Allosteric LFA-1 Inhibitors Modulate Natural Killer Cell Function

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ABSTRACT

Natural killer (NK) cells are believed to play an important role in a variety of disease pathologies, including transplant rejection and autoimmunity. None of the therapeutic modalities currently available are known to potently interfere with NK cell activity. Here we demonstrate for the first time that low molecular weight inhibitors of the integrin lymphocyte function-associated antigen-1 (LFA-1) readily block NK cell adhesion, activation, and NK cell-mediated cytolysis in vitro, in contrast to other immunosuppressive agents. These effects were independent of the type of allosteric mechanism by which LFA-1 inhibition was achieved. In addition, we describe a simple, nonradioactive whole-blood assay that should be suitable to monitor NK cell activation in clinical practice. Taken together, our study underlines the importance of LFA-1 in NK cell effector functions and indicates that allosteric LFA-1 inhibitors may become important tools to further elucidate the therapeutic potential of NK cell modulation in immunological diseases.

Natural killer (NK) lymphocytes are part of the innate immune system and play an established role in the surveillance and elimination of virus-infected or malignant cells. Upon detection of sensitive target cells, NK cells are activated, release the cytotoxic content of their lytic granules, and produce inflammatory cytokines, predominantly interferon-γ (Vivier et al., 2008). NK cells can also act as regulators of adaptive immunity by providing stimulatory signals for dendritic cells, macrophages, and T cells (Hanna and Mandelboim, 2007; Vivier et al., 2008). These properties of NK cells imply a broad role in immunity and a potential involvement in disease pathologies. Clinical observations and results from experimental animal models suggest that NK cells contribute to autoimmunity and the rejection of allogeneic bone marrow, organ allografts, and xenografts (Rocha et al., 2003; Young, 2004; Johansson et al., 2005). In particular, in the context of transplant rejection down-regulation of NK cell function may be beneficial. However, clinical studies indicate that established immunosuppressive drugs fail to reliably control NK cell activity (Vampa et al., 2003; Fildes et al., 2008). Thus, pathways different from those targeted by these drugs should be investigated to identify a new strategy of NK cell modulation.

There is increasing evidence that the αβ heterodimeric adhesion receptor LFA-1 (also designated αLβ2 or CD11a/CD18) might be a suitable target to control NK cells during immune responses (Bryceson et al., 2006). LFA-1 belongs to the β2 integrin family and is expressed on all leukocytes, including NK cells. NK cells also express lower levels of other β2 integrins, including Mac-1 (CD11b/CD18) and CD11c/CD18 (Bryceson et al., 2006). Intracellular signaling or cations are required to convert LFA-1 from a non–ligand-binding state to a ligand-binding state (Shimaoka and Springer, 2004). The major ligand for LFA-1 is intercellular adhesion molecule 1 (ICAM-1), which is expressed on both leukocytes and endothelial cells. In T cells, LFA-1 controls migration and costimulation (Graf et al., 2007). In NK cells, LFA-1 has been shown to be involved in adhesion and cytolysis (Schmits et al., 1996; Barber et al., 2004; Osman et al., 2007). To date, the anti-LFA-1 monoclonal antibody (mAb) efalizumab is approved for use in patients with psoriasis, and several small molecule LFA-1 inhibitors are under preclinical evaluation for the treatment of autoimmune disease and transplant rejection (Shimaoka and Springer, 2004; Leonardi et al., 2008). However, the effect of these inhibitors on NK cell adhesion and effector function has not been fully investigated.

In this study, we address for the first time the effect of two small-molecule LFA-1 inhibitors with different modes of actions on NK cell adhesion, activation, and NK cell-mediated cytolysis in vitro. LFA878 is known to selectively inhibit the type of allosteric mechanism by which LFA-1 inhibition was achieved. In addition, we describe a simple, nonradioactive whole-blood assay that should be suitable to monitor NK cell activation in clinical practice. Taken together, our study underlines the importance of LFA-1 in NK cell effector functions and indicates that allosteric LFA-1 inhibitors may become important tools to further elucidate the therapeutic potential of NK cell modulation in immunological diseases.

ABBREVIATIONS: NK, natural killer; BSA, bovine serum albumin; CFSE, 5(6)-carboxyfluorescein diacetate N-succinimidyl ester; CsA, cyclosporine A; DMSO, dimethyl sulfoxide; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; LFA-1, lymphocyte function-associated antigen-1; mAb, monoclonal antibody; mTOR, molecular target-of-rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; PI, propidium iodide; TBS, Tris-buffered saline.
LFA-1 by binding to an allosteric site (l-site) in the I (inserted) domain of LFA-1 (Weitz-Schmidt et al., 2004). The I domain contains the ligand binding site of LFA-1 and is located on the α chain (Shimaoka and Springer, 2004). In contrast, XVA143 is believed to interact with the I-like domain of the β chain of LFA-1, near a regulatory interface with the α chain (Shimaoka et al., 2003). By binding to this interface, the compound seems to block the transmission of activation signals across the LFA-1 α/β heterodimer, which is crucial for the conversion of the LFA-1 I domain into a high-affinity, ligand binding state. Moreover, XVA143 is not a specific inhibitor of LFA-1. The compound also inhibits other β2 integrins, including Mac-1 (Weltenbach et al., 2002; Shimaoka et al., 2003).

Furthermore, we compare the effects of the LFA-1 inhibitors with those of the calcineurin inhibitor cyclosporine A (CsA) (Borel and Kis, 1991), the molecular target-of-rapamycin (mTOR) inhibitor everolimus (Sedrani R et al., 1998) (Novartis, Basel, Switzerland), and the anti-interleukin (IL)-2 receptor mAb basiliximab (Halloxy, mTOR inhibitor everolimus (Sedrani et al., 1998), and the anti-interleukin (IL)-2 receptor mAb basiliximab (Halloxy, mTOR inhibitor everolimus (Sedrani et al., 1998), and the anti-interleukin (IL)-2 receptor mAb basiliximab (Halloxy, mTOR inhibitor everolimus (Sedrani et al., 1998). Inhibition of the HMG-CoA reductase enzyme lovastatin (Endo, 2008) was included in the study because this statin has been described to directly inhibit LFA-1 by interfering with the l-site and reduce NK cell-mediated cytolysis (Cutts et al., 1989; Weitz-Schmidt et al., 2001).

Materials and Methods

Cell Cultures. The NK-92 cell line and the K562 cell line were obtained from the American Type Culture Collection (Manassas, VA). NK-92 cells were maintained in minimal essential medium-α supplemented with 0.2 mM inositol (Sigma, St. Louis, MO), 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid (Fluka, Buchs, Switzerland), 12.5% horse serum, 12.5% fetal bovine serum, 6 mg/ml interleukin (IL-2) (Sigma), 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate. Human K562 cells were maintained in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum, 1% non essential amino acids, and 1% GlutaMAX. All reagents were obtained from Invitrogen (Carlsbad, CA) if not otherwise indicated.

Compounds and Antibodies. LFA878 (Weitz-Schmidt et al., 2004), XVA143 (Shimaoka et al., 2003), CsA (Borel and Kis, 1991), everolimus (Sedrani R et al., 1998) (Novartis, Basel, Switzerland), and lovastatin (Endo, 2008) (Sigma) were dissolved at 10 mM in DMSO (Sigma). The stock solutions were further diluted in DMSO and then in assay buffer allowing for equal DMSO concentrations (<0.5%) in all samples of the experiment. Basilimab (Halloran 2004) (Novartis) was dissolved in distilled water and further diluted in assay buffers. The mouse anti-human LFA-1 mAb HI 111 (BD Biosciences, San Jose, CA) was diluted in assay buffer.

Adhesion to Immobilized ICAM-1. The NK-92 cell/ICAM-1 adhesion assay was essentially performed as described previously (Kallen et al., 1999). In brief, 96-well flat bottom plates (Costar; Corning Life Science, Acton, MA) were coated and blocked with recombinant ICAM-1 mouse Cs fusion protein in carbonate buffer (5 μg/ml), blocked with Tris-buffered saline (TBS) containing 1.5% bovine serum albumin (BSA) and 0.5% Tween, and rinsed with TBS containing 1.5% BSA. NK-92 cells in TBS containing 1.5% BSA, 2 mM MgCl₂, 2 mM MnCl₂, and 5 mM D-glucose monohydrate (binding buffer) were labeled with 10 μg/ml 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxyethyl ester for 30 min at 45 min in 37°C in the dark. The labeled cells were centrifuged, resuspended in binding buffer, transferred to the ICAM-1-coated plates (1 × 10⁵ cells/well), and incubated with the compounds at 37°C for 30 min. The plates were gently washed with binding buffer, and bound cells were quantified by measuring fluorescence using a Wallac VICTOR2 microplate reader (PerkinElmer Life and Analytical Sciences, Waltham, MA).

Homotypic Adhesion. To assess the effect of inhibitors on NK-92 cell aggregate formation, the cells were washed with phosphate-buffered saline (PBS), resuspended in NK-92 cell culture medium by gently pipetting up and down, and transferred in 96-well flat-bottomed polypropylene microtiter plates (Costar; Corning Life Science) (5 × 10⁶ cells/well in a volume of 100 μl). Then the inhibitors were added, and cells were allowed to aggregate at 37°C in 5% CO₂ for 1.5 h. The cultures were examined for homotypic aggregation using an inverted microscope (Axiovert 200 M; Carl Zeiss Inc., Thornwood, NY) connected to a charge-coupled device camera (AxioCam MRm). Representative pictures of each well were taken at approximately the same location within each well at 100× magnification. Aggregates larger than 1.2 cm on a printout of 26.4 × 19.7 cm were counted. For assessing disaggregation, resuspended NK-92 cells were allowed to form aggregates in 96-well plates for 2 h at 37°C in 5% CO₂ before the inhibitors were added to the cultures. At 30 and 60 min, the number of aggregates were quantified as described above.

Cytotoxicity Assay. A flow cytometry-based method using NK-92 cells as effector cells and K562 cells as target cells was used to measure NK cell cytotoxicity. K562 cells were resuspended in NK-92 culture medium (assay buffer) and fluorescently labeled with 150 nM 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) (Fluka) for 3 min at room temperature. CFSE-labeled cells were washed three times with PBS and adjusted to 1 × 10⁵ cells/ml assay buffer. NK-92 cells, rinsed once with PBS, were resuspended in assay buffer at 5 × 10⁵ cells/ml. NK-92 cell suspension (100 μl), K562 cell suspension (100 μl) and diluted test agents (20 μl) were added to 96-well flat-bottomed polypropylene microtiter plates (Costar; Corning Life Science) to achieve an effector-to-target cell ratio of 50:1. The samples were incubated for 4 h at 37°C in 5% CO₂. After incubation, the cell suspensions were transferred to micronic tubes (Mironic, Frankfurt, Germany), and permeabilized cells were stained by the addition of propidium iodide (PI) (Roche, Indianapolis, IN) diluted in assay buffer (200 μl) to a final concentration of 0.5 μg/ml. The cell suspensions were incubated for 5 min at room temperature in the dark and then washed twice with PBS containing 0.5% BSA. To determine spontaneous lysis of target cells, samples without NK-92 cells were included in the experiments. All samples were analyzed by flow cytometry on a FACScan (BD Biosciences), using the CellQuest software (BD Biosciences) for data acquisition and analysis. 3000 K562 cells per sample were counted during acquisition. Target cells were gated by their forward and side scatter and fluorescence (CFSE-positive staining, FL-1). PI uptake was determined within the gated cells (PI-positive staining, FL-3). Cell death was expressed as the percentage of dead cells among the total target cells gated.

Whole-Blood NK Cell Activation Assay. Heparinized human blood (150 μl) and compounds diluted in K562 cell culture medium (25 μl) were added to 96-well round-bottomed polypropylene plates (Costar; Corning Life Science) and incubated for 30 min at 37°C. K562 cells were washed once in PBS and resuspended in K562 cell culture medium (1.2 × 10⁶ cells/ml). The cell suspension was transferred to the samples (30,000 cells/well) and incubated overnight (16–18 h) at 37°C in 5% CO₂. Then the following labeled mAbs were added to each well: 15 μl of anti-CD3-PerCP mAb, 4.5 μl of anti-CD69-PE mAb, 1.5 μl of anti-CD3-PerCP mAb, and 7.5 μl of anti-CD56-APC mAb (BD Biosciences). After an incubation of 20 min in the dark, the blood samples were transferred into 96-well Deepwell polypropylene plates (2.0 ml, conical bottom; BD Biosciences) containing 1.4 ml of FACS lysing solution (BD Biosciences). The samples were incubated with the FACS lysing solution for 10 to 15 min in the dark and centrifuged for 5 min at room temperature (1300 rpm, Heraeus Megafuge 1.0R; Thermo Fisher Scientific, Waltham, MA). The cell pellet was washed twice with PBS containing 0.5% BSA. Four-color flow cytometry data were acquired on a FACSCalibur (BD Biosciences).
Biosciences) using the CellQuest software and analyzed. At least 1500 NK cells or 20,000 T lymphocytes per sample were examined.

Cell Viability Assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent (Sigma) was used to determine the viability of compound-treated NK-92 cells. NK-92 cells in culture medium (45 µl) were transferred in 96-well flat-bottomed plates (3 x 10^3 cells/well). Compounds diluted in NK-92 cell medium (46 µl) were added and incubated for 4 or 24 h. Then 10 µl of MTT dissolved in PBS (5 mg/ml) was added to each well followed by an incubation of 2.5 h at 37°C. After the addition of 10% SDS in 0.01 M HCl (100 µl), the samples were incubated overnight at 37°C. The absorbance of each well was measured at a wavelength of 600 nm using the Wallac VICTOR 2 microplate reader.

Results

Effect of LFA-1 Inhibitors on NK-92 Cell Adhesion. NK-92 is an IL-2-dependent human NK cell line with functional and phenotypic characteristics of activated primary NK cells. NK-92 cells express both LFA-1 in an active state and the LFA-1 ligand ICAM-1 (Maki et al., 2001). The NK-92 cell line was used as a model system to study the effect of a blocking anti-LFA-1 mAb HI 111 and the small-molecule LFA-1 inhibitors LFA878 and XVA143 on NK cell adhesion and cytotoxicity. As shown in Fig. 1, the antibody and the selected compounds blocked the binding of NK-92 cells to immobilized ICAM-1 completely. Moreover, the small-molecule inhibitors reduced homotypic adhesion of NK-92 cells, which spontaneously occurred in culture (Fig. 2, A and B). It is interesting that the compounds were also able to disaggregate already existing NK-92 cell cluster in a time-dependent manner. Already after 30 min, the number of NK-92 cell aggregates was reduced significantly (Fig. 2, C and D) whereas after 60 min, complete disaggregation was noted (data not shown). In agreement with previous studies (Weitz-Schmidt et al., 2004), XVA143 was a more potent inhibitor of LFA-1-mediated cell adhesion than LFA878 (Figs. 1 and 2 and Table 1).

LFA-1 Inhibitors Block NK Cell-Mediated Target Cell Lysis. NK-92 cell-mediated target cell lysis was evaluated by flow cytometry. The flow cytometric analysis of cytolyis required a dual fluorescent staining of the target cells. As target cells, we selected the human leukemia cell line K562 (major histocompatibility complex class I−) because this cell line has been successfully used before in killing assays of different formats (Johann et al., 1995; Muldoon et al., 1997; Piriou et al., 2000). The green fluorescent dye CFSE was used to stain the target cells, whereas the red fluorescent dye PI was applied to discriminate between viable (no PI staining) and permeabilized (PI staining) target cells. An example of the flow cytometric analysis is shown in Fig. 3A, in which target cells in the top right quadrant are stained with PI and thus dead. Similar flow cytometry-based assays have been used before to detect NK cell killing (Johann et al., 1995; Piriou et al., 2000). The anti-LFA-1 mAb HI 111 inhibited NK-92 cell-mediated cytosis by 60% at the highest concentration tested (Fig. 3B). Almost complete inhibition of NK-92 cell-mediated killing was achieved in the presence of LFA878 and XVA143 (Fig. 3C). Consistent with results obtained in the adhesion assays, the inhibitory effect of XVA143 on NK cell killing was more pronounced than the effect of LFA878 (Fig. 3C and Table 1). To exclude the impairment of NK cell function due to potential compound-related toxicity, NK-92 cells alone were exposed to the small-molecule LFA-1 inhibitors for 4 and 24 h. According to the MTT test, LFA878 and XVA143 did not impair the viability of the NK-92 cells up to concentrations of 10 µM (data not shown). These results together suggested that the protective

![Fig. 1. Effect of an anti-LFA-1 mAb and allosteric LFA-1 inhibitors on NK-92 cell adhesion to ICAM-1. The adhesion of NK-92 cells to immobilized ICAM-1 was quantified in the presence of the anti-LFA-1 mAb HI 111 (△) (A) and LFA878 (○) and XVA143 (○) as described under Materials and Methods. Each point represents the mean ± S.D. value of triplicates. A representative of two independent experiments is shown. C, chemical structures of LFA878 and XVA143.](image-url)
effects observed for LFA878 and XVA143 in the killing assay were related to allosteric LFA-1 inhibition and prompted us to investigate the impact of the small-molecule inhibitors on primary human NK cells.

**LFA-1 Inhibitors Block NK Cell Activation in Whole Blood.** To avoid the isolation of human NK cells, the effect of LFA878 and XVA143 on NK cells was directly assessed in undiluted human blood. A flow cytometric assay was established, which measured the activation of NK cells upon the addition of K562 target cells. In this assay, NK cells were defined by their CD16+ CD56+ CD3− phenotype (Vivier et al., 2008), and NK cell activation was detected by quantifying CD69. CD69 is known to be one of the earliest cell-surface molecules expressed after the activation of NK cells, T cells, and other lymphocytes by different stimuli. Moreover, CD69 expression has been suggested to be a suitable marker for the cytolytic activity of NK cells (Clausen et al., 2003). We found that the degree of CD69 expression on NK cells in whole blood correlated with the number of K562 target cells added to the blood samples. In contrast, the activation status of T cells in the same samples remained unchanged in the presence of K562 cells (Fig. 4). This finding demonstrated the

![Graph showing the effect of different compounds on NK cell adhesion, cytolysis, and activation.](image)

**TABLE 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>NK-92/ICAM-1 IC50 μM</th>
<th>n</th>
<th>NK-92 Cytolysis IC50 μM</th>
<th>n</th>
<th>NK Cell Activation IC50 μM</th>
<th>n</th>
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<tbody>
<tr>
<td>CsA</td>
<td>&gt;10</td>
<td>2</td>
<td>1.09, 1.15</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Everolimus</td>
<td>&gt;10</td>
<td>2</td>
<td>&gt;10</td>
<td>2</td>
<td>&gt;10</td>
<td>2</td>
</tr>
<tr>
<td>Basiliximab</td>
<td>&gt;10 μg/ml</td>
<td>2</td>
<td>&gt;10 μg/ml</td>
<td>2</td>
<td>&gt;10 μg/ml</td>
<td>2</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>&gt;10</td>
<td>2</td>
<td>&gt;10</td>
<td>2</td>
<td>&gt;10</td>
<td>2</td>
</tr>
<tr>
<td>LFA878</td>
<td>4.1 ± 3.2</td>
<td>3</td>
<td>1.2 ± 0.2</td>
<td>3</td>
<td>2.6, 7.8</td>
<td>2</td>
</tr>
<tr>
<td>XVA143</td>
<td>0.017, 0.019</td>
<td>2</td>
<td>0.006 ± 0.002</td>
<td>3</td>
<td>0.020 ± 0.018</td>
<td>3</td>
</tr>
</tbody>
</table>

*In this assay, CsA (only) required a 20-h preincubation period.*
remarkable specificity of the assay signal for the NK cell-target cell interaction. Although some variability in the activation signal was noted from donor to donor (the CD69 expression varied from 8 to 30%), the flow cytometry assay was used to test the effect of compounds on NK cell responses. LFA878 and XVA143 were found to suppress K562 cell-induced CD69 expression in whole blood in a concentration-dependent manner (Fig. 5, A and B, and Table 1). The IC\textsubscript{50} values determined in the whole-blood assay correlated well with those measured in the NK-92 cell cytolysis assay (Table 1). This finding further validated CD69 as a suitable marker for NK cell-killing activity. Moreover, these data confirmed the inhibitory activity of LFA878 and XVA143 in primary NK cells.

Beyond the findings above, the stable performance, reproducibility, and specificity of the NK cell activation assay indicated that the method may be suitable for the ex vivo analysis of blood samples from treated subjects. By avoiding radioactivity, the newly established assay has a clear advantage over the conventional chromium release assays sometimes applied to assess NK cell cytotoxicity ex vivo (Muldoon et al., 1997).

**Effect of Immunosuppressive Drugs on NK Cell Adhesion, Killing, and Activation.** Furthermore, we compared the effects of the LFA-1 inhibitors on NK cell function with those of established immunosuppressive drugs and lovastatin. In contrast to the LFA-1 inhibitors, CsA, everolimus, basiliximab, and lovastatin did not prevent NK-92 cell adhesion to ICAM-1 (Table 1). Moreover, with the exception of CsA, the drugs had no effect on NK-92 cell-mediated cytolysis and target cell-induced NK cell activation in whole blood (Table 1). CsA was found to modestly inhibit the cytolytic activity of NK-92 cells at 10 \( \mu \text{M} \) (data not shown). Under modified conditions (pretreatment of NK-92 cells with CsA for 20 h), however, the effect of the drug on the cytolytic activity of NK-92 cells became more pronounced (Table 1). Consistent with the results described above, CsA also blocked NK cell activation after K562 stimulation (Fig. 5C and Table 1). In the whole-blood cultures, NK cells are exposed to the test compound for 16 to 18 h. The IC\textsubscript{50} values

![Fig. 3. Flow cytometric determination of NK-92 cell cytotoxicity in presence of LFA-1 inhibitors. A, a dual parameter dot plot shows living K562 target cells (bottom right quadrant) labeled with CFSE (FL-1) versus dead target cells (top right quadrant) labeled with PI (FL-3) in the absence of NK-92 cells (a), in the presence of solvent (b), and in the presence of the LFA-1 inhibitor XVA143 at a concentration of 0.3 \( \mu \text{M} \) (c). The experiment was performed with an effector-to-target cell ratio of 50:1 as described under Materials and Methods. B, NK-92 cell-mediated K562 target cell lysis was analyzed in the presence of the anti-LFA-1 mAb HI 111 (△). C, NK-92 cell-mediated K562 target cell lysis was assessed in the presence of indicated concentrations of LFA878 (●) and XVA143 (○) as described under Materials and Methods. Each point represents the mean value of triplicates ± S.D. A representative of three independent experiments is shown.](https://www.aspetjournals.org/doi/abs/10.1093/molpharm/76.3.359)
determined for CsA in the NK cell cytotoxicity and activation assay were comparable with those of the LFA-1 inhibitor LFA878 (Table 1).

Discussion

Contact and adhesion is an important prerequisite for NK cell effector functions (Bryceson et al., 2006; Osman et al., 2007). In the present study, we show for the first time that small-molecule inhibitors of LFA-1 with different allosteric modes of action prevent NK cell adhesion in two experimental systems. The compounds blocked both NK-92 cell adhesion to purified ICAM-1 and homotypic NK-92 cell interactions. Moreover, we report here for the first time that allosteric LFA-1 inhibitors readily reverse ongoing homotypic adhesion when added to NK-92 cell cultures (or cultures of the cell line Jurkat; S. Chreng, unpublished data). This result indicates that allosteric LFA-1 inhibitors are capable of converting high-affinity LFA-1 into low-affinity LFA-1. It remains to be evaluated whether this unique activity of the allosteric LFA-1 inhibitors could be of therapeutic potential in immune-mediated diseases. Moreover, the compounds almost completely abrogated NK cell-mediated cytolysis. This effect is believed to be a consequence of the strong antiadhesive activity of the inhibitors. It is very likely that the compounds prevent the optimal interaction between NK cells and target cells, which in turn leads to impaired triggering of NK cells.

Fig. 4. CD69 up-regulation on NK cells in human blood after the addition of K562 cells. K562 target cells were added to heparinized human blood samples as indicated and incubated overnight. After red blood cell lysis, CD69 expression on NK cells (CD56⁺, CD16⁺, CD3⁻) and T cells (CD3⁺) was quantified by multicolor flow cytometry as described under Materials and Methods. No target cells: CD69 expression on NK cells or T cells in the absence of target cells. Each bar represents the mean ± S.D. of triplicates. A representative of three independent experiments is shown. Each independent experiment involved a different blood donor.

Fig. 5. Effect of inhibitors on NK cell activation in human whole blood. Heparinized human blood samples were preincubated with the LFA-1 inhibitors LFA878 (A), XVA143 (B), and CsA (C) for 30 min before K562 cells were added. CD69 expression on NK cells were measured by flow cytometry after an incubation overnight as described under Materials and Methods. Each point represents the mean value of triplicates ± S.D. High ctrl, CD69 expression in the presence of solvent; low ctrl, CD69 expression in the absence of target cells. A representative of two to three independent experiments is shown. Each independent experiment involved a different blood donor.
cell-activating receptors (including LFA-1) and degranulation. Further investigation is required, however, to support this assumption. It needs to be noted that the activity described above of the inhibitors was assessed using an IL-2-dependent NK cell line with a phenotype of activated NK cells. To address the influence of the compounds on primary, human NK cells under more physiological conditions, we established an assay system allowing to specifically assess target cell-induced NK cell activation in human blood. Consistent with their effect on NK-92 cell adhesion and cytolysis, the LFA-1 inhibitors also suppressed NK cell responses in whole blood. Moreover, the result demonstrated that the compounds retain their activity in whole blood, an important parameter for pharmacological testing in vivo. Furthermore, we found that the αβ I-like allosteric inhibitor XVA143 blocked NK cell responses more potently than the L-site inhibitor LFA878. The better potency of XVA143 is most likely explained by the higher affinity of the compound toward LFA-1 (Welzenbach et al., 2002). It is unlikely that the higher affinity of the compound toward LFA-1 explains by the higher affinity of the compound toward LFA-1. It is unlikely that the super affinity of XVA143 is due to the inhibition of other β integrins on NK cells because these are absent (e.g., Mac-1 is not expressed on NK-92 cells) or expressed at low levels (Maki et al., 2001; Bryceson et al., 2006). Like the small-molecule LFA-1 inhibitors, a commercially available anti-LFA-1 mAb was able to completely block the adhesion of NK-92 cells to recombinant ICAM-1. The antibody also blocked NK-92 cell-mediated cytolysis, but only partially. Higher concentrations of the antibody may be required to achieve the effects observed for the small-molecule LFA-1 inhibitors. Taken together, our data strongly support the notion that LFA-1 is necessary for NK cell interactions with and the lysis of target cells and are in agreement with data published by other investigators (Barber et al., 2004; Perez et al., 2004; Bryceson et al., 2006).

Next we compared the effects of the allosteric LFA-1 inhibitors on NK cells with those of established immunosuppressive drugs and lovastatin. In contrast to LFA878 and XVA143, none of the compounds blocked cation-induced NK cell binding to ICAM-1. This result was expected for CsA, basiliximab, and everolimus, which interfere with pathways not relevant for cation-induced cell adhesion to ICAM-1. In the case of lovastatin, higher concentrations may be required to prevent the NK cell interaction with ICAM-1. It was shown previously thatLovastatin inhibited LFA-1-dependent T-cell adhesion to ICAM-1 with an IC50 value of 25 μM under similar conditions (Weitz-Schmidt et al., 2001). Moreover, the effect of the immunomodulatory compounds on cytolysis and NK cell activation was assessed. The impact of CsA on the cytocytic activity of NK cells was found to be marginal after short-term exposure (4 h). In agreement with data reported previously (Introna et al., 1981), longer exposure times (>16 h) were required to detect an inhibitory effect of CsA on NK cell function. This delayed onset of activity clearly distinguished the effect of CsA from the immediate effects of the LFA-1 inhibitors. A recent study suggests that calcineurin is involved in NK-92 cell lytic granule exocytosis (Pores-Fernando et al., 2008). Thus, it is possible that CsA blocked cytolysis by interfering with calcineurin function. On the other hand, this potential mechanism of action does not explain the long exposure times required for detecting CsA-related effects (Pores-Fernando et al., 2008). The IC50 values determined for CsA in the NK cell killing and the whole-blood activation assay were near the value for peak blood levels in patients (Linnebacher et al., 2008), implicating that CsA could have an effect on NK cells in patients. However, clinical studies in transplant recipients show that full-dose immunosuppression with CsA, steroid, azathioprine, and basiliximab did not have a detectable impact on NK cell-mediated cytolysis (Vampa et al., 2003). Moreover, other in vitro studies using different experimental designs suggest that CsA-treated NK cells retain their cytotoxicity against target cells (Wang et al., 2007; Wai et al., 2008). Further investigations are necessary to resolve these conflicting data. It is interesting that our experiments indicate that the anti-IL-2 receptor mAb basiliximab does not affect NK cell activation and killing in contrast to the LFA-1 inhibitors. Although IL-2 is described to play a crucial role in NK cell growth and proliferation (Becknell and Caligiuri, 2005), our data imply that it is less important for the cytolytic activity of the cells. This result is supported by the clinical study described above (Vampa et al., 2003). Likewise, everolimus failed to suppress NK cell activation and killing in vitro, providing evidence that the mTOR pathway does not contribute to the respective NK cell responses. Sirolimus (also known as rapamycin), a close derivative of everolimus with mTOR as a target, was shown to modestly inhibit NK cell killing in vitro but potently block NK cell proliferation in vitro and in vivo in a rat model of liver transplantation (Wai et al., 2008). These data together suggest that everolimus and sirolimus have a greater effect on the proliferation of NK cells than on NK cell-mediated cytolysis. Furthermore, our results indicate that neither the LFA-1 nor the HMG-CoA reductase-dependent activity ofLovastatin is sufficient to impact NK cell activation and killing. It should be noted, however, that HMG-CoA reductase-related effects ofLovastatin on these NK cell responses may become more evident in vitro after depletion of the cellular mevalonate pool (requires a preincubation with the statin for more than 3 days), the immediate downstream product ofHMG-CoA reductase (Cutts et al., 1989; Tanaka et al., 2007). On the other hand, a 6-month clinical investigation involvinglovastatin-treated healthy subjects did not find measurable effects on NK cell cytotoxicity ex vivo and thus supports our findings (Muldoon et al., 1997). The results described above demonstrate that allosteric LFA-1 inhibitors can be readily distinguished in vitro from established immunosuppressive drugs andLovastatin by their immediate and potent inhibitory effect on NK cell adhesion, activation, and cytotoxicity.

Taken together, the results of the present study indicate that small-molecule LFA-1 inhibitors with an allosteric mode of action could be used to further elucidate the role of NK cells in the rejection of allogeneic bone marrow and solid organ transplants. Thus, allosteric LFA-1 inhibitors may constitute a new starting point for the development of immunomodulatory agents that can target the harmful effects of NK cells in the rejection of allogeneic bone marrow transplants and solid allotransplants while preserving the beneficial effects of NK cells against cancer cells and viruses.

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References


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