 7-AAD. Cells were analyzed using a BD LSR II flow cytometer and BD FACSDiva software (BD Biosciences) in three independent cytotoxicity assays performed using NK cells from three different healthy donors in triplicates. Data were analyzed in FlowJo software (BD Biosciences). The gating strategy for flow cytometry analysis is shown in Supplementary Fig. 7. Data are presented as a mean of measurements with S.D. When applicable, data were statistically evaluated by one-way ANOVA with values of p < 0.05 considered statistically significant.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The refined coordinate and structure factor files for the X-ray crystal structures reported in this study have been validated by the Protein Data Bank (https://www.wwpdb.org) and deposited there under the accession numbers of https://doi.org/10.2210/pdb5MGR/pdb (NKR-P1_glyco), https://doi.org/10.2210/pdb5MGS/pdb (NKR-P1_deglyco), and https://doi.org/10.2210/pdb5MTG/pdb (NKR-P1:LLT1). Links to the other PDB entries in this paper are https://doi.org/10.2210/pdb62BDP/pdb, https://doi.org/10.2210/pdbB2CL8/pdb, https://doi.org/10.2210/pdb5YF7/pdb, https://doi.org/10.2210/pdb3T3A/pdb, https://doi.org/10.2210/pdb40OP/pdb, https://doi.org/10.2210/pdb4Q6K/pdb, https://doi.org/10.2210/pdb5J2S/pdb, and https://doi.org/10.2210/pdb6E7D/pdb. Diffraction data have been deposited in the SBGrid Data Bank under the codes 778 (NKR-P1_glyco; https://doi.org/10.15785/SBGRID/778); 779 (NKR-P1_deglyco, https://doi.org/10.15785/SBGRID/779) and 780 (NKR-P1:LLT1; https://doi.org/10.15785/SBGRID/780). Diffraction data from the SEC-SAXS experiment have been deposited to Mendeley Data (https://doi.org/10.17632/268ww2m4j31). The total output of the OLIGOMER analysis of the SEC-SAXS data is available as Supplementary Data 1. Source data are provided with this paper.

References

1. Vivier, E. et al. Innate or adaptive immunity? The example of natural killer cells. Science 331, 44–49 (2011).
2. Cerwenka, A. & Lanier, L. L. Natural killer cell memory in infection, inflammation and cancer. Nat. Rev. Immunol. 16, 112–123 (2016).
3. Yokoyama, W. M. & Plougastel, B. F. Immune functions encoded by the natural killer gene complex. Nat. Rev. Immunol. 3, 304–316 (2003).
4. Bartel, Y., Bauer, B. & Steinle, A. Modulation of NK cell function by genetically coupled C-type lectin-like receptor/ligand pairs encoded in the human natural killer gene complex. Front. Immunol. 4, 362 (2013).
5. Želenky, A. N. & Gready, J. E. The C-type lectin-like domain superfamily. FEBS J. 272, 6179–6217 (2005).
6. Rozbesky, D. et al. Re-evaluation of binding properties of recombinant lymphocyte receptors NKR-P1A and CD69 to chemically synthesized glycan and peptides. Int. J. Mol. Sci. 15, 1271–1283 (2014).
7. Lanier, L. L., Chang, C. & Phillips, J. H. Human NKR-P1A. A disulfide-linked homodimer of the C-type lectin superfamily expressed by a subset of NK and T lymphocytes. J. Immunol. 153, 2417–2428 (1994).
8. Spreu, J. et al. Interaction of C-type lectin-like receptors Nkp65 and KAC1 facilitates dedicated immune recognition of human keratinocytes. Proc. Natl Acad. Sci. USA 107, 5100–5105 (2010).
9. Welte, S., Kuttruff, S., Waldbauer, I. & Steinle, A. Mutual activation of natural killer cells and monocytes mediated by Nkp80-AICL interaction. Nat. Immunol. 7, 1334–1342 (2006).

10. Vogler, I. & Steinle, A. Vis-a-vis in the NKC: genetically linked natural killer cell receptor/ligand pairs in the natural killer gene complex (NKC). J. Innate Immun. 3, 227–235 (2011).

11. Aldemir, H. et al. Cutting edge: lectin-like transcript 1 is a ligand for the CD161 receptor. J. Immunol. 175, 7791–7795 (2005).

12. Asgharian, A. et al. Cutting edge: lectin-like transcript 1 is a ligand for the inhibitory human NKR-P1A receptor. J. Immunol. 175, 7796–7799 (2005).

13. Poggi, A., Costa, P., Tomasello, E. & Moreta, L. IL-12-induced up-regulation of NKR-P1A expression in human NK cells and consequent NKR-P1A-mediated down-regulation of NK cell activation. Eur. J. Immunol. 28, 1611–1616 (1998).

14. Exley, M., Porcelli, S., Furman, M., Garcia, J. & Balk, S. CD161 (NKR-P1A) costimulation of CD1d-dependent activation of human T cells expressing invariant V alpha 24 J alpha P1A. Eur. J. Immunol. 36, 2877–2886 (2006).

15. Bennett, I. M. et al. Deletion of lectin-like transcript 1, the ligand for natural killer receptor P1A, in Rheumatoid Arthritis. Ann. Rheum. Dis. 70, 1050–1056 (2011).

16. Fergusson, J. R. et al. CD161-dependent regulation of human T helper 17 cell development. Cell. Immunol. 350, 86–93 (2018).

17. Germain, C. et al. Induction of lectin-like transcript 1 protein cell surface expression by pathogens and interferon-gamma contributes to modulate immune responses. J. Biol. Chem. 286, 28141–28147 (2011).

18. Germain, C. et al. Cutting edge: lectin-like transcript 1 (LLT1) protein cell surface expression by pathogens and interferon-gamma contributes to modulate immune responses. J. Biol. Chem. 286, 28141–28147 (2011).

19. Ussher, J. E. et al. Cutting edge: lectin-like transcript 1 (LLT1) protein cell surface expression by pathogens and interferon-gamma contributes to modulate immune responses. J. Biol. Chem. 286, 28141–28147 (2011).

20. Mathewson, N. D. et al. Inhibitory CD161 receptor identified in glioma-infiltrating T cells by single-cell analysis. Cell 178, 1291–1298 (2021).

21. Poggi, A., Costa, P., Zocchi, M. R. & Moreta, L. Phenotypic and functional analysis of CD4+ NKR-P1A+ human T lymphocytes. Direct evidence that the NKR-P1A molecule is involved in transendothelial migration. Eur. J. Immunol. 27, 2345–2350 (1997).

22. Chalan, P. et al. Expression of Lectin-Like Transcript 1, the Ligand for CD161, in Rheumatoid Arthritis. PLoS One 10, e0132436 (2015).

23. Rosen, D. B. et al. Functional consequences of interactions between human NKR-P1A and its ligand LLT1 expressed on activated dendritic cells and B cells. J. Immunol. 180, 6508–6517 (2008).

24. Boles, K. S., Barten, R., Kumaresan, P. R., Trowsdale, J. & Mathew, P. A. Cloning of a new lectin-like receptor expressed on human NK cells. Immunogenetics 50, 1–7 (1999).

25. Roth, P. et al. Malignant glioma cells counteract antitumor immune responses through expression of lectin-like transcript-1. Cancer Res. 67, 3540–3544 (2007).

26. Mathew, S. O., Chaudhary, P., Powers, S. B., Vishwanatha, J. K. & Mathew, P. A. Overexpression of LLT1 (OICL, CLEC2D) on prostate cancer cells inhibits NK cell-mediated killing through LLT1-NKR-P1A (CD161) interaction. Oncotarget 7, 68650–68661 (2016).

27. Marrufo, A. M. et al. Blocking LLT1 (CLEC2D, OICL)-NKR-P1A (CD161) interaction enhances natural killer cell-mediated lysis of triple-negative breast cancer cells. Am. J. Cancer Res. 8, 1050–1063 (2018).

28. Germain, C. et al. Cutting edge: lectin-like transcript 1 is a marker of germinal center-derived B cell non-Hodgkin’s lymphomas dampening natural killer cell functions. Oncoimmunology 4, e1026503 (2015).

29. Zambrano-Zaragoza, J. F., Romo-Martinez, E. J., Duran-Avelar, M., Garcia-Magallanes, N. & Vibanco-Perez, N. Th17 cells in autoimmune and infectious diseases. Int. J. Inflamm. 2014, 651503 (2014).

30. Afzali, B. et al. CD161 expression characterizes a subpopulation of human regulatory T cells that produces IL-17 in a STAT3-dependent manner. Eur. J. Immunol. 43, 2042–2054 (2013).

31. Billerbeck, E. et al. Analysis of CD161 expression on human CD8+ T cells defines a distinct functional subset with tissue-homing properties. Proc. Natl Acad. Sci. USA 107, 3006–3011 (2010).

32. Smith, J. A. & Colbert, R. A. Review: The interleukin-23/interleukin-17 axis in spondyloarthritides pathogenesis: Th17 and beyond. Arthritis Rheumatol. 66, 231–241 (2014).

33. Brucklacher-Waldert, V., Stuenner, K., Kolster, M., Wolt hausen, J. & Tolosa, E. Phenotypical and functional characterization of T helper 17 cells in multiple sclerosis. Brain 132, 3329–3341 (2009).

34. Estrada-Capetillo, L. et al. Induction of Th17 lymphocytes and Treg cells by monocyte-derived dendritic cells in patients with rheumatoid arthritis and systemic lupus erythematosus. Clin. Dev. Immunol. 2013, 584303 (2013).

35. Michalak-Storna, A. et al. Serum levels of selected Th17 and Th22 cytokines in psoriatic patients. Dis. Markers 35, 625–631 (2013).

36. Germain, C. et al. Characterization of alternatively spliced transcript variants of CLEC2D gene. J. Biol. Chem. 285, 35207–35215 (2010).

37. Li, Y., Wang, Q., Chen, S., Brown, P. H. & Mariuzza, R. A. Structure of Nkp65 bound to its keratinocyte ligand reveals basis for genetically linked recognition in natural killer gene complex. Proc. Natl Acad. Sci. USA 110, 11505–11510 (2013).

38. Bauer, B., Spreu, J., Rohe, C., Vogler, I. & Steinle, A. Key residues at the membrane-distal surface of KACL, but not glycosylation, determine the functional interaction of the keratinocyte-specific C-type lectin-like receptor KACL with its high-affinity receptor Nkp65. Immunology 145, 114–123 (2015).

39. Kamishikiryo, J., Fukuhara, H., Okabe, Y., Kuroki, K. & Maenaka, K. Molecular basis for LLT1 protein recognition by human CD161 protein (NKR-P1A/KLRB1). J. Biol. Chem. 286, 23823–23830 (2011).

40. Kita, S. et al. Crystal structure of extracellular domain of human lectin-like transcript 1 (LLT1), the ligand for natural killer receptor-P1A. Eur. J. Immunol. 45, 1605–1613 (2015).

41. Aguilar, O. A. & Maenaka, K. A viral Immunoevasin controls innate immunity by targeting the prototypical natural killer. Cell Receptor Fam. Cell 169, 58–71 (2017).

42. Balaji, G. R. et al. Recognition of host C1r-b by the inhibitory NKR-P1B receptor provides a basis for missing-self recognition. Nat. Commun. 9, 4623 (2018).

43. Skalova, T. et al. Four crystal structures of human LLT1, a ligand of natural killer NKR-P1I, in varied glycosylation and oligomerization states. Acta Crystallogr. D. Biol. Crystallogr. 71, 578–591 (2015).

44. Blaha, J., Pachl, P., Novak, P. & Vanek, O. Expression and purification of soluble and stable ectodomain of natural killer cell receptor LLT1 through high-density transfection of suspension adapted HEK293S GnT1-(-) cells. Prot. Expr. Purif. 109, 7–13 (2015).

45. Vanek, O. et al. Soluble recombinant CD69 receptors optimized to have an exceptional physical and chemical stability display prolonged circulation and remain intact in the blood of mice. FEBS J. 275, 5589–5606 (2008).

46. Kolenko, P. et al. The high-resolution structure of the extracellular domain of human CD69 using a novel polymer. Acta Crystallogr. F. Struct. Biol. Cryst. Commun. 65, 1258–1260 (2009).

47. Skalova, T. et al. Mouse Clr-g, a ligand for NK cell activation receptor NKR-P1F: crystal structure and biophysical properties. J. Immunol. 189, 4881–4889 (2012).

48. Brown, J. et al. Structure of the fungal beta-glucan-binding immune receptor dectin-1: implications for function. Protein Sci. 16, 1042–1052 (2007).
49. Vanek, O. et al. Production of recombinant soluble dimeric C-type lectin-like receptors of rat natural killer cells. Sci. Rep. 9, 17836 (2019).
50. Blaha, J. et al. High-level expression and purification of soluble form of human natural killer cell receptor NKR-P1 in HEK293S GnTI cells. Protein Expr. Purif. 140, 36–43 (2017).
51. Rother, S. et al. The c.503T>C polymorphism in the human KLRB1 gene alters ligand binding and inhibitory potential of CD161 molecules. PLoS One 10, e0135682 (2015).
52. Skorepa, O. et al. Natural killer cell activation receptor Nkp46 Oligomerization depends on its N-Glycosylation. Cancers 12, 1988 (2020).
53. Davis, D. M. et al. The human natural killer cell immune synapse. Proc. Natl. Acad. Sci. USA 96, 15062–15067 (1999).
54. Pageon, S. et al. Superresolution microscopy reveals nanometer-scale reorganization of inhibitory natural killer cell receptors upon activation of NKGD2. Sci. Signal. 6, ra62 (2013).
55. Stamper, C. C. et al. Crystal structure of the B7-1/CTLA-4 complex that inhibits human immune responses. Nature 410, 608–611 (2001).
56. Schwartz, J. C., Zhang, X., Fedorov, A. A., Nathenson, S. G. & Almo, S. C. Structural basis for co-stimulation by the human CTLA-4/B7-2 complex. Nature 410, 604–608 (2001).
57. Bhatia, S., Sun, A., Almer, N. S., Nathenson, S. G. & Hodes, R. J. Dynamic equilibrium of B7-1 dimers and monomers differentially affects immunological synapse formation and T cell activation in response to TCR/CD28 stimulation. J. Immunol. 184, 1821–1828 (2010).
58. Reeves, P. J., Callewaert, N., Contreras, R. & Khorana, H. G. Structure and function in rhodopsin: high-level expression of rhodopsin with restricted and homogeneous N-glycosylation by a tetracycline-inducible N-acetylglucosaminyltransferase I-negative HEK293S stable mammalian cell line. Proc. Natl Acad. Sci. USA 99, 13419–13424 (2002).
59. Grueninger-Leitch, F., D’Arcy, A., D’Arcy, B. & Chene, C. Deglyco- sylation of proteins for crystallization using recombinant fusion protein glycolipids. Protein Sci. 5, 2617–2622 (1996).
60. Kabsch, W. Xds. Acta Crystallogr. D. Biol. Crystallogr. 66, 125–132 (2010).
61. Evans, P. R. & Murshudov, G. N. How good are my data and what is the resolution? Acta Crystallogr. D. Biol. Crystallogr. 69, 1204–1214 (2013).
62. Long, F., Vagin, A. A., Young, P. & Murshudov, G. N. BALBES: a molecular-replacement pipeline. Acta Crystallogr. D. Biol. Crystallogr. 64, 125–132 (2008).
63. Li, Y. et al. Structure of natural killer cell receptor KLRG1 bound to E-cadherin reveals basis for MHC-independent missing self recognition. Immunity 31, 35–46 (2009).
64. McCoy, A. J. et al. Phaser crystallographic software. J. Appl. Crystallogr. 40, 658–674 (2007).
65. Kolenko, P. et al. Molecular architecture of mouse activating NKR-P1 receptors. J. Struct. Biol. 175, 434–441 (2011).
66. Vagin, A. & Teplyakov, A. Molecular replacement with MOLREP. Acta Crystallogr. D. Biol. Crystallogr. 66, 22–25 (2010).
67. Murshudov, G. N. et al. REFMAC5 for the refinement of macromolecular crystal structures. Acta Crystallogr. D. Biol. Crystallogr. 67, 355–367 (2011).
68. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. Acta Crystallogr. D. Biol. Crystallogr. 66, 486–501 (2010).
69. Rozboksz, D. et al. High-level expression of soluble form of mouse natural killer cell receptor NKR-PIC(B6) in Escherichia coli. Proteins, Exp. Purif. 77, 178–184 (2011).
70. Schuck, P. Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling. Biophys. J. 78, 1606–1619 (2000).
71. Brautigam, C. A. Calculations and publication-quality illustrations for analytical ultracentrifugation data. Meth. Enzymol. 562, 109–133 (2015).
72. Schuck, P. On the analysis of protein self-association by sedimentation velocity analytical ultracentrifugation. Anal. Biochem. 320, 104–124 (2003).
73. Scheuermann, T. H., Padrick, S. B., Gardner, K. H. & Brautigam, C. A. On the acquisition and analysis of microscale thermophoresis data. Anal. Biochem. 496, 79–93 (2016).
74. Petoukhov, M. V. et al. New developments in the program package for small-angle scattering data analysis. J. Appl. Crystallogr. 45, 342–350 (2012).
75. Konarev, P. V., Volkov, Y. V., Skolova, A. V., Koch, M. H. J. & Svergun, D. I. PRIMUS: a Windows PC-based system for small-angle scattering data analysis. J. Appl. Crystallogr. 36, 1277–1282 (2003).
76. Li, Z., Michael, I. P., Zhou, D., Nagy, A. & Rini, J. M. Simple pigment transposon-based mammalian cell expression system for inducible protein production. Proc. Natl. Acad. Sci. USA 110, 5004–5009 (2013).
77. Ovsey, M., Krizek, P., Borkovec, J., Svindrych, Z. & Hagen, G. M. ThunderSTORM: a comprehensive ImageJ plug-in for PALM and STORM data analysis and super-resolution imaging. Bioinformatics 30, 2389–2390 (2014).
78. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675 (2012).
79. Thompson, R. E., Larson, D. R. & Webb, W. W. Precise nanometer localization analysis for individual fluoroscent probes. Biophys. J. 82, 2775–2783 (2002).
80. Andronov, L., Orlov, I., Lutz, Y., Vonesch, J. L. & Klaholz, B. P. ClusterViSu, a method for clustering of protein complexes by Voronoi tessellation in super-resolution microscopy. Sci. Rep. 6, 24084 (2016).
81. Blaha, J., et al. Human NKR-P1/LLT1 SEC-SAXS raw data. Mendeley Data, V1, https://data.mendeley.com/datasets/268ww2m4j3/1 (2022).
82. Lovell, S. C. et al. Structure validation by Calpha geometry: phi, psi and Cbeta deviation. Proteins 50, 437–450 (2003).
83. Madeira, F. et al. Search and sequence analysis tools services from the EMBL-EBI in 2022. Nucleic Acids Res. 50, W276–W279 (2022).
84. Robert, X. & Gouet, P. Deciphering key features in protein structures with the new ENDascript server. Nucleic Acids Res. 42, W320–W324 (2014).

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**Author contributions**

J.B., O.S., B.K., S.P., E.P., and C.A. contributed to protein expression and purification; J.B. and Y.Z. performed the protein crystallization; Y.Z. and K.H. performed the X-ray diffraction measurements; J.B., T.S., J.S., T.K., J.Du., and J.Do. contributed to the data processing and model refinement; O.S. performed the SEC-SAXS data measurement; J.B. and T.S. performed the SAXS data analysis; O.V. performed the analytical ultracentrifugation measurements and analysis; J.B. and B.K. acquired and analyzed the dSTORM data; B.K., V.G., and D.C. performed the NK cytotoxicity assay; J.B., T.S., J.Do., and O.V. designed the experiments and wrote the manuscript with critical input from J.H.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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