# Molecules and Structures Involved in the Adhesion of Natural Killer Cells to Vascular Endothelium

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## Summary

The present study was designed to define molecules and structures involved in the interaction of natural killer (NK) cells with the vascular endothelium in vitro. Resting and interleukin 2 (IL-2)-activated NK cells were studied for their capacity to adhere to resting and IL-1-treated human umbilical vein endothelial cells (EC). In the absence of stimuli, NK cells showed appreciable adhesion to EC, with levels of binding intermediate between polymorphs and monocytes. The binding ability was increased by pretreatment of NK cells with IL-2. Using the appropriate monoclonal antibody, the $\beta_2$ leukocyte integrin CD18/CD11a was identified as the major adhesion pathway of NK cells to unstimulated EC. Activation of EC with IL-1 increased the binding of NK cells. In addition to the CD18-CD11a/intercellular adhesion molecule pathway, the interaction of resting or IL-2-activated NK cells to IL-1-activated EC involved the VLA-4 ($\alpha_4\beta_1$)-vascular cell adhesion molecule 1 receptor/counter-receptor pair. No evidence for appreciable involvement of endothelial-leukocyte adhesion molecule was obtained. Often, NK cells interacted either with the culture substrate or with the EC surface via dot-shaped adhesion structures (podosomes) protruding from the ventral surface and consisting of a core of F-actin surrounded by a ring of vinculin and talin. The identification of molecules and microanatomical structures involved in the interaction of NK cells with EC may provide a better understanding of the regulation of NK cell recruitment from blood, their extravasation, and their migration to tissues.

Leukocyte recruitment from the blood compartment is a crucial determinant for the induction and expression of immunity and inflammation. Leukocyte extravasation involves adhesion to the endothelial lining of blood vessels and response to tissue-derived chemotactic signals. Endothelial cells (EC)[1] play an active role in the control of leukocyte recruitment by producing cytokines active on leukocytes and by expressing, in a regulated fashion, membrane proteins that are substratum for adhesion of circulating cells (for review see references 1–5). Several of these molecules have been recently identified and sequenced, including intercellular adhesion molecule 1 and 2 (ICAM-1 and -2) (6, 7), endothelial-leukocyte adhesion molecule 1 (ELAM-1) (8), and vascular cell adhesion molecule 1 (VCAM-1, also known as INCAM 110) (9, 10). The expression of ICAM-1, ELAM-1, and VCAM-1 is regulated by bacterial products (e.g., LPS) and cytokines (1–5).

Different leukocyte populations recognize "activated" EC by means of different receptor/counter-receptor pairs. Polymorphs (PMN), but not lymphocytes, recognize ELAM-1 (8), whereas lymphocytes, via the integrin $\alpha_4\beta_1$ (VLA-4), bind to VCAM-1 (10, 11). VCAM-1 is also recognized by monocytes, which may also bind to ELAM-1 (2).

Cells with NK activity act as a first line of resistance against foreign cells, microbes, and possibly tumors (for review see references 12 and 13). Most blood NK activity is mediated by $CD3^-$, $CD16^+$, $CD56^+$ effectors. These cells, which also mediate antibody-dependent cytotoxicity, have the TCR genes in germline configuration and do not express a CD3/TCR complex (12, 13). The mechanisms involved in the regulation of extravasation of NK cells and their fate in tissues are poorly understood. NK cells migrate promptly in vitro (14–17) and adhere to and kill EC upon activation (18–21).

The present study was designed to identify the molecular structures involved in adhesion of resting and activated NK cells to resting and activated EC. We show that unstimulated NK cells have binding levels to resting EC intermediate

[1] *Abbreviations used in this paper:* EC, endothelial cells; ELAM, endothelial-leukocyte adhesion molecule; ICAM, intercellular adhesion molecule; RPHD, rhodamine-labeled phalloidin; VCAM, vascular cell adhesion molecule.


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between PMN and monocytes, and this involves the CD18/CD11a adhesion pathway. Upon activation of NK cells and/or EC, augmented binding involves also the $\alpha_4\beta_1$/VCAM-1 interaction pathway. A structural feature of NK cell interaction with EC is the formation of "podosomes", which represent dot-shaped protrusions of the cellular ventral membrane provided with adhesive properties and formed by a peculiar cytoskeletal architecture. Podosomes have been identified in a number of normal and transformed cells (22–25; reviewed in 26), and are believed to be rapidly modulated adhesion structures involved also in motility processes and in controlling the passage through anatomical barriers, including the basement membrane (25, 27). Podosomes have been described also in normal and malignant blood cells (28, 29) and found to be associated with the $\beta_2$/CD18 integrin complex.

## Materials and Methods

*Preparation of Leukocyte Populations.* Normal human NK cells were isolated on discontinuous Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden) as previously described (14). Low-density cells were further depleted of monocytes by plastic adherence and thereafter checked morphologically for the presence of large granular lymphocytes (12, 13). In part of the experiments, NK cells were depleted of monocytes and/or T lymphocytes by treatment with saturating amounts of mAb anti-CD14 (kind gift of Dr. J. Griffin, Dana Farber Cancer Institute, Boston, MA) and/or anti-CD3 (Becton Dickinson & Co., Mountain View, CA) and Dynabeads (Unipath, Italy); after two cycles of Dynabeads, the resulting population contained <0.5–2% $CD14^+CD3^+$ cells and >70–80% $CD16^+$ and/or $CD56^+$ cells, by FACS® analysis (Becton Dickinson & Co.). Purified preparations of monocytes were separated on Percoll gradients as reported (30). PMN were isolated by Ficoll-Hypaque gradient (Biochrom KG, Berlin, FRG) and red cells were lysed by hypotonic shock.

*Activation of NK Cells.* NK cell preparations were resuspended at $2 \times 10^6$/ml in RPMI 1640 (Biochrom KG) plus 10% FCS (Hyclone Laboratories, Logan, UT), referred as complete medium, and were activated by incubation with 100 U/ml IL-2 (Glaxo Imb., Geneva, Switzerland) for 24 h. At the end of the culture period, cells were washed with saline and resuspended in complete medium for labeling. Control NK cells were incubated in medium alone. Adhesion of NK cells cultured in medium was not different from that of freshly isolated cells.

*Preparation of EC.* Human EC were obtained from umbilical vein and cultured as previously described (31). Routinely, we used confluent cells ($10^5$/2-$cm^2$ culture well) between the first and fourth passage maintained in 199 medium with 20% newborn calf serum (Hyclone Laboratories) supplemented with endothelial cell growth supplement (50 $\mu$g/ml; Collaborative Research, Inc., Lexington, MA) and heparin (100 $\mu$g/ml; Sigma Chemical Co., St. Louis, MO). The purity of EC cultures was checked by expression of factor VIII antigen and found to be >99% positive.

*Antibodies (Functional Assays).* mAbs used in this study were obtained through the courtesy of the following persons: two mAbs directed against the common $\beta_2$ subunit (CD18) of leukocyte integrin, clone CLB54-LFA 1/1 (IgG1) (Dr. R. Van Lier CLB, Amsterdam, The Netherlands) and clone 10F12 (Dr. J. Ritz, Dana Farber Cancer Research Institute, Boston); mAb clone CLB-LFA 1/2 (IgG2a) directed against the $\alpha_L$ subunit (CD11a) of LFA1 (Dr. R. van Lier); mAb clone 44a (IgG2a) directed against the $\alpha_M$ subunit (CD11b) of Mac-1/CR3 (Dr. R. Todd, Ann Arbor, MI); mAb clone L29 (IgG1), recognizing the $\alpha_X$ subunit (CD11c) of p150,95 (Dr. L. Lanier, Becton Dickinson & Co.); mAb anti-ICAM-1, clone LB2 (IgG2b) (Dr. N. Hogg, ICR, London, UK) and clone 6.5B5 (IgG1) (Dr. D. Haskard, Guy's Hosp., London, UK); mAb anti-VLA4, clone HP2/1 (IgG1) (Dr. F. Sanchez-Madrid, University of Madrid, Madrid, Spain); mAb anti-VCAM-1, clone 4B9 (IgG1) (Dr. J. Harlan, University of Washington, Seattle, WA); and clone E1/6 (IgG1) (Dr. M. Bevilacqua, Harvard Medical School, Boston, MA); and mAb anti-ELAM-1, clone H4/18 (IgG1) (Dr. M. Bevilacqua). These reagents have previously been shown to recognize functionally relevant epitopes (32–40).

Results shown in Fig. 4–7 refer to mAb CLB54-LFA1/1 for CD18; LFA1/2 for CD11a; 44a for CD11b; L29 for CD11c; LB2 for ICAM-1; 4B9 for VCAM-1; HP2/1 for VLA-4; and H4/18 for ELAM-1, but similar data were obtained with the alternative appropriate reagents directed against the same structure.

mAbs were used as ascites after dialysis at a previously determined optimal concentration of 1/300, or as hybridoma supernatant diluted 1/5 to 1/10. mAbs recognizing leukocyte structures were incubated during the adhesion assay; mAbs recognizing structures on EC were pre-incubated with the EC monolayer for 20 min at 37°C and left in the medium during the adhesion assay. In each experiment, isotype-matched antibodies were used as negative control: mAb IgG1 (clone 21, Thy-2D3, anti-CD8), mAb IgG2a (clone 19, Thy-5D, anti-CD4), and mAb IgG2b (clone 24T6G12, anti-CD5).

*Antibodies (Immunofluorescence Assays).* The localizations of vinculin and talin were, respectively, assayed with a mAb to vinculin (41) from Bio Makor, Rehovot, Israel (VIN 11-5; no. 6501) and a mAb crossreacting with human talin, clone 8D4 (42), obtained from Dr. K. Burridge, University of North Carolina, Chapel Hill, NC. mAbs anti-VLA-4 B-5G10, to $\alpha_4$ (11) and anti-$\beta_1$, and A-1A5 (43) were a kind gift of Dr. M. Hemler, Dana Farber Cancer Institute.

*Adhesion Assay.* Adhesion of leukocytes to EC was studied as described previously (30). EC were grown to confluence in flat-bottomed 96-well trays and, in some experiments, were activated for 20 h with 10 ng/ml of IL-1$\beta$ (sp act, $8 \times 10^6$ U/$\mu$g) (Sclavo, Siena Italy) unless otherwise specified.

Leukocytes were resuspended in complete medium at $10^7$/ml and labeled with 100 $\mu$Ci $^{51}$Cr (sodium chromate; Amersham International, Amersham, UK) at 37°C for 1 h. At the end of the incubation, the cells were washed with medium and were resuspended at $10^6$/ml; then, 0.1 ml was dispensed to each well and incubated for 30 min at 37°C. At the end of the incubation, the wells were carefully washed three times with PBS + 1% FCS; adherent cells were solubilized with 0.2 ml of 0.025 M NaOH and 0.1% SDS, and radioactivity was counted in a gamma counter. Results are presented as percentage of adherent cells ± SD, with three to five replicates/group. Levels of statistical significance were calculated by Student's *t* test. In one series of experiments, HT29 colon carcinoma cells were used as reference population, in as much as the augmented binding of these cells to IL-1-activated EC is mediated via ELAM-1 (see Results).

*Preparation of NK Cells for Analysis of Adhesion Structures.* Adhesion of IL-2-activated NK cells was studied both on uncoated glass coverslips as well as on coverslips coated by a pre-formed EC monolayer. Cells were processed as previously described (28). Briefly, coverslip-attached cells were fixed in PBS containing 3% paraformaldehyde and 2% sucrose (pH 7.6) for 5 min at room temperature and permeabilized in Hepes-Triton buffer (20 mM Hepes, pH

7.4, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, and 0.5% Triton X100) for 5 min at 0°C, and finally rinsed in PBS. To show F-actin, fixed and permeabilized cells were stained with 2 μg/ml rhodamine-labeled phalloidin (R-PHD; Sigma Chemical Co.) for 30 min at 37°C. Indirect immunofluorescence experiments were performed as reported (28). Briefly, the primary antibody was layered on fixed and permeabilized cells and incubated in a humid chamber for 30 min. After rinsing in 0.1% BSA in TBS, coverslips were incubated in the appropriate rhodamine-tagged secondary antibody (Dakopatts, Glostrup, Denmark) for 30 min at 37°C in the presence of 2 μg/ml of fluorescein-labeled phalloidin (F-PHD; Sigma Chemical Co.), rinsed, and mounted in Mowiol 4-88 (Hoechst, Frankfurt/Main, FRG).

Observations were carried out in an Axiophot photomicroscope (Carl Zeiss, Inc., Thornwood, NY) equipped for epifluorescence and interference reflection microscopy. Fluorescence images were recorded on Kodak T-Max 400 films exposed at 1,000 ISO and developed in Kodak T-Max developer for 10 min at 20°C.

## Results

*Adhesion of Resting and Activated NK Cells to EC.* Leukocytes are heterogeneous in their ability to adhere to EC in the absence of stimuli, with PMN exhibiting minimal and monocytes high "spontaneous" binding. As shown in Fig. 1 *A*, NK cells had intermediate levels of spontaneous adhesion to EC with 16 ± 8% binding (mean ± SD; range 5–25%, of five experiments), compared with 40 ± 13 of monocytes (five experiments) and 7 ± 3% of PMN (seven experiments), as measured after 30 min. Similar results were obtained at all time points examined (Fig. 1 *B*).

We have studied the influence of stimuli on the interaction of NK cells with EC. IL-2 and IL-1 were used as prototypic activation signals for NK cells and EC, respectively (5, 13). As shown in Fig. 2, NK cells exposed to IL-2 (100 U for 24 h) showed a 2.5-fold increase in binding to EC, with 41 ± 13% adhesion. Similarly, when EC were exposed to IL-1 (10 ng/ml for 20 h), stimulated binding of resting NK cells (37 ± 12% in five experiments) was observed. The maximal level of binding (49 ± 7%) was observed when both NK cells and EC were stimulated with the appropriate respective stimulus. The experimental conditions used for these experiments were chosen after preliminary experiments had shown that they were optimal for NK cell adhesion to EC. Optimal activation of NK cells for binding was observed with IL-2 concentrations ranging from 100 to 1,000 U/ml, consistent with previous results and with the constitutive expression of the intermediate affinity IL-2 receptor by these cells (13). Maximal increase of the capturing capacity of NK cells was observed at IL-1 concentrations >0.5 ng/ml, as for other functions of EC (e.g., 5,30,31).

Distinct adhesion molecules induced in EC by IL-1 are expressed with distinct time courses (1–5). Cytokine-induced ELAM-1 expression is maximal at 4–6 h and declines thereafter, whereas expression of ICAM-1 and VCAM-1 is sustained for 24 h or longer (8–10). The distinct temporal appearance of these three adhesion molecules in IL-1-stimulated EC was confirmed in experiments preliminary to the present study (not shown). For the above reasons, in a series of experiments, we examined the time course of increased binding of NK cells to IL-1-treated EC. As shown in Fig. 3, where results of three experiments are presented, sustained binding to IL-1-treated EC was already detected at 4 h, with no increase or decrease up to 24 h.

*Adhesion Molecules Involved in Binding to Resting EC.* The role of different adhesion structures in binding of NK cells to EC was then examined. Representative results are presented in Fig. 4–7. The interaction of unstimulated NK cells with resting EC was first examined. Fig. 4, *A* and *B* shows one typical experiment. Anti-CD18 mAb caused 80 ± 12% inhibition of binding (mean ± SD of five experiments). When the role of the three different $\beta_2$ integrins ($\alpha_L\beta_2$ or LFA-1;

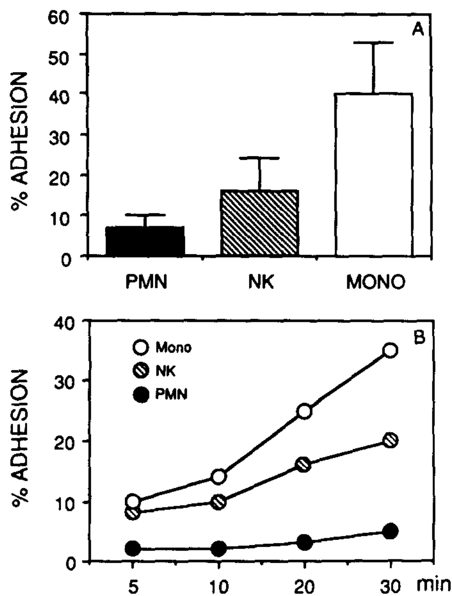


**Figure 1.** Leukocyte adhesion to monolayers of human EC. Percent adhesion of $^{51}$Cr-labeled polymorphs, monocytes, and NK cells was determined after 30-min incubation (*A*) or at earlier time points (*B*). Values represent mean ± SD of five to seven different experiments.

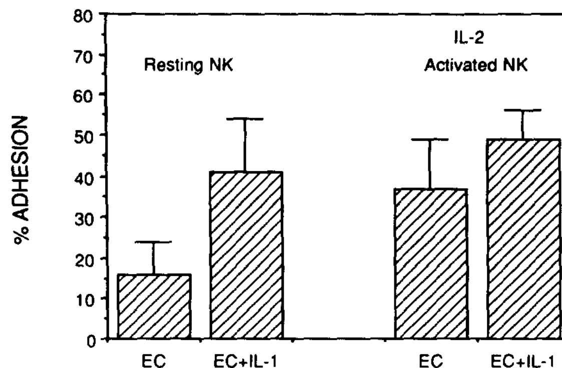


**Figure 2.** Adhesion of resting or IL-2-activated NK cells (100 U/ml of IL-2 for 24 h) to resting EC or pretreated for 24 h with 10 ng/ml of IL-1. Percent adhesion was determined after 30-min incubation at 37°C of $^{51}$Cr-labeled cells. Values represent mean ± SD of four different experiments.

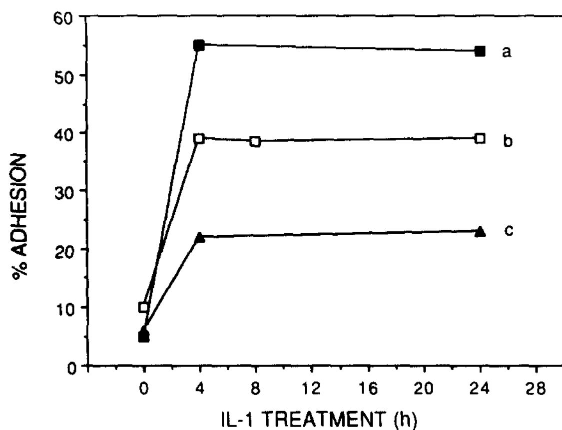


**Figure 3.** Kinetic analysis of IL-1 activation of EC. Percent NK cell adhesion was determined with $^{51}$Cr-labeled cells. *a*, *b*, and *c* represent independent experiments. SD were always <3–5% and were omitted for clarity.

$\alpha_M\beta_2$ or Mac 1; $\alpha_X\beta_2$ or p150,95) was studied, only anti-CD11a/LFA-1 antibodies inhibited binding (67 ± 10%). It must be emphasized that the anti-CD11b and anti-CD11c mAbs used in this study recognize functionally relevant epitopes (34–36). Anti-ICAM-1, anti-ELAM-1, anti-VLA-4, and anti-VCAM-1 had no consistent and significant effect.

Anti-CD18 and anti-CD11a mAbs caused a 57 ± 24% and 32 ± 7% inhibition, respectively, of the increased binding of IL-2-activated NK cells to resting EC (mean of four experiments, one shown in Fig. 4 *D*). Anti-ELAM-1 and anti-VLA-4 (*C*) had no inhibitory activity, while anti-ICAM-1 caused 36 ± 7% inhibition (range 30–46%) in four experiments, and its effect was not significant in one.

*Adhesion Molecules Involved in Binding to Activated EC.* The interaction of resting NK cells with IL-1-stimulated EC was then analyzed (Fig. 5, *A* and *B*). As for the binding to resting EC, anti-CD18 and anti-CD11a mAbs caused substantial inhibition of adhesion (45.6 ± 11% and 44 ± 5%, blocking in four experiments, one of which is shown in Fig. 5 *B*). Interestingly, the interaction of IL-2-activated NK cells with IL-1-treated EC was inhibited by these mAbs to a lesser degree (31 ± 6% and 24 ± 2% inhibition, respectively, four experiments), as compared with any other cell combination (Fig. 5 *D*).

Anti-ICAM-1 mAb caused appreciable inhibition of binding of both resting and activated NK cells with 36 ± 3% and 22 ± 10% inhibition, respectively (Fig. 5, *A* and *C*). Anti-ELAM-1 mAb had no substantial effect on binding to activated EC (Fig. 5, *A* and *C*). These results were obtained with anti-ELAM-1 mAb, clone H4/183, and clone BBIGE8 (results not shown, courtesy of Dr. J. Gordon, BBL, Oxford, UK). The results presented in Figs. 4 and 5 refer to EC pretreated with IL-1 for 20 h. It was important to evaluate the involvement of ELAM-1 after 4–6 h of exposure to IL-1, when expression of this structure is maximal (8). As illustrated by the experiment presented in Fig. 6 *A*, at no time point did anti-ELAM-1 mAb influence binding of NK cells to activated EC. It is noteworthy that under these conditions, anti-ELAM-1 inhibited the augmented binding of colon carcinoma cells HT29 (80% blocking) to IL-1-treated EC (Fig. 6 *B*).

Anti-VLA-4 and anti-VCAM-1 mAbs appreciably reduced binding (36% and 25% inhibition) of unstimulated NK cells to activated EC (Fig. 5 *A*). These mAbs did not substantially affect the adhesion of activated NK cells; a representative experiment is shown in Fig. 5 *C*.

In an effort to obtain better identification of the role of the VLA-4 adhesion pathway, combinations of anti-CD18 and anti-VLA-4 mAbs were studied (Fig. 7). After inhibition of the CD18 adhesion pathway, the blocking effect of

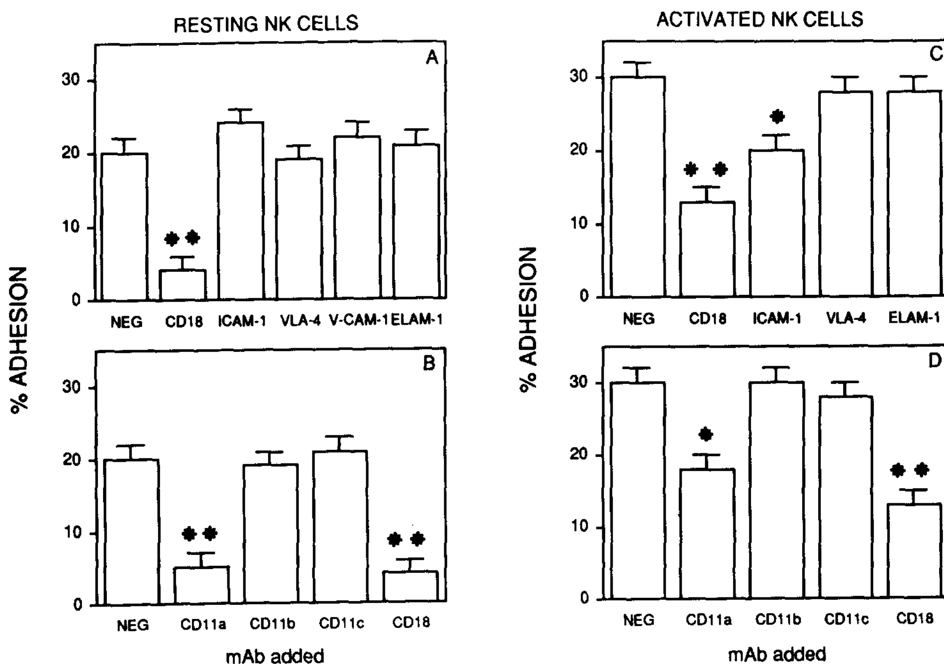


**Figure 4.** Inhibition of NK cell adhesion to resting EC by individual mAb. $^{51}$Cr-labeled resting NK cells (*A* and *B*) and IL-2-activated NK cells (*C* and *D*) were incubated for 30 min on EC monolayers in the presence of optimally diluted mAb. Anti-VCAM-1, anti-ELAM-1, and anti-ICAM-1 mAbs were pre-incubated for 20 min on EC alone. Values represent mean ± SD of three to five replicates. (*) Statistically significant at $p < 0.05$ vs. negative control. (**) Statistically significant at $p < 0.01$ vs. negative control.

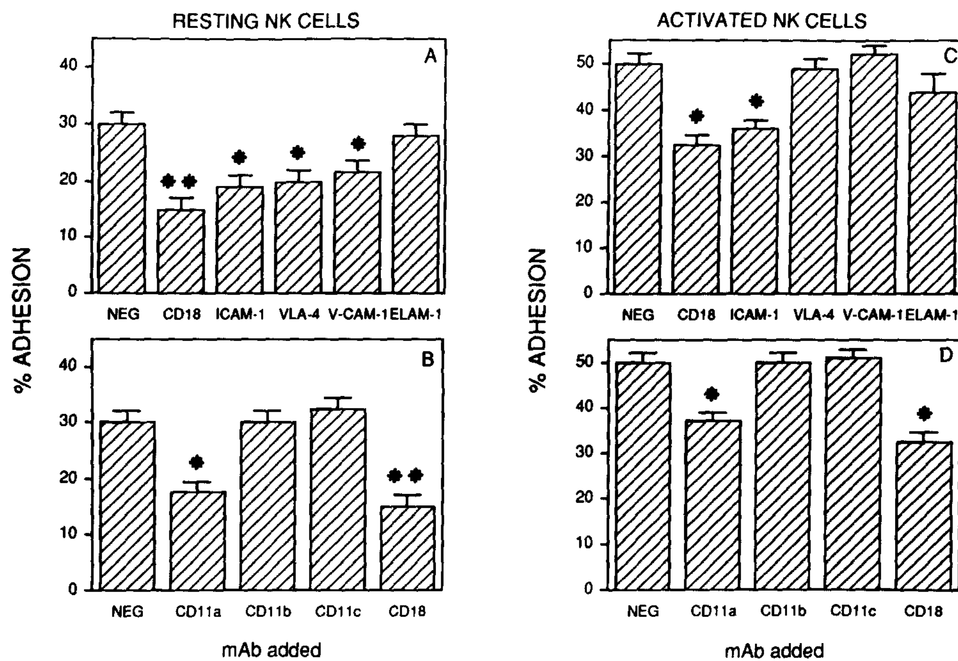


**Figure 5.** Inhibition of NK cell adhesion to IL-1-activated EC by individual mAb. Resting NK cells (*A* and *B*) and IL-2 activated NK cells (*C* and *D*) were incubated for 30 min on EC monolayers in the presence of optimally diluted mAb. Values represent mean ± SD of three to five replicates. (*) Statistically significant at $p < 0.05$ vs. negative control. (**) Statistically significant at $p < 0.01$ vs. negative control.

anti-VLA-4 mAb on binding of resting (Fig. 7 *A*) and activated (*B*) NK cells to IL-1-treated EC was clearly and consistently detectable. Combinations of anti-ICAM-1 with anti-CD18 mAb were not different from those obtained with anti-CD18 alone.

To identify a possible role of ELAM-1, a mAb directed against this molecule was added to combinations of anti-VLA-4 and CD18. Alto under these conditions, anti-ELAM-1 mAb was devoid of appreciable activity (Fig. 7 *B*).

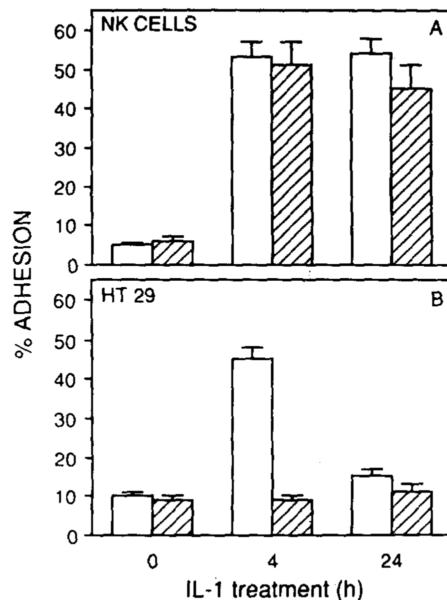


**Figure 6.** Inhibition of NK cell and colon carcinoma (HT29) cell adhesion on activated human EC by anti-ELAM-1 mAb. Adhesion assay was performed at different time points after IL-1 activation of EC. Open bars and hatched bars refer to control and anti-ELAM-1-treated cultures, respectively.

These results indicate that CD18/CD11a and VLA-4 are important pathways of adhesion of NK cells to endothelium. Expression of $\beta 2$ integrins on NK cells has been extensively characterized (13, 37). It was important to analyze the expression of VLA-4 on resting and IL-2-activated NK cells. As shown in Fig. 8, where one representative experiment of three performed is shown, NK cells expressed VLA-4, an observation that confirms a recent report (44). Expression of VLA-4 was not appreciably altered after IL-2 activation, as judged by fluorescence intensity.

*Adhesion Structures Involved in the NK-EC Interaction.* The adhesion of IL2-activated NK cells was studied by fluorescence microscopy upon seeding cells on unstimulated and IL-1-stimulated EC monolayers. The stimulation of EC with IL-1 increased the number of adherent NK cells by at least threefold. These data were obtained by observing cells stained with R-PHD, which binds to F-actin. Many NK cells displayed irregular shape and membrane ruffles, indicating that they were undergoing active locomotion on the EC monolayer (Fig. 9, *a* and *b*). EC-adherent NK cells showed strong membrane positivity for the CD11a/LFA-1 integrin complex, as shown by CD18/$\beta_2$ mAb (Fig. 9 *c*) and an $\alpha_L$/CD11a mAb (Fig. 9 *d*). Also VLA-4 was expressed on NK cell membrane, although at a somewhat weaker extent (Fig. 7 *e*). In any case, these integrin heterodimers were not expressed by the underlying EC, as expected, but a control with a $\beta_1$ mAb (A-1A5) showed that other $\beta_1$ integrins were expressed by EC (data not shown; e.g. reference 45).

10–20% of the NK cells that had firmly adhered to the EC monolayers displayed a peculiar distribution of F-actin in 0.2–0.4-$\mu$m punctate dots (Fig. 9, *a* and *b*, e.g., at arrowheads), which were identical to the dot-like close contact adhesion sites that we had previously described in many normal and transformed cell types (22–25) and denoted as podosomes.

Podosomes were also displayed by NK cells seeded on glass

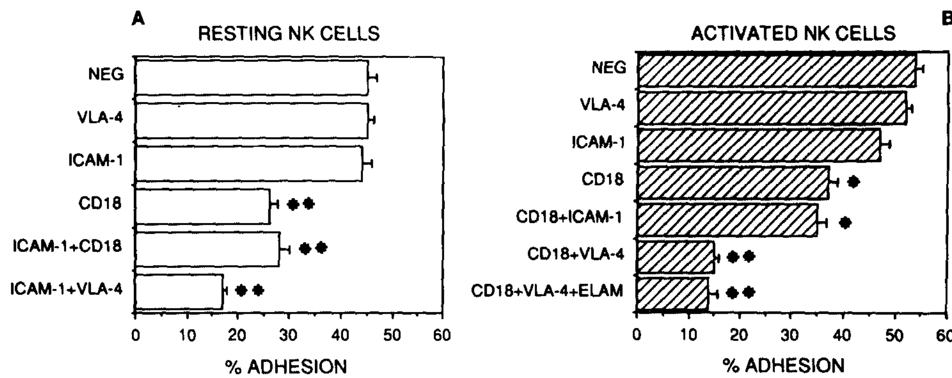


**Figure 7.** Inhibition of NK cell adhesion to IL-1-activated EC by combinations of mAbs. Resting NK cells (*A*) and IL-2-activated NK cells (*B*) were incubated on EC monolayers in the presence of optimally diluted mAbs. Values represent mean ± SD of three to five replicates. (*) Statistically significant at $p < 0.05$ vs. negative control. (**) Statistically significant at $p < 0.01$ vs. negative control.

(not shown). Under these conditions, the podosomes of NK cells were better observed in the absence of any interfering signal from underlying EC. We could confirm that NK cell podosomes displayed the typical ring-like distribution of vinculin, talin, and CD18 already described in other cell types (22–25). We could not obtain a good high resolution pattern of vinculin and talin on NK cells seeded on EC because the latter are very rich in vinculin and talin and gave a strong background signal (45).

## Discussion

The present study identifies receptor/counter-receptor systems and structures involved in the interaction of NK cells with EC. Unstimulated NK cells adhered significantly to resting EC, with binding capacity intermediate between that of PMN and monocytes. It was already reported that among nonphagocytic lymphoid cells, NK cells show increased ability to adhere to EC (18–20).

In this study, the $\beta_2$ leukocyte integrin CD18/CD11a (LFA 1) was identified as the main adhesion structure involved in the NK/EC interaction in the absence of stimuli. Inhibition of lymphocyte binding by anti-CD11a mAb has already been reported for T cells (46, 47), and in one report for cells with NK characteristics (18). Members of the leukocyte $\beta_2$ integrin family are known to play an important role in adhesion of PMN and monocytes to EC. Binding of monocytes

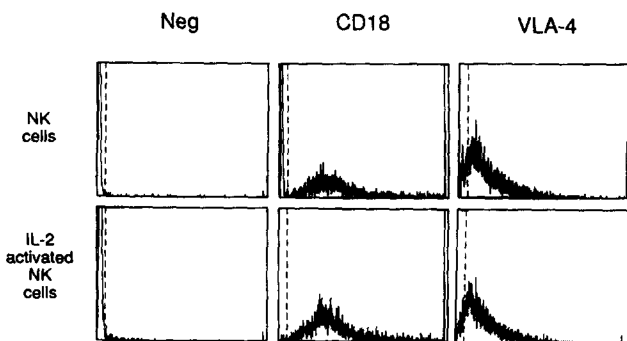


**Figure 8.** Flow cytometric analysis of resting and IL-2-activated (24 h) NK cells stained with anti-CD18 and anti-VLA-4 mAb.

and PMN to EC involves, in addition to CD11a, also CD11b and, to a very minor degree, CD11c (p150, 95) (30, 34). NK cells express CD11b (Mac 1) and CD11c/p150,95 (13, 37), but we failed to identify a functional role for these structures in binding of these cells to EC. This apparent discrepancy in Mac-1 involvement between phagocytes and NK cells may reflect a difference in the amount of this molecule expressed on the surface as well as a difficulty in identifying a role for minor adhesive components in the presence of relatively low levels of binding.

The $\beta_2$ integrin CD18/CD11a recognizes ICAM-1 and ICAM-2 (6, 7). ICAM-2 is constitutively expressed by EC, whereas ICAM-1 is present at relatively low levels and is increased by cytokines (1, 7). Since anti-ICAM-1 mAb had no effect on binding of resting NK cells to unstimulated EC, we infer that the main receptor/counter-receptor system involved in this interaction is LFA-1/ICAM-2.

Activated NK cells have increased ability to adhere to resting EC. These cells have been shown to kill EC (19, 21), a phenomenon that may be important in the vascular leak syndrome associated with lymphokine-activated killer/IL-2 therapy (48). As for resting NK cells, the CD18-dependent pathway accounted for 60% of binding of activated NK cells to unstimulated EC.

A major objective of the present study was to identify adhesion pathways involved in the interaction of NK cells with IL-1-activated EC, which have increased capacity to capture leukocytes. As for binding to resting EC, anti-CD18/CD11a inhibited adhesion. Binding of NK cells to activated EC was also consistently reduced by anti-ICAM-1, an observation that reflects the increased expression of this molecule in EC exposed to IL-1 (1, 2). It is of interest to highlight the fact that $\beta_2$ integrin antibodies caused less inhibition of binding to IL-1 treated than to resting EC (45 ± 12% vs. 80 ± 11%; $p < 0.01$). The level of such inhibition was inversely proportional to the extent of EC activation and this finding suggests the involvement of other IL-1-induced adhesion mechanisms.

VCAM-1 is a recently identified adhesion molecule induced on endothelium by cytokines and LPS (9, 10). The leukocyte receptor for VCAM-1 is $\alpha_4\beta_1$/VLA-4 (CD49d) (11). VCAM-1 is an adhesion molecule recognized by normal and leukemic T and B lymphocytes as well as by monocytes and myeloid leukemic lines (9, 10). Anti-VLA-4 and anti-VCAM-1

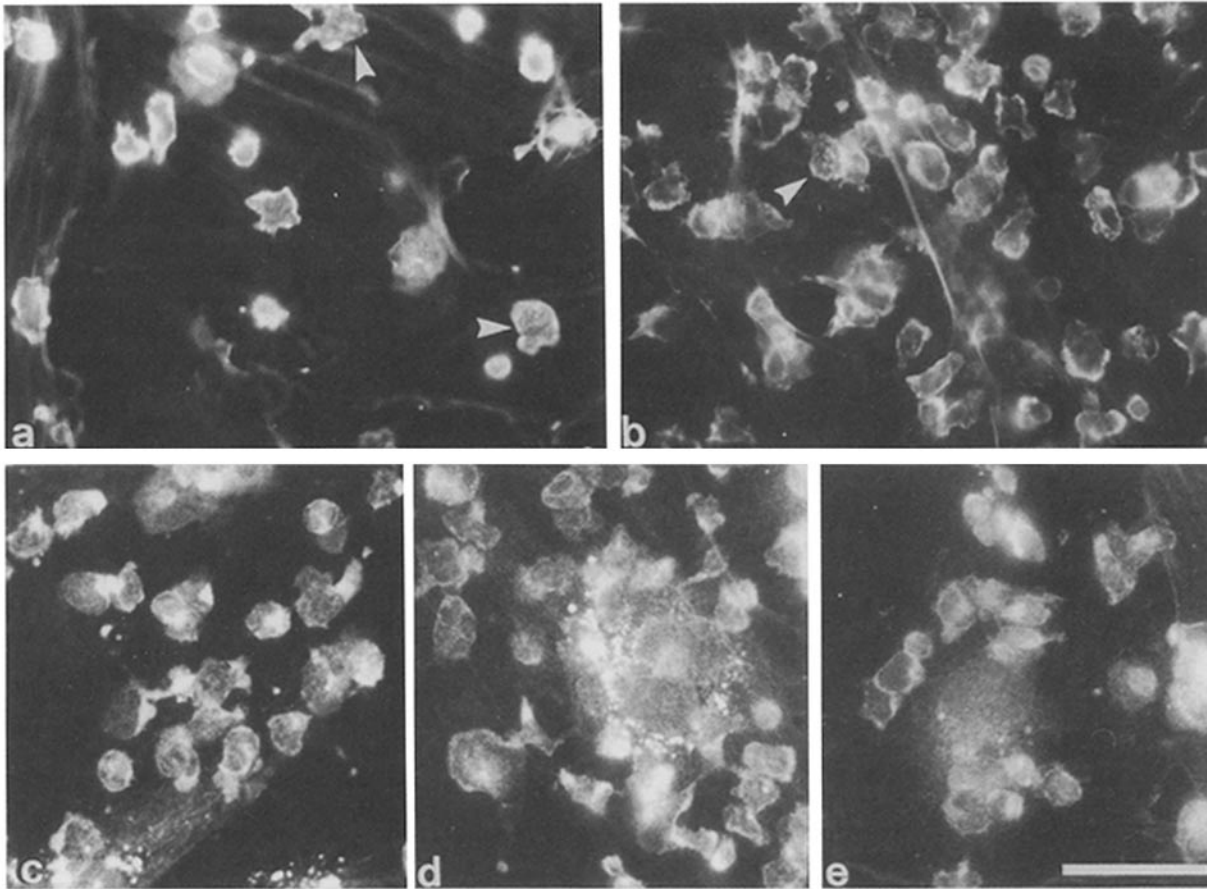


**Figure 9.** Fluorescence detection of NK cells on EC monolayers by means of F-actin staining with R-PHD. The focus was adjusted on NK cells and, therefore, the underlying EC are only barely visible. Some of the NK cells display dots of F-actin (e.g., at arrowheads) both on unstimulated (*a*) and IL-1 stimulated (*b*) EC monolayers. *c*, *d*, and *e*, respectively, show the immunofluorescence localization of CD18/$\beta_2$, CD11a/$\alpha_L$, and VLA-4/$\alpha_4$ on NK cells that had adhered on IL-1-stimulated EC cell monolayers. Bar denotes 15 $\mu$m.

mAbs had some, but modest, effect on NK binding to activated EC when used alone. In contrast, when the CD18 adhesion pathway was blocked by an appropriate mAb, a role for the VCAM-1/VLA-4 receptor/counter-receptor interaction was clearly identified and accounted for ~34% of binding of activated NK cells to IL-1-treated EC (Fig. 7). Blocking of CD18 was also required to highlight the role of VLA-4 in a recent study on binding of monocytes to EC (2, 49).

ELAM-1 binds neutrophils and some leukemic myeloid cells, but not lymphocytes and lymphocytic lines (8). The capacity of ELAM-1 to bind monocytes is not clearly defined (39, 50), but recently, it was found that mononuclear phagocytes recognize ELAM-1 expressed in CHO cells (Carlos, T.M. et al., manuscript submitted for publication). In the present study, anti-ELAM-1 did not affect the interaction of NK cells with EC. Moreover, we did not find a role for this protein combining anti-ELAM-1 mAb with anti-CD18 (± anti-VLA-4), a condition that demonstrated the role played by VLA-4/VCAM-1. Two anti-ELAM-1 mAbs were used, under conditions in which they blocked the augmented binding of colon carcinoma cells (HT29) to IL-1-activated EC (Fig. 6) and PMN (not shown). Thus, these observations suggest that ELAM-1 is not an important structure for the adhesion of NK cells to vascular EC.

In the presence of saturating concentrations of anti-CD18 and anti-VLA-4 mAbs (± anti-ELAM-1), adhesion of a substantial proportion of resting and activated NK cells (25–30%; Fig. 7) on activated EC was clearly detectable. Conceivably, the interesting possibility exists that unknown adhesion pathways, other than those identified in the present study (CD18/CD11a-ICAM and VLA-4-VCAM-1), may play a role in the interaction of NK cells with vascular EC.

The results presented here identify adhesion molecules and structures involved in the interaction of NK cells with vascular EC. Relatively little information is available on the regulation of NK cell extravasation and localization in tissues. NK cells can be identified morphologically and functionally in the peritoneal cavity of mice early after the intraperitoneal inoculation of bacterial agents (51). Cells with NK activity and phenotype accumulate during virus infection in the liver and at sites of virus replication (52–57). Early after transplantation, NK cells can be identified in allografted tissues (55). After administration of biological response modifiers, NK cells localize in certain tissues, particularly in the liver

(56). On the other hand, NK cells generally represent a minor proportion of lymphoid cells infiltrating tumor tissues (13, 57).

The adhesion of NK cells to EC or to other culture substrates involves the appearance of peculiar dot-like adhesion structures called podosomes (22–25), which have already been described in other lymphoid cells (28) and in normal and malignant monocytes (29). Podosomes are not displayed by all adherent NK cells but only by a variable proportion of them. Such irregular distribution may just reflect the fact that podosomes are also involved in extracellular proteolysis (27) and may appear only in those cells that are actively engaged in attacking extracellular structures and particularly in making their way across basement membranes (25).

As already shown in B cell chronic lymphocytic and monocytic leukemia (28, 29), transmembrane $\beta_2$ integrins are codostributed with, and presumably linked to, cytoskeleton molecules like vinculin and talin, an occurrence that has also been shown for $\beta_3$ integrins in human osteoclasts (58). Since podosomes are considered rapidly modulated adhesion structures (59) involved also in cell locomotion and in the penetration of anatomical barriers (27), they may play a role in NK extravasation. The identification of molecules and microanatomical structures involved in the interaction of NK cells with EC may provide a better understanding of the regulation of NK cell recruitment from blood, their extravasation, and their migration to tissues.

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