



# Nanoscale restructuring of the immune synapse with an engager enhances NK cell function

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Engagers are antibody-based therapies which bind immune cell receptors and a target cell ligand. Next-gen engagers typically bind two activating receptors, but the effect of this on immune synapse formation and signaling is unknown. Here, we coligated activating receptors CD16a and NKG2D on natural killer (NK) cells with a CD33-binding anti-acute myeloid leukemia (AML) engager. Superresolution microscopy revealed that coligating CD16a and NKG2D with a single molecule triggered their nanoscale coclustering. This enhanced phosphorylation of CD3 $\zeta$ , ZAP70, and SLP-76 which augmented secretion of IFN- $\gamma$  and TNF- $\alpha$ , by NK cells from healthy donors and AML patients. Thus, in addition to connecting immune cells to target cells, the clinical promise of engagers results from their ability to manipulate the nanoscale architecture of the immune synapse.

natural killer cells | immune synapse | superresolution microscopy | receptor clustering | NKG2D

Natural killer (NK) cells are vital for our defense against cancer and many infectious diseases (1), and have roles in autoimmune diseases, reproductive complications, transplantation, and malaria (2, 3). As well as killing diseased cells, they also regulate other immune cells via direct contact and cytokine secretion (4, 5). Thus, understanding NK cell activation has the potential to aid the development of new medicines in a wide range of diseases. The capacity of NK cells to target and destroy cancer cells without prior sensitization makes NK cell-based immune therapies particularly promising in treating hematological malignancies such as acute myeloid leukemia (AML) and various solid tumors such as lung, ovarian, and pancreatic cancer (6, 7).

NK cell responses are governed by a balance of signals from a variety of germ-line encoded activating and inhibitory surface receptors (8). Different activating receptors trigger distinct intracellular signaling pathways, and elicit specific effector functions, with synergistic effects arising when multiple receptors are coactivated (9–11). CD16a (Fc $\gamma$ RIIIA) is one of the primary activating receptors on mature NK cells, specifically binding the Fc portion of antibodies to elicit antibody-dependent cellular cytotoxicity (ADCC) of opsonized target cells (12, 13). CD16a associates with the immunoreceptor tyrosine-based activation motif (ITAM)-bearing signaling protein CD3 zeta (CD3 $\zeta$ ). Upon CD16a engagement, kinases are recruited and CD3 $\zeta$  is phosphorylated.

NKG2D is another major activating receptor on NK cells, and some T cells, which triggers NK cell responses against target cells exhibiting stress-induced ligands on their cell membrane, including many cancer cells (14, 15). Upon ligation, the NKG2D signaling transducer DAP10 recruits phosphoinositide 3-kinase (PI3K) and growth factor receptor-bound protein 2 (Grb2). While CD16a can activate NK cells when triggered alone (16), NKG2D seems to require some level of coactivation to elicit effector functions (16, 17).

Superresolution microscopy has revealed a nanoscale rearrangement of NK cell receptors at the cell surface upon cellular activation (18–20). An emerging idea is that the activation and inhibition of immune cells is influenced by the nanoscale proximities of receptor clusters. For instance, we have previously found that upon ligation with ULBP2, surface nanoclusters of NKG2D coalesce with clusters of IL-15 receptor, leading to synergistic signaling between these two receptors (18).

Engagers come in various forms, as bi- or trispecific antibodies, sometimes called BiKEs, TriKEs, TriNKETs, or ANKETs (21, 22). NK cell engagers are particularly attractive because they exhibit lower toxicity compared to T cell engagers, which are often associated with cytokine release syndrome and neurotoxicity (23). For example, an antibody targeting NKG2D and SLAM family member 7 (SLAMF7) exhibited therapeutic efficacy in pre-clinical models of multiple myeloma (24) and an NK cell engager targeting Nkp46 in combination with CD16a and CD123 on AML cells has also proved to be effective (23).

## Significance

Immune engagers are an important cancer immunotherapy which work to target immune cells to cancer cells. However, how these engagers influence the immune synapse at the nanoscale is unknown. Here, we show that coengaging natural killer cell receptors CD16a and NKG2D with a single molecule restructures the immune synapse to enhance activation signaling. This work highlights the importance of nanoscale receptor organization in immune function and provides insight into how engager therapies work.

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Importantly, the physical and biological consequences of concurrent engagement of multiple activating receptors within a single engager are relatively unexplored. To address this, we examined CC-96191, an NK cell engager that simultaneously targets CD16a and NKG2D on NK cells and CD33 on AML cells (25). CD33 is present on blasts and leukemic stem cells in 85 to 90% of AML patients (26, 27). We compared the engager to variants with non-functional silent arms: Fc-silent, NKG2D-silent, their combination ((NKG2D+Fc)-silent), and CD33-silent, as well as bivalent antibodies targeting NKG2D and CD33 separately. We report that this engager causes a nanoscale reorganization of the immune synapse, not seen with the combination of NKG2D- and CD16a-silent variants, which ligate the same receptors but with two separate molecules. This leads to enhanced synaptic signaling and functional outcomes. These effects were seen in NK cells from healthy donors as well as AML patients. Broadly, this study establishes that engagers can amplify immune activation signals through nanoscale coclustering of multiple activating receptors.

## Results

### Engager Boosts NK Cell Activation Against CD33+ Target Cells.

To determine whether the simultaneous activation of CD16a and NKG2D by a single molecule enhances NK cell function, we set out to compare the fully functional engager, CC-96191, with a variety of modified forms of the same molecule. Specifically, we utilized a Fc-mutant variant (Fc-silent), engineered to lack CD16a binding, a variant which lacked the ability to ligate NKG2D (NKG2D-silent) and a third variant which could not ligate CD33 (CD33-silent). Prior research using surface plasmon resonance (SPR) has validated that the engager CC-96191 binds to CD33, NKG2D, and CD16a (25). Throughout the current study, we also used a combination of the Fc-silent and NKG2D-silent variants to ligate the exact same activating receptors as the fully functional engager, but via two separate molecules (Fig. 1*A*). In this way, we could ascertain the consequences of ligating two distinct receptors with one molecule. In addition, we compared activation with an anti-NKG2D antibody (clone A49), and an anti-CD33 antibody (clone I07). This comparison would determine whether bivalent binding itself enhances receptor clustering and stronger downstream signaling.

We initially tested for NK cell activation by evaluating the expression of surface CD69, a well-established marker of immune cell responses (28). Primary NK cells and EOL-1 cells were cocultured for 4 h in the presence of the different antibodies, engagers, and variants. The engager induced the highest level of CD69 expression (Fig. 1*B*). In contrast, coculturing cells with a combination of the Fc-silent and NKG2D-silent variants of the engager, or with anti-NKG2D or anti-CD33 antibodies, did not significantly increase CD69 expression (Fig. 1*B*).

One outcome of NK cell activation is the release of cytokines, including IFN- $\gamma$  and TNF- $\alpha$  (29). The engager induced the highest proportion of NK cells to produce IFN- $\gamma$  and TNF- $\alpha$  when cocultured with EOL-1 for 4 h (Fig. 1 *C* and *D* and *SI Appendix, Fig. S1 A and B*). Additionally, assessing IFN- $\gamma$  and TNF- $\alpha$  secretion by ELISA after a 16 h coculture with EOL-1 revealed a 4-fold and 3.5-fold increase, respectively (Fig. 1 *E* and *F*). Cytokine secretion was not enhanced when cells were incubated with a combination of the Fc-silent and NKG2D-silent variants of the engager, or with anti-NKG2D or anti-CD33 antibodies (Fig. 1 *E* and *F*). Even with an increased concentration of the combination of the Fc-silent and NKG2D-silent variants, there was no discernible boost in cytokine secretion (*SI Appendix, Fig. S1 C*). In contrast, cytokine secretion was high in the presence of the engager

at all tested concentrations. Moreover, pairing NKG2D-silent with an anti-NKG2D antibody, or CD33-silent with anti-CD33 failed to elicit the comparable enhancement in cytokine secretion observed with the engager (*SI Appendix, Fig. S1 D*).

Thus, using the engager CC-96191 to simultaneously trigger two activating receptors, activates NK cells, and induces cytokine secretion, better than using a combination of molecules which engage the same receptors.

A key outcome of synapse formation is degranulation, which leads to secretion of cytotoxic proteins. In the presence of the engager and CD33<sup>+</sup> target cells, more NK cells degranulated, evidenced by surface expression of CD107a ( $26 \pm 7\%$ ), compared to  $13 \pm 6\%$  when engaging the same two receptors but with separate molecules (Fig. 1*G* and *SI Appendix, Fig. S2 A*). Activation with other variants was slight; incubation with NKG2D-silent, Fc-silent, or CD33-silent variants, resulted in  $12 \pm 5\%$ ,  $11 \pm 8\%$ , or  $8 \pm 4\%$  of cells degranulating, respectively (Fig. 1*G*).

To determine the cytotoxicity induced by the engager, we compared the killing of the tumor cell line EOL-1 by NK cells in the presence of the engager or a combination of NKG2D-silent and Fc-silent versions of the engager. The engager enhanced the killing of EOL-1 ( $49.6 \pm 9\%$ ) more than using the combination of the two NKG2D-silent and Fc-silent versions ( $39 \pm 8\%$ ) (Fig. 1*H*). Next, we transduced GFP-CD33 into the Raji cell line, which otherwise lacks expression of CD33 (*SI Appendix, Fig. S2 B*). The engager induced more killing ( $48 \pm 8\%$ ) of CD33-expressing Raji cells compared to the combination of NKG2D-silent and Fc-silent versions ( $36 \pm 12\%$ ) (Fig. 1*I*). Consistent with these results, the engager has previously been shown to kill a range of human AML cell lines in a CD33-dependent manner while sparing normal monocytes (25).

Interestingly, IFN- $\gamma$  secretion was also significantly enhanced when the cells were activated via the engager compared to its variants at the 4-h time point, which was the same time point used to assess cytotoxicity (*SI Appendix, Fig. S2 C*). Thus, the engager CC-96191 causes a potent activation of NK cells. This happens to a greater extent than when ligating the same activating receptors with separate molecules.

**Superresolution Microscopy Reveals Synaptic Nanoclusters of CD16a and NKG2D.** Having established the superior functional potency of the engager in activating NK cells, we next aimed to explore the underlying mechanism of action at the immunological synapse. To do so, we used a glass-supported planar lipid bilayer (PLB) system presenting mobile CD33 ligands to mimic the dynamic surface of target cells (Fig. 2*A* and *SI Appendix, Fig. S3 A*). This allowed us to visualize receptor organization and synaptic structures in a controlled setting and to determine whether coengagement by the engager facilitates nanoscale reorganization of CD16a and NKG2D.

NK cells were plated on PLBs containing ICAM-1-His and CD33-His ligands, in the presence of the engager, or Fc- or NKG2D-silent variants, or their combination. To assess the functional ability of this bilayer system, IFN- $\gamma$  secretion was measured following an overnight incubation with NK cells. The engager induced a minimum of 4.5-fold increase of IFN- $\gamma$  secretion compared to its variants or their combination (Fig. 2*B*). Thus, CD33 on a PLB can efficiently activate NK cells in the presence of the engager, similarly to CD33 expressing target cells (Fig. 1*E*).

To mechanistically understand this, human primary NK cells were plated onto PLBs containing ICAM-1 and CD33, in the presence of the engager or its variants, for 20 min, before being fixed, stained for NKG2D and CD16a, and imaged initially by TIRF microscopy, followed by direct stochastic optical reconstruction

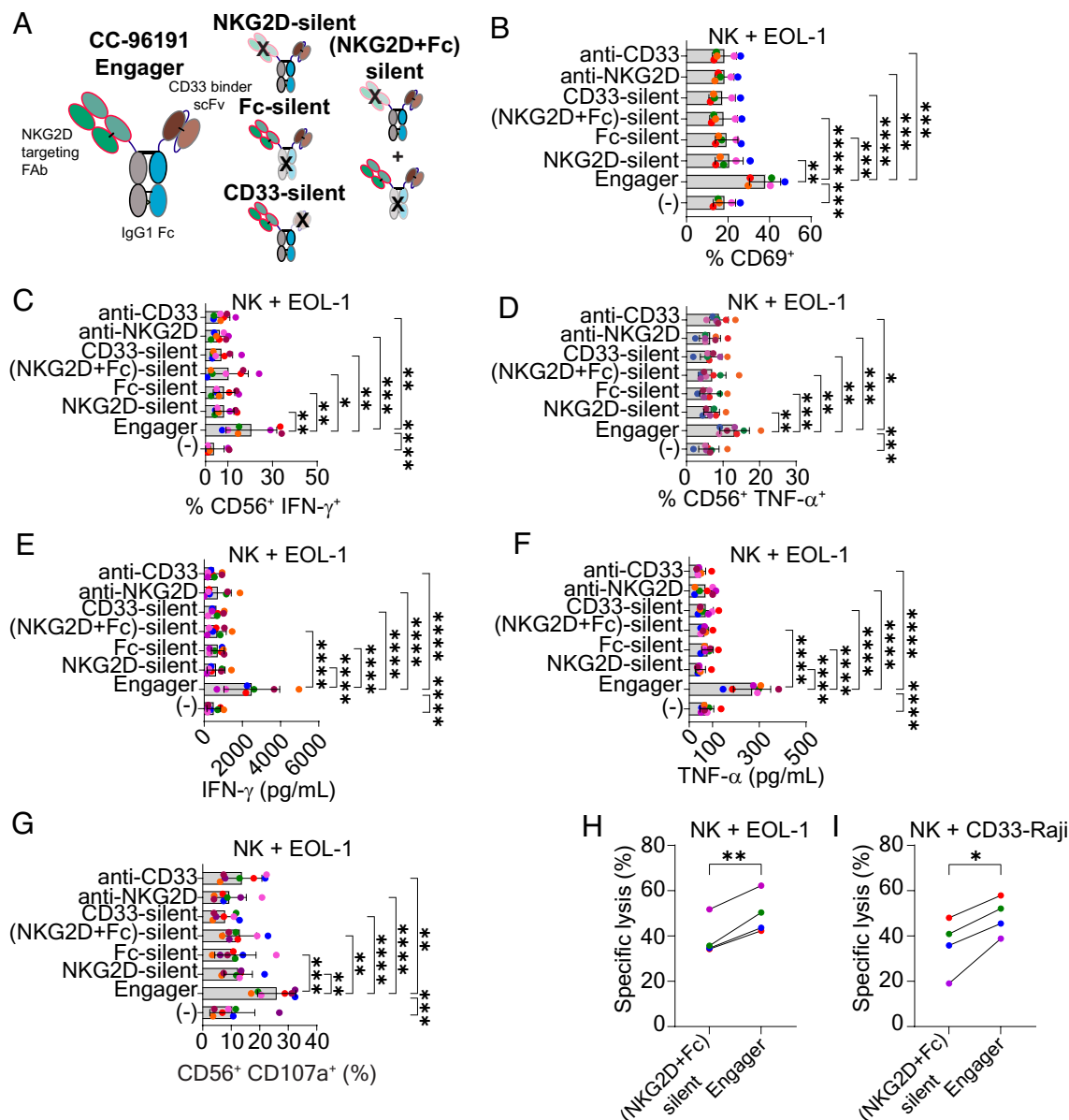


**Fig. 1.** Coligation of CD16a and NKG2D by an engager enhances NK cell activation more than ligating the same activating receptors with separate molecules. (A) Schematic illustrating the fully functional engager, CC-96191, and its variants: CD33-silent (does not bind CD33), Fc-silent (does not bind CD16a), NKG2D-silent (does not bind NKG2D), and the combination of NKG2D-silent and Fc-silent, denoted as “(NKG2D + Fc) silent.” (B) Percentage of CD69<sup>+</sup> activated NK cells after 4 h coculture with EOL-1 (n = 5). (C and D) Percentage of CD56<sup>+</sup> NK cells producing IFN-γ (C) and TNF-α (D) after coculture with EOL-1 (n = 7). (E and F) IFN-γ (E) and TNF-α (F) secretion from NK cells after 16 h coculture, measured by ELISA (n = 7). (G) Percentage of CD56<sup>+</sup> NK cells with surface expression of CD107a, marking degranulation, after 4 h coculture with EOL-1 cells and the engager or its variants (n = 7). (H and I) Specific lysis of EOL-1 (H) and CD33-transduced Raji (I) cells by NK cells treated with the engager or (NKG2D + Fc)-silent versions of the engager (n = 4 donors). Data shown as mean ± SD. Statistical analysis for panels B–G was performed using ANOVA followed by Dunnett’s test, for panels H and I paired t test was performed: \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001, ns: not significant.

microscopy (dSTORM) (30). TIRF microscopy revealed that efficient colocalization of NKG2D and CD16a at the immune synapse required the presence of CD33 on the PLB, and that copresentation of ICAM-1 with CD33 did not significantly enhance receptor clustering or synapse formation (SI Appendix, Fig. S3B). To assess receptor organization at the nanoscale, we next employed dSTORM imaging of CD16a and NKG2D at the immune synapse. To ensure accurate and high-quality localization, we applied fluorophore-specific thresholds based on photon count, sigma, and localization uncertainty distributions (SI Appendix, Fig. S3C). We then performed Ripley’s-based clustering analysis (31) on 3 × 3 μm regions to assess the nanoclustering of these receptors. This procedure assesses clustering by examining the number of localizations within increasing concentric radii (32). CD16a and NKG2D

localizations were clustered in all conditions to a different extent (SI Appendix, Fig. S3D and E). To quantitatively compare clustering between conditions, Ripley’s H-functions were computed for individual cells across all groups. Peak clustering radii were extracted from each cell’s H(r) curve and statistically compared, revealing that the NK engager condition consistently displayed significantly greater clustering than other conditions (SI Appendix, Fig. S3F and G).

Getis and Franklin’s local point pattern analysis was then used to quantitatively define nanoclusters (density and binary maps; Fig. 2C and D). In the presence of the engager, the density for CD16a at the synapse, corresponded to 750 ± 287 localizations/μm<sup>2</sup>, significantly higher than any other condition. In detail, cells activated with Fc-silent activation showed a density of 200 ± 145

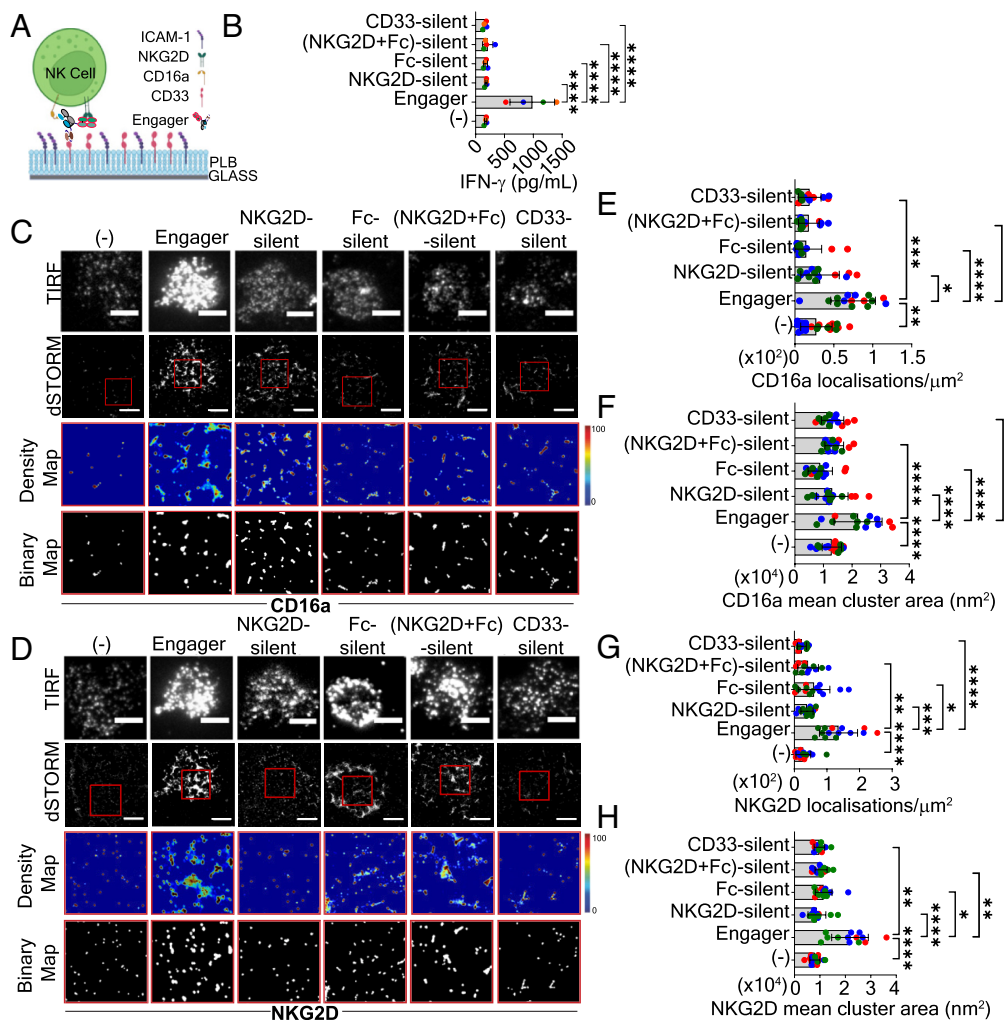


**Fig. 2.** Superresolution microscopy reveals that the engager induces larger nanoclusters of CD16a and NKG2D. (**A**) Schematic illustration of primary human NK cells interacting with a glass-supported lipid bilayer (PLB) functionalized with ICAM-1-His (200 molecules/ $\mu\text{m}^2$ ) and CD33-His (400 molecules/ $\mu\text{m}^2$ ), in the presence of the engager. (**B**) Secretion of IFN- $\gamma$  when human primary NK cells were incubated for 16 h at 37 °C on a CD33-enriched PLB, measured by ELISA, in the presence of engagers and variants ( $n = 4$ ). (**C** and **D**) Representative dSTORM images of CD16a (**C**) and NKG2D (**D**) on NK cells after 20 min interaction with PLBs containing ICAM-1-His (200 molecules/ $\mu\text{m}^2$ ) and CD33-His (400 molecules/ $\mu\text{m}^2$ ). Top row: TIRF images (Scale bar, 5  $\mu\text{m}$ ); second row: dSTORM images (Scale bar, 2  $\mu\text{m}$ ); third row: Getis and Franklin density maps of regions highlighted by red boxes (3  $\mu\text{m} \times 3 \mu\text{m}$ ; Scale bar, 0.5  $\mu\text{m}$ ); bottom row: binary maps of identified clusters. (**E** and **F**) Quantitative analysis from representative images shown in (**C**)  $n \geq 15$  from 3 donors. (**E**) Mean localization density of CD16a at the contact interface, in the presence of the engager or variants, as indicated. (**F**) Mean cluster area of CD16a ( $\text{nm}^2$ ). (**G** and **H**) Quantitative analysis of dSTORM as represented in (**D**),  $n \geq 14$  cells from 3 donors. (**G**) Mean localization density and (**H**) mean cluster area of NKG2D. Data are mean  $\pm$  SD. Statistical significance was determined using one-way ANOVA with Dunnett's multiple comparisons: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , ns: not significant.

localizations/ $\mu\text{m}^2$ , those activated with NKG2D-silent had a density of  $323 \pm 247$  localizations/ $\mu\text{m}^2$ , and those activated with the combination exhibited a density of  $180 \pm 145$  localizations/ $\mu\text{m}^2$  (Fig. 2E). The localization density for NKG2D was  $1,350 \pm 580$  localizations/ $\mu\text{m}^2$  in cells activated with the engager. In contrast, cells activated with Fc-silent showed a density of  $600 \pm 480$  localizations/ $\mu\text{m}^2$ , those activated with NKG2D-silent had a density of  $388 \pm 188$  localizations/ $\mu\text{m}^2$ , and those activated with the combination exhibited a density of  $421 \pm 316$  localizations/ $\mu\text{m}^2$  (Fig. 2G). Thus, dSTORM reveals a far higher density of CD16a and NKG2D recruited to the synapse by the engager.

Quantification of binary nanocluster maps (Fig. 2C and D, bottom rows) showed that the engager caused CD16a and NKG2D to assemble into nanoclusters with a mean area of  $22,100 \pm 8,400 \text{ nm}^2$  and  $21,800 \pm 7,100 \text{ nm}^2$ , respectively (Fig. 2F and H). Stimulation with Fc- or NKG2D-silent variants resulted in significantly smaller nanoclusters, with a mean area of  $8,900 \pm 4,200 \text{ nm}^2$  or  $13,100 \pm 5,500 \text{ nm}^2$ , respectively for CD16a, and  $11,500 \pm 3,300 \text{ nm}^2$  or  $8,800 \pm 3,500 \text{ nm}^2$ , respectively for NKG2D. The combination of Fc- and NKG2D-silent variants resulted in nanocluster sizes of  $13,800 \pm 3,300 \text{ nm}^2$  for CD16a and  $10,450 \pm 2,370 \text{ nm}^2$  for NKG2D (Fig. 2F and H). When analyzed using median nanocluster sizes, the engager-induced clustering remained statistically significant across donors (SI Appendix, Fig. S3H). This is consistent with Ripley's analysis showing that while CD16a and NKG2D are preclustered at the immune synapse across all conditions, engagement with the

engager CC-96191 specifically promotes the growth and enrichment of these nanoclusters. Thus, the engager impacts the nanoscale organization of CD16a and NKG2D by increasing their clustering density and radius.

**Engager Promotes the Coalescence of CD16a and NKG2D Nanoclusters.** The clustered organization of CD16a and NKG2D raises the question of whether these receptors are colocalized or arranged in distinct membrane domains. To test this, primary human NK cells were again plated onto PLBs containing ICAM-1 and CD33 with the engager or its variants, for 20 min, then fixed, stained with anti-NKG2D-Alexa Fluor-647 and anti-CD16-Alexa Fluor-488, and imaged using two-color dSTORM (Fig. 3A).

To quantitatively assess the degree of colocalization between CD16a and NKG2D in dSTORM images, we employed coordinate-based colocalization (CBC) analysis. CBC assigns a correlation coefficient to each localization within a defined radial distance, ranging from  $-1$  (indicating perfect segregation) to  $+1$  (indicating perfect colocalization) (33, 34). As a positive control, CD16a was stained with a primary anti-CD16 mAb conjugated with AF488, which was followed by an isotype-specific secondary antibody conjugated with AF647. This resulted in  $35 \pm 18\%$  of localizations having correlation coefficients between 0.5 and 1. The negative control encompassed the same data (from the positive control), but with the XY coordinates of one channel being swapped and it had  $4 \pm 3\%$  of localizations that had correlation coefficients between 0.5 and 1 (SI Appendix, Fig. S4A–C). To confirm the

specificity of the clustering observed, we imaged NK cell receptors NKG2D and CD16a, which do not interact, across all experimental conditions. Neither receptor exhibited condition-dependent changes in nanoscale organization, with similar cluster areas observed throughout (SI Appendix, Fig. S4D). Additionally, CBC analysis of these receptors showed no change in pairwise distances or colocalization levels across conditions (SI Appendix, Fig. S4E), reinforcing that the nanoclustering effects are specific to the activating receptors NKG2D and CD16a. This underscores the specificity of nanoclustering driven by the NK engager. These serve as negative biological controls, supporting the selective clustering of CD16a and NKG2D.

In comparison, the engager resulted in a substantial colocalization between CD16a and NKG2D, threefold higher than any other condition. Specifically,  $28 \pm 13\%$  of localizations had correlation coefficients between 0.5 and 1 (Fig. 3B and C). In contrast, activation with CD33-silent ( $13 \pm 5\%$ ), Fc-silent ( $13 \pm 4\%$ ), NKG2D-silent ( $12 \pm 5\%$ ), or their combination ( $13 \pm 5\%$ ) showed significantly lower correlation, comparable to untreated cells ( $11 \pm 5\%$ ) (Fig. 3C).

As an alternative measure, the nearest neighbor distance (NND) between nanoclusters of CD16a and NKG2D was used. A positive control exhibited a NND of  $36 \pm 33$  nm, while a negative control showed a NND of  $257 \pm 117$  nm (SI Appendix, Fig. S4C). Activation via the engager resulted in an NND between nanoclusters of CD16a and NKG2D of  $38 \pm 15$  nm. In contrast, stimulation with NKG2D-silent ( $97 \pm 67$  nm), Fc-silent ( $115 \pm 30$  nm), or their combination ( $100 \pm 44$  nm) yielded larger NNDs (Fig. 3D), demonstrating significantly less colocalization.

Taken together, two-color dSTORM established that the engager CC-96191 promotes the coalescence of NKG2D and CD16a nanoclusters at the immune synapse.

**Coligation of CD16a and NKG2D Promotes the Recruitment and Coalescence of pCD3 $\zeta$  and pZAP70.** CD16a, which lacks an intrinsic signaling domain, associates with CD3 $\zeta$  to transduce signals. When CD16a is engaged, tyrosines in the ITAM motifs in the cytoplasmic portion of CD3 $\zeta$  are phosphorylated by Src family kinases, such as Lck and Fyn. This creates docking sites for SH2 domain-containing proteins, including ZAP-70.

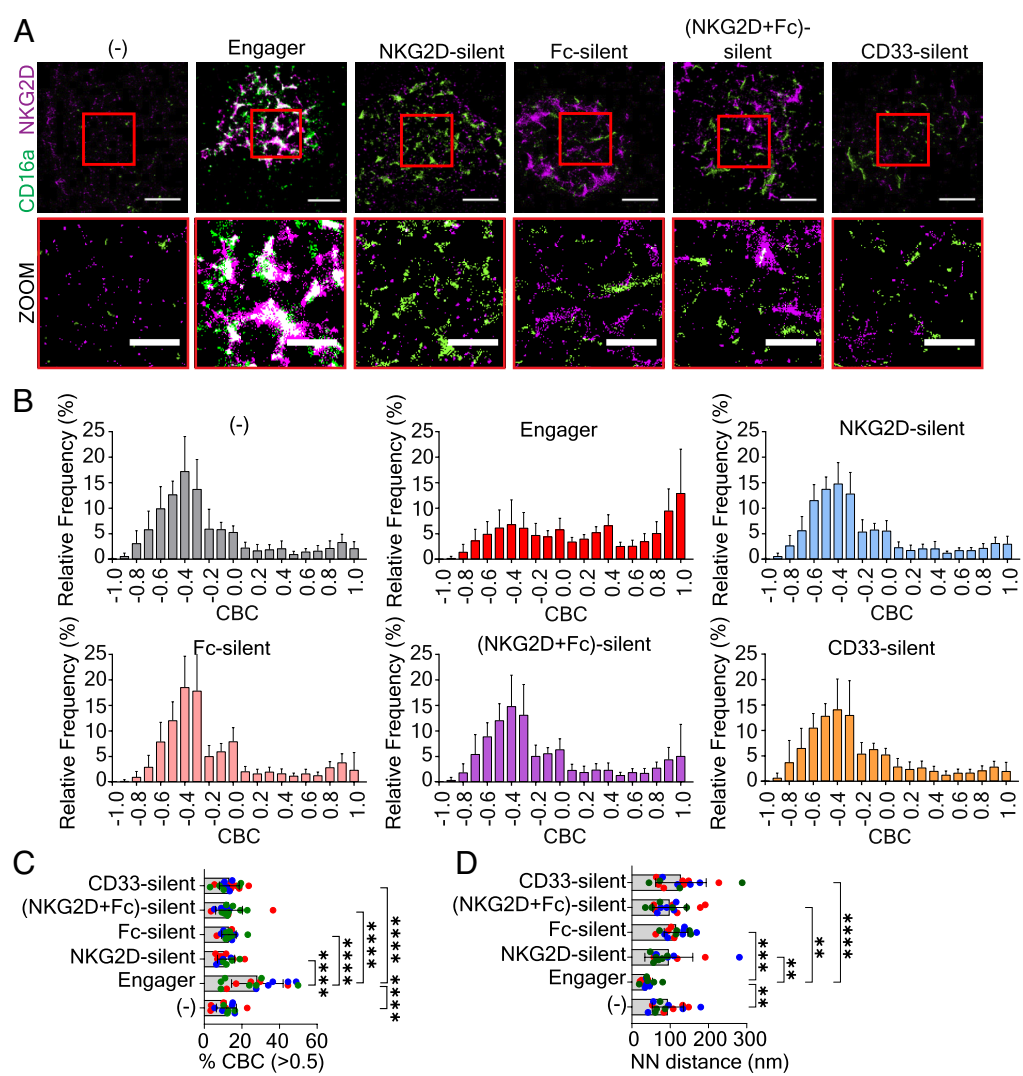


**Fig. 3.** Engager coalesces CD16a and NKG2D nanoclusters. (A) Representative two-color dSTORM images of CD16a (green) and NKG2D (magenta) on human primary NK cells interacting with PLB containing 200 molecules/ $\mu\text{m}^2$  ICAM-1 and 400 molecules/ $\mu\text{m}^2$  CD33-His. (Scale bar, 2  $\mu\text{m}$ .) The bottom row shows enlarged images of the red-boxed area. (Scale bar, 0.5  $\mu\text{m}$ .) (B) Coordinate-based colocalization (CBC) histograms of the single-molecule distributions of the colocalization of CD16a and NKG2D for cells, as represented in (A). Data are from  $n \geq 15$  cells from 3 donors. Bars represent means  $\pm$  SD. (C) Mean ( $\pm$ SD) fraction of localizations with a score  $>0.5$  from CBC analysis ( $n \geq 14$  cells from 3 donors). (D) Nearest neighbor distance (NND) analysis for clusters of NKG2D and CD16a, from imaging data represented in (A). Symbol represents the median NND of all paired single-molecule localizations from one cell. All data are presented as mean  $\pm$  SD. Statistical significance was determined using one-way ANOVA with Dunnett's multiple comparisons: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.000$ , ns: not significant.

Thus, to investigate the impact of coengaging CD16a and NKG2D with the engager on downstream signaling events, we used PLBs incorporating CD33 to stimulate NK cells and assessed CD3 $\zeta$  and ZAP70 phosphorylation. Incubation of primary NK cells with the engager resulted in CD3 $\zeta$  and ZAP70 phosphorylation in multiple dense nanoclusters, with the two signaling proteins being triggered in close proximity (Fig. 4A). The localization density for pCD3 $\zeta$  in cells activated with the engager was  $2,000 \pm 1,000$  localizations/ $\mu\text{m}^2$ . In contrast, cells activated with Fc- or NKG2D-silent, or the combination of both, showed densities of  $1,000 \pm 830$ ,  $750 \pm 600$ , and  $1,250 \pm 980$  localizations/ $\mu\text{m}^2$ , respectively (Fig. 4B, Left panel). Similarly, for pZAP70, cells activated with the engager had a higher localization density of  $1,100 \pm 900$  localizations/ $\mu\text{m}^2$ , compared to  $575 \pm 400$  for Fc-silent,  $900 \pm 700$  for NKG2D-silent, and  $600 \pm 350$  localizations/ $\mu\text{m}^2$  for the combination (Fig. 4B, Right panel). Ripley's-based clustering analysis revealed that pCD3 $\zeta$  and pZAP70 localizations were preclustered across all tested conditions, engager treatment led to increased cluster enrichment and growth (SI Appendix, Fig. S5 A–D), indicating enhanced nanoscale organization of signaling molecules upon synaptic engagement.

Quantification also revealed significant differences in the size of nanoclusters formed. The engager produced the largest nanoclusters, at  $19,300 \pm 6,300$  nm $^2$  for pCD3 $\zeta$ , and  $12,100 \pm 2,800$  nm $^2$  for pZAP70. The NKG2D-silent version formed large nanoclusters of pCD3 $\zeta$  with an area of  $14,900 \pm 9,300$  nm $^2$  while the Fc-silent version generated smaller nanoclusters ( $10,500 \pm 3,800$  nm $^2$ ). Coactivation with a combination of NKG2D- and Fc-silent variants formed nanoclusters of pCD3 $\zeta$ , at  $17,500 \pm 6,600$  nm $^2$  and pZAP70 of  $7,900 \pm 2,600$  nm $^2$  (Fig. 4C). Thus, the engager causes a significant increase in the density and nanocluster size of key signaling molecules at the immune synapse. This is consistent with Ripley's analysis showing cells engaged with the engager exhibited larger nanocluster radii compared to NKG2D-silent, Fc-silent, or their combination (SI Appendix, Fig. S5 A–D). CBC analysis revealed that the engager significantly promoted colocalization of these signaling nanoclusters (Fig. 4D). With the engager,  $27 \pm 9\%$  of localizations had correlation coefficients between 0.5 and 1, indicating strong colocalization (Fig. 4D and E). In contrast, activation with Fc-silent ( $8 \pm 4\%$ ), NKG2D-silent ( $18 \pm 9\%$ ), their combination ( $9 \pm 3\%$ , purple bars), or CD33-silent ( $12 \pm 5\%$ , orange bars) showed much lower colocalization (Fig. 4D and E). Engaging CD16a alone, with the

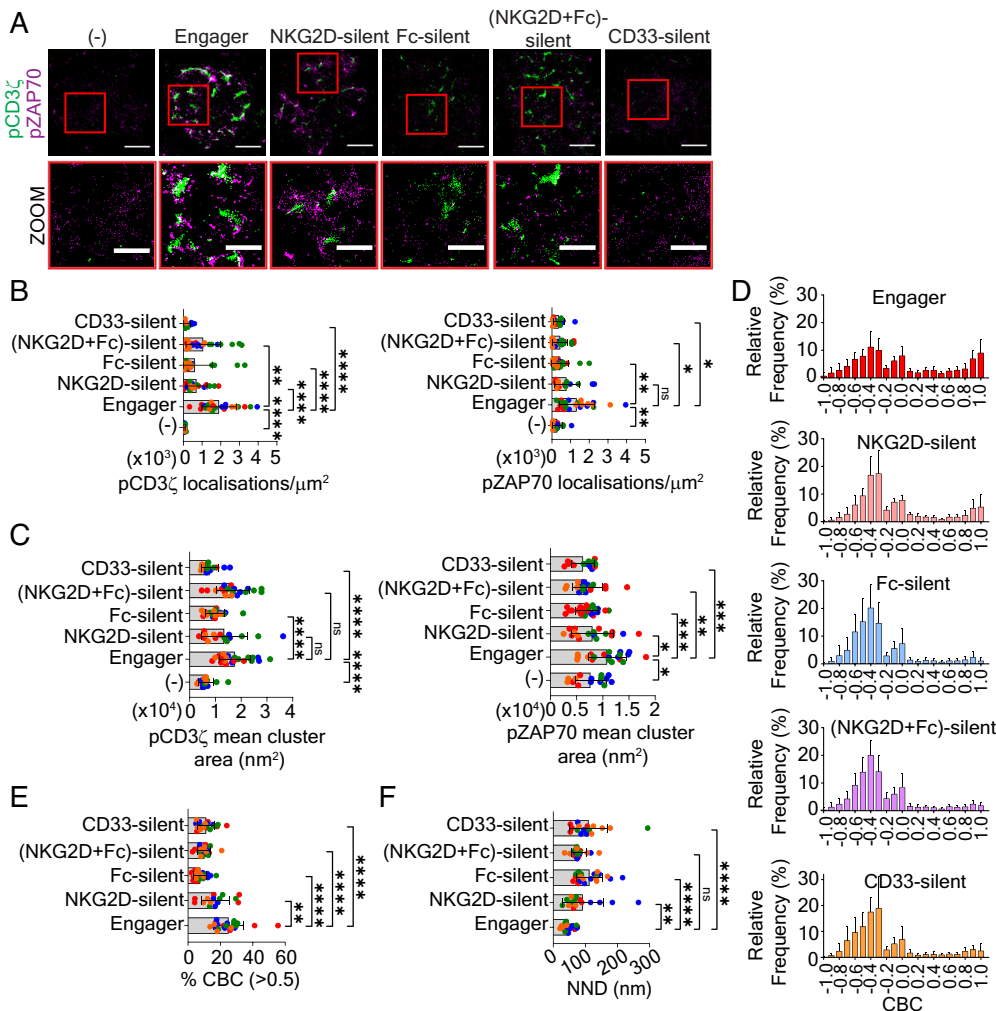


**Fig. 4.** CD16a and NKG2D coligation enhances nanoclustering of their associated membrane-proximal signaling molecules. (A) Representative two-color dSTORM images of pCD3 $\zeta$  and pZAP70 on NK cells after 20 min interaction with PLBs enriched with CD33 and ICAM-1, in the presence of the engager or its variants. (Scale bars, top row: 2  $\mu\text{m}$ , enlarged images: 0.5  $\mu\text{m}$ .) (B and C) Quantification of dSTORM data showing pCD3 $\zeta$  and pZAP70 localization density (B) and cluster area (C) ( $n \geq 11$  cells, 3 donors). (D) CBC histograms for pCD3 $\zeta$  and pZAP70. (E) Fraction of localizations with CBC score  $>0.5$ . (F) NND analysis of pCD3 $\zeta$  and pZAP70 localizations. Data presented as mean  $\pm$  SD. Statistical significance was assessed using Kruskal-Wallis tests and one-way ANOVA with Dunn's multiple comparisons: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , ns = not significant.

NKG2D-silent variant, increased colocalization of pCD3 $\zeta$  and pZAP70 compared to other single engagements (Fig. 4 *D* and *E*). Measurements of the NND between pCD3 $\zeta$  and pZAP70, shows that the engager brought these two signaling molecules closest together, with an average distance of  $51 \pm 15$  nm. The combination of Fc-silent and NKG2D-silent versions had them at  $77 \pm 13$  nm apart, and individually, the Fc-silent or NKG2D-silent variants resulted in distances of over 100 nm (Fig. 4*F*). Altogether, these results demonstrate that, by ligating two activating receptors within one molecule, the engager enhances the recruitment, coalescence, and activation, of membrane-proximal signaling molecules CD3 $\zeta$  and ZAP70 within synaptic nanoclusters.

**CD16a and NKG2D Coligation Enhances Downstream Signaling.** SLP-76 is especially important for NK cell cytotoxicity mediated by CD16a and NKG2D (35). dSTORM imaging revealed that cells activated with the engager exhibited a 2-fold higher localization density ( $270 \pm 145$  localizations/ $\mu\text{m}^2$ ) for pSLP-76 compared to cells activated via the combination of NKG2D- and Fc-silent variants ( $73 \pm 48$  localizations/ $\mu\text{m}^2$  and  $35 \pm 28$  localizations/ $\mu\text{m}^2$ , respectively). The combination of NKG2D- and Fc-silent variants triggered a density of  $147 \pm 102$  localizations/ $\mu\text{m}^2$  (Fig. 5 *A* and *B*). Cells engaged with the engager showed the largest nanocluster radii (Fig. 5*C*).

pSLP-76 assembled into nanoclusters with a mean area of  $18,900 \pm 6,900$  nm $^2$  in the presence of the engager (Fig. 5*C*). Stimulation with the Fc-silent variant resulted in significantly smaller nanoclusters, with a mean area of  $7,700 \pm 3,400$  nm $^2$ . Stimulation with NKG2D-silent produced clusters with a mean area of  $11,700 \pm 5,800$  nm $^2$  similar to using the combination of NKG2D- and Fc-silent variants ( $12,100 \pm 7,300$  nm $^2$ ; Fig. 5*C*). Spatially, pSLP-76 nanoclusters colocalized with NKG2D nanoclusters upon stimulation with the engager (Fig. 5*D*). Specifically,  $24 \pm 10\%$  of localizations in the engager condition had correlation coefficients between 0.5 and +1, indicating strong colocalization (Fig. 5 *D* and *E*). In contrast, activation with Fc-silent ( $8 \pm 5\%$ ), NKG2D-silent ( $8 \pm 4\%$ ), their combination ( $12 \pm 9\%$ ), or CD33-silent ( $6 \pm 3\%$ ) showed much lower colocalization (Fig. 5 *D* and *E*). This finding was corroborated by measuring the NND between pSLP-76 and NKG2D, where the engager brought these molecules closest, with an average distance of  $50 \pm 40$  nm. In contrast, stimulation via a combination of Fc- and NKG2D-silent variants brought them to  $90 \pm 50$  nm, and separate engagement with Fc-silent or NKG2D-silent resulted in distances of over 100 nm [ $113 \pm 66$  nm for NKG2D-silent and  $130 \pm 48$  nm for Fc-silent; (Fig. 5*F*)]. Thus, the engager significantly increases the density and size of pSLP-76 nanoclusters, compared to separate engagement of CD16a and NKG2D.

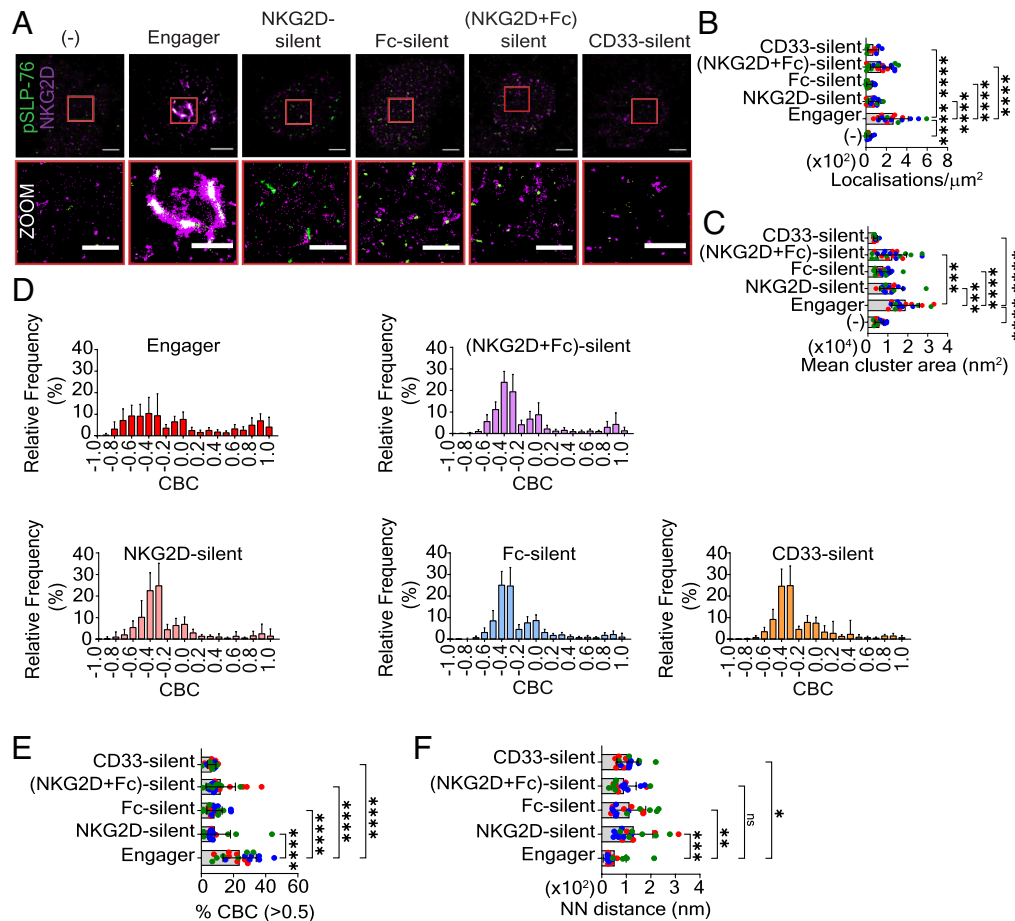


**Fig. 5.** Engager enhances downstream signaling. (A) Primary human NK cells interacted with PLBs loaded with ICAM-1-His (200 molecules/ $\mu\text{m}^2$ ), and CD33-His (400 molecules/ $\mu\text{m}^2$ ) for 20 min. Representative 2-color dSTORM images of pSLP-76 and NKG2D are shown (Scale bar, 2  $\mu\text{m}$ ), with the red-boxed region enlarged (Scale bar, 0.5  $\mu\text{m}$ ). (B and C) Quantification of dSTORM data showing pSLP-76 localization density (B) and cluster area (C) ( $n \geq 10$  cells from 3 donors). (D) CBC histograms of pSLP-76 and NKG2D colocalization. (E) Fraction of localizations with CBC score  $>0.5$ . (F) NND analysis of pSLP-76 and NKG2D clusters. Symbol represents the median NND of all paired single-molecule localizations from one cell. Data presented as mean  $\pm$  SD. Statistical significance was assessed using one-way ANOVA with Dunnett's multiple comparisons: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , ns: not significant.

**Engager Augments NK Cell Activity Against AML blasts.** CD33 is expressed on blasts and leukemic stem cells in the vast majority of AML patients (26, 36). The engager CC-96191 has been shown to augment NK cell activation against AML cells evidenced by elevated expression of CD69 (25), when compared to treatment with Lintuzumab, a therapeutic anti-CD33 monoclonal antibody (37).

Here, we tested the potential of the engager CC-96191 to activate NK cells within a complex population of immune cells, such as all peripheral blood mononuclear cells (PBMCs). When the engager was added to PBMCs isolated from healthy donors, no cytokine secretion was observed (Fig. 6A), even though a small fraction of cells in healthy donors express CD33 (SI Appendix, Fig. S6 A and B). Coculturing PBMCs with the CD33-expressing AML cell line EOL-1, followed by treatment with the engager at various E:T ratios revealed that 5:1 ratio was optimal to induce IFN- $\gamma$  secretion (SI Appendix, Fig. S6C). The engager triggered higher cytokine secretion compared to incubation with the Fc-silent, NKG2D-silent, or a combination of both, variants (Fig. 6A). This effect was also observed when CD8<sup>+</sup> T cells were depleted prior to coculture; the remaining PBMCs still exhibited enhanced cytokine responses

across different E:T ratios (SI Appendix, Fig. S6C), indicating that CD8<sup>+</sup> T cells were not responsible for this activity.

Thus, the engager CC-96191 is highly effective in complex populations of immune cells, more so than a combination of reagents ligating the same receptors in separate molecules.

Next, we tested PBMCs from AML patients, where CD33-positive cells, or primary blasts, ranged in frequency between 40 and 70% (SI Appendix, Fig. S6B). The engager significantly enhanced secretion of IFN- $\gamma$  and TNF- $\alpha$  compared to the NKG2D-silent, Fc-silent, or both variants combined (Fig. 6B). This suggests the engager CC-96191 has significant potential to boost the anti-AML NK cell response.

We next employed dSTORM to assess the synaptic organization of receptors. 78  $\pm$  6% NK cells from PBMCs of AML patients expressed NKG2D while 42  $\pm$  18% of cells displayed CD16a at the cell surface (SI Appendix, Fig. S6 D and E), a reduction consistent with previous studies (38, 39). dSTORM revealed a 3-fold increase in the localization density for CD16a and NKG2D in NK cells activated with the engager compared to stimulation with Fc- or NKG2D-variants, or their combination (Fig. 6D). Specifically, the localization density for CD16a was 1,480  $\pm$  1,000

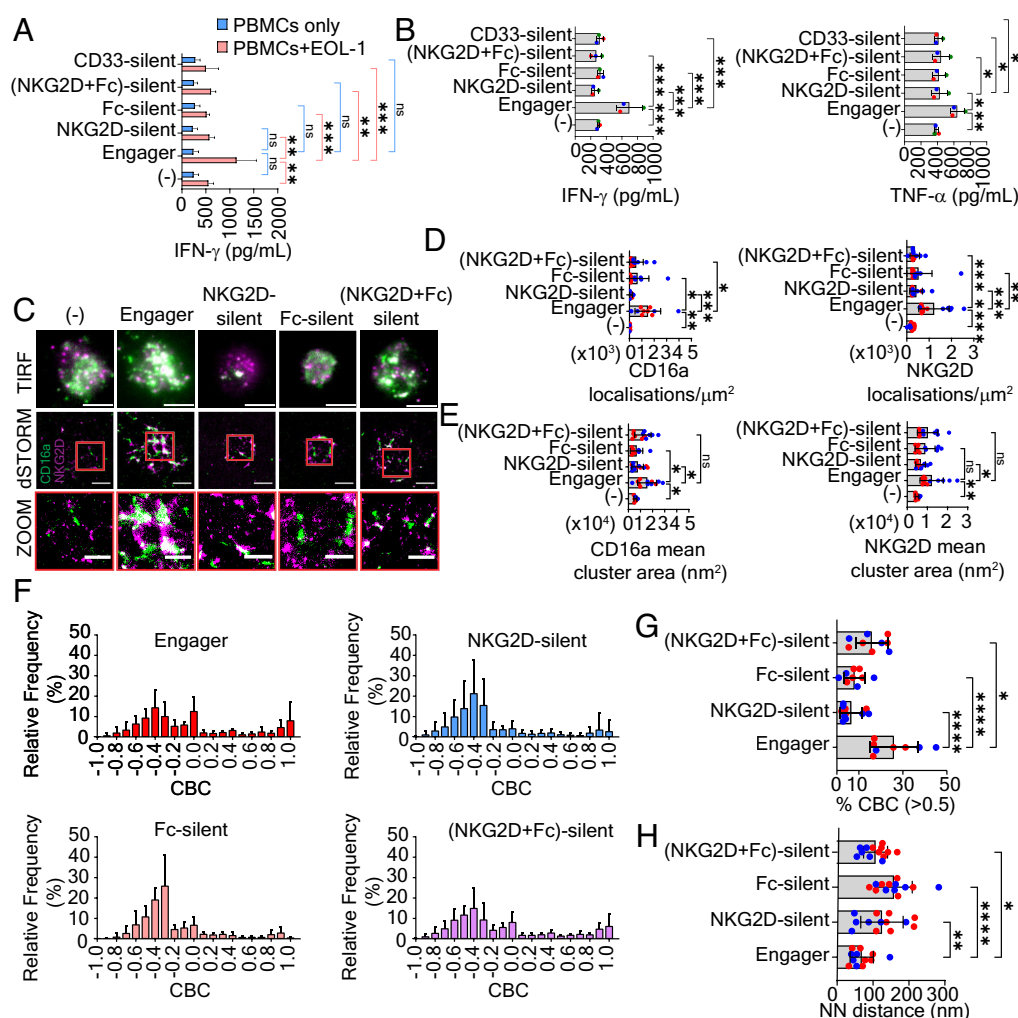


**Fig. 6.** Engager enhances NK cell activation and CD16a-NKG2D colocalization in AML-derived NK cells. (A) Secretion of IFN- $\gamma$  by PBMCs from healthy donors cultured alone or with EOL-1 cells (E:T ratio 5:1) for 16 h in the presence of the engager or its variants (n = 3). (B) IFN- $\gamma$  (Left) and TNF- $\alpha$  (Right) secretion from AML patient PBMCs after 16 h coculture with the engager or its variants (n = 3), measured by ELISA. (C) Representative TIRF images of AML patient NK cells stained for CD16a (green) and NKG2D (magenta), with corresponding dSTORM images. [Scale bar, 5  $\mu$ m (enlarged 3  $\mu$ m  $\times$  3  $\mu$ m)]. (D and E) dSTORM quantification of localization density (D) and cluster area (E) for CD16a and NKG2D in NK cells exposed to the engager or variants (n  $\geq$  9 cells, 2 AML patients). (F) CBC histograms for CD16a-NKG2D colocalization. (G) Fraction of localizations with CBC scores >0.5. (H) NND analysis of CD16a-NKG2D localizations. Statistical significance was determined using one-way ANOVA with Dunnett's multiple comparisons. In panels A, two-way ANOVA with multiple comparisons was performed: \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001, ns: not significant.

localizations/ $\mu\text{m}^2$  with the engager, compared to  $640 \pm 428$ ,  $130 \pm 110$ , and  $530 \pm 480$  localizations/ $\mu\text{m}^2$  for Fc-silent, NKG2D-silent, and the combination, respectively (Fig. 6D). For NKG2D, the engager induced a localization density of  $1,200 \pm 680$  localizations/ $\mu\text{m}^2$ , significantly higher than  $620 \pm 500$  for Fc-silent,  $420 \pm 280$  for NKG2D-silent, and  $350 \pm 200$  localizations/ $\mu\text{m}^2$  for the combination (Fig. 6D).

Quantification of binary nanocluster maps indicated significant differences in nanocluster sizes for CD16 and NKG2D in AML-derived NK cells activated with the engager versus other conditions. For CD16a, the engager induced the largest nanoclusters with an area of  $15,400 \pm 7,700 \text{ nm}^2$ . This was larger than nanoclusters formed by the Fc-silent ( $6,700 \pm 5,100 \text{ nm}^2$ ), NKG2D-silent ( $7,600 \pm 4,700 \text{ nm}^2$ ), and their combination ( $12,000 \pm 6,700 \text{ nm}^2$ ; Fig. 6E). Similarly, the engager induced the largest nanoclusters of NKG2D ( $12,100 \pm 5,800 \text{ nm}^2$ ), compared to those formed by NKG2D-silent ( $6,600 \pm 2,500 \text{ nm}^2$ ), Fc-silent ( $8,800 \pm 5,000 \text{ nm}^2$ ), and the combination ( $10,100 \pm 5,000 \text{ nm}^2$ ; Fig. 6E).

The engager enhanced the colocalization of CD16a and NKG2D, evidenced by CBC analysis (Fig. 6F). Specifically,  $26 \pm 11\%$  of localizations had correlation coefficients between 0.5 and 1 with the engager, indicating strong colocalization (Fig. 6F and G). In contrast, the NKG2D-silent ( $6 \pm 5\%$ ), or the Fc-silent variant ( $8 \pm 5\%$ ), or their combination ( $16 \pm 7\%$ ), showed lower colocalization (Fig. 6F and G). Engagement of CD16a and NKG2D via two separate molecules did increase the colocalization of their target receptors, but not to the extent of the engager (Fig. 6F and G). In the presence of the engager, the NND between NKG2D and CD16a nanoclusters was  $70 \pm 37 \text{ nm}$ , compared to  $100 \pm 40 \text{ nm}$  with the Fc-silent and NKG2D-silent combination, and  $124 \pm 59$  and  $158 \pm 51 \text{ nm}$  with NKG2D-silent and Fc-silent, respectively (Fig. 6H).

These findings underscore that coligation of CD16 and NKG2D by the engager CC-96191 enhance activation in NK cells derived from AML patients, driving increased receptor colocalization and synaptic restructuring as observed in healthy donors.

### Fresh NK Cells Show Poor Cytokine and Killing Responses.

While NK cells from AML patients showed enhanced cytokine production and synaptic coclustering of NKG2D and CD16a in the presence of the engager, the overall response remained modest. This could be due to insufficient cytokine priming, such as the absence of IL-2. To test this, we examined cytokine production, target cell killing, and receptor reorganization at the immune synapse in freshly isolated NK cells from healthy donors. Despite clear engager-induced coclustering of CD16a and NKG2D (SI Appendix, Fig. S7 A and B), these freshly isolated NK cells produced only low levels of IFN- $\gamma$  and displayed minimal cytotoxicity (SI Appendix, Fig. S7 C and D). This contrasted with the robust functional responses observed in IL-2-cultured NK cells (SI Appendix, Fig. S7 E and F), consistent with the well-established importance of cytokine-mediated priming in boosting the outcomes of cell–cell interactions.

## Discussion

Antibody-based therapies typically target individual receptors (40, 41) or a combination of receptors (42). NK cell engagers concurrently bind multiple proteins to link tumor cells and NK cells (43, 44). Importantly, however, the impact of ligating two activating receptors on NK cells with a single molecule on NK cell immune synapse assembly, nanocluster organization, and downstream signaling integration remains poorly understood.

NK cell receptors, including both activating and inhibitory receptors are expressed at the cell surface within nanoscale clusters (18–20). Receptor nanocluster size and composition affect immune cell activation and inhibition (19, 20, 45). The dynamic nature of receptor nanoclusters can influence signaling by segregating inhibitory receptors away from activating ones (46). Conversely, synergistic signaling can be triggered when distinct receptor nanoclusters coalesce (18). Here, we use superresolution microscopy (30), to determine the mechanism underpinning enhanced NK cell activation upon coligation of CD16a and NKG2D by the engager CC-96191, and thus whether engaging two activating receptors with one molecule would consequentially impact their spatial proximity. In detail, CD16a and NKG2D were spaced  $38 \pm 15 \text{ nm}$  apart when ligated by the engager CC-96191, in comparison to over  $100 \text{ nm}$  apart when the same receptors were engaged but with a combination of engager variants, in which one arm was disabled. This receptor clustering led to an enrichment of phosphorylated CD3 $\zeta$ , ZAP70 and SLP-76 within receptor nanoclusters. Thus, the engager triggers a specific nanoscale restructuring of the immune synapse, boosting membrane-proximal and downstream phosphorylation events.

The engager led to increased killing of CD33-expressing cells, as evidenced by Raji cells, which are deficient in CD33 but, when transduced with CD33, were killed more effectively by the engager than by the combination of the NKG2D-silent and Fc-silent versions of the antibody. Functionally, the coengagement of NKG2D and CD16a by the engager CC-96191 amplified NK cell activation in several ways. IFN- $\gamma$  and TNF- $\alpha$  secretion increased 4-fold and 3.5-fold, respectively, in primary NK cells in the presence of EOL-1 cells, a CD33-expressing cell line (Fig. 1). This increase in cytokine production occurred due to more NK cells being activated and greater cytokine production per cell, consistent with enhanced activating signals. In almost every experiment, the engager outperformed the combination treatment of Fc- and NKG2D-silent variants, which would ligate the same receptors but via two separate molecules. Importantly, the engager CC-96191 activated NK cells from both AML patients and healthy donors, even when part of a complex mixture of PBMCs. However, in freshly isolated, non-cytokine-primed NK cells, the engager-induced coclustering of CD16a and NKG2D alone was insufficient to trigger robust cytokine production or cytotoxicity, despite clear receptor reorganization at the immune synapse (SI Appendix, Fig. S7). This suggests that additional priming, such as via IL-2, may be required to fully unlock effector functions downstream of receptor coengagement, consistent with previous reports on priming NK cell responses with IL-2 (47, 48). Together, these data establish that the nanoscale proximity of activating receptors, triggered by ligating them within one molecule, enhances NK cell responses in a context-dependent manner, with optimal activation requiring both spatial coengagement and appropriate priming signals.

Our findings indicate that coligation of CD16a and NKG2D by the same engager molecule drives their spatial convergence at the synapse, which in turn concentrates downstream signaling components and promotes synergistic activation. While CD16a is a strong activator through its ITAM-associated Fc $\gamma$ R pathway, NKG2D provides complementary signaling via the DAP10-PI3K-LAT/SLP-76 axis, which is critical for amplifying and sustaining NK cell activation. Our data suggest that engaging both pathways simultaneously in close proximity helps more NK cells surpass the activation threshold required for cytotoxicity. This may be particularly important under conditions where ligand density or another determinant of synapse structure are otherwise suboptimal, or in microenvironments where immune cell activity is inhibited.

These data lead to an important mechanistic insight into how immune cell engagers can boost responses. Namely, that nanoscale colocalization of activating receptors boosts signaling. One feasible mechanism is that one receptor signal would bring the LAT/SLP-76 signalosome (49) into close proximity to the other activating receptor, and vice versa, establishing positive feedback. It seems likely that these findings would be generalizable to other combinations of activating receptors, but perhaps not all. It is already established that different NK cell receptors exhibit specific synergies with each other. For example, NKG2D synergizes with another activating receptor, 2B4 (16, 17). Additionally, receptors like NKP30, NKP44, and NKP46 play crucial roles in NK cell activation (50), yet their cooperative effects with other receptors can vary based on the cellular context and the specific ligands present on target cells (51). Therefore, an important next goal would be to establish which activating receptors would synergize with which other activating receptors, when triggered to colocalize via an engager.

In the engager CC-96191, the relative proximity of activating receptor binding sites is fixed by the backbone architecture of the antibody-based engager. This may not be optimal. A systematic investigation of whether the nanoscale separation of engager binding domains effects outcomes would be very interesting. Then, the structure of the engager could be deliberately altered to position binding domains at an optimal distance apart. Resolving these issues, which stem directly from the results presented here, would lead to improving the design of next-gen engagers.

## Methods

**Primary Cell Isolation.** Blood leukocyte cones were obtained from the National Blood Transfusion Service in Manchester and London, UK, following all appropriate ethical guidelines (Manchester License: 05/Q0401/108 and 22/SW/0076 for Imperial College London). Using a Ficoll gradient through density centrifugation, PBMCs from healthy adult donors were isolated from leukocyte cones. To obtain human primary NK cells, negative magnetic selection (Miltenyi Biotec) was used. Isolated NK cells were cultured at  $1 \times 10^6$  cells/ml in a clone medium: Dulbecco's Modified Eagle Medium [DMEM] medium, containing 30% F12 Ham, 10% human serum, 1% nonessential amino acids, 2 mM L-glutamine, 50 U/ml penicillin/streptomycin (all from Sigma-Aldrich), 50  $\mu$ M 2-mercaptoethanol, and 1 mM sodium pyruvate (both Gibco). Clone media was supplemented with 200 U/ml recombinant IL-2 (Gibco). NK cells were rested and used 6 d after the addition of IL-2, unless otherwise mentioned, at 37 °C in 5% CO<sub>2</sub> tissue culture incubators. Similarly, CD8<sup>+</sup> T cells were isolated from PBMCs by negative magnetic selection (Miltenyi Biotec). The remaining PBMCs, depleted of CD8<sup>+</sup> cells, were washed three times with complete media before being used for experiments.

**Cell Lines.** EOL-1 and Raji cell lines were maintained in complete media consisting of RPMI-1640 Medium supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, and 50 U/ml Penicillin-Streptomycin (all from ThermoFisher Scientific), at 37 °C in 5% CO<sub>2</sub> tissue culture incubators.

**CD33 Transduction Into Raji Cells.** HEK293T cells were transfected with a CD33 lentiviral backbone plasmid, along with the VSV-G coding plasmid pMD2.G and the packaging plasmid psPAX2 (Trono, Addgene #12259, #12260) using the TransIT<sup>®</sup>-Lenti Transfection Reagent (Mirus). Nontissue culture-treated plates were coated overnight at 4 °C with 15  $\mu$ g/mL retronectin (Takara), dissolved in PBS. After coating, the plates were washed and subsequently coated with viral particles. Viral particles were harvested by collecting the media from 293T cells 48 h posttransfection, passing it through a 0.45  $\mu$ m filter to remove any detached cells, and spinning the plates at 2,000  $\times$  g for 1 h at 32 °C. The plates were then incubated at 37 °C in 5% CO<sub>2</sub> for an additional 4 h. Afterward, the media was removed and replaced with 4 mL of 0.25  $\times 10^6$  cells/mL of Raji cells (ATCC) in RPMI medium supplemented with 10% FBS and penicillin-streptomycin. The cells were incubated at 37 °C in 5% CO<sub>2</sub> for 48 h. After the incubation, successfully transduced cells were sorted based on GFP and CD33 expression.

**AML Samples.** Cryopreserved peripheral blood mononuclear cells from patients with AML were obtained from the MCRC Biobank, The Christie NHS Foundation

Trust, collected under MCRC Biobank Research Tissue Bank Ethics (ref: 18/NW/0092). Samples were defrosted and allowed to recover in complete media overnight before being used directly on experiments that involved AML PBMCs. Alternatively, AML NK cells were isolated using negative magnetic selection (Miltenyi) and cultured overnight in clone media supplemented with 200 IU rhIL-2 before use in experiments.

**Flow Cytometry.** To determine the levels of cell surface proteins on NK cells or AML cells, the cells were initially washed with PBS and then stained with live/dead discriminator dyes and incubated for 20 min at 4 °C. Following this, the cells were washed with FACS buffer (1% FBS in PBS) and subsequently blocked for 20 min at 4 °C using 1% human serum and FACS buffer. Antibodies were then added to the cells and left to incubate for 30 min at 4 °C. After washing, the cells were fixed with a final concentration of 2% PFA (ThermoFisher Scientific) in PBS for 20 min at room temperature (RT). Antibodies used for cell surface staining included FITC or PE anti-CD69 (FN50); BV421, PE, or AF647 anti-CD33 (WM53); BV421 or AF647 anti-NKG2D (1D11); AF488 or AF647 anti-CD16a (3G8); PE or AF647 anti-CD3 (OKT3); BV421 or APC anti-CD56 (HCD56), all from BioLegend.

For intracellular staining, following the staining of surface proteins, cells were washed, and then fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions. Intracellular cytokine staining using AF647 anti-IFN- $\gamma$  (25723, R&D Systems) and anti-TNF- $\alpha$  AF488(clone Mab11, Biolegend), was performed, and samples were incubated for 30 min at 4 °C. This was followed by a Perm/Wash (BD Biosciences) and readied for flow cytometry, and all analysis was carried out with the FlowJo software (v10.4.2; FlowJo).

**Degranulation Assay.** Degranulation was assessed by culturing NK and EOL-1 cells at 1:1 ratio in the presence of the engager or its variants at a concentration of 2  $\mu$ g/mL for 4 h at 37 °C in 1:1 clone:complete media, which contained 1/1000 dilution of brefeldin A (Fisher Scientific) and monensin (BD Biosciences) and PE-Cy7 anti-CD107a (H4A3; BioLegend). The cells were washed with PBS and stained with a dead cell marker at 4 °C for 20 min. Following blocking in 2% human serum for 20 min at 4 °C, cells were washed and stained with PE-Cy7 anti-CD107a mAb, BV421 anti-CD56 mAb, and PE anti-CD33 mAb for 30 min at 4 °C. After that, the cells were fixed in 2% PFA/PBS for flow cytometric analysis. 1  $\mu$ g/mL PMA and Ionomycin (Sigma) was used to stimulate NK cell degranulation via soluble stimulation and was added to NK cells 1 h before the end of the assay. NK cell degranulation was quantified by measuring the percentage of CD107a expressed on the surface of NK cells.

**Cytotoxicity Assays.** For Annexin V/PI cytotoxicity assay, NK cells were labeled with the CFSE for 20 min at 37 °C. After washing with PBS, NK cells were cultured with EOL-1 or CD33-transduced Raji cells (E:T, 1:1) for 4 h at 37 °C in a V-bottom 96-well plate. To quantify cell death, cells were stained with Annexin V (BioLegend) in Annexin V binding buffer (2.5 mM CaCl<sub>2</sub>, 140 mM NaCl, and 10 mM HEPES) for 15 min at RT and washed once with binding buffer. After this, propidium iodide (PI) (ThermoFisher Scientific) was added at a concentration of 1  $\mu$ g/mL before immediate flow cytometric analysis. Specific killing was determined by Annexin V/PI staining and normalized to target cells incubated without NK cells.

For calcein-AM cytotoxicity assay, NK cells were resuspended in NK-Cytox media: phenol red-free RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 50 to 55  $\mu$ M 2-mercaptoethanol, sodium pyruvate, 10 mM HEPES, non-essential amino acids, and Glutamax. EOL-1 target cells were labeled with 1  $\mu$ M Calcein-AM for 30 min at 37 °C, washed three times with PBS, and resuspended in NK-Cytox media. NK cells and Calcein-labeled EOL-1 cells were cocultured at a 1:1 E:T ratio in 96-well V-bottom plates and incubated overnight at 37 °C. Following incubation, 100  $\mu$ L of supernatant was carefully collected without disturbing the cells, and Calcein fluorescence was measured to quantify target cell lysis. Specific cytotoxicity was calculated relative to maximal release controls treated with 2% Triton X-100.

**Quantification of Ligand Loading on Lipids.** Liposomes were generated by extrusion with the lipids: Ni-NTA (1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid) succinyl]) and DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), and the extruder obtained from Avanti Polar Lipids, Inc. Bilayers. 5  $\mu$ m silica beads (SS05N; Bangs Laboratories, Inc) were washed three times with ddH<sub>2</sub>O and then PBS before incubating them with liposome solutions containing 75% DOPC and 25% Ni-NTA, in a 3:1 ratio (lipids:beads). The mixture was vortexed and blocked with the blocking solution (1% BSA; Sigma, in PBS) before incubating it. To quantify ligand loading, bead suspensions were aliquoted

to individual wells of a V-bottom 96-well plate and added His-tagged CD33 (R&D Systems), or His-ICAM-1 (Sino Biological) conjugated to Alexa Fluor 647 or atto488 (in house) at varying concentrations starting from 0.1 up to 1  $\mu\text{g}/\text{mL}$ . The ligands were incubated with the lipid suspension for 1 h at 37 °C. Quantum MESF beads (647 or 488; Bangs Laboratories, Inc.) were used in flow cytometry to measure protein density for each batch of lipids and proteins (34, 52).

**Construction of PLBs on Glass Coverslips.** PLBs were generated on 8-well Lab-Tek II chamber slides (Nunc). Wells were cleaned by adding 1 M HCl (Sigma-Aldrich) in 70% ethanol for 30 min at RT and then washed five times with sterile ddH<sub>2</sub>O. Following this, wells were washed with 0.45  $\mu\text{m}$  filtered 10 M NaOH (Thomas Scientific) for 15 min at RT, then washed five times with sterile ddH<sub>2</sub>O, and were finally dried under an argon stream. 200  $\mu\text{L}$  of liposome solutions (75%:25% DOPC:Ni-NTA) were added to each well and washed by sequentially adding and removing 500  $\mu\text{L}$  PBS five times to remove excess lipid. Wells were blocked with 1% BSA supplemented with 100  $\mu\text{M}$  Nickel Sulfate (Sigma-Aldrich) for 20 min at RT (34, 52). Unconjugated His-tagged ICAM-1 (200 molecules per  $\mu\text{m}^2$ ) and His-CD33 (400 molecules per  $\mu\text{m}^2$ ) were then added for 1 h at RT. Excess ligands were removed by washing wells with PBS five times. The wells were then equilibrated with multiple washes of the media before being used for cellular incubation and further experiments.

**Assessing the Mobility of Ligands on Constructed PLB.** To evaluate the mobility of ligands on PLB, His-tagged CD33 directly conjugated to Alexa Fluor 647 was added to a preblocked PLB-coated well. The concentration of the ligand tested varied from 100 to 1,200 molecules per  $\mu\text{m}^2$ . His-CD33-AF647 was added to the PLB and incubated for 20 min at RT. After incubation, excess His-CD33-AF647 was removed by washing the wells with PBS five times. The mobility of the ligand was then tested using FRAP microscopy (Leica TCS SP8). Small regions were bleached using 100% 660 nm STED laser and scanned every second for 1.5 min with a WLL of 650 nm. To determine mobility, the recovery within the bleached region over time was compared to other regions that had not been bleached. A minimum of 90% recovery was detected within  $T_{1/2} \sim 10$  s.

**ELISA.** To measure the levels of IFN- $\gamma$  and TNF- $\alpha$  in cell supernatants, an ELISA was performed in two different ways. For ELISAs on PLB, the PLB was constructed as previously described, but this time on 18-well chambers (Ibidi). Unconjugated His-tagged CD33 was added to appropriate wells at a density of 400 molecules/ $\mu\text{m}^2$ . 10<sup>5</sup> NK cells were then added, followed by adding the engager or its variants at a concentration of 2  $\mu\text{g}/\text{mL}$ , and cells were allowed to bind to the CD33-PLB overnight. In the second way, isolated NK cells (50,000 cells) were cocultured with the CD33-rich EOL-1 cell line (50,000) in a 1:1 ratio in 96-well V bottom plates. The engager antibody or its variants were added to the relevant wells at a concentration of 2  $\mu\text{g}/\text{mL}$ , and cells were allowed to form conjugates overnight. For PBMCs isolated from healthy donors, cocultures with EOL-1 cells were performed at varying E:T ratios of 1:1, 5:1, and 10:1 to assess optimal cytokine responses. In some experiments, CD8<sup>+</sup> T cells were depleted using magnetic isolation, and the remaining PBMCs were used under the same E:T titration conditions. Unless otherwise indicated, results shown were obtained at the 5:1 ratio. In all cases, supernatants were collected the next day, and ELISA was performed following the manufacturer's recommendation (DuoSet kits).

Briefly, Nunc MaxiSorp™ 96-well plates (ThermoFisher Scientific) were coated with human capture IFN- $\gamma$  or TNF- $\alpha$  antibody in PBS overnight at 4 °C. The excess antibody was then washed off by rinsing the wells three times with washing buffer: PBS with 0.05% Tween20 (Sigma-Aldrich). Next, the wells were blocked with blocking buffer (1% BSA + 0.05% Tween in PBS, 0.22  $\mu\text{m}$  filtered) for 1 h at RT with gentle shaking. Once blocked, samples and standards (rh IFN- $\gamma$  or TNF- $\alpha$ ) were added and left to incubate for 2 h at RT with gentle shaking. After three washes with the washing buffer, 1  $\mu\text{g}/\text{mL}$  detection antibody was added and incubated for 1 h at RT. The wells were washed again three times with the washing buffer before streptavidin-HRP was added and left to incubate at RT for 30 min. The wells were rinsed three more times before adding TMB solution (Thomas Scientific) to each well until the color developed, after which it was stopped with 0.5 N H<sub>2</sub>SO<sub>4</sub> and optical density at 450 nm was measured using [CLARIOstar Plus spectrophotometer (BMG Labtech)].

**Sample Preparation for Microscopy.** Primary NK cells were plated onto Lab-Tek II imaging chambers coated with His-tagged ICAM-1 and CD33-PLB, and the engager or its variants were added simultaneously with the cells. The mixture was allowed to interact with CD33 ligand on the PLB for 20 min at 37 °C. Then

cells were fixed with paraformaldehyde to a final concentration of 4% at RT for 20 min. The 20 min incubation time was selected based on a preliminary time-course analysis of synapse formation, where recruitment of key receptors such as NKG2D and CD16a was monitored at 2, 5, 10, 20, and 30 min. This analysis demonstrated that stable and significant receptor enrichment at the immune synapse consistently occurred by 20 min, making it an optimal time point for assessing synaptic organization across all experimental conditions (SI Appendix, Fig. S8). To prevent desiccation of the lipids, 200  $\mu\text{L}$  of the PBS was always left in the well, and 500  $\mu\text{L}$  of PBS was added and removed serially to wash the cells. If permeabilization was required, cells were treated with 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 10 min at RT. After that, the samples were blocked with a blocking buffer (3% BSA + 1% human serum in PBS) for 1 h at RT. Samples were then stained with AF647-Anti-NKG2D (1D11 at 1  $\mu\text{g}/\text{mL}$ , BioLegend) + AF488 anti-CD16a (clone 3G8 at 1  $\mu\text{g}/\text{mL}$ , BioLegend) in the blocking buffer for 1 h at RT or at 4 °C overnight. To image signaling proteins, samples were stained with atto488-anti-pCD3 $\zeta$  (K25-407.69, R&D Systems, labeled in house), AF647 anti-pZAP70 (Abcam/CS), or AF488 anti-pSLP-76 (225501, R&D Systems). Samples were washed three times with PBS and then imaged in TIRF mode using the NIKON inverted Ti2 microscope. For dSTORM imaging, PBS was replaced with 0.22  $\mu\text{m}$ -filtered STORM imaging buffer that was refreshed regularly to maintain a low-oxygen environment for the fluorophores. The buffer contained 560  $\mu\text{g}/\text{mL}$  glucose oxidase, 34  $\mu\text{g}/\text{mL}$  catalase, 25 mM glucose (all from Sigma-Aldrich), and 1%  $\beta$ -mercaptoethanol (ThermoFisherScientific).

**Microscopy: Image Acquisition.** For dSTORM, an inverted Nikon Ti Eclipse microscope featuring a TIRF 100 $\times$  oil immersion lens (NA, 1.49) and an electron-multiplying charge-coupled device (EMCCD) camera (Andor iXon Ultra 897), along with focus stabilization (perfect focus system) were used. A 170-mW 647-nm laser (Agilent Ultra High-Power Dual Output Laser) with an 18-ms exposure was used. EM gain was set at 17 MHz-16-bit, EM gain multiplier at 300, and conversion gain multiplier at 3. Two-color STORM imaging was performed sequentially, imaging 647 first, followed by 488. The 647-nm laser was used at 30 to 50% power for 10,000 frames, followed immediately by the 488-nm laser (at 50 to 70% power) for another 10,000 frames.

**Single-Molecule Localization Microscopy Data.** STORM images were processed using ThunderSTORM software (53). The raw images were filtered using the B-spline method of wavelet filter at order 3 and scale 2 to eliminate any noise. Initial events detection was determined by the local maximum localization method, with a peak intensity threshold set to 2 times the SD of the F1 wavelet and 8-neighborhood connectivity for each pixel screened. Subpixel event localization was calculated using the integrated Gaussian point-spread function and maximum likelihood estimator, with a fitting radius of 5 pixels and an initial sigma of 1.6 pixels (34).

Localizations were filtered based on intensity, sigma, and localization uncertainty. These thresholds were chosen based on the distribution of localization parameters for each dye (SI Appendix, Table S1). To ensure high-quality localization, fluorophore-specific thresholds were applied based on distributions of photon counts, sigma values, and localization uncertainties.

We acknowledge that dSTORM data can be affected by technical artifacts that may appear as false clustering, such as multiple fluorophores conjugated per antibody (degree of labeling), antibody-induced clustering, and fluorophore blinking. To minimize these effects, we used directly labeled primary antibodies with carefully optimized concentrations to reduce background signal. Additionally, we applied stringent localization filtering criteria based on intensity, point spread function sigma, and positional uncertainty (SI Appendix, Table S1), and implemented merging of potential reblinking events using dye-specific spatial and temporal thresholds to avoid overcounting fluorophore localizations. The degree of labeling (DoL) for each antibody is reported in SI Appendix, Table S2.

To correct for sample drift, the dataset was processed using the cross-correlation of images from three bins at a magnification scale of 5. The occurrence of reblinking events was minimized by merging filtered and drift-corrected events that appeared within 50 nm and 20 frames for the 647 channel, and within 20 nm and 10 frames for the 488 channel.

Clustering analysis of single molecule localization data was conducted utilizing custom MATLAB scripts accessible via Github repositories (<https://github.com/quokka79/RegionFinder>; <https://github.com/quokka79/ClusterFields>). In brief, cluster quantification was performed using a cutoff radius to define nanoclusters

of CD16a and NKG2D receptors from dSTORM data. To select an appropriate cutoff, we conducted a sensitivity analysis across multiple radii (20, 30, 40, 60, and 80 nm) to ensure robustness of our findings. Variations in cluster size according to each stimulation were consistent across all tested thresholds. The primary cutoff radius of 30 nm was chosen based on two criteria: 1) previously published receptor nanocluster sizes measured by dSTORM in immune synapses (54, 55), and 2) the lateral spatial resolution limit of our dSTORM system (~30 nm). This choice ensures clusters represent true spatial organization rather than localization noise.

**CBC and NND Analyses.** X-Y coordinate list from both the 488-nm and 647-nm dSTORM channels was used. The correlation function was calculated for each 647-nm channel localization to each 488-nm channel localization, which can vary from  $-1$  (perfectly separated) to  $+1$  (perfectly colocalized), with 0 indicating no correlation. The correlation coefficients were plotted as a histogram of occurrences with 0.1-binning. To determine the NND, 647-nm channel localization was compared to its closest localization from the 488-nm channel, and the median NND between localizations was plotted per cell (34).

**Statistical Analysis.** Statistical analyses and graphical data presentations were performed using GraphPad Prism 10 (version 10.2.1). The specific statistical tests

used are detailed in the figure legends. The figure legends and text also specify the exact data represented in the graphs (e.g., mean, SD). Data were assessed for normality using the "Normality and Lognormality Tests" option in GraphPad Prism software. Data that followed a normal distribution were analyzed using parametric tests, whereas nonnormally distributed data were analyzed using nonparametric tests. Sample sizes were not predefined using any statistical method. The experiments were not randomized and were not blinded.

**Data, Materials, and Software Availability.** All study data are included in the article and/or [SI Appendix](#).

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