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Coexpression of CD58 or CD48 with Intercellular Adhesion Molecule 1 on Target Cells Enhances Adhesion of Resting NK Cells

Domingo F. Barber[1] and Eric O. Long[2]

The β_2 integrin LFA-1 (CD11a/CD18) mediates adhesion of lymphocytes to cells expressing ICAM. The strength of this adhesion is regulated by different signals delivered by cytokines and chemokines, and by the TCR in the case of T cells. To determine the receptor-ligand interactions required for adhesion of resting NK cells, *Drosophila* cells expressing different combinations of ligands of human NK cell receptors were generated. Expression of ICAM-1 alone was sufficient for an adhesion of resting NK cells that was sensitive to inhibitors of *src* family kinase and of phosphatidylinositol 3-kinase. Binding of resting NK cells to solid-phase ICAM-1 showed similar signaling requirements. A pulse of either IL-2 or IL-15 to resting NK cells resulted in strongly enhanced, actin-dependent adhesion to insect cells expressing ICAM-1 alone. Coexpression of either LFA-3 (CD58) or CD48 with ICAM-1 resulted in strong adhesion by resting NK cells, even in the absence of cytokines. Therefore, receptors for LFA-3 and CD48 on resting NK cells strengthen the adhesion mediated by LFA-1. *The Journal of Immunology*, 2003, 170: 294–299.

Natural killer cells circulate in the blood of healthy individuals. Signals are required to activate normal, resting NK cells to migrate to sites of infection and to acquire effector functions, such as production of IFN-γ and cellular cytotoxicity (1). The rapid response of resting NK cells to cytokines is well documented. Type I IFN, produced during virus infections, stimulates the proliferation of NK cells and augments their cytotoxic activity. IL-15, secreted by a number of different cell types, activates NK cell proliferation, cytotoxicity, and cytokine production (2). NK cells secrete large amounts of IFN-γ in response to IL-12 and IL-18, which are produced during infections by various intracellular pathogens (3). NK cells are recruited to sites of infections by chemokines, such as macrophage-inflammatory protein-1α, during liver infection by mouse CMV (4). In addition to signals received from soluble mediators, NK cells are also activated through cell contact by receptors that recognize ligands on other cells. However, little is known about activation of resting NK cells by such receptors.

For practical reasons, most studies on NK-target cell interactions have relied on NK cells expanded in vitro by continuous stimulation with IL-2 or IL-15. The cytotoxicity of such activated NK cells is induced by several activating receptors that bind ligands expressed at the surface of other cells. For instance, human NK-sensitive tumor cells express various combinations of ligands of the activation receptors NKp30, NKp44, and NKp46 (5). Expression of MICA, MICB, and UL16 binding protein molecules on stressed cells or tumor cells leads to activation of NK cells through the receptor complex NKG2D/DAP10 (6). Some receptors act as costimulators of natural cytotoxicity. For instance, CD2, which binds to LFA-3 (CD58), has long been known to costimulate NK cell cytotoxicity (7, 8). 2B4 (CD244), which binds to CD48, costimulates activation signals delivered through the immunoreceptor tyrosine-based activation motif (ITAM)[3]-containing signaling subunits associated with CD16 and NKp46 (9), and through receptor KIR2DL4 (10). Conjugate formation between NK cells and target cells is a prerequisite to target cell lysis and is critically dependent on engagement of the β_2 integrin LFA-1 (11), a heterodimer of CD11a and CD18 which binds ICAM-1, ICAM-2, and ICAM-3. LFA-1-dependent adhesion of NK cells to target cells is inhibited by killer cell Ig-like receptors upon binding to MHC class I ligands on the target cells (12).

To test how specific receptor-ligand interactions activate resting NK cells during cell contacts, we expressed ligands of human NK cell receptors in *Drosophila* cells. Unlike mammalian cells, insect cells do not express a multitude of ligands for adhesion and activation receptors of human NK cells, and are therefore well suited to study the contribution of individual receptors to NK cell activation. As a first step, we investigated the requirements for adhesion of resting NK cells to a target cell. Adhesion of T cells to APC depends on LFA-1 and on TCR signals that regulate the affinity and avidity of LFA-1 for its ICAM ligands (13–16). T cell adhesion can be strengthened by signals from coreceptors CD2 and CD28 (17, 18). Adhesion of NK cells to target cells is also dependent on LFA-1 (11). Using transfected insect cells, we now show that expression of ICAM-1 is sufficient to initiate a signal-dependent adhesion of resting NK cells which can be greatly enhanced either by a pulse of IL-2 or IL-15, or by coexpression of LFA-3 or CD48 with ICAM-1, even in the absence of cytokines.

Materials and Methods

Cells

Drosophila Schneider cell 2 (SC2; a gift from L. Teyton, Scripps Research Institute, La Jolla, CA) was maintained in Schneider's medium with L-glutamine (Life Technologies, Rockville, MD) plus 10% FBS (Life Technologies). Human NK cells were isolated from peripheral blood using an

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[3] Abbreviations used in this paper: ITAM, immunoreceptor tyrosine-based activation motif; PI3K, phosphatidylinositol 3-kinase.

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NK cell isolation kit (Miltenyi Biotec, Auburn, CA). Activated NK cells were expanded in Iscove's modified essential medium (Life Technologies), supplemented with 10% human serum (Life Technologies), 2 mM glutamine (Life Technologies), 100 U/ml rIL-2 (Hoffmann-LaRoche, Nutley, NJ), and 10% purified human IL-2 (Hemagen, Columbia, MD). Resting NK cells were resuspended in the same medium without IL-2, and were used within 1 or 2 days after isolation, only when <5% positive for the activation markers CD25 and CD69. rIL-15 was from PeproTech (Rocky Hill, NJ).

Antibodies

For FACS analysis and cell sorting, the following directly conjugated mouse mAbs were used: R-PE-conjugated (R-PE)-CD2 (clone RPA-2.10), R-PE-CD11a (clone Hl11), R-PE-CD18 (clone 6.7), R-PE-CD25 (clone M-A251), R-PE-CD54 (clone HA58), CyChrome-CD54 (clone HA58), R-PE-CD58 (clone 1C3), and FITC-conjugated CD58 (clone 1C3) (BD PharMingen, San Diego, CA). R-PE-CD3 (clone UCHT1), FITC-CD48 (clone J4-57), R-PE-CD56 (clone N901), and R-PE-CD69 (clone FN50), and R-PE-2B4 (clone C1.7) were from Beckman Coulter (Miami, FL). FITC-CD58 (clone TS2/9.1.4.3) was from Ancell (Bayport, MN). Isotype-matched PE-, FITC-, or CyChrome-conjugated mAbs were from BD PharMingen.

Expression of human proteins in Drosophila *SC2 cells*

cDNA clones for LFA-3 (CD58), CD48, and ICAM-1 (CD54) were obtained by RT-PCR amplification from total RNA isolated from the human cell line 721.221 using the RNeasy kit (Qiagen, Valencia, CA). The following primer pairs were used: LFA-3, 5′-ATTGGATCCCGAGCATGG TTGCTGGGAGC-3′ and 5′-AAGTCGACTAGTTCAATTGGAGTTGGT TCTGTC-3′; CD48, 5′-ATTGGATCCTCTAGCCAGGCTCTCAACTGT CT-3′ and 5′-AAGTCGACTAGTGTGAGGAGCATGATCACCAACAG-3′; and ICAM-1, 5′-ATTGGATCCGAGCTCCTCTGCTACTCAGAGTTG-3′ and 5′-AAGTCGACTAGTATCTGACTGAGGACAATGCCCTGT-3′. PCR products were gel-purified using the Wizard PCR system (Promega, Madison, WI), cloned into pGEM-T-Easy (Promega), from which they were subcloned using *Bam*HI/*Sal*I sites into the insect expression vector pRmHa3 (19) (a gift from L. Teyton) under control of the metallothionein promoter. All cDNAs were verified by sequencing. Three million SC2 cells (1×10^6/ml) in a well of a six-well plate were transfected with a total of 30 μg of recombinant DNA and 1 μg of the selection vector pNeofly (20) (a gift from L. Teyton) using a calcium phosphate transfection kit (Invitrogen, Carlsbad, CA). Stable transfectants were selected and maintained in 0.5 mg/ml G418 (Life Technologies). Expression of the transfected cDNAs was induced by incubation for 24 h with 1 mM $CuSO_4$. SC2 cells expressing the transfected cDNAs were selected by fluorescence-activated cell sorting (FAST Systems, Gaithersburg, MD). Expression of transfected human cDNAs was monitored before every experiment by flow cytometry.

ICAM-1 binding and adhesion assays

Soluble ICAM-1 was produced as a fusion protein with the Fc portion of human IgG1, using the ICAM-1-receptor globulin construct (a gift of W. Kolanus, University of Munich, Munich, Germany) after transfection into COS/7 cells (21). Flat-bottom 96-well plates were coated with goat anti-human IgG (Fcγ-specific) Ab at 1 μg/well for 90 min at 25°C, blocked overnight with 1% BSA in PBS, incubated with purified ICAM-1-receptor globulin for 30 min at 24°C, and washed with PBS. NK cells were labeled with 12 μg/ml bisbenzimide H33342 fluorochrome trihydrochloride (Calbiochem, La Jolla, CA) for 30 min at 37°C, collected by centrifugation, resuspended in HBSS, and plated at 1.5×10^5/well. Where indicated, cells were incubated with different inhibitors 45 min before, and during the binding assay. Cells were then allowed to adhere for 30 min at 37°C. Unbound cells were washed off with 300 μl of HBSS three times. The proportion of bound cells was calculated by resuspending the cells in 100 μl of 2% paraformaldehyde in PBS and using a fluorescence plate reader (Victor^2 1420 multilabel counter; Wallac, Gaithersburg, MD). The signal of 1.5×10^5 cells/well at 490 nm represents 100% of binding. Each determination was conducted in triplicate. Conjugate formation between NK cells and SC2 cells was determined as described (12), with the following modifications: SC2 cells were resuspended in HBSS medium (BioSource, Camarillo, CA) plus 5% FBS, and 4×10^5 cells were mixed with 1×10^5 effector cells in a final volume of 200 μl. Insect cells grow at temperatures below 30°C and undergo a heat shock reaction above 32°C. However, the short incubation times (up to 1 h) at 37°C of the assays used here were not sufficient to cause detectable increase of propidium iodide uptake in insect cells.

Actin polymerization

NK cells (1×10^6) and SC2 cells (1×10^6) were mixed in a 400-μl final volume in 12 × 75 mm round-bottom polystyrene tubes (BD Falcon, Franklin Lakes, NJ), and centrifuged at 4°C for 3 min at 300 rpm ($25 \times g$). Samples were placed at 37°C for various times. The reaction was stopped by brief vortexing. Cells were fixed by adding 400 μl of 8% paraformaldehyde in Dulbecco's PBS for 20 min at room temperature, and permeabilized by adding 800 μl of 4% paraformaldehyde in PBS plus 0.25% Triton X-100, for 20 min at room temperature. After three washes with Dulbecco's PBS/0.5%BSA, the cells were resuspended in 100 μl of washing buffer and kept at room temperature for 20 min. Each sample was incubated for 30–60 min at 4°C in the dark with 5 μl of Oregon green-conjugated phalloidin (Molecular Probes, Eugene, OR) and 10 μl of PE-conjugated anti-CD18. After three washes, samples were analyzed by FACS. NK cells were gated by side scatter and by PE fluorescence.

Results

Binding of resting NK cells to ICAM-1 requires signaling

Resting NK cells, freshly isolated from peripheral blood, were compared with IL-2-activated NK cells for their ability to bind to ICAM-1 using two different assays. First, fluorescently labeled NK cells were added to plates coated with goat anti-human IgG Fc to which soluble ICAM-1, produced as a fusion protein with the Fc portion of human IgG1, was bound. The proportion of cells that remained bound after washing was determined with a fluorescence plate-reader. About 20% of resting NK cells and 80% of activated NK cells remained attached (Fig. 1*A*). Second, adhesion of NK cells (labeled with a green dye) to insect SC2 cells (labeled with a red dye) expressing human ICAM-1 was determined by a conjugation assay using two-color flow cytometry. Conjugate formation with resting NK cells peaked at 20 min, with ~20% of NK cells in conjugates with SC2-ICAM-1 cells (Fig. 1*B*). In contrast, within 5 min, ~60% of activated NK cells had already formed conjugates with SC2-ICAM-1 cells (Fig. 1*B*). No conjugate formation was detected with untransfected SC2 cells and either activated or resting NK cells.

The slower conjugate formation of resting NK cells with SC2-ICAM-1 cells suggested a dependence on signal transduction. To test this, NK cells were pretreated with inhibitors of *src* family tyrosine kinase PP1, phosphatidylinositol 3-kinase (PI3K; Wortmannin), actin polymerization (cytochalasin D), and microtubule polymerization (colchicine) before, and during the binding assays. Binding of activated NK cells to solid-phase ICAM-1 was barely

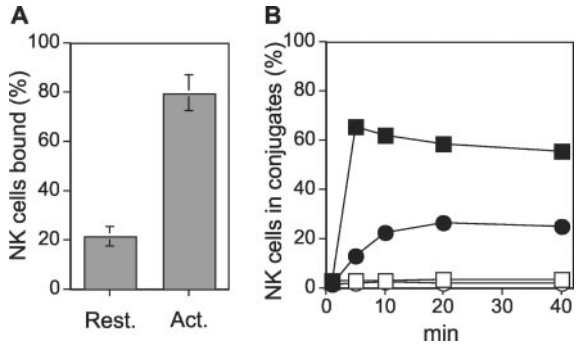

FIGURE 1. Binding of resting and IL-2-activated NK cells to plate-bound ICAM-1 and to SC2-ICAM-1 cells. *A*, Resting and activated NK cells were loaded with a green dye and added to plates coated with ICAM-1. Binding is expressed as the fraction of NK cells that remained bound after three washes. Bars indicate the SD ($n = 4$ independent experiments). *B*, Resting (○, ●) and activated (□, ■) NK cells labeled with a green dye were incubated with SC2 cells (○, □) and SC2-ICAM-1 cells (●, ■) labeled with a red dye. Conjugate formation was determined by flow cytometry and is represented as the fraction of NK cells that shifted into two-color conjugates.

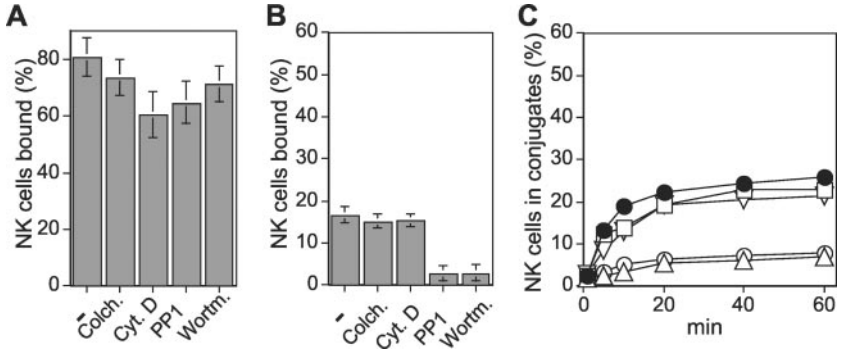

FIGURE 2. Binding of resting NK cells to ICAM-1 requires signal transduction. Activated (*A*) and resting (*B*) NK cells, preincubated for 45 min with the indicated inhibitors, were tested for binding to ICAM-1 on plates, as in Fig. 1, in the continuous presence of inhibitors. Inhibitor concentrations were 20 μM colchicine, 20 μM cytochalasin D, 30 μM PP1, and 400 nM Wortmannin. Bars indicate the SD (n = four independent experiments). *C*, Resting NK cells either untreated (●) or treated for 45 min with colchicine (□), cytochalasin D (▽), PP1 (△), or Wortmannin (○) were tested for conjugate formation with SC2-ICAM-1 cells in the continuous presence of inhibitors. Inhibitor concentrations were as in *A*.

affected by these inhibitors (Fig. 2*A*). In contrast, binding of resting NK cells was severely inhibited by PP1 and Wortmannin, but not by cytochalasin D and colchicine (Fig. 2*B*). Adhesion of resting NK cells to SC2-ICAM-1 cells was also inhibited by PP1 and Wortmannin (Fig. 2*C*). Adhesion of activated NK cells was not sensitive to these inhibitors (data not shown). These results suggest that binding of resting NK cells to ICAM-1 requires *src* family kinase and PI3K signals. These signals may be transmitted directly by LFA-1 upon binding to ICAM-1. However, it is formally possible that a constitutive, basal activity of *src* family kinase and PI3K in resting NK cells is required for LFA-1 binding to ICAM-1. We conclude that the binding of resting NK cells to ICAM-1 is signaling-dependent, and differs from that of IL-2-activated NK cells.

The rapid binding of IL-2-activated NK cells to ICAM-1, and its insensitivity to the inhibitors tested suggest that LFA-1 may be in a constitutively high affinity/avidity state on these cells. The rapid conjugate formation shown here with SC2-ICAM-1 cells is similar to that observed with the NK-sensitive human B cell line 721.221 (Ref. 12 and data not shown). NK cells express uniformly high levels of LFA-1, as shown by staining with mAbs to CD11a and CD18 (Fig. 3). The surface level of LFA-1 is very high on resting NK cells and is even higher on activated NK cells.

Binding of resting NK cells to ICAM-1 is enhanced by a pulse of IL-2 or IL-15

The stronger binding to ICAM-1 and the greater adhesion to SC2-ICAM-1 cells by IL-2-activated NK cells (Fig. 1) could be due to a stable alteration in the adhesive properties of IL-2-activated NK cells, to a specific signal transmitted by IL-2 and IL-15, or to a combination of both. We tested whether a 45-min pulse with 100 U/ml of IL-2 or IL-15 was sufficient to alter adhesion by resting NK cells. IL-2 and IL-15 each enhanced the binding of resting NK cells to ICAM-1 on plates (Fig. 4*A*) and to SC2-ICAM-1 cells (Fig. 4*B*). Adhesion to SC2-ICAM-1 cells occurred faster and reached higher levels in the presence of these cytokines. Binding of IL-2-pulsed resting NK cells to solid-phase ICAM-1 and adhesion to SC2-ICAM-1 cells were strongly inhibited by PP1, Wortmannin, and cytochalasin D (Fig. 4, *C* and *D*). Colchicine had only a minor effect: it slowed down the initial phase of adhesion to SC2-ICAM-1 cells (Fig. 4*D*). Similar results were obtained with these inhibitors during IL-15-induced adhesion to SC2-ICAM-1 cells (data not shown). Thus, not only *src* family kinase and PI3K activity but also actin polymerization appears to be an important requirement for the cytokine-induced binding to ICAM-1. These results show that IL-2 and IL-15 enhance adhesion of resting NK cells through an actin-dependent pathway that does not overcome the requirement for *src* family kinase and PI3K activity.

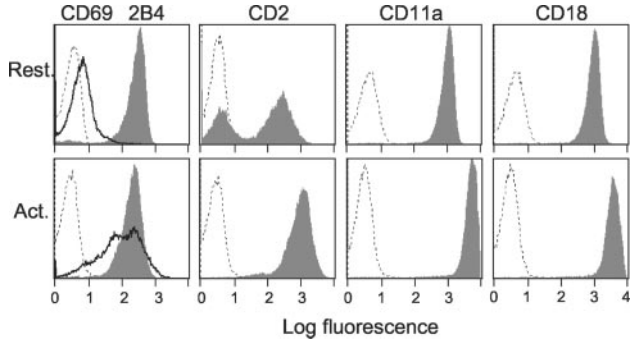

FIGURE 3. Cell surface expression of NK cell receptors. Resting (Rest.) and activated (Act.) NK cells were stained with mAbs to determine expression of the indicated receptors. The dotted lines represent staining with isotype controls, and shaded histograms represent staining for the indicated receptors, except for the staining of CD69 which is represented by a thick line in the first panel.

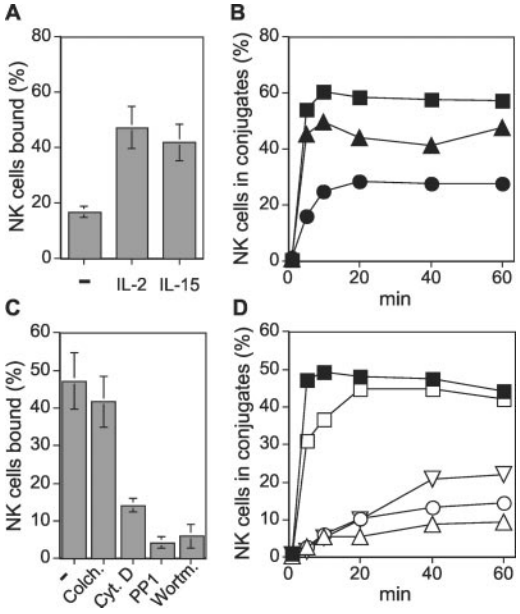

FIGURE 4. A pulse of IL-2 and IL-15 enhances binding of resting NK cells to ICAM-1. *A–B*, Resting NK cells (●) were incubated with 100 U/ml IL-2 (■) or 100 U/ml IL-15 (▲) for 45 min and tested for binding to plate-bound ICAM-1 (*A*) or to SC2-ICAM-1 cells (*B*) as described in Fig. 1. *C–D*, Resting NK cells preincubated for 45 min with a combination of 100 U/ml IL-2 and either nothing (■), 20 μM colchicine (□), 20 μM cytochalasin D (▽), 30 μM PP1 (△), or 400 nM Wortmannin (○) were tested for binding to ICAM-1 on plates (*C*) and for conjugate formation with SC2-ICAM-1 cells (*D*) as described in Fig. 1. Inhibitors were maintained during the binding assays. Error bars in *A* and *C* represent the SD in four independent experiments.

Coexpression of LFA-3 or CD48 with ICAM-1 on target cells enhances adhesion of resting NK cells

To test whether other receptors on resting NK cells could contribute to adhesion, we generated *Drosophila* SC2 cell transfectants that expressed human LFA-3 (CD58) and CD48, either alone or in combination with ICAM-1 (Fig. 5). Cell sorting was used to obtain SC2 cells with matched levels of expression of the same ligand on different transfectants. CD2 and 2B4 (CD244), the receptors for LFA-3 and CD48, respectively, are expressed on NK cells. Whereas expression of 2B4 is uniformly high, and comparable between resting and activated NK cells, CD2 expression is higher on activated NK cells (Fig. 3). A minority fraction of resting NK cells, which varies among individuals, is negative for CD2 surface expression (Fig. 3). Very little, if any, conjugate formation occurred between resting NK cells and SC2 cells expressing only LFA-3 or CD48 (Fig. 6*A*). However, coexpression of either LFA-3 or CD48 with ICAM-1 resulted in a much higher level of adhesion than that obtained with SC2-ICAM-1 cells (Fig. 6*A*). Conjugates increased gradually for 40 min, reaching ~60% of resting NK cells after incubation with SC2-ICAM-1/LFA-3 and SC2-ICAM-1/CD48 cells. In contrast, as shown in Fig. 1, activated NK cells formed conjugates very rapidly with SC2-ICAM-1 cells. Coexpression of LFA-3 or CD48 with ICAM-1 resulted in minimal enhancement of adhesion by activated NK cells (Fig. 6*B*). As observed with resting cells, activated NK cells formed very few, if any, conjugates with insect cells expressing only LFA-3 or CD48 (Fig. 6*B*). We conclude that, even in the absence of exogenous signals such as cytokines, resting NK cells are characterized by their ability to adhere to target cells expressing ICAM-1, and to adhere strongly to target cells that coexpress LFA-3 or CD48 with ICAM-1.

The enhanced adhesion of resting NK cells to SC2-ICAM-1/LFA-3 and to SC2-ICAM-1/CD48 cells was inhibited by PP1 (Fig. 7), as adhesion to SC2-ICAM-1 cells was, in the absence or presence of IL-2 (Figs. 2 and 4). In contrast, adhesion to SC2-ICAM-1/LFA-3 and to SC2-ICAM-1/CD48 cells was not as sensitive to Wortmannin and was only partially sensitive to cytochalasin D (Fig. 7). The effect of Wortmannin was mainly to reduce the number of conjugates at all time points whereas cytochalasin D caused a delay in conjugate formation. Partial sensitivity to the microtubule inhibitor colchicine was also observed (Fig. 7). Therefore, the signaling requirements for adhesion of resting NK cells to target cells expressing ICAM-1 together with either LFA-3 or CD48 are different from those for adhesion to ICAM-1 alone and for adhesion to ICAM-1 in the presence of IL-2. These results suggest that CD2 and 2B4, upon engagement by ligand on target cells, provide signals that enhance LFA-1-mediated adhesion.

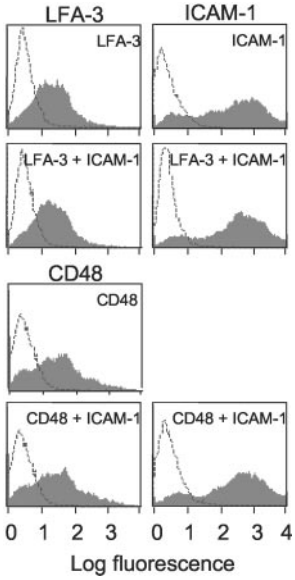

FIGURE 5. Cell surface expression of human molecules on transfected SC2 cells. SC2 cells, transfected with cDNAs encoding the molecules indicated within each panel, were stained with Abs specific for the molecules indicated above each panel. A PE-conjugated mAb specific for ICAM-1 (*right panels*) and FITC-conjugated mAbs specific for LFA-3 or CD48 (*left panels*) were used. SC2 cells express either one or two of the human proteins, as indicated in each panel. Dotted lines represent controls with isotype-matched PE- or FITC-conjugated mAbs.

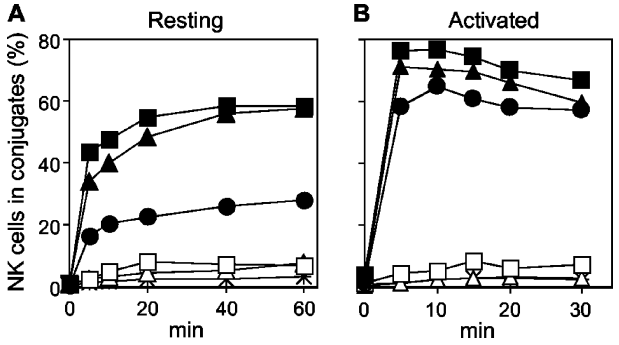

FIGURE 6. Engagement of CD2 and 2B4 by their ligands on target cells increases LFA-1-dependent adhesion of resting NK cells. Adhesion of resting NK cells (*A*) and of activated NK cells (*B*) to SC2 cells was determined as in Fig. 1. Untransfected SC2 cells (asterisks) and SC2 cells expressing ICAM-1 (●), LFA-3 (□), CD48 (△), or ICAM-1 in combination with LFA-3 (■) or CD48 (▲) were tested.

A 45-min pulse of IL-2 or IL-15 accelerated conjugate formation between resting NK cells and SC2-ICAM-1/LFA-3 and SC2-ICAM-1/CD48 cells (Fig. 8, *A* and *B*). The maximal number of conjugates was reached within 10 min, instead of 40 min in the absence of cytokine. Further evidence for a cooperation between cytokine signals and coreceptor engagement was obtained by quantitating polymerized F-actin (Fig. 8*C*). Phalloidin binding to fixed, permeabilized cells was determined by flow cytometry. Some variability in the assay may have been contributed by phalloidin binding to SC2 cells. Therefore, the only interpretable changes in F-actin were those observed between samples that formed a similar number of conjugates. In the absence of IL-2, no significant change in F-actin content was observed after incubation of resting NK cells with any of the SC2 cells. In contrast, incubation of IL-2-pulsed resting NK cells with SC2-ICAM-1/LFA-3 and SC2-ICAM-1/CD48 cells resulted in a doubling of F-actin content (Fig. 8*C*). This increase was significantly greater than that observed with SC2 and SC2-ICAM-1 cells, or with the double-transfected SC2 cells incubated with resting NK cells that had not been pulsed with IL-2. Therefore, combined signals from activation receptors CD2 or 2B4 and the IL-2R led to enhanced actin polymerization in resting NK cells.

Discussion

The interaction of ICAMs and β_2 integrins such as LFA-1 is a regulated process that controls leukocyte migration and function (22). Expression of ICAM-1 is up-regulated on endothelial cells during inflammatory responses. Specific signals in T cells and neutrophils, delivered by various receptors for cytokines, chemokines, and for ligands on other cells, regulate the affinity and avidity of β_2 integrins for ICAM. In this study, we investigated the regulation of

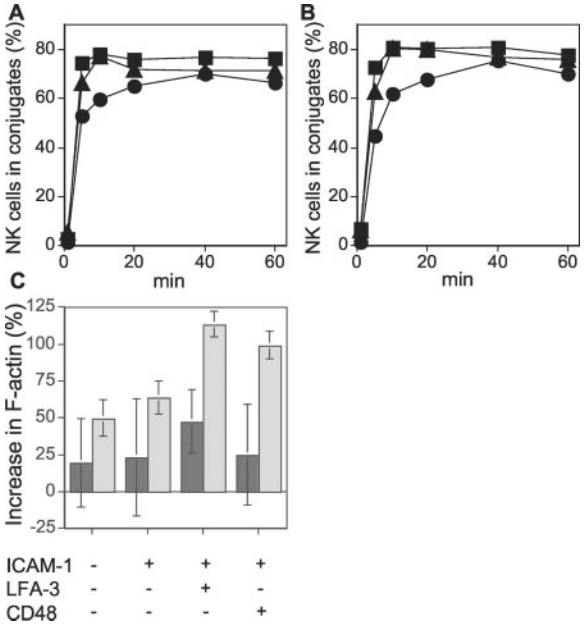

FIGURE 8. Adhesion of resting NK cells induced by combined signals from the receptors for IL-2 or IL-15, and for LFA-3 or CD48. Resting NK cells (●) pulsed with 100 U/ml IL-2 (■) or 100 U/ml IL-15 (▲) for 45 min were tested for adhesion to SC2 cells expressing ICAM-1 plus LFA-3 (*A*) or ICAM-1 plus CD48 (*B*). *C*, F-actin was quantitated by phalloidin staining of resting NK cells (darker shading) after a 45-min incubation with SC2 cells expressing the human molecules indicated (+) below the panel. The same was done with resting NK cells that were pulsed for 45 min with 100 U/ml IL-2 (lighter shading). NK cells were gated by side scatter and by the fluorescence of PE-conjugated anti-CD18. Phalloidin staining was determined by flow cytometry and is expressed as the increase of the mean fluorescence intensity over that obtained with NK cells alone. Error bars represent the SD in three independent experiments.

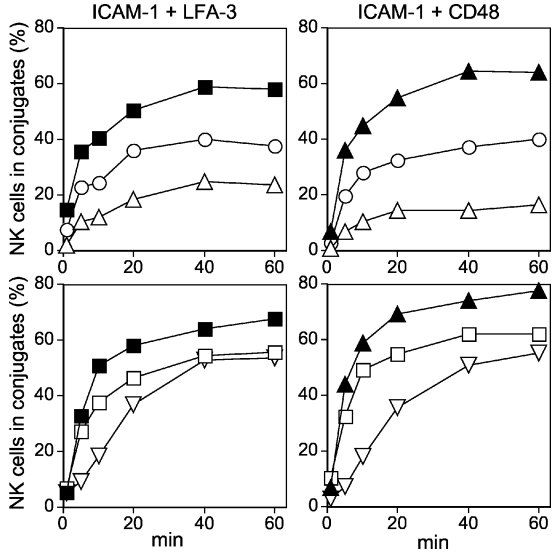

FIGURE 7. Inhibition of resting NK cell adhesion to SC2 cells expressing ICAM-1 and LFA-3 or ICAM-1 and CD48. Adhesion of resting NK cells to SC2-ICAM-1 plus LFA-3 cells (■) or SC2-ICAM-1 plus CD48 cells (▲) was tested after preincubation for 45 min with 30 μM PP1 (△), 400 nM Wortmannin (○), 20 μM colchicine (□), and 20 μM cytochalasin D (▽). The *top* and *bottom panels* represent two independent experiments. Inhibitors were maintained at the same concentration during the adhesion assay.

adhesion of resting NK cells to ICAM-1. We found that resting NK cells have ICAM-binding properties different from those of activated NK cells, and from those reported for T cells. Resting NK cells bound to ICAM-1 and formed conjugates with insect cells expressing ICAM-1 alone, in the absence of exogenous signals. This binding to ICAM-1 is an active process that was sensitive to inhibitors of *src* family kinases and of PI3K.

Adhesion of resting NK cells to ICAM-1 was enhanced by two different types of receptors. First, IL-2- or IL-15-pulsed resting NK cells adhered much more strongly than resting NK cells. This enhanced adhesion was sensitive to inhibitors of *src* family kinases, PI3K, and actin polymerization. Therefore, in response to a signal from IL-2 or IL-15, resting NK cells up-regulate their ICAM-1 binding properties through an actin-dependent mechanism that does not bypass the requirement for *src* family kinase and PI3K signals. In contrast, activated NK cells, which were expanded in IL-2 for several days, bound very strongly to ICAM-1 by a mechanism that was not sensitive to inhibitors of *src* family kinases and of PI3K. Therefore, the different adhesive properties of IL-2-activated NK cells must be due to stable alterations during expansion in IL-2. Second, resting NK cells adhered strongly to SC2 cells that expressed either LFA-3 or CD48 with ICAM-1. This enhanced adhesion could be due simply to the additive contribution of a second receptor-ligand interaction without specific signals, or it may require signals delivered by CD2 and 2B4 that enhance adhesion.

Our results support a signaling role of CD2 and 2B4 in adhesion to ICAM-1 by resting NK cells. Adhesion of resting NK cells to SC2-ICAM-1/LFA-3 and to SC2-ICAM-1/CD48 cells was only partially sensitive to inhibition of PI3K and of actin polymerization. The sensitivity of the CD2 and 2B4-dependent signals to PP1 indicated that these receptors deliver a *src* family kinase-dependent signal that reduces the dependence of adhesion to ICAM-1 on PI3K and actin polymerization. By comparison, the marked contribution of IL-2 and IL-15 to ICAM-1 binding, which is not due to an additive receptor-ligand interaction, did not overcome inhibition of *src* family kinases and PI3K, but had the additional requirement for actin polymerization. Signals through CD2 and 2B4 were also evident when measuring actin polymerization in resting NK cells incubated with SC2 cells expressing ICAM-1 with LFA-3 or CD48. Only with the combination of 1) coengagement of LFA-1 with CD2 or 2B4 and 2) an IL-2 or IL-15 signal was actin polymerization significantly increased.

A role of CD2 in adhesion of resting NK cells had been suggested by Ab blocking experiments (23). Adhesion of resting NK cells to a T lymphoma was blocked partially by anti-CD18, and less so by anti-CD2; but complete inhibition was obtained by blocking both receptors. In addition, the adhesion necessary for Ab-dependent cell-mediated cytotoxicity by a human NK clone was contributed by a combination of CD2 and CD18, whereas CD16 was not necessary for conjugate formation (24). 2B4 has been described as a costimulatory receptor that enhances signals from ITAM-containing receptors (9). In this study, we show that it can provide a signal that enhances adhesion through β_2 integrin, a process that is most likely ITAM independent.

The signal-dependent adhesion of resting NK cells to insect cells expressing ICAM-1 alone suggests that β_2 integrin itself can signal upon binding ICAM-1 to up-regulate its own adhesion. However, the possibility of an evolutionarily conserved structure on insect cells that is recognized by a receptor on human NK cells cannot be excluded. If such a receptor were to exist, it is unable to provide the signals that resting NK cells receive through the IL-2/IL-15 receptor, CD2, or 2B4. The transfected insect cells described here provide a useful system to dissect the contribution of

individual ligand-receptor interactions to the activation of NK cells. The very large number of activation and inhibitory receptors on NK cells, and the fairly ubiquitous expression of their ligands on various cell types, complicates the analysis of the role of specific receptors in NK cell responses. Additional ligands of NK cell receptors can be expressed in SC2 insect cells to study their contribution to different types of NK cell responses.

The physiological importance of NK cell regulation by cell contact is illustrated by the activation of resting NK cells by human dendritic cells. Within 1 wk of coculture, resting NK cells underwent several cell divisions and began producing IFN-γ (25). The unique ability of resting NK cells to initiate adhesion upon contact with ICAM-1-expressing cells, in the absence of exogenous signals, may be relevant to their important role as innate effector cells.

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