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2B4 Engagement Mediates Rapid LFA-1 and Actin-Dependent NK Cell Adhesion to Tumor Cells as Measured by Single Cell Force Spectroscopy

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Adhesion to tumor target cells is essential for initiation and execution of cellular cytotoxicity. In this study, we use single cell force spectroscopy to determine the exact biophysical values of the interaction forces between NK cells and tumor cells. We show that engagement of the activating NK cell receptor 2B4 can rapidly mediate an increase in the force necessary to separate NK cells from tumor cells, starting from 1 nN and increasing to 3 nN after only 120 s tumor cell contact. This early adhesion was mediated by the integrin LFA-1 and dependent on the actin cytoskeleton. The ability of NK cells to rapidly adhere to tumor target cells is consistent with their function in innate immune responses. Our data further suggest that a killing decision is already made within 120–300 s of tumor cell contact, supporting the essential function of cell adhesion during the early phase of cellular cytotoxicity. *The Journal of Immunology*, 2011, 186: 2757–2764.

Natural killer cells are important effector cells of the innate immune system and are involved in immune reactions against viral infections and cancer (1). Cell–cell contact is essential for triggering NK cell cytotoxicity (2). In this contact, activating and inhibitory NK cell receptors can interact with their respective ligands on the target cell to form the immunological synapse. This initiates a signaling cascade, resulting in the activation of NK cell effector functions (3). Cell–cell contact is equally important during the following effector phase. Lytic granules are directed toward the immunological synapse and are exocytosed into the contact area between the NK and target cell, which results in the directed lysis of the latter (4). Target cell adhesion is thus vital for NK cell cytotoxicity. It is mediated by integrins and closely regulated by the signals that originate from NK cell activating and inhibiting receptors.

Integrins are a family of well-characterized cell adhesion proteins (5, 6). Integrin-mediated cell adhesion is regulated by the expression level, clustering, and activation state of the integrins. NK cells express the integrins LFA-1 ($\alpha_L\beta_2$; CD11a-CD18) (7) and macrophage receptor 1 ($\alpha_M\beta_2$; CD11b-CD18). These interact

with ICAMs. The binding activity of integrins is mediated by inside-out signaling, where signals of other activating receptors increase integrin binding affinity by adopting an extended conformation of extracellular domains of the integrins (5, 6). It was shown that different activating NK cell receptors harbor the potential to induce the high-affinity conformation of LFA-1 (8, 9). The inside-out signaling has mostly been studied in T cells and was shown to involve PKC, talin, the small GTPases Ras and Rap1, as well as an association of actin with LFA-1 (10).

NK cell activating receptors include the natural cytotoxicity receptors NKp30, NKp46, and NKp44, as well as NKG2D, DNAM-1, and the members of the SLAM-related receptors 2B4, NTB-A, and CRACC (3). 2B4 is important for the coactivation of NK cells against target cells of hematopoietic origin, as its ligand CD48 is widely expressed by these cells (11). Engagement of activating receptors by their respective ligands on target cells is thought to be important for LFA-1-mediated NK cell adhesion. The coengagement of inhibitory NK cell receptors that recognize MHC class I on target cells can interfere with this process and block firm NK cell adhesion at an early time point (12). This allows for an effective control of NK cell activation by inhibitory receptors and may be important to enable the rapid scanning of various target cells by a single NK cell (13). Although micro-mechanics determine cell fate and function as much as molecular factors do, very little is known about the biophysics and forces that drive vital processes such as NK cell adhesion. In the past, this has been hampered at least in part by the availability of suitable high resolution methods, which would enable the investigation of these parameters on the single-cell level under physiological conditions in real time. With atomic force microscopy (AFM)-driven single cell force spectroscopy (SCFS) a method became available that fulfills these requirements (14–16). The instrumentation is based on a flexible tip, the cantilever, with a reflective coating on its back from which a laser beam is deflected onto a photosensitive diode. The tip can be moved with nanoscale precision by piezo elements. By this means, the instrument can be used as a forklift to bring cells into contact with each other and to separate them again in a controlled manner. Being essentially an optical lever, the slightest movements of this tip are detected by the photo diode. From the cantilever's spring

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Abbreviations used in this article: AFM, atomic force microscopy; LatA, latrunculin A; PPI, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine; SCFS, single cell force spectroscopy; smlCAM-Fc, soluble multimeric ICAM-1:Fc F(ab')₂.

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constant, absolute interaction forces up to the single molecule level can be determined. Until now, the biophysics and interacting forces between NK cells and tumor cells have not been investigated by SCFS. In this study, we use SCFS to determine the influence of the activating NK cell receptor 2B4 on the early adhesion processes of NK cells. We show that 2B4 engagement induces a rapid LFA-1- and actin-dependent NK cell adhesion to tumor target cells. In some contacts this adherence is already reduced again as early as 120 s after target cell contact, suggesting a rapid scanning of tumor targets by NK cells.

Materials and Methods

Reagents and cells

Latrunculin A was purchased from Enzo Life Sciences, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP1) was obtained from BIOMOL (Hamburg, Germany). Syk inhibitor IV (2-(7-(3,4-dimethoxyphenyl)-imidazo[1,2-*c*]pyrimidin-5-ylamino)-nicotinamide) was purchased from EMD Biosciences. The Abs used were anti 2B4 (clone C1.7; BioLegend), anti-CD18 (clone TS1/18; BioLegend), and mouse IgG1 as a control (clone MOPC-21, Sigma-Aldrich). PE-Cy5-labeled anti-CD56 (clone MEM-188) and FITC-conjugated anti-CD58 Ab (clone 1C3) were purchased from BD Pharmingen, and goat anti-human F(ab')₂ fragments were obtained from Jackson ImmunoResearch Laboratories. ICAM-1::Fc was purchased from R&D Systems, and anti-CD2 (clone RPA-2.10) Ab was purchased from Biozol. Cell lines used in this study were HeLa cells, retrovirally transduced with pBABEplus-CD48 and empty pBABEplus vector, respectively, cultured in DMEM containing 10% FCS, 1% penicillin/streptomycin, and 1 mg/ml puromycin. NK92-C1 cells, stably transfected with IL-2, were cultured in MEM α containing 12.5% FCS, 12.5% horse serum, 1% penicillin/streptomycin, and 0.1% 2-ME.

FACS-based conjugate assay

FACS-based conjugate formation was carried out as described earlier (12). Briefly, NK92-C1 cells and target cells were labeled with PKH26 or PKH67 (both Sigma-Aldrich), respectively, and resuspended in cold IMDM medium. Effector cells (5×10^4) were mixed with target cells in 100 μ l medium at an E:T ratio of 1:2, centrifuged for 2 min at $20 \times g$, and coincubated at 37°C for the indicated intervals. Subsequently, cells were vortexed, fixed with 4% paraformaldehyde in PBS, and measured on a FACScan. NK cells in conjugate were determined as double-positive events.

Multiwell conjugate assay

HeLa cells were seeded at 3×10^4 cells per well in 50 ml in a 96-well plate and grown overnight at 37°C/5% CO₂. Then, 3×10^5 NK cells/ml were labeled with 2 mM carboxyfluorescein diacetate (Molecular Probes) in Dulbecco's PBS for 30 min at 37°C/5% CO₂. Staining reaction was stopped with 9 ml IMDM medium containing 10% FCS and 1% Pen-Strep (all Invitrogen), washed, and resuspended to a final concentration of 3×10^5 cells/ml. NK cells (50 μ l) were added in triplicates at each time point to seeded HeLa cells. As a background, 50 μ l medium was added to three HeLa-containing wells. The plate then was centrifuged for 2 s at $20 \times g$, stopped with minimal break, and incubated at 37°C/5% CO₂. This procedure was repeated for the whole kinetic. In the final step, NK cells were added, spun down, and initial fluorescence of all wells was determined with a fluorescence microplate reader (Beckman Coulter 1420 Victor² multilabel counter) equipped with standard fluorescence filters. After the last time point the plate was sealed, inverted, centrifuged at $20 \times g$ for 2 s, and stopped with minimal break. The liquid containing nonadhering NK cells was completely removed, wells were once washed with 150 μ l IMDM without centrifugation, refilled with 100 μ l IMDM, and fluorescence was determined. Percentage NK cells in conjugate were calculated as $[(\text{fluorescence}_{\text{end}} - \text{fluorescence}_{\text{background}})/(\text{fluorescence}_{\text{start}} - \text{fluorescence}_{\text{background}})] \times 100$. The mean of all three values then was determined and SE calculated.

Chromium-release assay

HeLa cells were grown to midlog phase, and 5×10^5 cells were labeled in 100 μ l IMDM containing 10% FCS and 1% penicillin/streptomycin with 100 μ Ci (3.7 MBq) ⁵¹Cr for 1 h at 37°C. Cells were washed twice with IMDM medium and resuspended at 5×10^4 cells/ml in CTL medium. Effector cells were resuspended in IMDM medium and, where indicated, preincubated with Abs (10 μ g/ml final concentration) for 15 min at 25°C.

After preincubation, effector cells were mixed with 5000 labeled target cells/well in a U-bottom 96-well plate. Maximum release was determined by incubation of target cells in 1% Triton X-100 solution. For spontaneous release, targets were incubated without effectors in CTL medium alone. All samples were done in triplicates. Plates were incubated for 4 h at 37°C. Supernatant was harvested, and ⁵¹Cr release was measured in a gamma counter. Percentage specific release was calculated as $[(\text{experimental release} - \text{spontaneous release})/(\text{maximum release} - \text{spontaneous release})] \times 100$. The ratio between maximum and spontaneous release was at least 4 in all experiments.

SCFS

The basic mode of function of AFM and SCFS have been explained in detail elsewhere (15, 17). The data presented in this study were acquired with a NanoWizard II AFM equipped with a CellHesion stage (JPK Instruments, Berlin, Germany). The system was mounted on an inverted optical microscope (Axiovert; Zeiss). V-shaped tipless silicon nitride cantilevers with a nominal spring constant of 0.06 N/m were used (NP-O; Veeco, Camarillo CA). The cantilevers were functionalized using Cell-Tak reagent (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. Prior to use each cantilever was calibrated individually using the thermal noise method provided with the AFM control software. The experiment itself was set up as follows. One day before the experiment HeLa cells were seeded onto round glass coverslips with a diameter of 22 mm. For the experiment the coverslips were mounted into the temperature controlled perfusion chamber of the AFM (BioCell; JPK instruments, Berlin, Germany), overlaid with HBSS supplemented with 25 mM HEPES (pH 7.4) and kept at a temperature of 37°C. Subsequently, NK cells were flushed into the BioCell using the implemented perfusion system and attached to the cantilever by slightly touching one NK cell with a force of 0.8 nN for 30 s and retracting again. For blocking experiments NK cells were preincubated with Abs for 2 min at 37°C and then added into the chamber in the Ab solution. For force measurements the NK cell was lowered onto a HeLa cell until a contact force of 0.8 nN was reached. Contacts were maintained for set lengths of time as stated in the *Results*. For contacts maintained >10 s, only one force measurement was performed per NK cell. The speed of extension and retraction of the cantilever was set to 5 μ m/s. Measurements were performed in closed loop and constant height mode. Analysis of the resulting data were done using the JPK-IP software package (JPK Instruments, Berlin, Germany).

Ligand-complex-based adhesion assay

ICAM-Fc (1/80) and goat anti-human F(ab')₂ fragments (1/20) were coincubated for at least 30 min in the dark to generate soluble multimeric ICAM-1::Fc F(ab')₂ (smICAM-Fc) complexes. smICAM-Fc (2.5 μ l) was added immediately before transferring the cells to 37°C for 10 min. Then, 2×10^5 NK92-C1 cells per sample were resuspended in PBS containing 0.5% BSA, supplemented with 1 mM CaCl₂/2 mM MgCl₂ where indicated. When coincubated with target cells, NK92-C1 cells were stained for CD56 for 15 min at room temperature prior to use. For Src kinase inhibition, NK92-C1 cells were preincubated for 30 min at 37°C with 20 μ M PP1 or 40 nM Syk inhibitor IV for Syk kinase inhibition in PBS/BSA buffer containing Ca/Mg. Inhibitor concentrations were prepared according to the manufacturer's suggestions. For PMA stimulation cells were resuspended in PBS/BSA buffer containing Ca/Mg. PMA (4 μ M) was added before transferring the cells to 37°C. Mg/EGTA stimulation was performed by resuspending the cells in PBS/BSA buffer and supplementing with 10 mM MgCl₂ plus 1 mM EGTA. For target cell stimulation, HeLa cells were dissolved with trypsin-free cell dissociation buffer and resuspended in PBS/BSA buffer containing Ca/Mg, and NK92-C1 cells were stimulated with HeLa cells for 10 min at 37°C in a final volume of 25 μ l at an E:T ratio of 1:2.

Latrunculin A treatment

NK92-C1 cells were incubated in Dulbecco's PBS with 10 μ M latrunculin A (LatA) for 30 min prior to AFM experiments. Cells were then washed three times in HBSS supplemented with HEPES to remove residual LatA.

Results

SCFS setup

Studying the interaction between two cells with the AFM requires one cell being anchored to an inert surface such as a glass coverslip while the second cell is attached to the cantilever in the manner of a forklift (Fig. 1A). For our SCFS experiments, we attached the

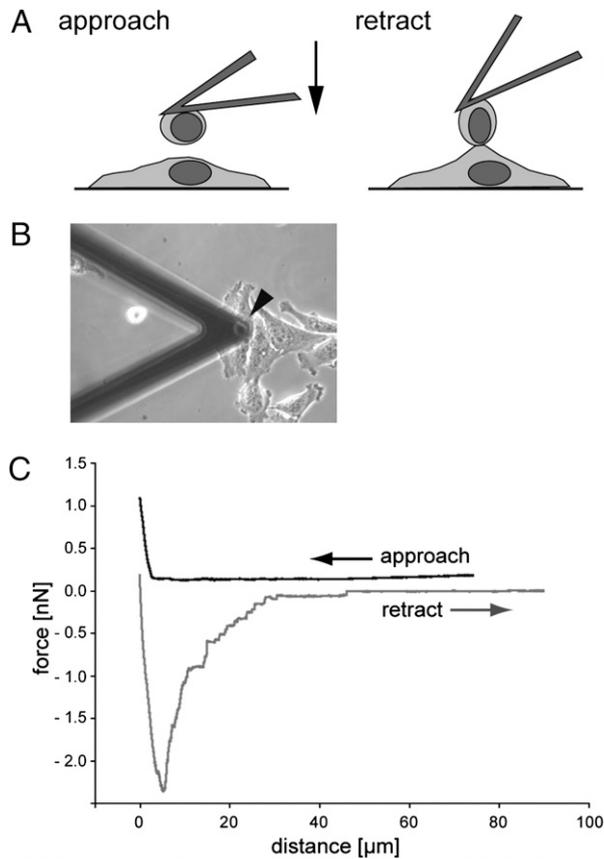


FIGURE 1. Setup of AFM experiments. *A* and *C*, Schematic setup and representative SCFS force–distance plot. A NK92-C1 cell is attached to the flexible cantilever and lowered onto a HeLa cell grown on a coverslip. When the cantilever approaches the surface, the distance between the two cells decreases until they touch each other. Upon contact of the two cells, the cantilever is bent until a given loading force is reached (black line). When the cantilever is retracted (gray line), the interaction between the two cells again bends the cantilever downward, which is registered as a negative force on the force–distance plot, which is gradually released as the cells separate. *B*, Phase contrast image. A single NK92-C1 cell (black arrowhead) attached to the V-shaped cantilever (visible by the lighter cell borders) is in contact with a HeLa cell.

human NK cell line NK92-C1 to the cantilever of the AFM (Fig. 1*B*, black arrowhead) and brought it into contact with an adherent HeLa cell. Fig. 1*C* shows a representative example of an SCFS force plot. During the approach the force remains constant while the distance between the cantilever and the coverslip surface decreases to the point when the two cells touch and the cantilever is bent to a given load (black line). By reversing the cantilever's motion, the interaction of the cells bends the cantilever toward the surface. This is accordingly registered as a negative force on the force–distance plot, which is gradually released as the molecular bonds and membrane tethers between the cells break (gray line). The lowest point of the retraction curve is a measure of the absolute interaction force (maximum unbinding force) needed to separate the cells in newtons. It is a measure for the peak force needed to initiate de-adhesion events. Upon overcoming this absolute interaction force by the pull of the cantilever, the NK cell will start to detach from the HeLa cell. The area contained by the force versus distance curve provides the unbinding work in joules. Other than the maximum unbinding force, the unbinding work is, strictly speaking, not an independent parameter. It results from the amplitude and number of unbinding events and when these occur during the retraction of the cantilever. The longer it takes to

completely separate the two cells, and the more molecule bonds that need to be broken, the higher is the work of interaction. The unbinding work thus provides a good measure to evaluate force–distance curves beyond the maximum unbinding force. By convention, deflection of the cantilever toward the surface is recorded by the AFM as a “negative” force. However, because force and work are absolute measures, these will be represented as positive values in the following figures to facilitate comprehension and comparison of the results.

2B4 mediates NK cell cytotoxicity and adhesion

To study the influence of the activating NK cell receptor 2B4 on NK cell adhesion, we stably transfected HeLa cells with the 2B4 ligand CD48. Homogeneous expression of CD48 was confirmed by FACS analysis (Supplemental Fig. 1). Whereas control transfected HeLa cells (HeLa-mock) were killed to a very minor extent by the human NK cell line NK92-C1 (Fig. 2*A*), HeLa-CD48 target cells were killed in a 2B4-specific fashion (Fig. 2*B*).

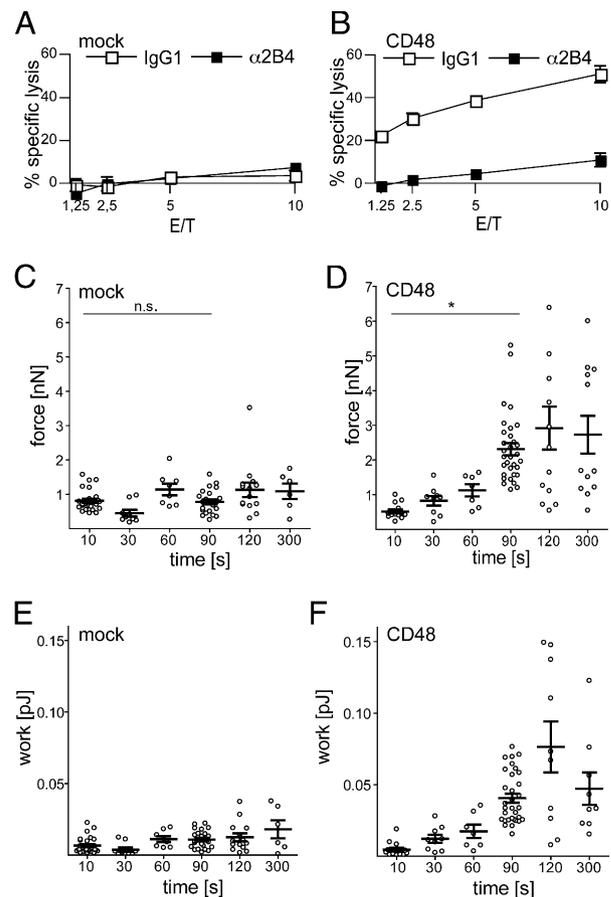


FIGURE 2. 2B4 induces increased adhesion to CD48-expressing targets. HeLa cells transfected with (*A*) control plasmid or (*B*) CD48 were used as targets for NK92-C1 cells in a standard chromium-release assay. Control IgG and blocking anti-2B4 Abs were used as indicated at a final concentration of 10 μg/ml. Values represent triplicates ± SD, $n = 3$. *C* and *E*, Using the AFM a single NK92-C1 cell was brought into contact with a mock-transfected HeLa cell for the indicated time spans ranging from 10 to 300 s. The (*C*) force or (*E*) work needed to separate the two cells again was determined and depicted in the graph for each individual experiment as a dot plot. Horizontal bars represent mean ± SEM. *D* and *F*, The same experiments were performed with CD48-expressing HeLa cells. The difference in detachment force between HeLa-mock and HeLa-CD48 after 90 s was statistically significant ($p < 0.05$). Data were analyzed using one-way ANOVA and Bonferroni's multiple comparison test. * $p < 0.05$.

In human cells, CD48 can also be bound by its low-affinity receptor CD2 that is expressed by NK92-C1 cells (Supplemental Fig. 2). Additionally, HeLa cells endogenously express CD58, the human high-affinity ligand for CD2 (Supplemental Fig. 2). To assess the role of CD2 in our system, we included a blocking anti-CD2 Ab in our assay. We observed a slight decrease in killing of HeLa target cells by NK92-C1 cells regardless of CD48 expression (Supplemental Fig. 2). This suggests that the CD2–CD58 interaction plays only a minor role in the killing of HeLa cells by NK92-C1 and that CD2 has no major contribution to the enhanced lysis of HeLa-CD48.

Using SCFS we then compared the force necessary to separate NK92-C1 cells from HeLa-mock or HeLa-CD48. NK92-C1 cells were attached to the cantilever and brought into contact with HeLa-mock or HeLa-CD48 target cells with a defined initial interaction force of 0.8 nN. Adhesion was then allowed to progress for defined time spans ranging from 10 to 300 s before the cantilever was retracted again and forces upon separation were measured. Detachment forces between NK92-C1 and HeLa-mock stayed at ~ 1 nN at all time points (Fig. 2C), consistent with the fact that this interaction did not result in target cell killing (Fig. 2A). At early time points of up to 60 s the force needed to separate NK92-C1 cells from HeLa-CD48 did not differ from that of HeLa-mock cells (Fig. 2D). However, starting at 90 s contact the detachment force increased significantly to an average of 3 nN until ~ 120 s and stayed comparably high until 300 s contact. As a second measure of adhesion we also analyzed the work needed to separate the cell couple, which can be deduced from the area enclosed by the retract curve. This analysis produced a similar result and also showed a specific increase starting at ~ 90 s only for the contact between NK92-C1 and HeLa-CD48 target cells (Fig. 2E, 2F). These data directly demonstrate that the engagement of 2B4 can result in an early increase of NK cell adhesion to CD48-expressing target cells. Whereas 2B4 stimulation resulted in a 3-fold increase of the forces necessary to separate the cells, it led to a 7-fold increase in the work necessary for the separation. Interestingly, the events recorded for 300 s contact to HeLa-CD48 target cells seemed to fall into two categories. Either very low detachment forces comparable to the ones for HeLa-mock target cells were recorded or very high detachment forces of up to 6 nN were measured.

2B4-mediated NK cell adhesion is not detectable in a standard conjugate assay

The adhesion between NK cells and target cells is typically measured in a FACS-based conjugate assay (12). In this assay NK cells and target cells are labeled with different fluorophores. Conjugates are detected after different incubation periods as double fluorescent events. Although we saw a clear 2B4-mediated NK cell adhesion in our SCFS experiment (Fig. 2), we could not detect any increased adhesion of NK92-C1 cells to HeLa-CD48 compared with HeLa-mock target cells (Fig. 3A). One explanation for this discrepancy is that in this FACS-based assay NK cells and target cells are incubated together in suspension, whereas in the SCFS measurement the HeLa cells are adherent. To investigate this possibility we developed an adhesion assay suited for adherent target cells (see *Materials and Methods*). Using this assay we could confirm the 2B4-mediated NK cell adhesion to HeLa-CD48 cells (Fig. 3B). This demonstrates that for adherent tumor cells the classical suspension-based conjugate assay is not suitable for measuring NK cell adhesion. For these tumor targets it is essential to keep them in their adherent state to accurately measure NK cell–tumor interactions.

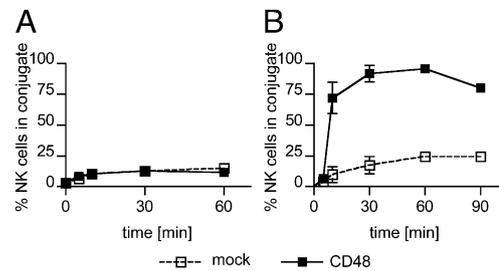


FIGURE 3. NK92-C1 cell adhesion to adherent growing HeLa cells is dependent on CD48 expression. *A*, PKH red stained mock or CD48-transfected HeLa cells and PKH green stained NK92-C1 were mixed at an E:T ratio of 1:2. After coincubation for the indicated time points, cells were extensively vortexed and fixed with 2% PFA. Conjugates were determined as double-positive events by FACS analysis. Shown is one representative out of five independent experiments. *B*, Mock or CD48 transfected HeLa cells (3×10^4 /well) were used in a multiwell adhesion assay. NK92-C1 cells were added in an E:T ratio of 1:2 and were allowed to adhere for the indicated times. Values represent triplicates \pm SEM, $n = 4$.

Adhesion is dependent on both 2B4 and CD18

To directly determine the influence of CD48 expression on the induction of adhesion in our system, the interaction between CD48 and 2B4 was blocked using an anti-2B4 Ab. This treatment reduced the force and work necessary to separate NK92-C1 and HeLa-CD48 cells by $\sim 50\%$ compared with the use of a control Ab, whereas adhesion to HeLa-mock cells remained unaffected (Fig. 4). This demonstrates that the specific interaction between 2B4 and CD48 is necessary for the increased adhesion to HeLa-CD48 cells. However, these experiments cannot show whether the increased adhesion between NK92-C1 and HeLa-CD48 is simply a result of the adhesive force of the 2B4–CD48 interaction, or if

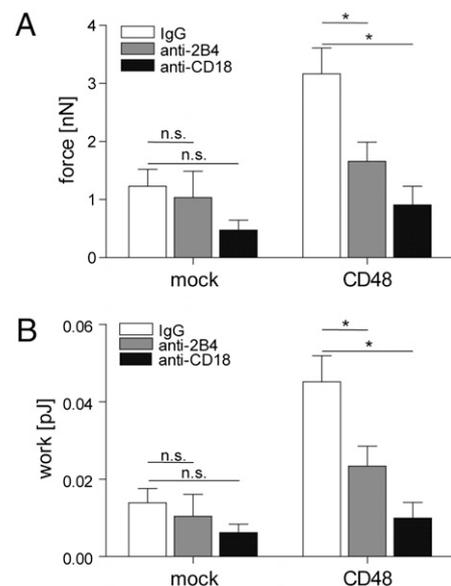


FIGURE 4. Adhesion of NK cells to CD48-expressing HeLa cells is dependent on 2B4 and CD18. *A*, NK92-C1 cells were preincubated with the indicated Ab for 2 min at 37°C and added into the reaction chamber of the AFM. Then SCFS experiments were performed with a contact duration of 90 s. Control IgG (10 μ g/ml, open bars), anti-2B4 (10 μ g/ml, gray bars), and anti-CD18 (20 μ g/ml, black bars) were added as indicated. Shown is the mean \pm SEM of four to nine individual experiments per condition. *B*, The work required to break the cellular connection as calculated from the obtained data shows a similar pattern. Data were analyzed using one-way ANOVA and Bonferroni's multiple comparison test. A p value < 0.05 was considered significant. * $p < 0.05$.

this interaction also influences the activity of adhesion receptors by inside-out signaling. Target cell adhesion of NK cells is mainly dependent on LFA-1 (CD18/CD11a). To test whether 2B4-mediated NK cell adhesion is dependent on LFA-1, we used a blocking anti-CD18 Ab to interfere with NK cell target cell adhesion induced after 90 s of interaction (Fig. 4). This treatment reduced the low detachment forces measured for HeLa-mock cells, suggesting that this background adhesion is already dependent on LFA-1. The increased detachment forces necessary to separate NK92-C1 and HeLa-CD48 cells was greatly affected by blocking LFA-1 and was now comparable to HeLa-mock targets. These data demonstrate that the 2B4-mediated NK cell adhesion is dependent on LFA-1 and suggest that 2B4 mediates this adhesion through inside-out signaling affecting the ability of LFA-1 to interact with its ligand ICAM-1.

2B4 engagement induces LFA-1 activation

To directly investigate changes in binding activity of LFA-1, we performed ligand-complex-based adhesion assays (18). This assay uses smICAM-Fc complexes to detect affinity and avidity changes in LFA-1 in a FACS-based assay (Fig. 5A). As NK92-C1 cells produce their own IL-2, we first assayed the baseline activity of LFA-1 on these cells. To establish background binding, we performed the staining in the absence of Ca^{2+} and Mg^{2+} as LFA-1 needs these divalent cations for its binding to ICAM-1. Compared to this background staining, we only detected a slight binding of the smICAM-Fc complexes to NK92-C1 cells in the presence of Ca^{2+} and Mg^{2+} (Fig. 5B). This indicated that LFA-1 on NK92-C1 cells is in a preactivated state, although the amount of preactivation is minor.

We then stimulated the NK92-C1 cells by coinubation with HeLa-mock or HeLa-CD48 (Fig. 5C). In agreement with Fig. 3, both target cell lines induced an increased smICAM-Fc binding to NK92-C1. However, when coinubated with CD48-expressing target cells the binding of smICAM-Fc was markedly increased. This indicates that the engagement of 2B4 by CD48 can directly affect the binding activity of LFA-1. To further investigate the signals necessary for this process, we used the Src-kinase inhibitor PP1 to block early 2B4-mediated signals (19) or a Syk inhibitor to interfere with ITAM-dependent LFA-1-mediated outside-in signals as a control (20). Src-kinase inhibition by PP1, although only minimally affecting LFA-1 binding activity induced by the coculture of NK92-C1 with HeLa-mock cells, completely abrogated the effect mediated by CD48-transfected HeLa cells (Fig. 5D). In contrast, Syk inhibition showed no effect. These data demonstrate that 2B4-mediated signals induce LFA-1 activation in a Src-kinase-dependent manner.

The actin cytoskeleton is indispensable for NK cell adhesion

2B4 signaling is dependent on a reorganization of the actin cytoskeleton (21), and inside-out signals affecting LFA-1 binding activity in T cells are also actin-dependent (22). We therefore wanted to determine the role of the actin cytoskeleton in target cell adhesion of NK cells. NK cells were preincubated with Latrunculin A prior to target cell contact. To avoid an influence on target cells, NK cells were extensively washed after LatA treatment and used immediately. Binding of LatA to G-actin inhibits its polymerization and therefore sequesters monomeric actin, leading to a general loss of the actin cytoskeletal structure. This treatment had already an effect on the forces necessary to separate NK cells from HeLa-mock targets (Fig. 6A). Also, the 2B4-mediated increase in detachment forces induced by HeLa-CD48 targets was

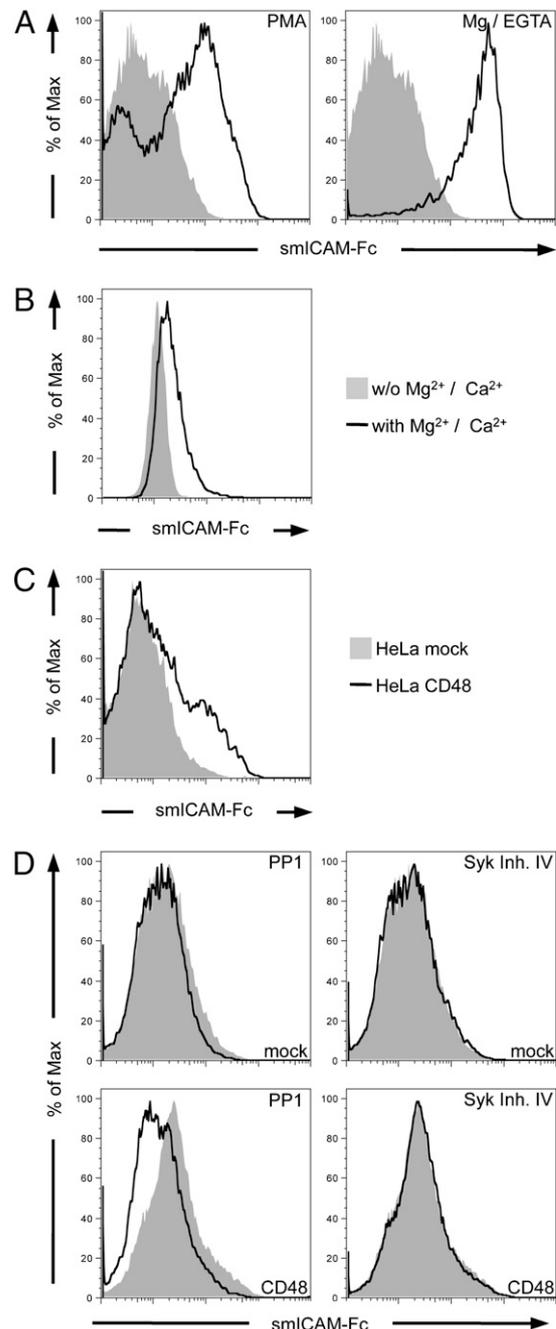


FIGURE 5. 2B4 signals induce LFA-1 activation. LFA-1 activation was investigated by binding of smICAM-Fc complexes after 10 min stimulus as indicated. *A*, NK92-C1 cells were incubated in the presence of 4 μM PMA or 10 mM MgCl_2 /1 mM EGTA (black lines). Controls were supplemented with DMSO (gray areas) ($n = 3$). *B*, NK92-C1 cells were incubated in buffer containing either 1 mM CaCl_2 and 2 mM MgCl_2 or PBS alone without further stimulation ($n = 2$). *C*, NK92-C1 cells were incubated with mock or CD48-transfected HeLa cells at an E:T ratio of 1:2 ($n = 6$). *D*, NK92-C1 cells were preincubated with 20 μM PP1, 40 nM Syk inhibitor IV (black lines), or DMSO (gray area) as control. HeLa-mock or HeLa-CD48 cells were then added at an E:T ratio of 1:2. Shown is one representative experiment out of four.

almost completely blocked by LatA pretreatment of the NK cells. Interestingly, there was still a slight and reproducible difference in detachment forces between HeLa-mock and HeLa-CD48 after LatA treatment. These data show that the actin cytoskeleton is indispensable for 2B4-dependent but also for 2B4-independent NK cell adhesion to target cells.

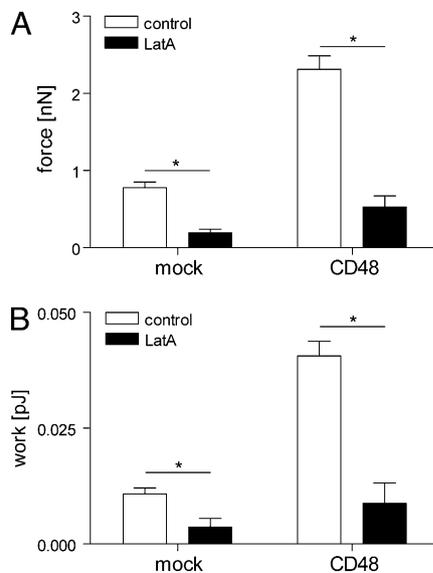


FIGURE 6. The actin cytoskeleton is indispensable for NK cell adhesion. *A*, SCFS experiments were performed with or without preincubation of the NK92-C1 cells with 10 μ M LatA to measure the unbinding forces of NK92-C1 HeLa cell couples. After preincubation for 30 min cells were washed twice and added into the AFM. The contact time was set to 90 s. *B*, The same experiments were analyzed for the unbinding work. Shown is the mean \pm SEM of four to six individual experiments. Open bars indicate control-treated NK92-C1; filled bars indicate LatA-treated NK92-C1. Statistical significance was determined using an unpaired *t* test. **p* < 0.05.

Discussion

NK cell adhesion to tumor target cells is an essential event for the initiation and effector phase of cellular cytotoxicity. In this study, to our knowledge we have reported for the first time the precise biophysical values for the interaction forces between NK cells and tumor target cells. Most AFM approaches have focused on isolated receptor ligand interactions either using purified molecules or cells interacting with immobilized purified ligands (23, 24). Other SCFS experiments have only concentrated on short-time effects, such as a 10-s contact between endothelial cells and Jurkat T cells (25). In this study, interaction forces of only 100 pN were reported. In our approach we deliberately measured also longer interaction times to specifically investigate the dynamics of NK cell adhesion and to allow for inside-out signaling to occur. For this setup we could not use the same cell attached to the cantilever for repeated measurements as done in the above-mentioned studies. Instead, we chose to use a fresh pair of NK cells and HeLa cells for each measurement >10 s interaction time. This approach is very work intensive, as it requires the disassembly of the setup after each measurement, and it allows only for a limited number of measurements. Also, homogeneous cell populations are necessary to make these individual measurements comparable. We therefore chose to work with a well-defined human NK cell line NK92-C1 and stably transfected HeLa target cells.

Recently, the peptide-specific interaction forces between CD4⁺ T helper cells and APCs were determined by SCFS (26). In this study the initial interaction forces were \sim 1 nN, which matches very well with our findings. However, for T cells these interaction forces did not change after 120 s of cell contact, and a specific increase was only evident after 15 min of contact. This is in contrast to the early increase in NK cell adhesion, which was evident already after 90 s cell contact. NK cells may therefore be much faster than T cells in establishing cellular adhesion, which is in line with their quick responses during innate immune reactions and that has been suggested to be essential for their effective

cytotoxic activity (27). In T cells maximal adhesion forces of \sim 14 nN were measured after 30 min contact time (26). In our experimental setup it was not possible to make accurate measurements after such long contact times, possibly because NK cells can already kill their target cells within this time frame. Also, the maximal adhesion forces of NK cells were \sim 3 nN and did not increase between 120 and 300 s in our experiments. This suggests that NK cells may only need \sim 2–5 min to establish target cell contact and that these contacts are less firm compared with the ones of T helper cells. This difference may be due to the different nature of the contacts studied. Whereas the NK cell contacts studied here will lead to a cytotoxic response, T helper cell contacts with APCs are not cytotoxic but result in the activation of Ag-specific T cells. It will therefore be interesting to compare the adhesion of NK cells and cytotoxic T cells. However, to our knowledge there are currently no quantitative data about the adhesive forces between cytotoxic T cells and target cells available.

To correctly measure the adhesion of NK cells to adherent tumor cells it was necessary to keep the tumor cells in their adherent state. The classical FACS-based conjugate assay failed to detect this adhesion, probably because NK and target cells are kept in suspension during this assay. The adherence of tumor cells could be important to provide polarization of the cell, which may be necessary for effective NK cell adhesion. Additionally, adherent tumor cells start to form clumps while in suspension during the incubation period of the FACS-based conjugate assay, thereby greatly reducing their ability to form contacts with NK cells. Additionally, NK–tumor cell conjugates within these clumps are no longer accessible for the FACS-based measurement. In this respect our setup of the SCFS mimics the physiological situation, as the tumor cells are kept in their adherent state during the measurement. In comparison with other adhesion assays, SCFS has the advantage of being able to very accurately detect detachment forces already within seconds of cellular contact on a single cell basis and under physiological conditions. To the best of our knowledge, we were therefore able to show for the first time in this study that the engagement of 2B4 can already result in an increase in NK cell adhesion as early as 90 s after target cell contact (Fig. 2), which was not detectable in a standard adhesion assay (Fig. 3). This activity of 2B4 nicely fits with its coactivating function in NK cells, where coengagement of 2B4 can synergistically enhance NK cell activation mediated by other activating receptors (28). It was recently shown that the synergism between 2B4 and NKG2D-mediated NK cell activation is a result of increased Vav-1 phosphorylation (29). The early 2B4-mediated increase in NK cell adhesion may result in enhanced interaction between other activating receptors and their ligands and could therefore be the functional basis of this increased Vav-1 phosphorylation and the synergistic activation of NK cells.

The 2B4-mediated increase in NK cell adhesion was LFA-1–dependent. This demonstrates that inside-out signaling is very fast and its effects can be detected as early as 90 s after cell contact. This fast kinetic is in line with a rapid initiation of 2B4 signaling, as 2B4 phosphorylation can be detected as early as 30 s after receptor engagement (21, 30). 2B4 phosphorylation is also actin-dependent (21), which could explain why the 2B4-mediated increase in NK cell adhesion was abrogated by inhibitors of actin polymerization. However, actin also plays an important role in the 2B4-independent adhesion of NK cells to tumor target cells. The β_1 and β_2 integrins are linked to the actin cytoskeleton through talin, which has been shown to be essential for inside-out signaling (31). Additionally, LFA-1 in NK cells has the unique ability to transmit its own inside-out signal to other LFA-1 molecules (32). However, this signal by itself was not sufficient in our

experimental setup, as we did not observe any increase in adherence to HeLa-mock cells.

Our ligand-complex-based adhesion assay experiments point toward a direct role of 2B4 in mediating inside-out signals. 2B4-mediated signaling is dependent on Src-family kinases such as Fyn, which is recruited to the cytoplasmic tail of 2B4 via the adapter SAP (11). This results in the activation of PLC γ and PKC, which can in turn activate Rap1, a known regulator of inside-out signaling (33, 34). Fyn can additionally promote the phosphorylation of ADAP and SKAP-55 (35), which are also involved in inside-out signaling. This suggests that Fyn activity may be important for 2B4-mediated inside-out signaling, which was supported by our finding that PP1 can inhibit 2B4-mediated LFA-1 activation.

LFA-1 engagement on NK cells induces phosphorylation of the Wiskott-Aldrich syndrome protein, a known regulator of the actin cytoskeleton (36). Our data are in line with the important role of the actin cytoskeleton for LFA-1-mediated adhesion, as inhibition of actin polymerization greatly reduced the adhesion to HeLa-CD48. Consistent with the baseline activation of LFA-1 in NK92-C1 cells it also reduced adhesion to HeLa-mock cells. Interestingly, even when actin polymerization was blocked we still observed a difference in detachment forces between HeLa-CD48 and HeLa-mock cells (Fig. 6). This difference might reflect the adhesive force of the 2B4-CD48 interaction and suggests that this interaction can also directly contribute to NK cell target cell adhesion, as was suggested earlier (37). LFA-1 is not only important for NK cell adhesion, but also contributes to granule polarization, which is necessary for directed degranulation (38). It is therefore likely that 2B4 and LFA-1 signals synergize for the effective lysis of HeLa-CD48 cells in our system.

Actin polymerization is also important for the clustering of surface receptors, and clustering of integrins contributes to cellular adhesion by creating high-avidity interactions. We observed a 7-fold increase in the work needed to separate NK92-C1 and HeLa-CD48 cells compared with only a 3-fold increase in the necessary separation force. Therefore, the interaction between NK92-C1 and HeLa-CD48 targets may not only increase LFA-1 affinity through inside-out signaling, which would contribute to the force necessary to separate the cells. This interaction could also induce the clustering of LFA-1, inducing a high-avidity interaction between the cells, requiring more work to separate the cells. Our data would therefore indicate that the interaction between NK92-C1 and HeLa-CD48 cells influences LFA-1 affinity and avidity to induce a strong interaction between these cells.

The adhesive forces between NK cells and HeLa-CD48 targets after 300 s of contact fell into two distinct categories. About half of these contacts showed very low adhesive forces that were not significantly different from the basic adhesion to HeLa-mock cells. The other half showed high adhesive forces of >4 nN. This trend was already visible after 120 s contact. As we used a homogeneous population of NK and target cells (see Supplemental Fig. 1), this finding is surprising. However, even in this homogeneous population not every contact between an NK and a target cell results in cytotoxicity, as we only observed $\sim 50\%$ lysis of HeLa-CD48 targets at an E:T ratio of 10:1 (Fig. 2B). Therefore, slight differences within the NK cell population, for example, cell cycle, expression level of receptors, and signaling molecules (39), may contribute to the decision whether a contact is sufficient to fully activate the NK cell and result in the lysis of the target cell. Interestingly, our data suggest that this decision is already made between 120 and 300 s after target cell contact. Lytic contacts would retain high interaction forces, whereas nonlytic contacts would lose their adhesion, resulting in the early separation of NK

and target cell. Such behavior has already been described for the inhibition of NK cell activation by inhibitory receptors (12). The engagement of these receptors can effectively interfere with the early adhesion of NK cells to target cells, resulting in a shortening of the contact time. To the best of our knowledge, our data suggest for the first time that also without inhibitory receptor signaling the killing decision of NK cells is made at a very early time point and already affects target cell adhesion. This may be important to enable NK cells to effectively scan many target cells within a short time frame. Although the killing decision may be made during the first 5 min of target cell contact, longer interactions are needed to deliver the lethal hit. Therefore, it was not possible to follow the fate of a target cell after we separated it from the NK cell after 5 min of contact to directly correlate high interaction forces with target cell lysis.

In conclusion, to the best of our knowledge, the present study describes for the first time the biophysical values for the interaction forces between NK cells and tumor target cells and demonstrates how 2B4 engagement can enhance this adhesion. Adhesion is an essential event in cellular cytotoxicity. Our data suggest that even without inhibitory receptor signals the killing decision within a homogeneous NK cell population is already made within the first minutes of target cell contact. It will be interesting to study how individual NK cells that decided to kill or not differ from each other to understand the critical factors influencing cellular cytotoxicity.

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Disclosures

The authors have no financial conflicts of interest.

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