Research Article
Quantitative analysis of human NK cell reactivity using latex beads coated with defined amounts of antibodies

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Natural Killer (NK) cell responses are regulated by a variety of different surface receptors. While we can determine the overall positive or negative effect of a given receptor on NK cell functions, investigating NK cell regulation in a quantitative way is challenging. To quantitatively investigate individual receptors for their effect on NK cell activation, we chose to functionalize latex beads that have approximately the same size as lymphocytes with defined amounts of specific antibodies directed against distinct activating receptors. This enabled us to investigate NK cell reactivity in a defined, clean, and controllable system. Only CD16 and NKp30 could activate the degranulation of resting human NK cells. CD16, NKG2D, NKp30, NKp44, and NKp46 were able to activate cultured NK cells. NK cell activation resulted in the induction of polyfunctional cells that degranulated and produced IFN-γ and MIP-1β. Interestingly, polyfunctional NK cells were only induced by triggering ITAM-coupled receptors. NKp44 showed a very sensitive response pattern, where a small increase in receptor stimulation caused maximal NK cell activity. In contrast, stimulation of 2B4 induced very little NK cell degranulation, while providing sufficient signal for NK cell adhesion. Our data demonstrate that activating receptors differ in their effectiveness to stimulate NK cells.

Keywords: activating receptors · degranulation · natural killer cells · polyfunctional NK cells · quantitative analysis

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction
Cellular cytotoxicity and the production of cytokines and chemokines are the main effector functions of NK cells. Despite their name, NK cells are no unspecific killers of the innate immune system, but are a highly regulated, heterogeneous population of innate lymphocytes [1–3]. NK cells are regulated by soluble factors such as IL-15, IL-12, or IL-18 that can in turn activate cytokine production of NK cells, influence their proliferation and prime them for cytotoxicity. Cellular cytotoxicity is initiated by firm attachment of the NK cell to the target, thereby forming an immunological synapse [4]. Via a set of regulated steps, NK cells then release their cytotoxic granules containing perforin and granzymes to kill the target cell [5,6]. In addition to degranulation of cytotoxic granules, activated NK cells can also produce cytokines and chemokines and are therefore called “polyfunctional” NK cells [7]. Cellular cytotoxicity is coordinated by many different activating and inhibitory NK cell receptors that recognize diverse ligands.
on the target cell membrane [2,3]. Activating NK cell receptors include NKG2D, the natural cytotoxicity receptors (NCR) NKp30, NKp44, and NKp46, members of the SLAM-family receptors such as 2B4, the Fcy receptor CD16, and many others. Ligands for NKGD2 are upregulated on infected, stressed, or transformed cells and include the MHC class I homologous proteins MICA and MICB, and in humans the proteins ULBP1-6. NKp30 recognizes B7-H6, which is expressed on certain tumor cells. NKp46 and NKp44 were shown to interact with complement factor P [8] and platelet-derived growth factor (PDGF)-DD [9], respectively. However, the major cellular ligands that induce NK cell cytotoxicity via NKp46 and NKp44 are still unknown. 2B4 recognizes CD48, which is widely expressed in the hematopoietic system.

Freshly isolated, resting human NK cells cannot be efficiently activated by stimulation of one single activating receptor, with the exception of CD16. They need to be triggered by a combination of at least two activating receptors, with some combinations being more efficient than others [10,11]. Preactivated NK cells can respond to the stimulation of one single receptor, but also here synergisms between activating receptors can enhance the activating signal [12]. The signals of the activating NK cell receptors can be counterbalanced by inhibitory receptors such as the human killer cell Ig-like receptors (KIR) or the NKG2A/CD94 heterodimer that bind to classical MHC class I and the non-classical MHC class I HLA-E, respectively [13]. Many of the activating receptors such as the NCRs and CD16 signal via ITAM-containing partner chains. CD16, NKp30, and NKp46 interact with CD3-ζ and/or FcyR while NKp44 signals via DAP12. NKGD2 signals via the DAP10 adapter that contains an Ig tail tyrosine (ITT)-like phosphorylation motif [14] and 2B4, like all SLAM-family receptors possesses immunoreceptor tyrosine-based switch motifs (ITSM) in its cytoplasmatic tail [15]. All these tyrosine-based motifs rely on Src-family kinases for their phosphorylation when they are triggered and then induce an activating signaling cascade [16].

Vav1 has to be phosphorylated to perform its function as a guanine exchange factor (GEF) for small Rho family guanosine triphosphatases (GTPases) [17,18]. One result of this activation cascade is the induction of actin reorganization at the immunological synapse, mediating the recruitment of activating receptors to the contact area between the NK cell and the target cell [5]. Phosphoinositide 3-kinase (PI3K) is another signaling mediator known to be associated with activating NK cell receptors such as NKG2D, resulting in the phosphorylation and activation of the central mediator AKT [19]. Downstream of PI3K and other activating mediators the MAP kinase pathway is triggered, including ERK activation, which is required for degranulation [20]. Inhibitory signals can interfere with activating signals by stimulating the activity of the protein phosphatases SHP-1 and SHP-2 or the inositol phosphatase SHIP. These phosphatases are recruited to the immunoreceptor tyrosine-based inhibition motifs (ITIM) of inhibitory NK cell receptors when these receptors are engaged [21]. The balance of all activating and inhibitory signals combined determines whether the NK cells executes its effector functions or is not activated [22].

Due to the large variety of surface receptors, their overlapping signaling pathways and the even larger set of ligands, analyzing the decision-making process of NK cells is very complex. While we can determine the overall positive or negative effect of a given receptor on NK cell activity, investigating NK cell regulation in a quantitative way is more challenging. However, such a quantitative understanding of NK cell activation will be essential to understand and to predict the activity of NK cells against tumor cells. Possible methods to study this include antibody-mediated crosslinking, which allows to stimulate single receptors in isolation or defined combinations of receptors and to titrate the signal by varying the amount of antibodies. Using target cells transfected with specific ligands is less artificial but it is more difficult to titrate the amount of signal and the system can be affected by unknown receptor–ligand interactions. As an alternative we chose to functionalize latex beads that have approximately the same size as lymphocytes. Specific antibodies directed against activating NK cell receptors were coated onto these beads to quantitatively investigate the respective receptors for their effect on NK cell activation.

Results

Quantification of the number of antibodies coated onto latex beads

In order to study NK cell activation via defined surface receptors in a quantitative fashion, we established a reductionist approach by using aliphatic amine latex beads with a diameter of 10 µm. These cell-sized beads have a high density of amine groups attached to the terminus of an aliphatic six-carbon spacer arm. We established a protocol to achieve the most efficient loading of antibodies onto the beads by using a chemical crosslinker to covalently link antibodies to the bead surface. The spacer arm between the bead and the attached antibodies should minimize difficulties with steric hindrance and conformation since it has a greater freedom to rotate. With a flow cytometry-based quantification kit the exact amounts of antibodies—called antibody binding capacity (ABC)—bound to the beads was calculated. The kit contains setup and calibration beads with 5 bead populations, each bearing different but defined numbers of monoclonal antibody molecules on their surface (Supporting Information Fig. 1A and B). These values were used to generate a calibration curve to quantify the number of antibodies bound to our beads (Supporting Information Fig. 1D). Beads coated with any mouse IgG or cells stained with mouse IgG can be quantified with the QIFI kit. Using different antibody amounts for loading, we created beads with different epitope quantities on their surface (Supporting Information Fig. 1C and D). Due to the covalent bond between bead and protein these beads were very stable, enabling us to store beads from different loading experiments and to combine them later in co-incubation experiments with NK cells.

NK cells interact with beads coated with antibody against activating receptors

First, we investigated the interaction of NK cells with different beads by microscopy (Fig. 1A). We used cultured human NK cells
Figure 1. Interaction of NK cells with antibody-coated latex beads. (A) Cultured NK cells were incubated for 3h with beads coated with BSA (left) or αNKG2D (right) in the presence of an αCD107a antibody (magenta). Arrows indicate close interactions. Images were acquired with 40x magnification. Scale bar = 10 μm. (B) After 3 h co-incubation, cultured NK cells and the beads were analyzed by flow cytometry. Gates for beads and NK cells were defined in the scatter plot. Beads coated with antibodies against activating receptors (αNKG2D, α2B4), a non-binding (IgG) or a non-activating (αHLA) antibody were used. The NK cell receptors 2B4 (C), NKG2D (D), and NKG2A (E) were stained on the surface of NK cells and beads. 2B4 was stained with a polyclonal antibody and NKG2D with a different clone than that used for coating of the beads to avoid that epitope blocking interferes with the staining. One representative experiment of 2 for A and 2 for IgG and αNKG2D and 3 for αHLA and α2B4 coated beads with one individual donor per experiment in B–E is shown.

and either beads that were loaded with a high ABC of αNKG2D or beads loaded only with BSA (no Ab) and monitored them by microscopy. Beads that were coated with BSA only showed few contacts to NK cells and the contact area was small. If the antibody against the activating receptor NKG2D was present on the beads we observed many intense contacts. By including a fluorescently labeled antibody against CD107a in the assay, we confirmed that the αNKG2D coated beads induced specific degranulation of the NK cells. By flow cytometry, we observed that, depending on the activation of the NK cells, surface proteins were transferred from the NK cells to the beads (Fig. 1B–E). We gated on beads or NK cells only, employing their different scatter properties (Fig. 1B). Most prominently, the receptor that was stimulated by the coated antibody could be stained on the beads after 3 h of co-incubation, but also other surface receptors were transferred in an activation dependent manner. 2B4 could be stained on the beads, not only when 2B4 was triggered but also after NKG2D stimulation. However, only when 2B4 coated beads were used this caused a notable decrease of 2B4 on the NK cells (Fig. 1C). A considerable amount of NKG2D was transferred to the beads upon NKG2D stimulation, but also 2B4 stimulation caused a slight increase of NKG2D on the beads (Fig. 1D). Even the inhibitory receptor NKG2A, that is highly expressed on NK cells, could be stained faintly on the beads in an activation dependent way (Fig. 1E). This indicates that NK cells engage in a close contact with beads that are coated with an antibody against activating NK cell receptors. They did not interact in that way with beads that were coated with control antibody that does not bind (IgG) or not activate NK cells (αHLA). When the contact is severed, not only the receptor, but probably entire pieces of the NK cell membrane containing the different proteins were transferred to the beads.

Beads trigger activating signaling events in NK cells

Co-incubation of cultured NK cells with antibody coated beads also induced typical activating signaling events (Fig. 2). Phosphorylation of Vav1 was strongest upon NKG2D stimulation but also occurred with beads against the other activating receptors. All activating receptors except 2B4 caused AKT and Erk 1/2 phosphorylation. The CD3-ζ chain, a signaling adapter interacting with different activating NK receptors was strongly phosphorylated upon CD16 stimulation and showed only weak phosphorylation in the Nkp30 and Nkp46 stimulated samples. This confirms that the stimulation of defined activating NK cell receptors by antibody-coated beads induces known signaling pathways of these receptors.
Quantifying NK cell degranulation

After confirming the specific interaction of NK cells with the coated beads, we wanted to investigate the activation of the cultured NK cells in a quantitative way. Beads coated with different amounts of antibodies directed against 2B4, CD16, NKG2D, Nkp30, Nkp44, or Nkp46 were mixed with NK cells at a 1:1 ratio and NK cell degranulation was quantified by flow cytometry after 3 h. Since in some samples many NK cells were in conjugates with beads at the time of analysis, we had to adopt a gating strategy that included those NK cells while excluding single beads. The percentage of degranulating NK cells as a function of the ABC of the indicated antibodies is shown (Fig. 3A). Stimulation of all tested receptors could induce NK cell activation as evident by degranulation. For the stimulation of CD16, NKG2D, Nkp30, and Nkp46 already a few thousand antibodies per bead were sufficient to induce significant degranulation and maximum degranulation was reached at about 50 000 antibodies per bead. In contrast, stimulation of 2B4 induced only very weak NK cell degranulation, whereas αNKP44 coated beads reached maximal NK cell activation at about 10 000 antibodies per bead. To better compare the different stimulations, we determined the $B_{\text{max}}$ (maximal % of degranulating NK cells) and the $K_D$ (ABC that induces half-maximal degranulation) by fitting a curve to the values of each donor. For all antibodies a hyperbolic curve was fitted except for $\alpha$NKP44 that showed much better fit to a sigmoidal curve. A mean curve for all donors is displayed (Fig. 3A). Beads stimulating CD16, Nkp30, and Nkp46 induced the highest degranulation ($B_{\text{max}}$). While we could only obtain a maximal loading of the beads with about 30 000 ABC of the α2B4 antibody, this stimulation induced significantly lower degranulation compared to all other activating receptors tested (Fig. 3B). The low stimulation by $\alpha$2B4 was also apparent when comparing the $K_D$ values (Fig. 3C). The $K_D$ of 2B4 stimulation had a very high variability between donors, but on average it was much higher than all other receptors tested. NKP44 had the lowest $K_D$, indicating a very rapid increase of degranulation with low ABCs, which might also be a reason, why the hyperbolic curve did not fit properly.

To better judge the reactivity of the different receptors, we quantified the amounts of receptors per NK cell (Fig. 3D). For involves...
most receptors we detected about 1000–2000 epitopes per cell with the exception of CD16, which was expressed at higher levels (about 5000 epitopes per cell). Receptors with lower surface expression such as NKG2D and NKp46 could induce high degranulation, whereas 2B4 did not activate strong responses, although we detected higher expression levels compared to NKG2D and NKp46. This demonstrates that the expression level did not correlate with the activity of a given surface receptor but that the efficiency of the different receptors is more complex than pure stoichiometry. In the same assay, we also analyzed the number of conjugates formed between NK cells and beads (Supporting Information Fig. 2). For some receptors, the percentage of cells in conjugates was very heterogeneous between donors. The conjugate formation was quite low but nevertheless activation specific, since beads coated with the binding but non-activating αHLA class I antibody showed much fewer conjugates between beads and NK cells. The maximum number of conjugates was reached at very low ABCs (about 5000–10 000 ABC per bead). 2B4 stimulation caused a similar number of conjugates compared to other receptors despite inducing low degranulation. Therefore, the potential of a receptor to induce degranulation did not strictly correlate with its ability to induce conjugate formation.

Induction of polyfunctional NK cells

To further discriminate the activating potential of the different NK cell receptors, we examined not only the degranulation of cytotoxic granules, but also the production of the chemokine MIP-1β and the cytokine IFN-γ in response to beads loaded with the different antibodies. We were able to distinguish NK cells that showed activation of one, two, or all three effector functions at once by sequential gating (Supporting Information Fig. 3). Figure 4A shows the percentage of non-responding cells and cells conducting the different effector functions in response to beads loaded with high amounts of the respective antibody. The majority of responding cells was single positive for CD107a. Double positive cells were either MIP-1β+ and CD107a+ or IFN–γ+ and CD107a+. Triple positive cells were rare and almost exclusively caused by the stimulation of ITAM coupled receptors. Stimulation of CD16 and NKp30 caused a significant amount of triple positive cells (Fig. 4B). We performed the same experiment using beads with different ABCs of CD16 or NKG2D antibody. Notably, once the maximal activation of the NK cells was reached, the ratio between single, double, and triple-functional NK cells did not change with increasing ABC (Fig. 4C and D).
experiments with a total of six donors and for fresh NK cells data from
their target, stimulation of surface receptors with antibody
Coating the beads with a recombinant NK cell ligand

degranulation of fresh NK cells was analyzed after stimulation with
NKp30 stimulation by itself induced considerable activation. Next,
development was achieved by CD16 stimulation. Surprisingly also
individual donors showed a high variability in reaction strength, but as expected, the strongest degran-
activation with increasing ABCs (Fig. 6C), resulting in a similar $B_{\max}$
and a lower $K_D$ than stimulation with antibody coated beads (Fig.
6D and E).

Discussion

Using functionalized latex beads coated with specific antibodies
directed against activating NK cell receptors, we were able to quan-
titatively investigate individual receptors for their effect on NK cell
activation. To generate reliable results, we quantified the ABC on
each batch of beads and controlled the stability by repeatedly
quantifying the same batch. The coated beads proved to be very
stable. Even after several weeks of storage, we did not detect any
major changes of the ABCs. Therefore, it was possible to make
larger batches of coated beads, determine the ABC, and to select
beads to cover the desired range of ABCs for different experiments.
The loading efficiency of different antibodies varied significantly,
despite optimization of the protocol. This may be due to differ-
ences in the formulation of the antibodies or their amino acid
composition. Additionally, some steric issues can affect the func-
tionality of the antibodies coated to the beads. Depending on the
reactive groups that are accessible, antibodies can be linked to the
bead in different places and in different orientations, possibly
affecting the antigen binding part and the ABC quantification in
different ways. Due to differences in the amino acid sequence and
the functionality of the streptavidin coated beads and compare
them to the beads coated directly with antibody, we repeated the
degranulation experiments with NKG2D antibody coated onto the
beads (Fig. 6A) and included streptavidin coated beads loaded with
a biotinylated version of the same antibody (Fig. 6B). NK

cells degranulated depending on the ABCs of the beads as observed
before, but the biotinylated NKG2D antibody was less efficient in
stimulating the cells, showing a flatter curve resulting in similar
$B_{\max}$ but a much higher $K_D$ (Fig. 6D and E). Possibly the undirected
biotinylation of the antibody reduces the efficiency of the interac-
tion with the receptor. Interestingly, stimulation with recombinant
MICA was very efficient, showing a steeper increase of degranu-
lation with increasing ABCs (Fig. 6C), resulting in a similar $B_{\max}$
and a lower $K_D$ than stimulation with antibody coated beads (Fig.
6D and E).

Stimulation of freshly isolated, resting NK cells

So far, we analyzed the responses of cultured, IL-2 activated NK
cells, since they respond strongly to stimulation of single activating
receptors. For freshly isolated, resting NK cells it is described that
they need co-activation of defined receptor combinations for effi-
cient stimulation. An exception is CD16, which by itself can induce
strong activation of resting cells. To test this, freshly isolated NK
cells were stimulated with beads coated high ABCs of differ-
ent antibodies and degranulation was analyzed after 3 h (Support-
ing Information Fig. 4). Individual donors showed a high variabil-
ity in reaction strength, but as expected, the strongest degran-
ulation was achieved by CD16 stimulation. Surprisingly also
NKp30 stimulation by itself induced considerable activation. Next,
degranulation of fresh NK cells was analyzed after stimulation with
different ABCs of aCD16 (Fig. 5B) and compared to cultured NK
cells (Fig. 5A). Fresh NK cells showed a higher inter-individual
variation in response strength as maximal degranulation ($B_{\max}$)
differed considerably between experiments (Fig. 5C). $B_{\max}$ was
more homogeneous and slightly higher in cultured NK cells. On
the other hand, the $K_D$ was lower in fresh NK cells (Fig. 5D), indi-
cating that fresh NK cells are more sensitive and already show a
stronger response with lower ABCs. However, the differences of
both parameters were not very high, demonstrating that activation
via CD16 was extremely efficient also in fresh NK cells.

Coating the beads with a recombinant NK cell ligand

Since antibodies are usually generated to have a very high affini-
ity to their target, stimulation of surface receptors with antibody
coated beads might cause different effects than the stimulation
with natural ligands. We chose the NKG2D receptor to compare
these two ways of stimulation (Fig. 6). First, we produced and
purified a recombinant ligand for NKG2D. We used the extra-
cellular domain of MICA fused to a his-tag for purification and
an Avi-Tag for site-specific biotinylation [23]. This biotinylation
at the C-terminus of the protein enabled us to bind the ligand
in its correct orientation to beads that were coated with strepta-
vidin. We coated the latex beads using a constant concentration
of streptavidin, then used different amounts of biotinylated pro-
tein to generate beads with different ABCs. In order to control the
functionality of the streptavidin coated beads and compare
them to the beads coated directly with antibody, we repeated the
degranulation experiments with NKG2D antibody coated onto the
beads (Fig. 6A) and included streptavidin coated beads loaded with
a biotinylated version of the same antibody (Fig. 6B). NK

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lation with increasing ABCs (Fig. 6C), resulting in a similar $B_{\max}$
and a lower $K_D$ than stimulation with antibody coated beads (Fig.
6D and E).
Figure 6. Comparison of the degranulation of cultured NK cells upon stimulation with NKG2D antibody and the NKG2D ligand MICA. Latex beads were either coated directly with αNKG2D (A) or beads were first coated with streptavidin, and then incubated with biotinylated αNKG2D (B) or recombinant MICA containing a biotinylation at the C-terminus (C) and used in degranulation assays with cultured NK cells. After 3 h coincubation the NK cells were analyzed for degranulation by flow cytometry. The amount of CD107a positive NK cells is displayed as a function of the ABC. To the values of each donor (displayed as different symbols), a hyperbolic curve was fitted as before, but $B_{\text{max}}$ had to be constrained to 100% in the case of one donor stimulated with biotinylated αNKG2D. The parameters $B_{\text{max}}$ (D) and $K_D$ (E) are displayed for all donors including the mean value (horizontal lines) with SD and were used to compare the efficiency of the stimulation. For αNKG2D data of four donors tested in four individual experiments, for αNKG2D biotinylated data from two experiments with three donors and for MICA four donors tested in three experiments is shown.

not mobile and does not allow for clustering of ligands as it was observed for target cells [24]. We found in our experiments that NK cells make activation-specific contacts with the beads. We observed a lower amount of conjugates with a binding but non-activating antibody and even activation dependent transfer of receptors to the beads, similar to conjugates with tumor cells [25]. In our microscopic observations, we also observed activation-specific contacts. Some NK cells spread over beads that induced their activation, making contact to about half of the beads’ surface. But many stable contacts had a much smaller “synapse” area with an estimated diameter of $\approx 5 \mu m$, resulting in a contact area of $20 \mu m^2$. While cells responded already to low amounts of antibodies on the beads, for optimal activation the amount of antibodies per bead had to be higher than the amount of receptors on the NK cell. For the engagement of CD16, we determined a $K_D$ of about 8000 antibodies per bead for half-maximal stimulation. This translates to 25 antibodies/$\mu m^2$ bead surface. At an expression level of $\approx 5000$ receptors/NK cell the surface density of CD16 is about 16 receptors/$\mu m^2$. However, it is known that activating receptors can cluster at the synapse, resulting in much higher receptor concentrations. Assuming that all CD16 would cluster in the contact area, the density would increase to 250 receptors/$\mu m^2$, which is ten times higher than the antibody density of 25 antibodies/$\mu m^2$ for half-maximal stimulation. Therefore, while for an initial contact the density of antibodies needed to be higher than the receptor density on the NK cell, once the receptor clusters at the IS only a fraction of these receptors may need to be engaged for efficient NK cell activation.

Our results not only demonstrated quantitative differences between the receptors, but also some qualitative differences. 2B4 did not induce much NK cell degranulation, even in a range of ABCs where all the other activating receptors already induced clear degranulation. Vav1 was phosphorylated but other activating phosphorylation events were not induced by stimulation with α2B4 coated beads. However, the amounts of conjugates of α2B4 coated beads with NK cells were comparable to other activating receptors. Therefore, 2B4 stimulation seems to be a better stimulus for NK cell adhesion than for NK cell degranulation, which is in line with the fact that 2B4 can very efficiently induce inside-out signaling of integrins, resulting in strong activation of the binding activity of LFA-1 [10, 26].

Cells from different donors showed surprisingly homogeneous reaction patterns in response to the stimulation of a certain receptor. NKp30 was the only exception, which might be due to the expression of functionally different isoforms [27], which could differ between donors. NKG2D stimulation induced strong degranulation, but not many polyfunctional NK cells. Noticeable amounts of triple positive cells were induced only by stimulation of ITAM-coupled receptors such as CD16, NKp30, or NKp44. Interestingly, the ratio of single, double, and triple positive cells was not increased by further stimulation once the ABC for maximal stimulation was reached. It will be interesting to test if the fraction of polyfunctional cells can be increased, for example, by stimulating receptor combinations [7]. NKp44 was very efficient in stimulating NK cell degranulation and showed the lowest $K_D$. Interestingly, only small changes in the ABC were necessary to go from almost...
no NK cell degranulation to a maximal response. This switch-like behavior was unique among the receptors tested. It should be noted that NKp44 is not expressed by resting NK cells and its expression is only induced upon cytokine-mediated activation of NK cells [28]. This may explain why this receptor requires very little stimulation to fully activate NK cells.

Triggering only one receptor, we were able to stimulate freshly isolated, resting NK cells via CD16, confirming previous results [10, 11]. The response upon CD16 triggering was more heterogeneous between donors when using resting NK cells. This may be related to an in vivo pre-activation of NK cells in some donors or due to the expression of CD16 variants with higher Fc binding affinity [29]. Alternatively, NK cells may be inherently “tuned” for different responsiveness as previously shown for NK2D-mediated NK cell activation [30]. Interestingly, the sensitivity of NK cells toward CD16 stimulation was not much different between resting and cultured cells and resting NK cells even showed a trend for a lower $K_D$. This suggests that NK cells can efficiently react against antibody-coated target cells even in the absence of additional stimulation. Surprisingly, we also saw some responses of resting NK cells upon NKp30 stimulation, which demonstrates that CD16 is not the only receptor that can trigger the activation of resting NK cells.

While using antibody-coated beads enabled us to efficiently and specifically trigger different activating receptors on NK cells, future studies will have to be performed using known ligands for the different receptors. For the NK2D ligand MICA, we used a recombinant protein that was expressed in a human cell line to provide the correct posttranslational modifications and that contained an enzymatic biotinylation site at the transmembrane terminus [31,32]. This guaranteed that all molecules were bound in their correct orientation to the bead. Additionally, the binding between biotin and streptavidin is almost as stable as a covalent bond [33,34]. Stimulation of NK cells with MICA coated beads was very efficient. Compared to αNK2D fewer molecules of MICA were needed for half maximal stimulation, demonstrating that the natural ligand is more efficient in stimulating the NK2D receptor compared to antibodies. We were using the allele MICA 07, which is known for its high affinity to the receptor [35], but with our method we can now systematically compare different alleles and ligands for the same receptor. Additionally, when using recombinant biotinylated ligands, we can coat various combinations of ligands to one bead and quantify them separately. Ultimately, this will enable us to comprehensively analyze how individual NK cell receptors contribute to NK cell activation in a quantitative fashion in order to better predict NK cell responses against tumors and pathogen infected cells.

Material and Methods

Primary cells

PBMC were isolated from whole blood of healthy donors using lymphocyte separation medium (LSM) from PAN. Isolation of NK cells was done using the Dynabeads® Untouched™ Human NK Cell-Kit from Invitrogen. After isolation cells were either rested overnight in medium (IMDM medium with Glutamax™ by Gibco, 10 % FCS, 1% P/S) and used the next day as fresh NK cells, or taken in culture. For culture, the NK cells were put in 96 round bottom plates at a density of ~2 Mio/mL with 10° irradiated feeder cells (K562mb15-41BBL) per plate. In addition, 200 U/mL IL-2 and 100 ng/mL IL-21 were added. After one week, cells were re-stimulated with fresh feeder cells. Medium supplemented with 100 U/mL IL-2 and from the third week of culture with 50 U/mL IL-15 was exchanged twice a week and cells were split 1:2 if necessary.

Cell lines and production of recombinant protein

The cell line 293F FreeStyle™ (ThermoFisher Scientific) was used to produce recombinant proteins. Cells were maintained in F17 medium (Gibco®) supplemented with 0.05% Pluronic F-68 and 8 mM GlutaMAX™ as specified in the manual. Transfection of pMICA07-Avi-His (a kind gift of Prof. Dr. Alexander Steinle, Goethe-University, Frankfurt am Main), pDisplay BirA-ER, and pDisplay sBirA (both kindly provided by Dr. Jessica Hartmann, Paul-Ehrlich-Institut, Langen) using PEI was done simultaneously. To guarantee efficient biotinylation of the Avi-Tag, the medium was supplemented with 10 μM biotin after the transfection. The protein was purified from the supernatant after 4 days using Ni-NTA-beads.

Bead coating

Aliphatic Amine Latex Beads (Life Technologies) with a size of 10 μm were washed twice and resuspended in MES buffer (0.025 M) with a pH of 4.8 containing 0.1% SDS at 33 Mio beads/mL before the cross linker EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) was added to a final concentration of 10 mg/mL and incubated for 30 min on a tube rotator at room temperature. The beads were washed once and then the protein to be loaded (antibodies in various concentrations from 10 to 2000 ng or 0.5 μg streptavidin (Biolegend) per 1 Mio beads) was added in MES buffer without SDS for 1 h at room temperature. Antibodies used for coating all were mouse monoclonal IgGs: 2B4 (C1.7, IgG1), HLA class I (W6/32, IgG2a), NKp30 (p30-15, IgG1), NKp44 (P44-8, IgG1) (produced in our lab), CD16 (3G8, IgG1, BioLegend), IgG1 (MOPC 21, IgG1, Sigma-Aldrich), NK2D (149810, IgG1, R&D Systems), and Nkp46 (9E2, IgG1, BioLegend). Then 20 μg BSA per 1 Mio beads were added to all samples to saturate all reactive groups and the beads were incubated for additional 30 min. The beads were washed twice with PBS. If the beads were coated directly with antibodies, they were resuspended in storage buffer (PBS, 0.1 % Glycine, 0.1 % Sodium azide) at a density of 10 Mio per mL at this point. Streptavidin coated beads were incubated with different dilutions of the purified biotinylated ligands or biotinylated antibody for 20 min at room temperature, washed, and then resuspended in storage buffer. Beads were stored at 4°C and retained the coupled protein over several weeks.
Microscopy

Approximately 100 000 NK cells were placed in an eight-well chamber slide in medium without phenol red containing 1:100 CD107a-PE-Cy5 (BD Pharmigen™), mixed 1:1 with the respective beads and incubated at 37°C. Images were acquired with an EVOS FL Auto using an objective with 40× magnification. Phase contrast images and the Cy5 signal were merged using ImageJ software.

Western Blot

NK cells were mixed at a 1:1 ratio with the respective beads on ice, centrifuged at 500 × g for 20 s and put in a 37°C water bath for 2 min for stimulation. Afterward, cells were lysed and gel electrophoresis and western blotting were performed as described [36]. Phosphorylation of proteins of interest were detected with AKT, pS473 (D9E, Cell Signaling), CD3-z, pY174 (EP510Y, Epitomics), and actin (polyclonal, Sigma) antibodies. Phosphorylation of proteins of interest were detected with electrophoresis and western blotting were performed as described [36]. Phosphorylation of proteins of interest were detected with AKT, pS473 (D9E, Cell Signaling), CD3-z, pY174 (EP510Y, Epitomics), and actin (polyclonal, Sigma) was used as loading control.

Flow cytometry and bead quantification

Flow cytometry was performed according to published procedures [37]. Cells and beads were acquired on a BD LSR Fortessa, data were analyzed using FlowJo software (v10). The number of antibodies or ligands per bead was quantified with the QIFIKIT® (Dako) according to the manual. Antibody coated beads were stained directly with the same dilution (1:200) of secondary antibody (goat anti mouse-PE, Dianova) as the beads from the QIFIKIT. Beads loaded with MICA were incubated with 5 µg/mL anti-MICA (R&D systems) before staining with goat anti-mouse PE. Antibodies used for additional stainings were NKG2D-PE (1D11, BioLegend), NKG2A-PE (Z199, Beckman Coulter), goat anti human 2B4 biotinylated (polyclonal, R&D systems), and streptavidin-PE (Dianova).

Degranulation assay

Degranulation was measured after 3 h of coincubation of NK cells and beads at a 1:1 ratio in NK cell medium at 37°C with CD107a-PE-Cy5 (BD Pharmigen™).

Polymorphic NK cell assay

NK cells and beads were coincubated at a 1:1 ratio in NK cell medium at 37°C with CD107a-APC (1:200) (Biolegend) for 3 h, then 5 µg/mL Brefeldin A was added to prevent secretion of chemokines and cytokines and incubation was continued for another 3 h. The samples were stained with CD45-AF700 (1:500) (BD Pharmigen™) then fixed with 2 % paraformaldehyde for 10 min at room temperature and permeabilized using BD FACS Permeabilising solution 2. The cells were stained with the antibodies IFN-γ-PE (1:50) (Immunotools) and MIP-1β-FITC (1:20) (BD Pharmigen™).

Statistical Analysis

Statistical Analysis and calculations were performed using Graph Pad Prism Version 7. The tests performed are indicated in the figure legends. Significant P-values are indicated by asterisks: * indicates P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001. To quantify degranulation a hyperbolic curve with the formula \( y = \frac{B_{\text{max}} \times x}{K_y + x} \) or a sigmoidal curve with the formula \( y = \frac{B_{\text{max}}}{1 + \left(\frac{x}{K_y}\right)^2} \) was fitted to the results of each donor. The mean values of these parameters were then used to generate mean curves.

Conflict of Interest: The authors have no financial or commercial conflicts of interest.

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Abbreviations: ABC, antibody binding capacity; Bmax: maximal % of degranulating NK cells; KD: ABC inducing half-maximal degranulation; MIP-1β: macrophage inflammatory protein 1β.

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