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KHYG-1 and NK-92 represent different subtypes of LFA-1-mediated NK cell adhesiveness

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1. ABSTRACT

Novel cancer cellular therapy approaches involving long-term *ex vivo* IL-2 stimulated highly cytotoxic natural killer (NK) cells are emerging. However, adhesion properties of such NK cells are not very well understood. Herein, we describe the novel observation of permanently activated $alpha_L beta_2$ integrin leukocyte function-associated antigen (LFA)-1 adhesion receptor in long-term IL-2 activated NK cells and the permanent NK cell lines KHYG-1 and NK-92. We show that such cytokine activated NK effectors constitutively adhered to the LFA-1-ligand ICAM-1, whereas binding to the lower affinity ligand ICAM-3 required additional exogenous activating conditions. The results demonstrate an extended conformation and an intermediate affinity state for the LFA-1 population expressed by the NK cells. Interestingly, adhesion to ICAM-1 or K562 induced pronounced cell spreading in KHYG-1, but not in NK-92, and partially in long-term IL-2 stimulated primary NK cells. It is conceivable that such differential adhesion characteristics may impact motility potential of such NK effectors with relevance to clinical tumor targeting. KHYG-1 could be a useful model in planning future targeted therapeutic approaches involving NK effectors with augmented functions.

2. INTRODUCTION

Natural killer (NK) cells are important mediators of the innate immune response and possess the intrinsic capacity to spontaneously eliminate transformed cells, such as malignant or pathogen-infected cells. NK cell cytotoxicity does not require antigen priming, but instead is tightly regulated by an array of surface receptors with activating and inhibitory functions (1). NK cells can potentially directly target a broad range of malignancies, which renders them attractive candidates for adoptive immunotherapy (AIT; (2-5). Importantly, NK cell therapy does not seem to be afflicted with graft-versus-host disease (6, 7). Novel promising AIT approaches are emerging, which involve Good-Manufacturing Practices (GMP)-compliant long-term IL-2 stimulation and expansion of NK cells, to significantly increase effector potency and numbers (8-10). Another new concept involves clinical application of the permanent NK cell line NK-92 (ZRx101, ZelleRx; (11), which was originally isolated from a patient suffering from non-Hodgkin's lymphoma (12). NK-92 is highly cytotoxic and can be easily expanded and maintained *in vitro*. First Phase I/II trials were conducted using irradiated NK-92, to avoid engraftment of the transformed cells, which demonstrated safety for the procedure (3, 11, 13). In addition, another highly cytotoxic

permanent NK cell line, KHYG-1 (14), has been recently discussed as a potential future cell therapy candidate (3, 15, 16). Such innovative developments aim to overcome limitations, such as low cell numbers and moderate cytolytic potency of effectors, of traditional approaches. These involved short-term IL-2 stimulated Lymphokine Activated Killer (LAK) cells (17). However, not much is known about adhesion dynamics of long-term IL-2-stimulated NK effectors, generated for clinical applications. Adhesion characteristics may impact their migratory potential (extravasation) and ability of tumor targeting.

An important receptor with central roles in leukocyte adhesion is the heterodimeric integrin leukocyte function-associated antigen (LFA)-1 (subunits $alpha_L beta_2$, CD11a/CD18). The central paradigm holds that activation of LFA-1 is imperative for ligand binding. This is concluded from studies with alpha-beta T cells, where the integrin occurs in a low affinity bent form. LFA-1 activation could be experimentally achieved with divalent cations, such as magnesium (Mg^{2+}; (18), activating antibody KIM185, or both (19, 20). Such treatments give rise to an extended intermediate or extended high-affinity LFA-1 receptor (21, 22). The intermediate affinity LFA-1 has a closed “headpiece” that carries the ligand binding 'I' domain, whereas the high affinity LFA-1 is characterized by an open headpiece (23). These two affinity states could be distinguished through ligand binding experiments. Intermediate affinity LFA-1 binds effectively to intercellular adhesion molecule (ICAM)-1, but not to the lower affinity ligand ICAM-3, whereas high affinity LFA-1 binds both ligands (24). However, it has been recently demonstrated that in NK cells ICAM-1 could directly trigger major steps involved in tumor target lysis (25-27). Since LFA-1 was not further activated in these experiments, the results suggested a constitutively active LFA-1 receptor expressed on NK cells. To advance our understanding of spontaneous LFA-1-mediated adhesion in NK cells with relevance to clinical applications, we examined LFA-mediated adhesion characteristics of long-term IL-2 activated NK cells and the permanent IL-2 dependent model NK cell lines NK-92 and KHYG-1.

3. MATERIAL AND METHODS

3.1. Cell lines and culture

NK-92 (12), K562, and Jurkat cell lines were purchased from the American Type Culture Collection (ATCC, Manassass, VA) and KHYG-1 (14) from the Human Science Research Resources Bank (JCRB0156, HSRRB, Tokyo, Japan). NK-92 was cultured in XVivo 10 medium (Biowhittaker, Walkerville, MD), supplemented with 1.8 mM L-Serine, 0.6 mM L-Asparagine (Sigma-Aldrich, Saint Louis, MO), 3 mM L-Glutamine (Invitrogen, Carlsbad, CA), and 450 U/ml recombinant IL-2 (rhIL2, Proleukin, Chiron, Emeryville, CA; (28) and 10% (v/v) heat-inactivated (hi) Fetal Bovine Serum (FBS, HyClone, South Logan, UT); K562 and Jurkat were maintained in RPMI 1640 medium (Invitrogen), 2 mM L-Glutamine, and 10% (v/v) hi FBS, and KHYG-1 was cultured in RPMI 1640 medium, 2 mM L-Glutamine, 20% (v/v) hi FBS, 2 mM sodiumpyruvate (Invitrogen), and 450 U/ml rhIL-2 (Chiron). All cell lines tested negative for mycoplasma contamination using 4',6-Diamidino-2-Phenylindole (DAPI; DAPI containing antifade, EMD Biosciences, San Diego, CA) staining and confocal imaging (Zeiss, LSM 510 Meta confocal microscope).

3.2. NK cell and peripheral blood lymphocyte (PBL) isolation and culture

Buffy coats were obtained from healthy donors after informed consent. Peripheral blood mononuclear cells (PBMCs) were purified with density gradient centrifugation (Ficoll-Paque Plus, GE Healthcare, Waukesha, WI). Peripheral Blood Lymphocytes (PBLs) were obtained as the non-adherent PBMC fraction after 1-1.5 hrs plastic adherence (37°C, 5% CO_2 and humidified atmosphere). For efficient NK cell isolation from PBMC, a red blood cell lysis step was included using 1-2 consecutive standard red blood cell lyses in Ammonium Chloride Potassium (ACK) buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA), to minimize red blood cell contamination. Briefly, cell pellets were resuspended in at least 5 volumes of cold ACK buffer, thoroughly mixed for 1 min. by vortexing, and incubated for 6 min. at RT. Reactions were stopped by adding 8-10 volumes of wash buffer (0.5% hiFBS, 2 mM Ethylene Diamine Tetra-acetic Acid (EDTA) in phosphate-buffered-saline (PBS, pH 7.4), followed by centrifugation. 'Untouched' $CD3^-CD56^+$ human NK cells were then magnetically enriched (NK cell isolation kit, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions and allowed to rest over night (o/n) at 37°C, 5% CO_2, in humidified atmosphere. Routinely, purities were at least 90% $CD56^+CD3^-$ NK cells were achieved as determined by antibody surface staining (flow cytometry). Short-term culture was supported by RPMI1640, 2mM L-Glutamine and 10% hiFBS (1% Penicillin-Streptomycin (Invitrogen) and 225 U/ml rhIL-2 (where applicable) or AIM V® media (Invitrogen, containing glutamine and penicillin/streptomycin), 5% human AB serum (Lonza, Basel, Switzerland), 5% hiFBS (Hyclone), and IL-2 (1000-2000 U/ml).

For long-term expansion (several weeks as stated in the text), purified NK cells or LAK cells were cultured in AIM V®-conditions (see above; AIM V® is available as GMP-grade). After long-term cultures, NK cells formed a major part of the LAK population and were further purified with the NK isolation kit (Miltenyi) to achieve at least 90% purity. For NK cell $CD8^+/CD8^-$ subset isolation, the $CD56^+CD8^+/CD8^-$ NK cell isolation kit (Miltenyi) was used according to the manufacturer’s instructions. Importantly, the CD8-expressing NK cell subset has been previously found to contain a significant fraction of cells with low-density (dim) marker expression (29-31), which renders purity assessment by Flow cytometry (CD8-staining) challenging. However, the magnetic selection kit allows positive $CD8^+$-NK cell isolation and depleted $CD8^-$-NK cells were purified further in an additional column-depletion step. For the 2 donor samples studied here (NK53 and NK54; imaging), we assessed % $CD56^+CD3^-$-NK cell content in purified $CD56^+CD8^+$ and $CD56^+CD8^-$ NK cell subsets first, which ranged from 96%-99% $CD56^+CD3^-$ purities. As expected, positively selected $CD8^+$-NK cells contained a dim expressing fraction, however 98-99% of

the $CD56^{+}CD8^{-}$ subset NK cells were CD8-negative compared to matching isotype controls.

3.3. Reagents

Monoclonal antibody (mAb) anti-LFA-1 (CD11a), clone MHM24 (anti-α_L crosslinking studies was obtained from A. J. McMichael (John Radcliffe Hospital, Oxford, U.K.) and in addition mAb anti-CD18 (clone IB4; $beta_2$ integrin heterodimer-specific and function-blocking; (32, 33) was used for blocking studies; mAbs KIM127 and KIM185 (anti-$beta_2$, activating) were from M. K. Robinson (Celltech, Slough, UK); mAb (FITC)-labeled anti-CD11a, (FITC)-labeled anti-CD3, (PE)-conjugated anti-CD2 and (PE)-labeled anti CD56 were purchased from BD Biosciences (BD, San Jose, CA); ICAM-1/Fc and ICAM-3/Fc were prepared as described previously (34); (FITC)-labeled Annexin V was purchased from Biovision, Inc. (Mountain View, CA), 7-amino-actinomycin D (7AAD) were from Sigma-Aldrich, and FITC-conjugated Fluorospheres from Beckman Coulter (BC; Fullerton, CA, USA).

3.4. ICAM-1 and ICAM-3 binding assays and imaging

Adhesion assays on immobilized ICAMs were performed for all NK cells as previously described (24). Briefly, polysorb microtitre wells (Nunc, Rosklide, Denmark) were coated with 0.5 μg of goat anti-human IgG Fc-specific (Sigma) in 50 mM bicarbonate buffer (pH 9.2) o/n at 4°C. Non-specific binding sites were blocked with 0.5% (w/v) bovine serum albumin (BSA) in PBS for 1 h at RT. Wells were washed once with PBS, and each well was then coated with recombinant human ICAM-Fc (50 ng) in PBS for 2 h at RT. Wells were washed twice with wash buffer (RPMI containing 5% HI-FBS and 10 mM HEPES, pH 7.4) before use. Cells were labeled with 3.0 mM 2'7'-bis-(2-carboxyethyl)-5-(and-6) carboxyfluorescein-acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, OR) in wash buffer for 20 min. at 37°C. Each well was seeded with ~ 2 x 10^4 labeled cells in medium and incubated for 30 min. at 37°C in a humidified 5% CO_2 incubator. Unbound cells were removed by washing the wells 2X in wash buffer. The percentage of cells that adhered to each well was determined by measuring the fluorescence signal using a fluorescence plate reader (FL600; Bio-Tek Instruments, Winooski, VT). Activation of LFA-1 was achieved by incubating cells in medium containing either Mg/EGTA (5 mM $MgCl_2$ and 1.5 mM EGTA) or mAb KIM185 (10 μg/mL) or both. The mAb MHM24 (10 μg/mL) or mAb IB4 were used as blocking mAbs to verify LFA-1-mediated adhesion specificity.

For cell imaging experiments, cells that adhered to ICAM-coated wells or BSA only (as control) were imaged with an inverted phase contrast light microscope (20X objective; Olympus, 1X70) fitted with a CoolSNAP Monochrome camera. Images were presented using the MetaMorph® software (Molecular Devices, Downingtown, PA, USA).

3.5. Microscopic analyses: effector-target contact

For effector-target contact studies, effector cells (NK-92, KHYG-1) were labeled with the fluorescent cytoplasmic dye CellTracker™ Green (5-chloromethylfluorescein diacetate, CMFDA) or remained unlabeled (primary NK cells) and target K562 cells were labeled with Cell Tracker™ Orange (CMRA) according to the protocols provided by the manufacturer. Effector and target cells were then mixed at 5:1 E:T ratios, 1-2x10^5 E and 2x10^4 T, in 200 μl assay media (RPMI1640, 2 mM L-glutamine, 0.1% BSA and 225 U/ml rhIL-2), briefly centrifuged (1000 rpm, 1 min.) and coincubated at 37°C, 5% CO_2, humidified atmosphere for 10 min. Cells were pelleted, resuspended in 50-100 μl of assay medium, spotted on poly-lysine-coated slides and incubated for 30 min. at 37°C, 5% CO_2, humidified atmosphere to allow cells to settle on the slide. The medium was then carefully soaked off; cells were covered with DAPI-containing antifade reagent (EMD Biosciences)/coverslip and analyzed by confocal microscopy using the Zeiss LSM510 Meta or the Nikon A1Rsi (Nikon, Japan) confocal microscope. Quantitative analysis of homotypic NK-NK adhesions on target cell synapses (NK-92 and KHYG-1) was performed by randomly scanning the slides and imaging sufficient conjugates (100-200) for final analyses; for each effector NK cell only one target-conjugate was counted to not bias the results (in some cases NK cells formed conjugates with more than one target simultaneously).

3.6. Flow cytometry

Cells were stained in PBS-buffer, containing 1% (v/v) FBS, 5 mM EDTA (Flow buffer), and incubated according to the instructions provided with the respective antibodies. In general, incubations were 20 min. at room temperature (RT) in a 30-100 μl volume, using matching mouse isotype controls (BD). Analyses were carried out on viable cells, identified either by 7AAD (Sigma-Aldrich) staining or by forward - side scatter properties. Acquisition (10000 cells/ reaction) was performed using a flow cytometer FACS Calibur (BD), calibrated with CaliBRITE Beads (BD), and data were analyzed with CellQuest Software or FlowJo (BD). Mean fluorescence intensities (MFIs) to assess receptor expression levels were measured using CellQuest software.

3.7. Flow cytometric cytotoxicity assay

The flow cytometric cytotoxicity assay is based on previously published methods (15, 35). Briefly, effector NK cells and target cells (*e.g.*100,000 effectors and 10,000 targets per well for a 10:1 ratio; maximum 2x10^5 cells/well) were incubated in triplicate in 96-well U-bottom plates, in RPMI 1640, 2 mM L-glutamine, 0.1% BSA and 225 U/ml rIL-2 for 4 h at 37°C, 5% CO_2 in humidified atmosphere. As controls for the 0-hour time point, the effector and target cells were plated in parallel at identical concentrations (duplicate), but separated from each other, and pooled together at the time of harvest. Targets were labeled with the red fluorescent membrane dye PKH26 (Sigma-Aldrich) for 100% detection. At the end of incubation, cells were washed 2X with FACS-buffer and stained with Annexin V-FITC and 7AAD (according to the manufacturer's instructions). Flow cytometric acquisition

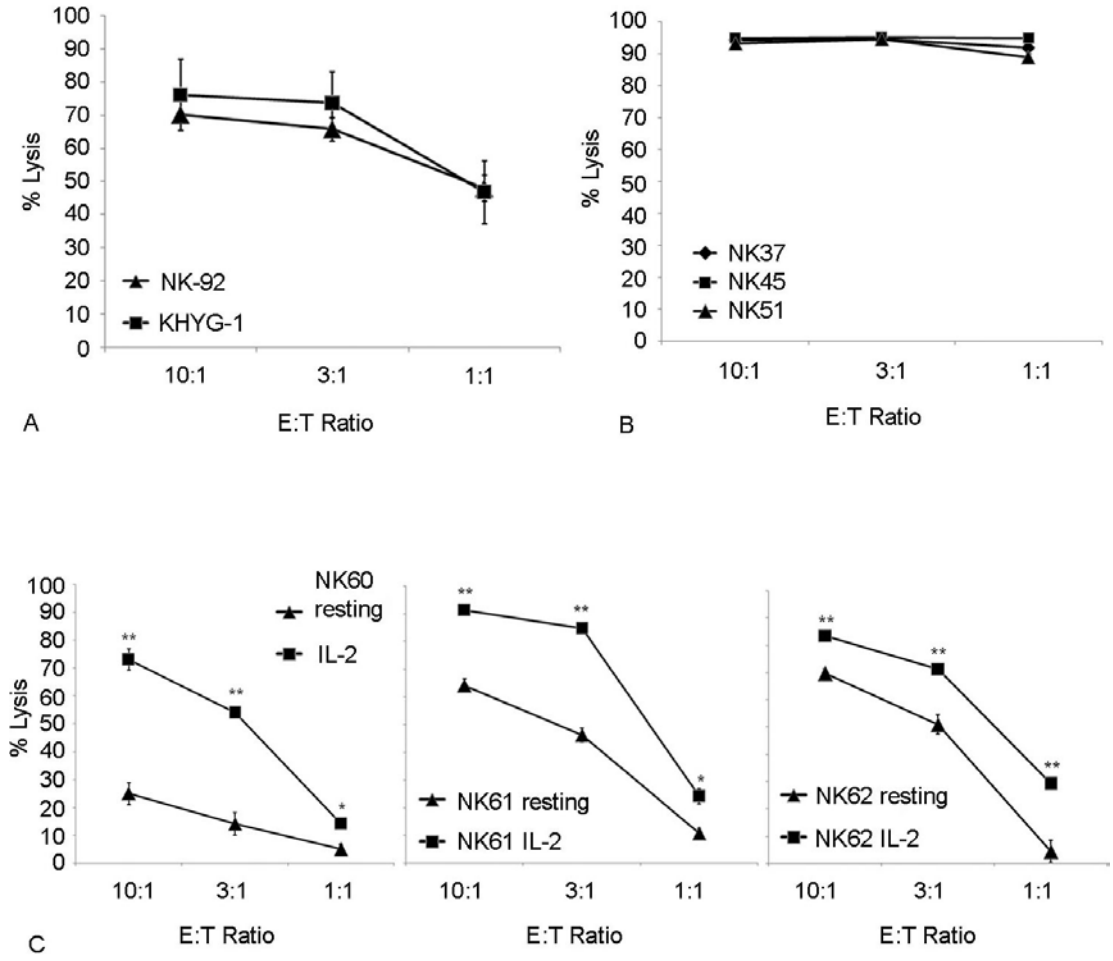

Figure 1. NK-92, KHYG-1 and long-term IL-2 activated NK cells are highly cytotoxic. (A) Flow cytometric cytotoxicity assay comparing NK-92 and KHYG-1 lysis of K562 in 4 hr assays at effector to target (E:T) ratios 10:1, 3:1, and 1:1; error bars represent SEM, 3 independent experiments (each experiment in triplicate). (B) Flow cytometric cytotoxicity assays with long-term IL-2 stimulated NK cells (3 donors, NK37, NK45, and NK51 at Day 21 of culture, $CD56^{+}CD3^{-}$ purity was at least 90%; each experiment in triplicate) against K562 in 4 hr assays. (C) Flow cytometric cytotoxicity assays with freshly isolated NK cells before and after o/n IL-2 stimulation (3 donors; $CD56^{+}CD3^{-}$ NK cell purity: NK60 = 73%, NK61 = 92%, and NK62 = 95%; each experiment in triplicate) against K562 in 4 hr assays. Statistical significance is indicated, where applicable * = p-value was smaller than 0.05; ** = p-value was smaller than 0.01 (Student t-test for each E:T ratio).

was standardized with Flow-Count™ Fluorospheres (BC). Viable target counts were determined by excluding debris, Annexin V- and 7AAD-positive target cells. Percent lysis was calculated according to the formula: [(mean viable target counts at time 0 h – mean viable target counts at time point (4 h)/ mean viable target counts at time 0 h) x 100] and the standard error of the mean (SEM) was calculated (15, 35).

3.8. Statistical analyses

Statistical analyses were performed using Excel software. Statistical significance for the results was determined by Analysis of Variance (ANOVA) or Student's T-test (t-test assuming equal variances), expressed as p-values and indicated in the text or Figures/Figure legends.

4. RESULTS

4.1. Long-term IL-2 stimulated NK cells/cell lines have superior cytotoxicity

We first measured cytotoxicity of KHYG-1, NK-92, and long-term IL-2 stimulated NK cells against the standard target cell line K562 (proof of principle) in 4 hr assays in a sensitive flow cytometric cytotoxicity assay (15, 35). Under the culture conditions chosen in this study, cytotoxicity was comparably high for KHYG-1 and NK-92. Figure 1A shows efficient target cell lysis, even at the low effector:target (E:T) ratio 1:1, with 47% +/-9% SEM by KHYG-1 and 48% +/- 4% SEM by NK-92. Long-term IL-2 stimulated primary NK cells, which were kept in culture for several weeks, even exceeded cytotoxicity of the cell lines, for 3 donors tested, with 92% +/-1% SEM (NK37), 95%

+/- 0.2% SEM (NK45), and 89% +/-1% SEM (NK51) K562 lysis at the lowest 1:1 ratio (Figure 1B). In comparison, cytotoxicity of purified resting NK cells (3 donors) remained markedly below the potential of the long-term IL-2 stimulated NK cells/ cell lines, even after short-term IL-2 induction. This was most pronounced at lower E:T ratios, with K562 lysis of 14% +/-2% SEM (NK60), 24% +/- 3% SEM (NK61), and 29% +/- 2% SEM (NK62) at E:T 1:1 *e.g.* (Figure 1C). These findings demonstrate the powerful effect of long-term cytokine stimulation on NK cell potency with great relevance to cancer immunotherapy.

4.2. LFA-1 is constitutively activated in NK effectors

We next assessed binding potential of KHYG-1, NK-92, and primary NK cells to immobilized ICAM-1 (CD54) ligand. As previously shown, the T-ALL cell line MOLT-4 requires activation with Mg/EGTA or activating mAb KIM 185 for significant ICAM-1 binding of cells (20). Similarly, the T-ALL cell line Jurkat used as a control in these experiments also required activation with Mg/EGTA or mAb KIM 185 for significant ICAM-1 binding (Figure 2A). The same was found for both, resting and o/n IL-2 stimulated PBL cells. However, in KHYG-1 60% +/-1% SD cells bound to ICAM-1 and in NK-92 68% +/-1% SD cells bound to ICAM-1 without additional activation (Figure 2A). In KHYG-1, cell binding to ICAM-1 increased after activation with Mg/EGTA to 94% +/-0% SD and after activation with mAb KIM185 to 91% +/- 0% SD. Similarly, in NK-92, binding to ICAM-1 increased to 84% +/- 5% SD after activation with Mg/EGTA and to 82% +/-3% SD after activation with mAb KIM185. In Jurkat, NK-92, and KHYG-1 addition of anti-LFA-1 blocking mAb (anti $alpha_L$), clone MHM24, almost completely abrogated ICAM-1 binding (maximum 3% remaining ligand binding), which demonstrated specificity for LFA-1receptor with ICAM-1 ligand interactions. However, a minor background activity remained in PBLs, which could not be blocked by clone MHM24. This was likely accounted by NK cells in the population. In fact, purified NK cells showed significant spontaneous ICAM-1 binding with 44% +/- 1% SD for resting and even higher for o/n IL-2 stimulated NK cells showed 55% +/- 1% SD (Figure 2B). Consistent with the findings for PBL described above, MHM24 blocked spontaneous ICAM-1 adherence only to a minor degree. However, blocking was achieved with function-blocking mAb anti-CD18 (IB4). This indicated involvement of other beta2 integrins, such as macrophage antigen-1 (CD11b), in ICAM-1 adherence of primary NK cells (Figure 2B; (36). In subsequent experiments involving primary NK cells mAb IB4 was therefore used for blocking,

Another LFA-1 ligand, ICAM-3 has been shown to bind the integrin with lower affinity than ICAM-1 in T cells. LFA-1 induction by a single agent, such as Mg/EGTA or KIM185, was sufficient to induce ICAM-1 binding, whereas 2 stimulants were required to allow binding to ICAM3 (20, 37). As shown in Figure 2C, KHYG-1 and NK-92 did not significantly bind to ICAM-3 without further activation, different from their ICAM-1 binding ability. However, ICAM-3 binding required activation with only a single agent, either Mg/EGTA or KIM185, in NK-92 and KHYG-1 and was further enhanced through combined Mg/EGTA and KIM185 stimulation. Binding specificity was confirmed as before using MHM24 as LFA-1 blocking control. Similarly, long-term IL-2 stimulated NK cells from 3 donors showed significant spontaneous adhesion to ICAM-1, on average 62% +/-4% SD, which increased to 73% +/-0.5% SD after activation with Mg/EGTA and to 69% +/- 1% SD after activation with mAb KIM185 (Figure 2D). Similar to the cell lines, a single agent was sufficient to induce ICAM-3 ligand binding. Combined Mg/EGTA and KIM185 stimulation did not further increase binding efficiency. Addition of β2-integrin function blocking mAB (anti-CD18, clone IB4) almost completely abrogated ICAM-1 and ICAM-3 binding in the experiments (Figure 2D). Furthermore, NK-92, KHYG-1, and long-term IL-2 stimulated NK cells (3 donors) showed constitutive binding of the activation reporter mAB KIM127. KIM127 shows selective binding to an extended LFA-1 conformation in which the antibody epitope is exposed as represented in intermediate or high affinity LFA-1 activation states (38, 39). Taken together, these findings reveal expression of an intermediate affinity LFA-1 in NK-92, KHYG-1, and long-term IL-2 stimulated NK effectors.

4.3. ICAM-1 binding induces cell spreading in KHYG-1, but not in NK-92 and partially in long-term IL-2 activated primary NK cells

Despite the observed similarities for the LFA-1 activation states in KHYG-1 and NK-92, phase contrast imaging revealed formation of pronounced focal adhesions or cell spreading in KHYG-1 (Figure 3A), but not in NK-92 (Figure 3B) upon ICAM-1 binding . Similar morphological changes have been previously described for ICAM-1 stimulated T cells (40) and phorbol ester stimulated PBLs (41). In long-term IL-2 stimulated NK cells, a subset of NK cells formed pronounced focal adhesions upon ICAM-1 adhesion (Figure 4). We hypothesized that the 'spreading' phenotype could represent a feature of the CD8 alpha-alpha -expressing NK cell subset, since KHYG-1, but not NK-92, is CD8 alpha-alpha-positive (data not shown; (14, 42). To test this hypothesis, $CD8^+$- and $CD8^-$-NK cells were purified from long-term IL-2 expanded NK cells (2 donors) and subjected to ICAM-1-adhesion. However, pronounced focal adhesions occurred partially in both NK cell subsets and were not restricted to one or the other (data not shown). The 'spreading phenotype' is consequently not a feature of the CD8-expressing NK cell subset. Instead, a proportion of long-term cytokine stimulated NK cells, responded to ICAM-1-induction with cell spreading, independent of CD8-expression.

To test relevance of such ICAM-1 induced morphological changes in a tumor-target contact setting, we performed experiments with the ICAM-1 expressing chronic myelogenous leukemia cell line K562 (41). Consistent with the findings for ICAM-1 ligand binding, KHYG-1, but not NK-92 formed focal adhesions in the target contact setting (Figure 5A). Furthermore, KHYG-1, but not NK-92, formed homotypic adhesions with

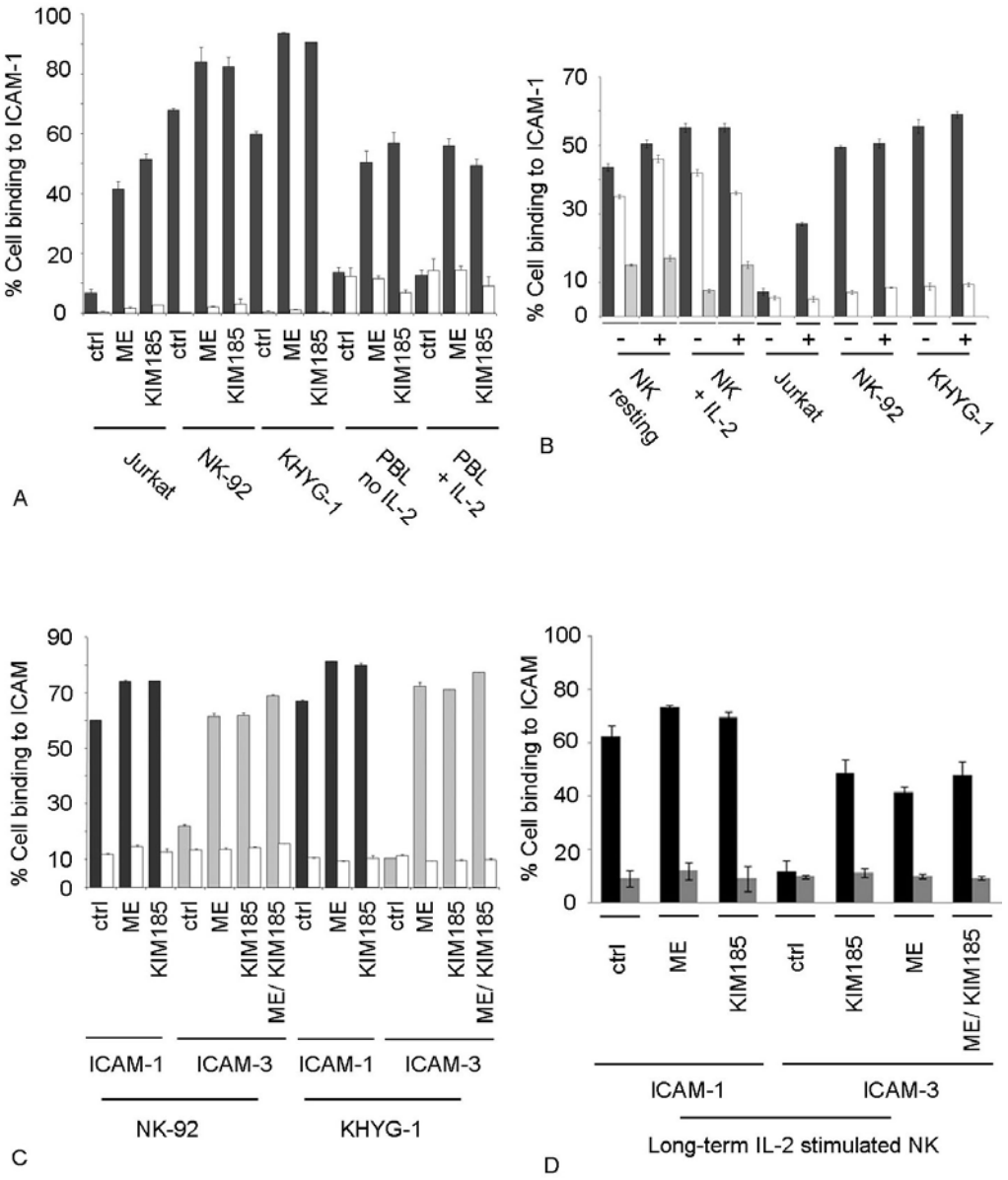

Figure 2. LFA-1 is constitutively activated in NK-92, KHYG-1, and NK cells (A) Cell binding assays to immobilized LFA-1-ligand, ICAM-1, comparing unstimulated control (ctrl), Mg/EGTA (ME) or mAb KIM185 (KIM185) stimulated KHYG-1 and NK-92 to Jurkat and resting or o/n IL-2 stimulated PBLs; black bars represent percent cell-binding to ICAM-1 and white bars ligand binding after mAb LFA-1 (clone MHM24) blocking; error bars represent SD (calculated from triplicates). (B) ICAM-1 binding comparing KHYG-1 and NK-92 to resting or o/n IL-2 stimulated purified primary NK cells and Jurkat (representative for 2 independent experiments); black bars represent percent ICAM-1 binding, white bars ligand binding after mAb anti-LFA-1 (clone MHM24) blocking, and gray bars represent percent ICAM-1 binding after mAb anti-CD18 (clone IB4) blocking; + = Mg/EGTA stimulation, - = unstimulated control cells; error bars represent SD calculated from triplicates. (C) Cell binding assays to immobilized ICAM ligands, comparing KHYG-1 and NK-92 cells; black bars represent percent ICAM-1 ligand binding, gray bars represent ICAM-3 ligand binding, and white bars ligand binding after mAb anti-LFA-1 (clone MHM24) blocking; error bars represent SD (triplicates). (D) Cell binding assays with long-term IL-2 stimulated NK from 3 different donors (NK24, Day 33, NK23, Day 26, and NK39, Day 28) to immobilized ICAM-1 or ICAM-3; black bars represent percent cell binding to ICAM-1- or ICAM-3, gray bars represent ligand binding after IB4 antibody blocking; error bars represent SD calculated from 3 different donor samples (each experiment performed in triplicate). The majority of NK cells express the Fc-receptor (FcgammaRIII) CD16, which was blocked in the experiments with heat-inactivated pooled human AB serum to exclude unspecific binding of ICAM-Fc, however KHYG-1 and NK-92 were CD16 negative (42).

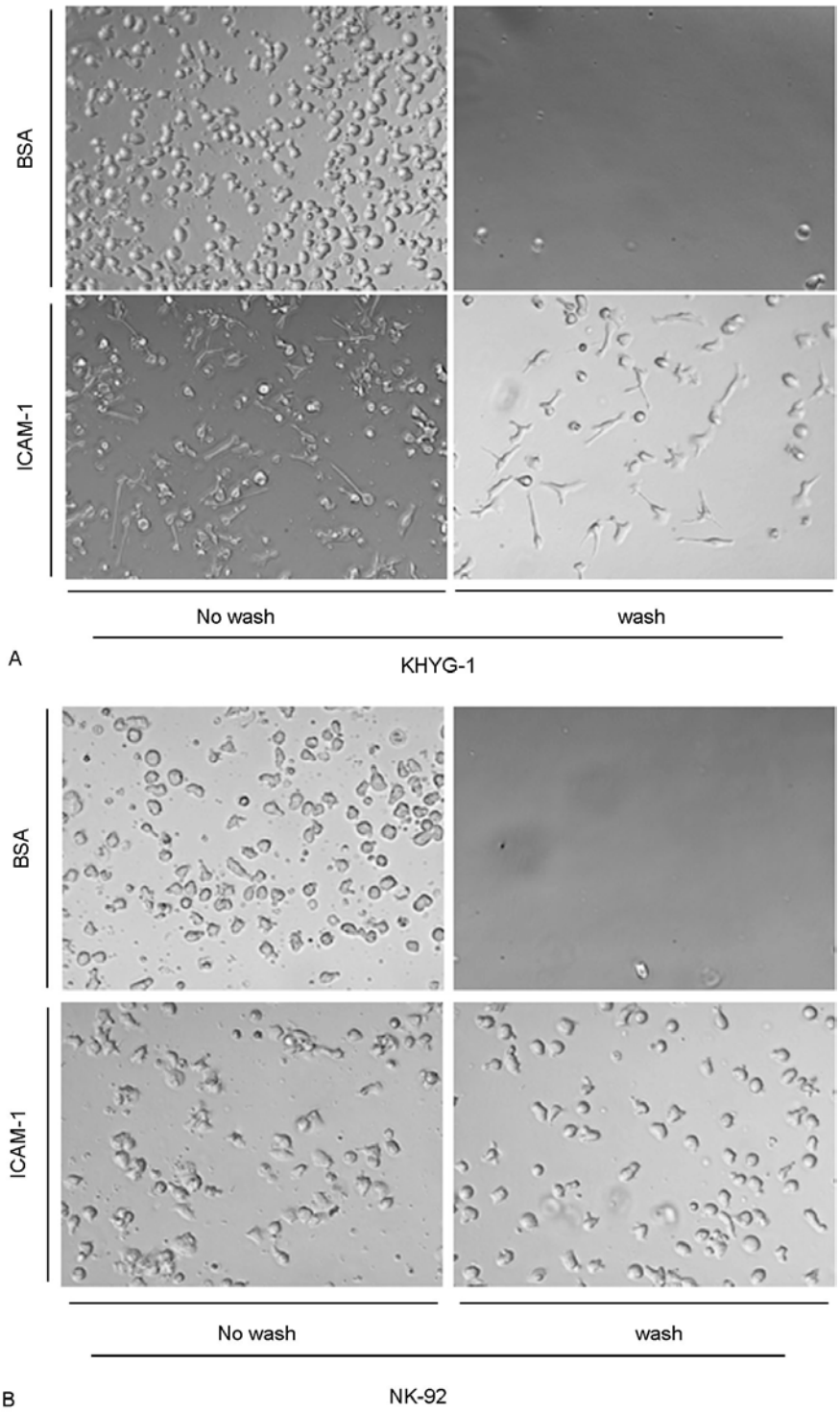

Figure 3. ICAM-1 binding induces cell spreading in KHYG-1, but not in NK-92. Phase contrast imaging of NK-92 and KHYG-1 before and after ICAM-1 binding, 'BSA only' as control, before (no wash) and after (wash) final washes (representative for 3 independent experiments).

neighbouring effector cells at the target synapse, a feature similarly described for primary CD8 alpha-alpha-expressing NK cells (31). A functional role for such NK-NK connections, mediated through CD8-MHC class I-interactions, in protection of NK cells from apoptosis after target contact (cytotoxicity induced cell death) has been discussed previously (31). Evaluation of 2 independent experiments and a total number of 211 KHYG-1-K562- and

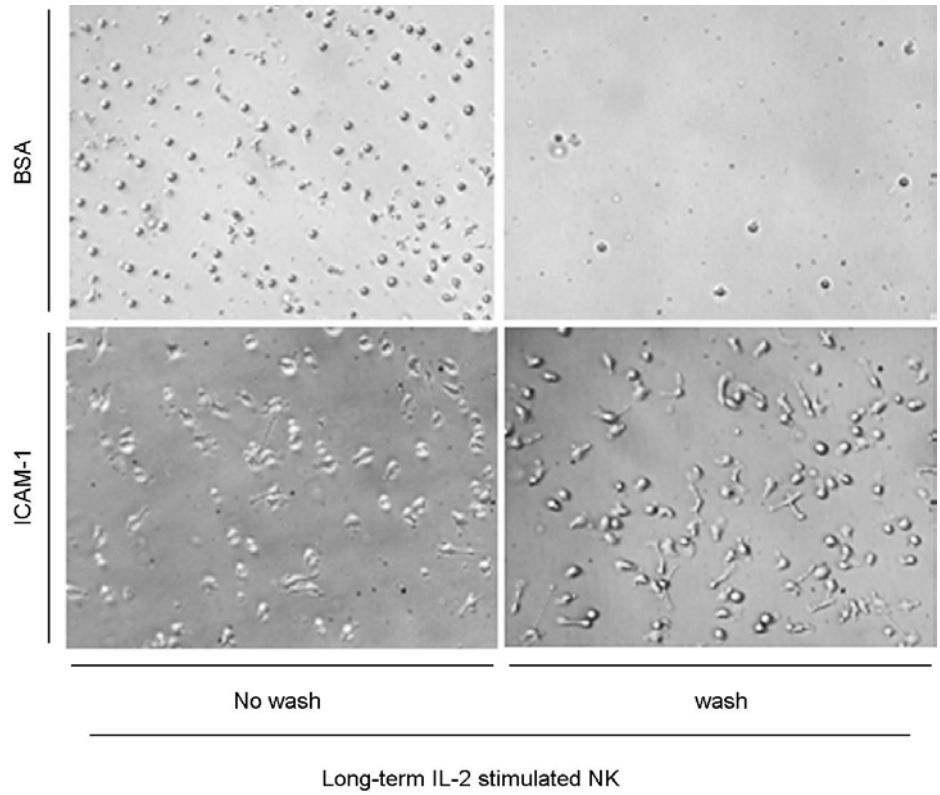

Figure 4. ICAM-1 binding induces cell spreading partially in long-term IL-2 stimulated NK cells. Phase contrast imaging of long-term IL-2 stimulated NK cells (NK39, Day 34, purity was at least 90%; representative for 3 donor samples) before and after ICAM-1 binding; 'BSA only' = control, before (no wash) and after (wash) final washes.

243 NK-92-K562-conjugates respectively (K562 does not express MHC class I), revealed that 61% (72/100 and 57/111) of KHYG-1 cells formed homotypic adhesions at the synapse compared to 12% NK-92 cells (13/105 and 16/138).

Consistent with the findings above, cell spreading could also be observed in a proportion of long-term IL-2 stimulated NK cells upon target encounter (Figure 5B). However, statistical evaluation for the occurrence of such 'spreaders' or their isolation is hampered by the lack of knowledge for other distinguishing features at this point. A cell line like KHYG-1 is therefore potentially of great value to further elucidating characteristics of such NK cell effectors.

5. DISCUSSION

Novel developments in cell manipulations, including efficient specific cell isolation and design of improved culture cocktails, which effectively support NK cell expansion, enable clinical trial protocols involving long-term expanded NK cells. A major benefit of such an approach lies in the generation of increased numbers of highly potent effectors for clinical scale-up (8, 43). Here we focused on adhesion characteristics mediated through the central leukocyte adhesion receptor LFA-1, of long-term IL-2 stimulated NK cells and the permanent NK cell lines NK-92 and KHYG-1. It had been previously established that LFA-1 could directly trigger all major steps required for lysis of a target cell in IL-2 stimulated NK cells by direct engagement of its ligand ICAM-1 (25, 27). These previous findings suggested an activated LFA-1 on these cells, because stimulation with divalent cations or specific activation antibodies as in alpha-beta T cells was not required for ICAM-1 ligand binding. Here we found in ligand binding assays that KHYG-1, NK-92, and primary NK cells directly adhered to ICAM-1, with increased binding after IL-2 stimulation. These ICAM-1 binding results were consistent with an activated state of LFA-1 in NK cells. The observed increased binding of IL-2 stimulated NK cells was potentially also reflected in increased cytotoxicity of IL-2 stimulated NK cells compared to resting NK cells (Figure 1; (44). However, long-term IL-2 stimulated NK cells, which exceeded cytotoxicity of KHYG-1 and NK-92, bound ICAM-1 with an efficiency that was comparable to the binding efficiency of the cell lines. Furthermore, roles for other IL-2 inducible NK cell activating receptors, such as NKp44, are well established in NK cell cytotoxicity (45) and in NK-92 an essential contributing role for CD44 in cytotoxicity has been previously established (46).

To further characterize LFA-1 affinity states in long-term IL-2 activated NK effectors, we exploited another LFA-1 ligand, ICAM-3 (19). This ligand is known to bind to LFA-1 with significantly less affinity (9-fold

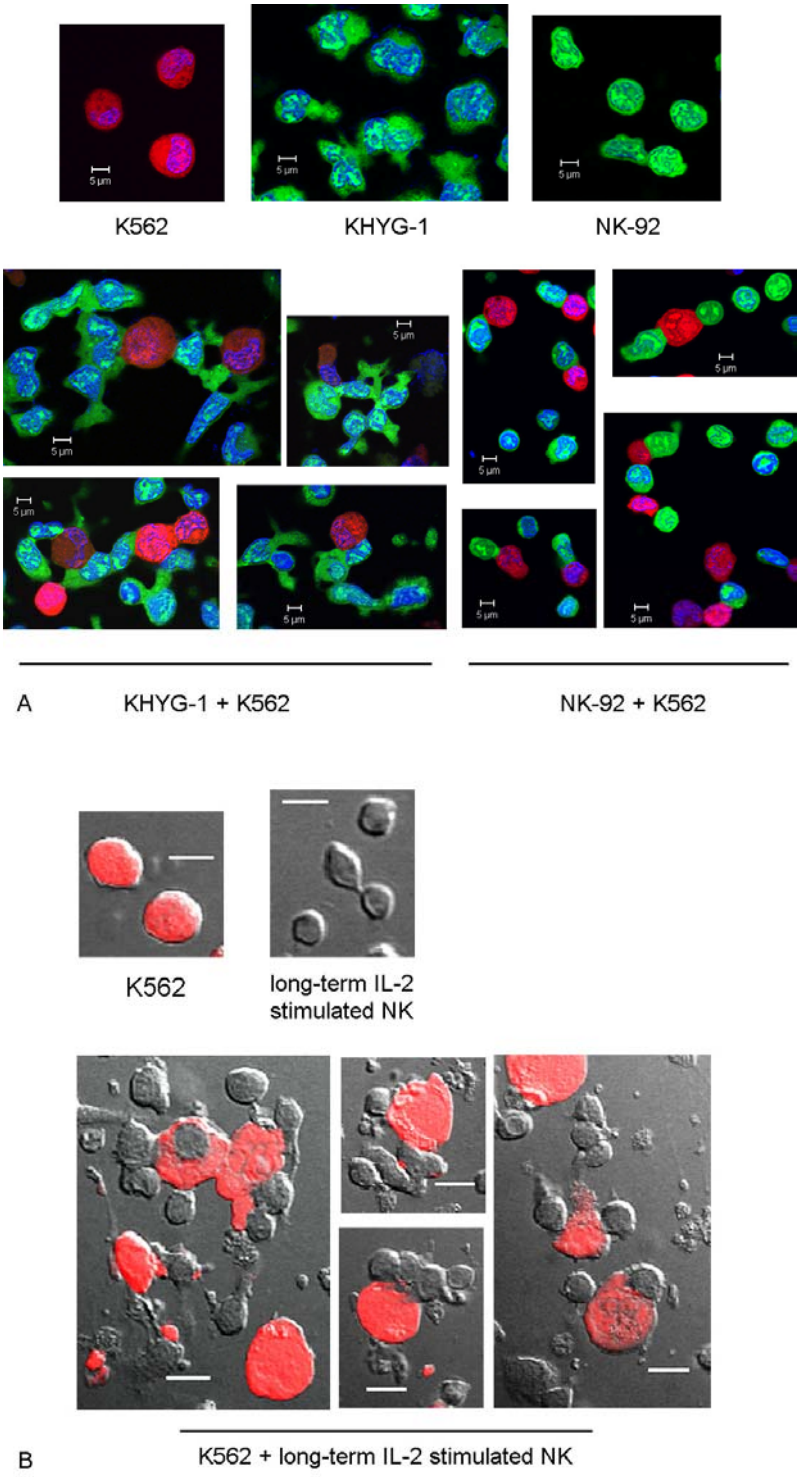

Figure 5. KHYG-1, NK-92 show different morphology upon K562 tumor target contact. (A) Tumor target contact experiments: confocal imaging of KHYG-1 and NK-92 (CellTracker Green, green) before (upper panel) and after (lower panel) K562 (CellTracker Orange, red) encounter; cells were counterstained with nuclear stain DAPI; bars measure 5 micrometers (representative for 2 independent experiments). (B) Confocal imaging of tumor target contacts involving K562 targets (CellTracker Orange, red) and long-term IL-2 stimulated NK cells (NK38, Day 28, at least 90% purity, representative for 2 independent experiments/donor samples; Differential Interference Contrast Microscopy, DIC); bars measure 10 micrometers.

lower) than ICAM-1 and required a high affinity LFA-1 conformation for binding (24, 47). Collectively our results strongly argued for an extended conformation and an intermediate affinity activation state for LFA-1 in KHYG-1, NK-92, and long-term IL-2 stimulated primary NK cells. Our data did not support a constitutive high affinity activation state in such NK effectors. Interestingly, studies with neutrophils had revealed that a transition from intermediate affinity, which supported rolling, to high affinity resulted in neutrophil arrest (48). Permanently high affinity activation of LFA-1 in NK cells would likely lead to arrest of the cells and consequently pose an obstacle to execution of other NK cell functions, such as cytotoxicity. Importantly, constitutive intermediate affinity states for LFA-1 were also inducible in T cells upon transfection with the cytoplasmic cytoskeleton protein talin-1 (49). It was shown that Talin-1 directly promoted activation of the integrin and a conformational change toward intermediate affinity (50, 51). We speculate that Talin-1 may be a candidate protein involved in maintaining constitutively activated LFA-1 in NK effectors (52). However, additional future molecular studies are necessary to elucidate underlying functional mechanisms.

Imaging studies revealed that ICAM-1 binding pronounced cell spreading in KHYG-1, but not in NK-92, and in a subset of long-term IL-2 stimulated NK cells. Cell spreading is a prelude to extravasation (53), and in fact it had been reported that KHYG-1 was isolated from an aggressive NK cell leukemia, with the leukemic NK cells invading all organs of the patient's body (14). It is unlikely that such 'spreader' NK cells were adherent-NK (A-NK) cells, which have been previously described for their ability to adhere to plastic upon short-term IL-2 stimulation (54, 55). The adherent NK subset in our study did not adhere to plastic, since cells were easily washed off from the wells, unless they were coated with ICAM-1 (Figures 3 and 4). Furthermore, KHYG-1 did not show tendency in culture to adhere to the bottom of the plastic flask.

We speculate that NK 'spreaders' may potentially be more primed to extravasate and to target inflamed or malignant tissue *in vivo*. This feature could be of important clinical relevance. It has been demonstrated in T cells that a decrease in mRNA levels of the Ras homolog gene family member H (RhoH) induced increased cell adhesion and spreading (56). However, isolation of the 'spreader' NK cells for molecular analyses may be challenging. Studies with NK-92 and KHYG-1 as model cell lines may be useful to further elucidate mechanisms involved in the differential adhesion response of IL-2 activated NK cells in the future.

In conclusion, in long-term IL-2 stimulated NK cells/ cell lines the integrin LFA-1 was identified to be constitutively activated in an intermediate affinity state. Strikingly, ICAM-1 stimulation induced cell spreading in KHYG-1 and in a population of long-term IL-2 stimulated NK cells, but not in NK-92. We hypothesize that NK cell 'spreaders' and 'non-spreaders' may differ in their motility potential. Administration of a 'spreader' NK type could be particularly advantageous for tumor targeting, for example in metastasized tumors, in adoptive immunotherapy.

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