SUPPORTING INFORMATION

Activation of human Natural Killer cells by graphene oxide-templated antibody nanoclusters

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Methods

Antibodies

Anti-humanCD16 (clone 3G8, Biolegend or BD Biosciences) and mouse IgG1 κ-isotype (clone MOPC-21 Ultra-LEAF grade, Biolegend or clone MOPC-21, BD Biosciences) were first fluorescently labelled with AlexaFLuor 546 and then biotinylated. Fluorescent labelling was carried out by reacting antibodies (5 mg/mL, concentrated using centrifugal filtration, 10 kDa cutoff, Amicon Ultra, Millipore), with Alexa Fluor 546 NHS ester (final concentration 0.148 mg/mL) for 1.5 h at room temperature in Dulbecco's Phosphate Buffered Saline (PBS) with $MgCl_2$ and $CaCl_2$ (275 – 304 mOs/kg, pH = 6.9-7.1). Unreacted Alexa Fluor 546 NHS ester was removed by centrifugal filtration with repeated rinsing. The mean number of fluorescent labels per antibody molecule was calculated by measuring UV-visible absorbance at 280 nm and 546 nm (NanoDrop 2000 spectrometer, Thermo Scientific) and using extinction co-efficients provided by the dye manufacturer. In a second stage, labelled antibodies were biotinylated by reaction with NHS-PEG_4-biotin (EZ-Link™, ThermoScientific, final concentration 0.512 mmol/L in PBS). The number of biotin groups per antibody was determined using the Pierce Biotin™ Quantification Kit, which briefly quantifies the number of biotin groups by measuring the change in UV-visible absorbance at 500 nm (VersaMax™ Microplate reader) arising from the displacement of 4′-hydroxyazobenzene-2-carboxylic acid from avidin by the biotinylated sample.

Synthesis of nanographene oxide (NGO)-templated molecular nanoclusters

Conjugation of NGO with 8-arm star-PEG-amine

NGO was purchased as an aqueous solution from 2D-Tech, Manchester, UK. Firstly, this solution was reacted with chloroacetic acid to generate -COOH groups on the surface (NGO-COOH). The as-received solution was diluted to 1 mg/mL, (ultrapure water 18.2 MΩ), and ultrasonicated for 1 hour to break up any aggregates before addition of chloroacetic acid (200 mg/mL) in combination with sodium hydroxide (240 mg/mL) which also serves as a base-wash to remove organic impurities created during the NGO synthesis. The reaction was continued for 4 hours under ultrasonication before the resulting NGO-COOH was purified by repeated rinsing with ultrapure water using a centrifugal filter (100 kDa cutoff, Amicon Ultra, Millipore) and the pH adjusted to ~7 using hydrochloric or sulfuric acid (1M). The presence of carboxylic acid groups was confirmed by the appearance of an IR absorbance at 1591 cm^{-1} (Fig. 1B). The second stage of the reaction conjugated 8-arm star-poly(ethylene glycol) amine with total M_w 40 kDa (star-PEG-amine, Creative PEGWorks. Star-PEG amine (20 mg/mL) was added to the NGO-COOH suspension with 5 mins ultrasonication before N-(3-methylaminopropyl-N'-ethylcarbodiiimide) was added (to final concentration 8 mmol/L) and left to react overnight with agitation. Unreacted EDC was quenched by addition of 2-mercaptoethanol (to final concentration 40 mmol/L), and the NGO-PEG product was purified by centrifugal filtration with repeated rinsing as above. If required an additional centrifugation (16000 g, 4 h) discarding the pellet was used to remove any remaining large aggregates leaving the stable product. For comparison of stability a conjugate of NGO with 4 arm star-PEG-amine (M_n 10 kDa, Sigma Aldrich) was produced using the same method with 2 mg/mL PEG and 4 mmol/L EDC, quenched with 20 mmol/L 2-mercaptoethanol.

Biotinylation and streptavidin coating of NGO-PEG construct

NGO conjugated with 8-arm star-PEG amine was biotinylated using an NHS-PEG_4-amine cross-linker (EZ-Link™, ThermoScientific) in PBS with $MgCl_2$ and $CaCl_2$ in accordance with the manufacturer's instructions. The product was purified by rinsing and centrifugal filtration as above. Biotinylation was confirmed using a HABA/avidin assay (Pierce Biotin™ Quantification Kit) as per the manufacturer's instructions except for an adjustment for NGO absorbance achieved by additionally measuring a non-biotinylated NGO-PEG sample. Streptavidin coating was carried out at large excess in order to minimize the possibility of cross-linking between NGO sheets. In a typical case, biotinylated NGO-PEG (0.6 mg/mL) was mixed with streptavidin (5 mg/mL) with thorough mixing. Excess streptavidin was removed through centrifugal filtration with repeated rinsing as above.

Binding of antibodies to streptavidin-coated NGO-PEG construct

Streptavidin-coated NGO-PEG (0.4 mg/mL) was mixed with biotinylated antibodies (to final concentration 0.418 mg/mL) in PBS with $MgCl_2$ and $CaCl_2$ and allowed to react overnight at 4 °C. The relative concentrations were calculated to allow the NGO construct to entirely deplete the antibody solution leaving no unbound antibody. The antibody-functionalized NGO-PEG was separated into fractions by centrifuging at 10,000g and extracting the pellet every 5 mins. Successive pellets corresponded to different size distributions of the NGO-mAb constructs, with the larger nanoclusters concentrating into the earlier pellets. In total four fractions were separated and fractions 1-3 resuspended producing samples with distinct size distributions as indicated in the main text and Fig 2E.

Bead-binding assays

Compensation Beads (coated with anti-mouse IgG1κ antibody, BD Biosciences). The coated beads were mixed 1:1 with uncoated beads and diluted with equal volumes of PBS. In binding assays, 50 μL were mixed with NGO-mAb (1 μL, 200 μg/mL, final concentration 3.92 μg/mL) molecular nanoclusters and their control antibodies and incubated using the same

conditions as used for cell binding assays below, after which they were washed with PBS, and analyzed using flow cytometry, again as for cell binding assays. In a negative control experiment, the beads showed no binding to a soluble antibody solution for which they have no affinity (polyclonal goat α-mIgG2b, Thermo Scientific).

Characterization of NGO-templated molecular nanoclusters

Thermogravimetric analysis (TGA)

TGA used a Mettler Toledo TGA0-DSC with sample masses of ~ 1 mg. Experiments were carried out in a N_2 atmosphere using a temperature range of 100 – 800 °C and a heating rate of 10 °C/min. PEG was entirely pyrolized between 300 – 440 °C.

Atomic force microscopy (AFM)

AFM measurements were made on a Bruker Innova microscope in Tapping Mode using aluminium-coated silicon probes NCHV-A (Bruker Nano). Samples were prepared by depositing a sample droplet onto a silicon wafer substrate and allowing to dry in the air.

Quantification of the number of antibodies per NGO-mAb nanocluster by UV-visible spectroscopy

The concentration of NGO sheets in a given sample was determined by UV-visible spectroscopy at 700 nm. An extinction coefficient at this wavelength for NGO-PEG was first determined by generating a calibration curve using a pre-weighed sample, with the ratio of NGO-PEG determined from TGA. In combination with the known sheet size from AFM, this gave the number concentration of NGO sheets. The concentration of antibody molecules was then determined using UV-visible spectroscopy at 546 nm with subtraction of the NGO-PEG background, based on the labelling of antibodies with AlexaFluor546 dye, (Fig. 1F). These concentration data were combined with the nanosheet size determined by AFM to indicate the number of antibody molecules per nanosheet. To ensure that no confounding free molecules

were present in cell experiments, a sample of each batch was removed and pelleted by centrifugation, with UV-visible spectroscopy on the supernatant confirming the absence of such free molecules. The experimental uncertainties derived from fitting the UV-visible data are estimated to introduce a standard error of ± 40 nm into the calculated antibody numbers. This is independent from the interquartile ranges quoted in the main text which characterize the statistical distribution of antibody numbers deriving from the distribution of NGO-mAb nanocluster sizes measured by AFM.

Primary human NK (pNK) cells

pNK cells were obtained from healthy donor peripheral blood concentrates (NHS Blood and Transplant) in the form of Leukopaks, with ethical approval. NK cells were isolated from the Leukopaks under negative magnetic selection (NK cell isolation kit, Miltenyi Biotec) and cultured in RPMI-1640 supplemented with 10% human serum, 1mM L-glutamine, 1mM sodium pyruvate, 1mM penicillin-streptomycin, 1mM MEM nonessential amino acids and 20 μM 2-mercaptoethanol (all ThermoScientific). For cell binding experiments, freshly isolated pNK cells were stimulated with 150 U/mL human recombinant IL-2 (Roche) and cultured for 6 days prior to use. For experiments investigating functional response to nanoclusters and antibodies, pNK cells were cultured in media with IL-2 for a total of 10 days before use with resuspension in fresh media containing IL-2 at days 7 and 9. Experiments carried out 1 day after the last IL-2 addition.

Cell-binding assays

The binding of NGO-constructs and soluble antibodies to pNK cells was assayed as follows. pNK cells were resuspended in flow buffer (2% FCS in PBS) and incubated with unconjugated antibodies and NGO-mAb constructs with concentrations adjusted so that the overall

concentration of antibody was 2 μg/mL in all cases. The solution was incubated for 20 mins at 4 °C.

Stimulation of pNK cells with NGO-mAb molecular nanoclusters

pNK cells were suspended at 1×10^6 cells/mL (100 μL aliquots) in R10 with: anti-LAMP-1-AlexaFluor647 or its isotype control, Golgi Plug (BD Biosciences) and Monensin (eBiosciences) (both at 1:1000 dilution) to ensure only pre-synthesized existing IFN-γ is secreted as described in the text. Stimulating NGO-mAb molecular nanoclusters and antibodies were added with a final overall concentration of 2 μg/mL antibody in all cases. Concentrations were determined using UV-vis absorbance, based on AlexaFluor546 labelling, with the soluble antibody taken from the same labelling batch as the NGO to ensure consistency. The cells were placed in 96-well-plates (Nunc Maxisorp) that had been prepared by washing with 0.05% Tween-20 in PBS, and briefly centrifuged (10s at 300g) to move them to the bottom of the wells. As a positive control to ensure that cells were capable of being stimulated, some cells were plated (with no added soluble antibodies or NGO-mAb reagent) onto a stimulating surface consisting of a well that had been coated with anti-CD16 antibodies (overnight incubation of 50 μL solution at 1 μg/mL): donors that did not fulfil this condition were discarded. Cells were incubated at 37 °C for 6 h prior to readouts of CD107a degranulation and IFN-γ secretion/expression. To confirm that the NGO-mAb nanoclusters did not impact cell viability Zombie Aqua cell viability stain (Biolegend) was added to distinguish live from dead cells. For cell viability plots gating on the lymphocyte population by FSC-A, SSC-A followed by gating for Zombie Aqua negative cells to establish percentage viability. Flow cytometry was carried out using a BD LSRFortressaTM and anaylsed using FlowJoTM version 10. The NGO-mAb nanoclusters did not significantly impact cell viability (Fig. S3).

CD107a [LAMP-1] degranulation assay

After 6h stimulation with NGO-mAb molecular nanoclusters or control antibodies, cells were removed by repeated pipetting, washed and resuspended in flow buffer (2% FCS in PBS) with a human serum blocking step. They were then stained with Zombie Aqua™ (Biolegend), anti-humanCD56-BV421 (clone HCD56, Biolegend) and anti-LAMP-1-AlexaFluor647 for 20 mins at 4 °C, washed with flow buffer (2% FCS in PBS) and fixed with PFA at a final concentration of 2%. Flow cytometry was measured using the same instrument and software as for cell-binding assays. For intracellular stains, cells were washed twice with Perm/Wash™ (BD Biosciences), blocked with human serum and then stained with anti-human IFN-γ mAb for 30 mins at 4 °C before being washed a further two times, fixed and analysed by flow cytometry.

IFN-γ secretion ELISA measurements

EISA plates (Nunc Maxisorp) were coated with anti-humanIFN-γ (clone NIB42, BD Biosciences) in carbonate buffer (Sigma Aldrich) and blocked (1% bovine serum albumin (BSA) and 0.05% Tween in PBS, blocking buffer). Cell supernatant (80 μL) was mixed with 2% BSA solution (2x PBS, 80 μL). In parallel, samples of human IFN-γ (BD Biosciences) diluted in blocking buffer between 0 – 500 pg/mL were prepared in duplicate to generate a calibration curves. The diluted supernatants were added to the ELISA plate (50 μL per condition, in triplicate) and incubated. After 1 h, biotinylated antihuman IFN-g (clone 4S.B3, BD Biosciences, 1 μg/mL), streptavidin horseradish peroxidase (BD Biosciences, 50 μL) and 3,3',5,5'-Tetramethylbenzidine ELISA substrate (Sigma Aldrich, 50 μL) were added in sequence and the reaction was halted with sulfuric acid (100 μL, 1 N). Absorbance was measured at 450 nm with a correction at 570 nm (Tecan Infinite 200 PRO microplate reader).

Statistical significance

Statistical analysis was carried out using Prism 7 software (Graphpad). Significance was determined by performing two-tailed, paired or unpaired as described, parametric t-tests with a threshold for significance of * $p > 0.05$, ** $p < 0.01$.

Supplementary Results: Antibody labelling of NK cells at different concentrations

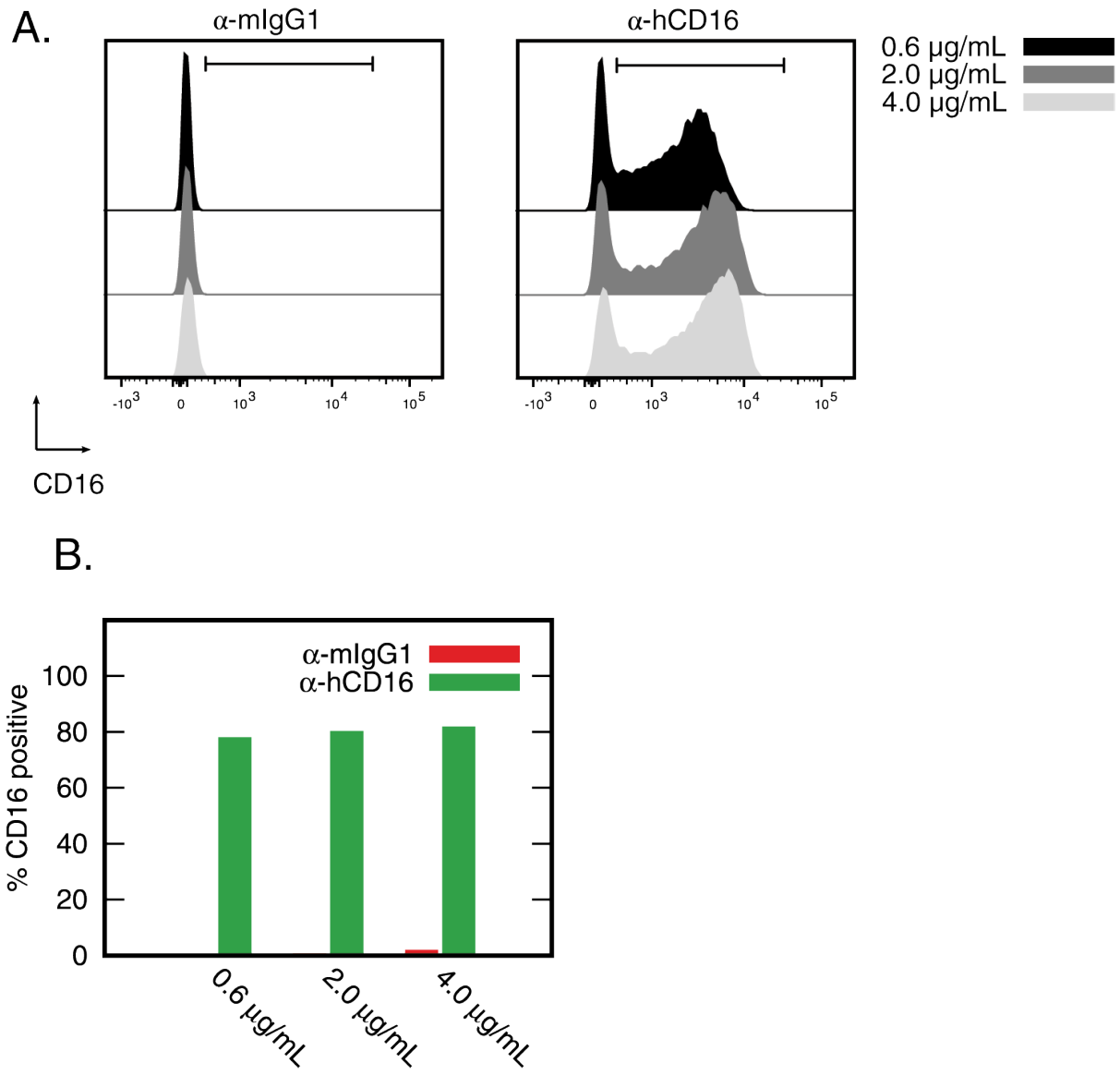

Fig. S1. Antibody labeling of NK cell surface CD16 with 2 µg/mL α-hCD16 is sufficient to saturate receptors (A.) Histograms of α-mIgG1 control and α-hCD16 at 0.6, 2.0 and 4.0 µg/mL each. α-hCD16 shows good separation of the CD16 positive NK cell population at all concentrations and does not increase significantly after 2 µg/mL. (B.) Quantification of the percentage CD16 positive cells at each antibody concentration. Between 2 µg/mL and 4 µg/mL % CD16 positive cells increases by only 1.5% however nonspecific binding, as shown by the

isotype control, increases by 1.2% showing that there is no benefit to using concentrations greater than 2 μg/mL.

Binding kinetics of NGO-mAb to NK cells

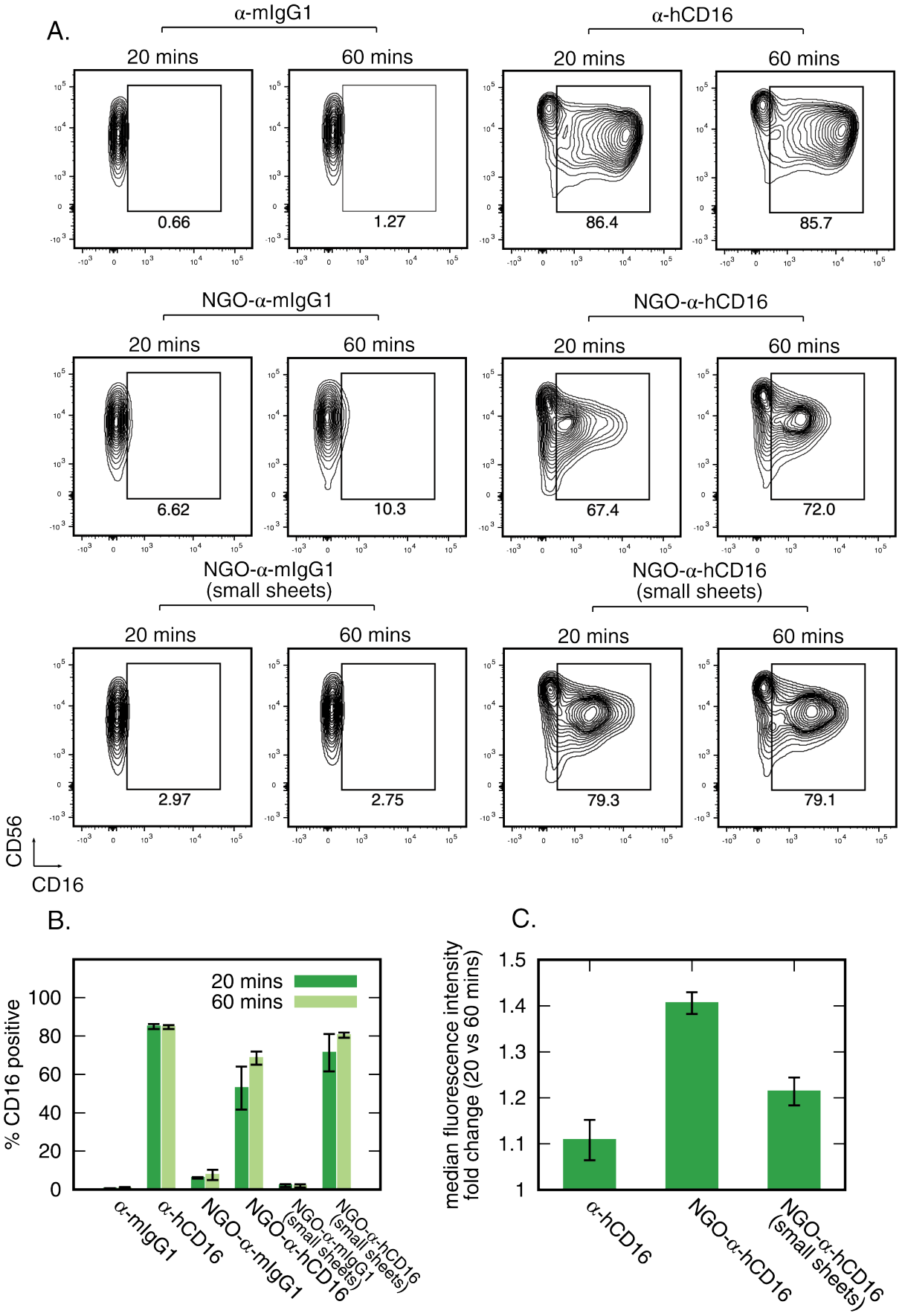

Fig S2. NGO-mAb of larger diameter show slower binding kinetics compared to NGO-mAb (smaller sheets). (A.) Representative flow cytometry plots of the binding of pNK cells by α-mIgG1, α-hCD16, NGO-α-mIgG1 and NGO-α-hCD16 at both 20 and 60 minute timepoints. The axis labelled CD16 represents the fluorescence intensity associated with the stimulating species in each case. (B.) Quantification of the percentage CD16 positive pNK cells when incubated for either 20 or 60 mins. Results are from 2 repeats, using independent donors. (C.) Fold change in the median fluorescence intensity of CD16 positive pNK from 20 to 60 mins of incubation. Data normalized to an FMO control by subtraction. To summarize the visible changes, α-hCD16 shows minimal change with time in binding of pNK cells as the median fluorescence intensity. NGO-α-hCD16 on the other hand shows a modest increase in the percentage of positive pNK cells but a large shift in the mean fluorescence intensity, indicating increased binding of the NGO-α-hCD16 at 60 mins. NGO-α-hCD16 (small sheets) show similar, but smaller shift in the mean fluorescence intensity, in following with their smaller size. Hence we see that the fold change increases as the size of the binding species increases which is an expected consequence of slower binding kinetics for larger species.

Cell viability controls

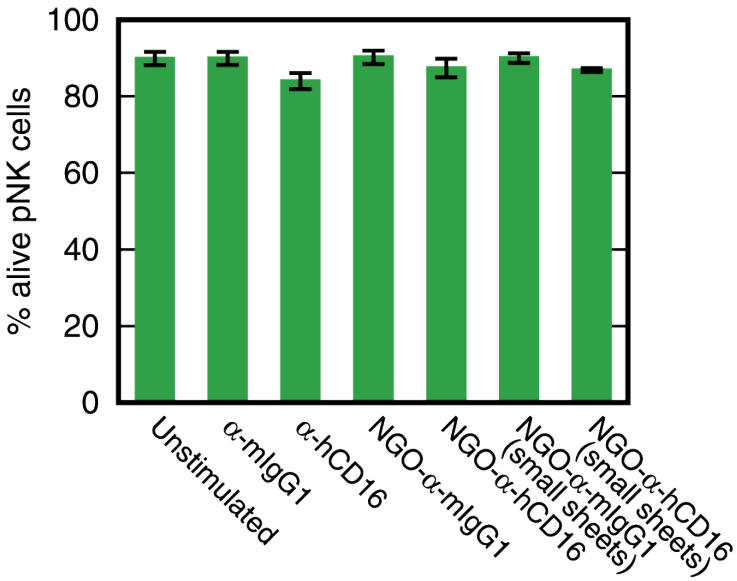

Fig. S3. NGO-mAb nanoclusters do not significantly impact pNK cell viability. There is no significant difference in pNK cell survival after 6 hrs incubation with NGO-mAb compared to unconjugated mAbs. Cell viability measured using Zombie aqua staining (positive staining equating to dead cell) after gating for pNK cell population by characteristic size using FSC-A, SSC-A plot.