NK Cell Activation by Dendritic Cells Is Dependent on LFA-1-Mediated Induction of Calcium-Calmodulin Kinase II: Inhibition by HIV-1 Tat C-Terminal Domain

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In this study, we show that binding to autologous dendritic cells (DC) induces a calcium influx in NK cells, followed by activation of the calcium-calmodulin kinase II (CAMKII), release of perforin and granzymes, and IFN-γ secretion. CAMKII is induced via LFA-1: indeed, oligomerization of LFA-1 leads to CAMKII induction in NK cells. Moreover, release of lytic enzymes and cytotoxic activity is strongly reduced by masking LFA-1 or by adding CAMKII inhibitors such as KN62 and KN93, at variance with the inactive compound KN92. NK cell-mediated lysis of DC and IFN-γ release by NK cells upon NK/DC contact are inhibited by exogenous HIV-1 Tat: the protein blocks calcium influx and impairs CAMKII activation elicited via LFA-1 in NK cells, eventually inhibiting degranulation. Experiments performed with synthetic, overlapping Tat-derived peptides showed that the C-terminal domain of the protein is responsible for inhibition. Finally, both KN62 and Tat reduced the extension of NK/DC contacts, possibly affecting NK cell granule polarization toward the target. These data provide evidence that exogenous Tat inhibits NK cell activation occurring upon contact with DC: this mechanism might contribute to the impairment of natural immunity in HIV-1 infection. The Journal of Immunology, 2002, 168: 95–101.

Natural killer cells represent one of the most potent effectors of natural immunity: they provide a first line of defense in the early phases of viral diseases, including HIV-1 infection (1, 2). Indeed, impairment of NK cell function, which is observed early in HIV-1-infected individuals, contributes to the progressive immunosuppression in AIDS (3–6). Natural cytotoxicity is initiated by adhesion receptors, such as the LFA-1, that drive NK cells and their targets to close contact and trigger NK cell activation (2, 7); these receptors elicit an intracellular free calcium concentration ([Ca2+]i) increase (2), mainly due to extracellular calcium influx (7–9), that is needed for the granule release involved in the delivery of lethal hit (8, 9). Recently, we reported that functional L-type calcium channels, whereby extracellular calcium can enter the cell, are present in NK cells and are required for NK cell activity (9). We have shown that exogenous Tat, an HIV-1 product that exerts many extracellular functions (10, 11), inhibits NK cell-mediated cytotoxicity of tumor targets by competing with L-type calcium channels expressed by these cells (9). With a similar mechanism, secretion of IL-12, a cytokine that up-regulates NK cell function, by dendritic cells (DC) is impaired by Tat (11, 12).

Interaction between NK cells and DC is likely to play a complex role in the control of innate immunity. Indeed, contact with DC activates NK cell cytolytic activity and IFN-γ secretion, thus amplifying one of the mechanisms of primary host defense against pathogens (13). In contrast, it has been recently proposed that DC can be killed efficiently by autologous NK cells (14–16); this process may contribute to the feedback regulation of immune responses (17, 18). In addition, NK/DC interaction is supposed to be relevant in the control of antiviral immunity, including in AIDS, in which DC are a potential virus reservoir (5, 19, 20); finally, production of IFN-γ by NK cells has been proposed to participate in the control of viral replication and in the amplification of the immune response (1, 6, 21).

In this study, we demonstrate that NK cell killing of autologous DC is mediated by the LFA-1-dependent activation of the calcium-calmodulin kinase II (CAMKII), resulting in degranulation and extracellular release of perforin and granzymes. Likewise, IFN-γ secretion occurring upon NK/DC contact or LFA-1 engagement is inhibited by blocking CAMKII.

Interestingly, in the presence of exogenous HIV-1 Tat, several events that follow NK/DC interaction are affected: the extent of cell to cell contacts is reduced; calcium entry and degranulation, elicited in NK cells by binding to DC, are inhibited. Finally, activation of CAMKII and IFN-γ secretion are blocked: these suppressive effects eventually lead to an impairment in the process of DC-mediated activation of NK cell functions.

Materials and Methods

Isolation and culture of CD3–CD16+ NK cells and DC

Highly purified CD3–CD16+ NK cells were obtained from PBL after immunodepletion of monocytes and T lymphocytes, as described (9), stimulated with 10 μg/ml PHA (Sigma, St. Louis, MO), and cultured in RPMI

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‡ Abbreviations used in this paper: [Ca2+]i, intracellular free calcium concentration; CAMKII, calcium-calmodulin kinase II; CD, dendritic cell; GAM, goat anti-mouse; NCR, natural cytotoxicity receptor; VPM, verapamil.
1640 medium supplemented with 10% FCS, 1 mM glutamine, 100 U/ml penicillin-streptomycin (all from Biochom, Berlin, Germany), and 100 U/ml IL-2 (Cetus, Emeryville, CA). Peripheral blood monocytes were isolated and cultured in the presence of 40 ng/ml GM-CSF plus 100 U/ml IL-4 for 10 days to obtain monocyte-derived DC (12).

**Cytolytic assay**

Cytolytic activity of activated NK cells against autologous DC, or against the K562 or 721.221 cell lines, was tested in a 4-h 51Cr release assay, as described (9), at different E:T ratios, from 40:1 to 1:1. In some experiments, NK cells were pretreated 15 min with 100 or 10 nM Tat alone or with CAMKII inhibitors: L-[N-(2-aminoethyl)-(4-methoxybenzenesulfonyl)]-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN62) or KN93 or the inactive compound L-[N(4-methoxybenzenesulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzamidane, phosphate KN92 (Calbiochem-Inalco S.p.A., Milan, Italy; 100 nM), or with a blocking anti-Fas mAb (ZB4, IgG; MBL, Naka-ku Nagoya, Japan), or with the anti-perforin mAb 8G9 (PharMingen, San Diego, CA), both at the concentration of 5 μg/ml. Results are expressed as percentage of cytoxicity, calculated as described (9).

**Measurement of perforin and granzyme release**

Granzyme release was measured in the supernatants or in cell lysates of NK cells, according to Shiver et al. (24), as previously reported (9). Briefly, supernatants or cell lysates (0.5% Triton X-100) obtained from 2-h cultures of 106 NK cells plus 106 DC, with or without 100 nM Tat or 100 nM Tat peptides, were added to 1 mM dithionitrobenzoic acid (Sigma) and 1 mM Na2-benzoylxyconvalin-1-lysin thiobiszylerster (Calbiochem), and the reaction run at room temperature for 30 min. In some experiments, NK cells were treated with the CAMKII inhibitors KN62 or KN93 (50 nM) or with the inactive analogue KN92 (50 nM). The OD were measured at 414 nm, and results were expressed as percentage of esterase release calculated as follows: (OD414 SN/OD414 CL + OD414 SN) × 100. Perforin release by NK cells was evaluated by flow cytometry after NK cell/DC interaction as reduction of perforin-positive NK cells. Cytoplasmatic staining was performed by indirect immunofluorescence, as described (9). Briefly, aliquots of 106 cells were fixed with 1% paraformaldehyde and permeabilized with 0.05% Triton X-100, stained with the anti-perforin mAb 8G9, followed by PE-conjugated antiisotype-specific goat anti-mouse serum (GAM; Southern Biotechnology Associates, Birmingham, AL). NK or DC were identified using the forward scatter and side scatter parameters. Samples were analyzed on a flow cytometer (FACSort; Becton Dickinson, Mountain View, CA) exciting PE at 488 nm, after calibration with CALIBRITE particles (Becton Dickinson), and gating to exclude debris. Data were analyzed using the Lysis II computer program (version 1.1).

**Single-cell analysis of calcium fluxes by video microscopy and ratio imaging**

Single-cell analysis of calcium fluxes was performed as described (9, 12). Briefly, DC cultured on round coverslips were loaded with 1 μM fura 2-AM (1 at 37°C), placed in a microincubator (Medical System, Oberkochen, Germany), and maintained at 37°C, 5% CO2, placed in a microincubator (Medical System, Greenlund, Germany), and maintained at 37°C (25). Single-cell analysis of calcium fluorescence was performed with the specific mAb 70H12 (IgG2a, 5 μg/ml), followed by GAM, as described (9), in the absence or presence of 100 to 10 nM Tat or 100 to 1 nM Tat peptides. Preliminary kinetics experiments showed that the optimal time for CAMKII activation was 12 min in NK/DC coculture and 4 min in LFA-1 oligomerization (Table I). CAMKII was measured with the CAMKII assay kit, using the specific substrate and [γ-32P]ATP, after immunoprecipitation with the specific anti-CAMKII Ab (Upstate Biotechnology, Lake Placid, NY) and chromatography. Results are expressed as cpm × 10−3 and are the mean ± SD from triplicate samples.

**Electron microscopy**

Effecter/Target cell interaction was evaluated by transmission electron microscopy as well, to this purpose, cells were fixed in 2% glutaraldehyde for 20 min at 4°C and washed three times in phosphate buffer. Postfixation was performed in 1% osmium tetroxide. Samples were then dehydrated in ethyl alcohol and propylene oxide and embedded in epon-aradite resin. Thin sections (80 nm) were then obtained, stained with uranyl acetate and lead citrate, and analyzed under a Zeiss CEM 902 electron microscope.

**IFN-γ production**

Cell-free supernatants were collected 24 h after NK/DC contact (4:1 ratio) or oligomerization of LFA-1 obtained with the specific mAb, or with GAM alone, as described above. In some experiments, NK cells were pretreated with the Fab’ of the 70H12 mAb (9) or of the isotype-matched TAI181H12 mAb (12), both at 5 μg/ml, CAMKII inhibitor KN62 (50 nM), or with HIV-1 Tat (50 nM). IFN-γ concentrations in culture supernatants were measured following the manufacturer’s recommended procedure using the human IFN-γ ELISA kit (R&D Systems, Minneapolis, MN), and results were expressed as nanograms/106 NK cells/milliliter. The limit of detection was 1 ng/ml.

**Results**

Degranulation and cytolytic activity occurring upon NK/DC contact are dependent on CAMKII activation

It is now accepted that DC activate NK cells, which, in turn, can lyse autologous DC (13–16). However, the signal transduction pathway(s) underlying NK cell activation in this system is still poorly defined. To investigate this point, we first studied the mechanism of lethal hit delivery by NK cells. Fig. 1A shows that an anti-perforin mAb blocked the cytotoxicity of DC by autologous NK cells, at variance with an anti-Fas mAb; this indicates that, in this system, lytic enzymes released upon calcium-dependent exocytosis of NK cell granules are the prevalent effectors of target cell damage, in agreement with previous observations that DC lysis by NK cells is inhibited by calcium chelators (14, 16). Granule exocytosis in epithelial cells is regulated by calcium-dependent activation of CAMKII (27, 28); thus, we asked whether this kinase was also involved in regulating degranulation in NK cells, as shown in Fig. 1B, NK cell treatment with the CAMKII inhibitors KN62 or KN93, but not with the inactive KN92, leads to the inhibition of perforin depletion, which occurs upon binding to DC. In keeping with this, the CAMKII inhibitors KN62 or KN93 impaired DC killing (Fig. 1C); this inhibition was dose-dependent.
calcium fluxes (Fig. 2A) and CAMKII activation, which is blocked by KN62 and by masking LFA-1, with the F(ab’)_2 of the specific mAb, thus inhibiting cell to cell contact (Fig. 2B). This suggests that CAMKII is induced through the engagement of LFA-1 integrin in NK cells during interaction with the target: indeed, CAMKII activation was also achieved by oligomerization of LFA-1 obtained with the specific mAb 70H12 (Fig. 2, C and D, hatched columns).

As HIV-1 Tat can impair NK cell activity by blocking calcium influxes through L-type calcium channels (9), we further investigated whether this protein could also affect LFA-1-triggered CAMKII activation. Of note, the addition of Tat to NK cells prevented CAMKII activation elicited by LFA-1 engagement (Fig. 2C), and this effect was neutralized by an anti-Tat antiserum (not shown). To determine which domain of HIV-1 Tat protein was involved in this inhibition, different Tat-derived synthetic peptides (22, 23) were used in LFA-1-dependent CAMKII activation assay. The C-terminal Tat peptide, spanning the 46–60 aa, exerted an inhibitory activity comparable with that of the whole Tat protein (Fig. 2C), which was still evident at 10 nM concentration (Fig. 2D). Conversely, the Tat(65–80) peptide, containing the basic domain of the protein, or the Tat(24–51) peptides, containing the cysteine-rich and the core domain of Tat (22), did not exert any effect (Fig. 2C). Finally, a slight inhibition was observed using the Tat(56–70) peptide, partially overlapped to Tat(65–80) (Fig. 2, C and D).

**FIGURE 2.** CAMKII in NK cells is triggered by binding to DC or LFA-1 engagement and inhibited by HIV-1 Tat. A, \([Ca^{2+}]_i\) increases were measured in NK cells upon interaction with DC (arrow), as described in Materials and Methods. Results are expressed as \([Ca^{2+}]_i\), nM, and are representative of four independent experiments. B, CAMKII activation in NK cells after coculture with DC, in the absence or presence of the F(ab’)_2 of the anti-LFA-1 mAb 70H12 (5 µg/ml) or an unrelated, isotype-matched mAb. C and D, Oligomerization of LFA-1 in NK cells was performed with the specific mAb 70H12 (5 µg/ml), followed by GAM, and incubation at 37°C for 5 min, in the absence or presence of 100 nM Tat or Tat peptides (C). D, Titration of Tat and Tat(65–80) or Tat(56–70) peptides. CAMKII was measured using the specific substrate and \(\gamma\text{-}32\text{P}\)ATP, after NK cell lysis and immunoprecipitation with the anti-CAMKII Ab. Results are expressed as cpm × 10^{-3} and are the mean ± SD from triplicate samples.

**FIGURE 1.** Degranulation and CAMKII activation are required for DC killing by NK cells. Cytolytic activity of NK cells against autologous DC (A, C, and D) or 721.221 (E) or K562 (F) target cells was assayed in a 4-h \({^{32}}\text{P}\)-release assay at the indicated E/T ratios, and results are expressed as percentage of \({^{32}}\text{P}\)-specific release (mean ± SD from four independent experiments). A, The anti-Fas (ZB4, □) or the anti-perforin mAb (8G9, △), at the concentration of 5 µg/ml, were added at the onset of the cytotoxic assay. C, Lysis of DC was tested in the absence or presence of 100 nM KN62, KN92, or KN93, or in the presence of saturating amounts of the anti-LFA-1 mAb 70H12 (5 µg/ml). D, NK cell-mediated lysis of autologous DC at 20:1 in the presence of 100, 10, 1 nM KN62 or KN92 or KN93. Nil: lysis in the absence of drugs. E and F, Cytolytic activity against 721.221 (E) or K562 (F) target cells was assayed without (nil) or with 100 nM KN62 or KN92 or KN93 or in the presence of saturating amounts of the anti-LFA-1 mAb 70H12 (5 µg/ml). D, NK cell-mediated lysis of autologous DC at 20:1 in the presence of 100, 10, 1 nM KN62 or KN92 or KN93. Nil: lysis in the absence of drugs. E and F, Cytolytic activity against 721.221 (E) or K562 (F) target cells was assayed without (nil) or with 100 nM KN62 or KN92 or KN93 or in the presence of saturating amounts of the anti-LFA-1 mAb. B, NK cells were coincubated 2 h with DC at the E/T ratio of 10:1, in the absence or presence of 50 nM KN62, KN92, or KN93, as indicated. Cells were then permeabilized and stained with the anti-perforin mAb (8G9), followed by PE-conjugated antiserotype-specific GAM serum. NK cells were identified using the forward scatter and side scatter parameters. Samples were run on a flow cytometer gated to exclude nonviable cells and debris. Data are expressed as percentage of perforin-positive NK cells, and they are the mean ± SD from four independent experiments.

(D), and the specificity of CAMKII involvement was confirmed by the finding that the inactive compound KN92 had no effect on NK cell cytolytic activity (Fig. 1, C and D). However, IL-2-cultured NK cells efficiently lysed HLA-I-negative target cells, represented by the lymphoblastoid 721.221 and the erythroleukemia K562 cell lines (Fig. 1, E and F), and this lysis was not affected by CAMKII inhibitors. Interestingly, the anti-LFA-1 mAb also was ineffective in blocking the killing of K562 (Fig. 1F) or 721.221 cells (Fig. 1E).

**LFA-1 engagement during NK/DC contact triggers CAMKII activation in NK cells: inhibition by HIV-1 Tat**

CAMKII is activated by extracellular calcium entry that induces calmodulin conformational changes, allowing its interaction with the kinase (28). Because calcium fluxes can be elicited via LFA-1, the β_2 integrin that mainly mediates NK cell binding to target cells (2, 7, 9), we investigated the role of LFA-1 triggering in CAMKII induction. Fig. 2 shows that interaction with DC elicits in NK cells calcium fluxes (Fig. 2A) and CAMKII activation, which is blocked by KN62 and by masking LFA-1, with the F(ab’)_2 of the specific mAb, thus inhibiting cell to cell contact (Fig. 2B). This suggests that CAMKII is induced through the engagement of LFA-1 integrin in NK cells during interaction with the target: indeed, CAMKII activation was also achieved by oligomerization of LFA-1 obtained with the specific mAb 70H12 (Fig. 2, C and D, hatched columns).
Contact with DC elicits CAMKII-dependent IFN-γ secretion by NK cells, which is inhibited by Tat

Another important antiviral tool is represented by IFN-γ, which is believed to be a mediator of NK cell cytotoxicity (21). IFN-γ can be produced and released by NK cells upon interaction with DC (13); therefore, we analyzed the effect of CAMKII inhibitors and of HIV-1 Tat on IFN-γ secretion upon NK/DC contact. As shown in Table II, coculture of NK cells and DC, as well as oligomerization of LFA-1, induced the secretion of IFN-γ, which was detectable in the culture supernatant by ELISA; interestingly, pretreatment of NK cells with KN62 or KN93, but not with KN92, inhibited the release of IFN-γ, raising the possibility that the calcium-dependent activation of CAMKII controls more than one effector mechanism in NK cells. Interestingly, a comparable inhibition was observed when NK cells were exposed to synthetic Tat (Table II).

HIV-1 Tat C-terminal domain blocks the early events that follow NK/DC contact

Fig. 3 shows that NK/DC interaction elicited an increase in the [Ca^{2+}]_i of NK cells starting from 5 min after their binding to DC; several waves of calcium fluxes were observed during the following 20–30 min, similarly to what was reported in NK/tumor cell interaction (29). This intracellular calcium rise was mainly due to extracellular calcium influx, as it was strongly reduced by the addition of the L-type calcium channel blocker VPM (Fig. 3A). Interestingly, a similar inhibitory effect was observed using synthetic Tat (Fig. 3B) and its C-terminal peptide Tat_{65–80} (Fig. 3C), at variance with the basic Tat_{46–60} peptide (Fig. 3D) or the other Tat-derived peptides (not shown).

Transmission electron microscopy showed that the first step (adhesion) of natural killing (Fig. 4A) was maintained in the presence of Tat (Fig. 4C). However, the extent and complexity of cell to cell contacts were strongly reduced upon exposure of NK cells to Tat (Fig. 4, C vs A). More importantly, in untreated NK cells, most granules were detectable along the membrane portion in contact with DC (Fig. 4, A and B), while in Tat-treated NK cells, granule were distributed throughout the whole cytoplasm (Fig. 4C). Interestingly, a similar effect was observed using the CAMKII inhibitor KN62 (Fig. 4D). The analysis of 100 cell to cell contacts for each sample showed that >80% of untreated NK cells had filopodia or irregular cytoplasmic processes that formed many interdigitations with the cytoplasmic protrusions of DC; moreover, electron-dense granules localized in the cytoplasm toward DC in >70% of NK cells. In Tat-treated NK cells, the number of such interdigitations and the length of filopodia were reduced by 50%, and granules were always spread in the cytoplasm. Altogether, these data suggest that calcium waves occurring upon NK/DC binding are needed for CAMKII activation, which, in turn, contributes to the stabilization of cell to cell contacts, granule polarization, and the delivery of the lethal hit: this mechanism is impaired by exogenous Tat.

Table II. IFN-γ secretion upon NK/DC contact or LFA-1 engagement is CAMKII dependent and inhibited by HIV-1 Tat

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NK/DC</th>
<th>LFA-1</th>
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<tbody>
<tr>
<td>Nil</td>
<td>25 ± 4</td>
<td>36 ± 5</td>
</tr>
<tr>
<td>GAM</td>
<td>ND</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>Anti-LFA-1 mAb</td>
<td>10 ± 3</td>
<td>ND</td>
</tr>
<tr>
<td>Ctr mAb</td>
<td>21 ± 3</td>
<td>ND</td>
</tr>
<tr>
<td>KN62</td>
<td>6 ± 3</td>
<td>10 ± 4</td>
</tr>
<tr>
<td>KN92</td>
<td>27 ± 3</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>HIV-1 Tat</td>
<td>8 ± 4</td>
<td>12 ± 5</td>
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</tbody>
</table>

*Cell-free supernatants were collected upon NK/DC contact or after oligomerization of LFA-1 obtained with the specific mAb as described in Materials and Methods. In some experiments NK cells were pretreated with or without GAM alone, or with the F(ab')2 of the 70H12 mAb or of the isotype-matched TA18H12 mAb (Ctr mAb), both at 5 μg/ml, or with the CAMKII inhibitor KN62 (50 nM) or with HIV-1 Tat (50 nM). IFN-γ concentrations in culture supernatants were measured using a human IFN-γ ELISA, and results are expressed as nanograms/10^6 NK cells/ml.

![FIGURE 3. HIV-1 Tat inhibits the [Ca^{2+}]_i increase elicited in NK cells by binding with DC. NK cells were loaded with 1 μM fura-2-AM, and fluorescence was monitored as described in Materials and Methods. [Ca^{2+}]_i increases in NK cells were measured upon binding of NK cells to DC (arrows), in the absence or in the presence of 10 μM VPM (A) or 100 nM HIV-1 Tat (B) or Tat_{65–80} peptide (C) or Tat_{46–60} peptide (D). Results are expressed as [Ca^{2+}]_i nM and are representative of four independent experiments.

![FIGURE 4. CAMKII inhibitors and HIV-1 Tat reduce the extension of intercellular contacts between NK cells and DC. NK cells were allowed to bind to autologous DC at the E:T ratio of 10:1 in the absence (A and B) or presence of 100 nM Tat (C) or 100 nM KN62 (D). Samples were fixed, dehydrated, and embedded in epon-araldite resin. Thin sections (80 nm) were then obtained, stained with uranyl acetate and lead citrate, and analyzed under a Zeiss CEM 902 electron microscope. A and D, ×12,000 magnification. B, ×10,000 magnification. C, ×8,000 magnification.]
Release of lytic enzymes by NK cells and DC killing are inhibited by the C-terminal domain of HIV-1 Tat

Synthetic Tat or its C-terminal peptide Tat20–80, at variance with the Tat-basic (Tat46–60) peptide, inhibited the release of granzymes from NK cells (Fig. 5A), as well as the intracellular perforin depletion (Fig. 5B), elicited by binding to DC, and they were effective up to 10 nM concentration (Fig. 5, A and B). These findings indicate that Tat inhibits the degranulation and enzyme release occurring upon NK cell/DC interaction and needed for NK cytolytic activity. Indeed, synthetic Tat significantly reduced the lysis of DC by NK cells, and this inhibition could be prevented by a neutralizing anti-Tat antiserum, at variance with normal rabbit Ig (Fig. 6A). Again, the C-terminal Tat45–80 peptide exerted an inhibitory activity on the lysis of autologous DC by NK cells (Fig. 6B) superimposable to that of the whole Tat protein (Fig. 6A), and this inhibition was detectable from 100 to 10 nM (Fig. 6C). Conversely, little or no inhibition of cytotoxicity was observed when the Tat20–80, Tat20–39, or Tat24–51 peptides were used (Fig. 6B). Finally, Tat66–70 peptide, partially overlapped to Tat45–80, slightly reduced (by 10%) NK-mediated DC killing at the highest concentration (100 nM; Fig. 6, B and C).

Discussion

Lysis of target cells by NK cells requires a number of steps: binding, calcium entry, and release of lytic enzymes (1, 2, 7). In this study, we identify a new, intermediate step, which follows cell to cell interaction and precedes degranulation, that is triggering of CAMKII via LFA-1. Furthermore, we provide evidence that secretion of IFN-γ by NK cells, following DC contact, also is CAMKII dependent. All these events are inhibited by exogenous HIV-1 Tat by blocking calcium entry and CAMKII activation. It is well known that variations in [Ca^{2+}]_i regulate many secretory events, such as neurotransmitter release and exocytosis of secretory granules (30); furthermore, increasing evidence indicates that Ca^{2+}-regulated exocytosis is not restricted to specialized secretory cells, but is present in many cell types, including hematopoietic cells (31). In immune cells, this regulated secretion provides a tight control over the delivery of highly bioactive effector proteins, thus representing a major mechanism of control of immune responses (32). However, the biochemical events that follow receptor signaling leading to exocytosis are still unclear. In epithelial tissues, granule secretion is induced by the activation of CAMKII (27, 28); in this study, we show that also in NK cells, degranulation needs the induction of this kinase, which is triggered by calcium mobilization via LFA-1 upon effector/target cell adhesion. Along this line, we found that release of IFN-γ by DC-activated NK cells is inhibited by blockers of CAMKII. Thus, the activation of CAMKII occurs in different cell types, possibly representing a common step in the control of exocytosis. However, the lysis of the lymphoblastoid 721.221 and the erythroleukemia K562 cell lines by IL-2-cultured NK cells was apparently independent of CAMKII activation as well as of LFA-1 engagement. Because CAMKII induction can be achieved in NK cells via LFA-1, it is possible that this enzyme is not triggered when LFA-1 is not the main surface structure involved in NK cell activation.

FIGURE 6. HIV-1 Tat C-terminal peptide inhibits NK cell-mediated DC lysis. A, NK cell-mediated target cell lysis was tested in a 4-h ^51Cr release assay, as described in Materials and Methods, at E:T ratios from 40:1 to 1:1. NK cells were untreated (nil, □) or pretreated 15 min with 100 nM Tat (■). The effect of a neutralizing anti-Tat antiserum (1/200 dilution, △) or of normal rabbit Ig (R Ig, 1/200 dilution, △) is also shown. B and C, Effect of the different Tat peptides (at 100 nM concentration in B, from 100 to 1 nM in D) on NK cell-mediated DC lysis at the E:T ratio of 20:1. Results are expressed as percentage of ^51Cr-specific release and are the mean ± SD from four independent experiments for each panel.

FIGURE 5. Degranulation and release of lytic enzymes are inhibited by HIV-1 Tat C-terminal peptides. A, Nα-benzoyloxycarbonyl-L-lysine thio-benzyl-esterase activity was measured in the supernatants and in cell lysates of NK cells activated by interaction with DC (E:T = 10:1), before or after treatment with 100 to 1 nM Tat or Tat peptides, as indicated. OD were measured at 414 nm. Results are expressed as percentage of granzyme release calculated as described (24), and they are the mean ± SD from four independent experiments. B, NK cells were coincubated 2 h with DC at the E:T ratio of 10:1, in the absence or presence of 100 to 1 nM Tat or Tat peptides, as indicated. Cells were then permeabilized and stained with the anti-perforin mAb (6G9), followed by PE-conjugated anti-isotype-specific GAM serum. NK cells were identified using the forward scatter and side scatter parameters. Samples were run on a flow cytometer gated to exclude nonviable cells and debris. Data are expressed as percentage of perforin-positive NK cells, and they are the mean ± SD from four independent experiments.
Our data show that the lysis of DC by NK cells is due to perforin and granzymes release: this mechanism of killing has been reported as an essential step in the control of primary virus infections, including HIV-1, by CTLs (33–35). In keeping with these reports, Fas/Fas ligand interaction is not involved in the NK-mediated lysis of DC. Of note, we found that Tat acts on natural killing by inhibiting the release of both perforin and granzymes, as mediated lysis of DC. Of note, we found that Tat acts on natural killing by inhibiting the release of both perforin and granzymes, which occurs upon NK/DC binding. It has been proposed that the lysis of DC by autologous NK cells is involved in the regulation of immune responses (14–16). This mechanism could play a role also in viral diseases, in which the killing of autologous DC can help the clearance of infected cells (5, 6); conversely, a decreased NK cell function would allow the survival of infected DC. Support to this hypothesis comes from the finding that NK cells can be isolated from influenza virus-infected lungs before the detection of virus-specific T lymphocytes in mice (36). Recently, hemagglutinins of influenza or parainfluenza virus have been identified as ligands for the recently described natural cytotoxicity receptor (NCR) NKp46 and reported to activate NK cells (37), thus confirming the antiviral potential of this lymphocyte population. This may be of relevance also in the case of HIV-1 infection, in which follicular DC represent a reservoir of the virus (19, 20).

We have reported that during interaction between NK cells and DC, several activating and inhibiting molecules, expressed on either cell types, can be engaged and determine the final functional result. In particular, ligation of NKp30 and NKp46 on those NK cells that express these receptors leads to autologous APC lysis through the activation of the phosphatidylinositol 3 kinase (38). Interestingly, the lysis of HLA-I-negative targets seems to depend on the engagement of NCR: indeed, activated NK cells brightly express NKp30 and NKp46 can lyse the 721.221 cell line, at variance with NK cells with a dull expression of NCR (39). In contrast, we found that this lysis is not dependent on CAMKII and on LFA-1 (different from NK cell-mediated lysis of DC). Thus, two enzymatic pathways can be responsible for the release of perforin and granzymes, possibly due to the preferential engagement of NCR or LFA-1 or other adhesion receptors.

Moreover, the interaction of NK cells with DC contributes to the amplification of innate immunity, before the onset of acquired immune responses: in particular, upon contact with DC, NK cells become cytotoxic, secrete lytic enzymes, and produce IFN-γ, one of the effectors of NK cell function (1, 2, 13, 21). Interestingly, we found that all these events are activated by CAMKII, triggered via LFA-1 upon NK/DC contact, and HIV-1 Tat can impair this key biochemical mechanism by interfering with calcium entry. Indeed, the effect of Tat on NK cell-mediated killing of DC is not due to the block of effector/target cell binding, as the number of NK cells adhering to DC is not decreased upon exposure to Tat. However, the extent of membrane engaged in cell to cell contacts is deeply reduced, and granules are spread in the cytoplasm, instead of being localized at the cell to cell contact, upon treatment of NK cells with Tat. As NK/DC binding induces a Ca2+ influx that is blocked by Tat or VPM, it is conceivable that calcium entry is needed to strengthen the interactions between NK cells and DC, and drive CAMKII activation. In the whole, our observations indicate that Tat interferes with the earliest signaling events that follow NK/DC by blocking one important biochemical mechanism that leads to NK cell activation. Thus, early release of Tat by HIV-1-infected neighboring cells might hamper the triggering of the effectors of innate immunity and delay the amplification of an efficient antiviral response.

In conclusion, we have described one of the biochemical mechanisms underlying the activation of NK cells; as DC are the professional APCs, this mechanism might also contribute to bridge innate with acquired immunity. Finally, our present data add new evidence for a role of extracellular Tat as a viral tool to escape a first-line immune response.

References


