HIV infection leads to a state of chronic immune activation and progressive deterioration in immune function, manifested most recognizable by the progressive depletion of CD4+ T cells. A substantial percentage of natural killer (NK) cells from patients with HIV infection are activated and express the natural cytotoxicity receptor (NCR) NKp44. Here we show that a cellular ligand for NKp44 (NKp44L) is expressed during HIV-1 infection and is correlated with both the progression of CD4+ T cell depletion and the increase of viral load. CD4+ T cells expressing this ligand are highly sensitive to the NK lysis activity mediated by NKp44+ NK cells. The expression of NKp44L is induced by the linear motif NH2-SWSNKS-COOH of the HIV-1 envelope gp41 protein. This highly conserved motif appears critical to the sharp increase in NK lysis of CD4+ T cells from HIV-infected patients. These studies strongly suggest that induction of NKp44L plays a key role in the lysis of CD4+ T cells by activated NK cells in HIV infection and consequently provide a framework for considering how HIV-1 may use NK cell immune surveillance to trigger CD4+ T cell death. Understanding this mechanism may help to develop future therapeutic strategies and vaccines against HIV-1 infection.

The underlying mechanisms of CD4+ T cell depletion during HIV infection may have important therapeutic implications and are still debated, principally because both uninfected and infected CD4+ T cells are destroyed. No experiments have explored the possibility that natural killer (NK) cells might be cytotoxic for CD4 target cells that express their specific activating ligands. NK cells both mediate immune reactions against infection and adversely affect some autoimmune diseases (1). The major activating receptors are NKG2D and the natural cytotoxicity receptors (NCRs) Nkp30, Nkp46, and Nkp44 (2). The particularity of Nkp44 is its total absence in fresh NK cells; it is only expressed progressively after their activation. This may explain, at least in part, the higher levels of cytolytic activity mediated by IL-2-activated NK cells (3). The cellular ligands of these NCRs have not yet been identified, but studies suggest that they are not expressed on normal tissues, but can be induced under certain pathological conditions, including viral infections (2).

Researchers are just beginning to understand the sophisticated interactions between viruses and NK cell receptors (4). These interactions are often caused by the enhanced functions of inhibitory MHC class I-specific NK cell receptors, such as the UL18 protein encoding by the human CMV (HCMV), which interacts with LIR-1/ILT-2, an inhibitory receptor on NK cells (5). Conversely, UL16, another HCMV protein, interacts with NKG2D (6). The hepatitis C virus E2 glycoprotein binds to CD81 on NK cells and thus inhibits NK cytotoxicity (7), whereas the poxvirus A39R protein interacts with the virus-encoded semaphorin protein receptor (VESPR) expressed by NK cells (8). The role of NK cells in the control of HIV infection is currently unclear. The increased NK activity reported in some HIV-exposed patients suggests that the NK cells may contribute to protection against infection (9). Conversely, several studies show alterations in the number and function of NK cells during HIV infection and progression to AIDS (10). However, the NK cell cytotoxicity level does not seem to be predictive of the disease course, like the relative preservation of NK activities in healthy AIDS patients with low CD4 cell counts (11). On the other hand, the NK activating receptor KIR3DS1, in combination with HLA-Bw4–80Ile allele, is associated with more rapid progression to AIDS (12).

Here, we offer evidence that NKp44L, a ligand of the activating receptor NKp44, is specifically induced on CD4+ T cells from HIV-infected patients and that this expression is correlated with the progressive loss of CD4+ T cells, increased viral load, and susceptibility to lysis by NKp44+ NK cells. We also report that the gp41 subunit of the HIV-1 Env protein plays a direct role in this process. Our results suggest that a specific highly conserved motif of the gp41 protein stimulates NK cells and may therefore play a role in the CD4+ T cell depletion that occurs during the progression of HIV-1 disease.

**Materials and Methods**

**HIV-1 Infected Donors.** Blood samples of 25 HIV-infected patients were obtained from consenting donors at Hôpital Pitié-Salpêtrière (Paris). For control purposes, leukocytes from 20 uninfected donors were obtained by leukapheresis from the hospital blood bank.

**Chronically HIV-Infected U2 Cells.** The human U2 cell line chronically infected with the HIV-1 Sf2 strain was obtained as described (13). Constitutive viral expression was detected through measurement of p24 antigen and of reverse-transcriptase activity.

**Constructions and Production of NCR Human Fc Fusion Proteins.** The sequences encoding the extracellular domain of NCR proteins were amplified by PCR from mRNA isolated from NK clones. These fragments were cloned into a mammalian expression vector containing the Fc portion of human IgG1 (14). The integrity of these constructs was confirmed by sequencing. These vectors were transfected into COS-7 cells by FUGENE-6 (Roche Diagnostics), and the fusion proteins were purified on protein A/G columns (Pierce).

**Production of Antibodies.** Monoclonal antibodies were obtained from BalB/c mice immunized by using the ClonCell-HY hybridoma cloning kit (StemCell Technologies, Vancouver). Anti-NKp44 mAb (44/8: IgG1) was obtained by mice injection of extracellular fragment of NKp44 and purified on protein A/G columns (Pierce). Anti-NKp44L mAb (7.1; IgM) was obtained by mice injection of irradiated acid-treated 721.221 cells, as

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Abbreviations: NK, natural killer; NCR, natural cytotoxicity receptor.

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described (15), and purified on mannan-binding protein columns (Pierce). Hybridoma screening reagent (Roche Diagnostics) and ELISA were used to test the specificity of each mAb, and their purity was confirmed by SDS/PAGE. Purified polyclonal anti-gp41-C146 peptide antibody was prepared by Epytop (Nîmes, France).

**Cytolymphometric Analysis.** Three-color FACS analysis was performed on freshly harvested PBMC. Isotype-matched Ig served as the negative control (Becton Dickinson). Cells were incubated for 1 h at 4°C with the appropriate antibody mixture. Erythrocytes were lysed by using the FACS lysing solution (Becton Dickinson). At least 20,000 leukocytes were analyzed on a FACSscan.

**Purification of CD4+ T Cells Expressing NKp44L.** The RosetteSepCD4+ enrichment kit (StemCell Technologies) was used to sort the CD4+ T cell fraction. CD4+ T cells expressing NKp44L were positively selected by a two-step magnetic separation using anti-IgM mouse-coated Dynabeads (Dynal). After the purification, the dying cells were eliminated by a gradient-Ficoll centrifugation.

**Isolation of Primary NK Cells and NK Cytotoxicity Assays.** NK cells were purified by using the NK cell enrichment antibody mixture (StemCell Technologies). NK cells were cultured in MyeloCult H5100 medium (StemCell Technologies) supplemented with 100 units of IL2 (Bohringer). The purity was evaluated by flow cytometry after staining with anti-CD3 (Becton Dickinson), anti-CD56 (Becton Dickinson), and anti-NKp46 mAbs. The cytolytic activity was assayed on triplicates during a 4-h 51Cr-release assay as described.

**Recombinant Vaccinia Virus Expressing HIV-1 Proteins.** CD4+ T cells were infected with wild-type vaccinia virus (WT) or with various recombinant vaccinia viruses at a multiplicity of infection of 20 plaque-forming units (pfu)/cell. Recombinant vaccinia viruses for HIVBRU proteins provided from Transgène (Strasbourg, France). The efficacy of vaccinia infection was verified with polyclonal anti-vaccinia FITC Ab (Tebu).

**AT-2 Inactivation of HIV-1 Particles.** To preserve the conformational and functional integrity of HIV particles, inactivation was done with 2,2’-dithiodipyridine (AT-2; Sigma) reagent on HIVBRU particles, as described (16). Inactivation of HIV particles was confirmed by an undetectable TCID50.

**Peptides and Pools of Peptides.** The synthetic 15-mers peptides were purchased from Epytop (Nîmes, France) or kindly provided by the Agence Nationale de Recherche sur le SIDA. HPLC profiles show that peptides were >80% pure. Peptide pools included 10 different peptides, and each peptide overlapped the previous continuous peptide for 11 residues.

**Results**

**NKp44L Is Specifically Expressed on CD4+ Cells During HIV-1 Infection.** Expression of ligands for NCR was analyzed by using Ig-fusion proteins on promonocytic U2 cells chronically infected by the HIVU2 strain. The similar expression of the NKp30-Ig and NKp46-Ig fusion proteins, in both uninfected and HIV-infected U2 cells (Fig. 1A), indicates that HIV infection does not modulate the expression of NKp30 and NKp46 ligands. In contrast, expression of the NKp44-Ig fusion protein was only observed on the HIV-infected cells. Around 40% of the HIV-infected U2 cells expressed a ligand for NKp44 (NKp44L) (Fig. 1A). To study the related function and expression of this ligand during HIV-1 infection, we tested a library of mAbs screened for their capacity to inhibit NK lysis. One of them, mAb 7.1, revealed an epitope expressed on NKp44L. Specific staining with this mAb was found in HIV-infected U2 cells, but not in uninfected U2 cells (Fig. 1B), and it yielded a level of staining similar to that of the NKp44-Ig fusion protein (Fig. 1A and B). Inhibition of the mAb 7.1 staining by this fusion protein confirmed that mAb 7.1 specifically interacts with NKp44L (Fig. 1C Upper). A control experiment showed no effect with the NKp46-Ig fusion protein (Fig. 1C Lower). Furthermore, when HIV-infected U2 cells were treated with the mAb 7.1, their NK-mediated lysis decreased sharply (as much as 40%) (Fig. 1D). Similarly, results were obtained with a Jurkat cell line chronically infected by the HIVBRU strain (data not shown). These data suggest that NKp44L is expressed during HIV-1 infection and that mAb 7.1 reacts specifically with NKp44L on HIV-1-infected cells.

To obtain further insight into the role of NKp44L during HIV infection, its expression was determined in PBMC from HIV-1-infected individuals. Cross-sectional analysis revealed that a fraction of CD4+ T cell, but not CD8+ cells, from HIV-infected patients, but not from the healthy controls, expressed NKp44L (Fig. 2A). These results were confirmed with the NKp44-Ig fusion protein (data not shown). The percentage of CD4+ T cells expressing NKp44L+ was significantly and inversely correlated
with the circulating CD4+ T cell count ($P < 0.002$; Fig. 2B), and also highly correlated with the HIV viral load ($P < 0.005$; Fig. 2C). In addition, a time course experiment of NKp44L expression in representative patients on highly active antiretroviral therapy confirmed the strong relation between CD4 count and the expression of NKp44L (Fig. 2D). These results indicate that the fraction of CD4+ T cells expressing NKp44L during HIV-1 infection is correlated with disease course.

**CD4+ NKp44L+ T Cells Are More Susceptible to NK Lysis.** Further documentation that autologous NK cells can target CD4+ T cells, which express NKp44L, during in vivo HIV infection, came from the expression of NKp44 on CD3+ CD56+ NK cells from HIV-infected patients. The proportion of NK cells expressing NKp44 was significantly higher among HIV-infected patients with <250 CD4+ cells per mm3 than among controls (Fig. 3A). This induction was concomitant with expression of the CD69 activation marker (data not shown).

We then tested the sensitivity of CD4+ NKp44L+ T cells to NK lysis. After purifying the CD4+ NKp44L+ and NKp44L+ T cell fractions from three different HIV-infected patients, with CD4 cell counts close to 250 per mm3, we assessed their ex vivo sensitivity to NK92, an NK cell line expressing NKp44, and IL2-activated purified autologous NK cells. As Fig. 3B shows, the NK92 cells efficiently killed CD4+ NKp44L+ T cells from all samples. NK lysis of the CD4+ NKp44L+ T cells remained close to the background level. The anti-NKp44 mAb strongly inhibited the lysis of the CD4+ NKp44L+ T cells by NK92 cells, by as much as 40%. Similar results were observed with autologous NK cells (Fig. 3C). In addition CD4+ T cells from these patients were sensitive to lysis with freshly purified autologous NK cells, tested in absence of IL2 activation (Fig. 3D). These data provide evidence that CD4+ T cells expressing NKp44L are highly sensitive to the NK lysis activity mediated by activated NKp44+ NK cells.

**NKp44L Is Induced on CD4+ T Cells by the HIV-1 Envelope Protein.** To assess the role of HIV-1 in the induction of NKp44L, purified CD4+ T cells from healthy donors were incubated with several concentrations of AT-2-inactivated HIV particles. As Fig. 4A

![Figure 2](image1.png)  
**Fig. 2.** Expression of NKp44L in CD4+ T cells from HIV-infected individuals is associated with disease stage. (A) Specific expression of NKp44L on CD4+ T cells in HIV-infected patients stained with mAb 7.1. The horizontal lines mark the mean value. NKp44L expression is reported as percentage of positive cells. (B) Inverse correlation of NKp44L expression in CD4+ T cells with peripheral blood CD4 cell count in HIV-infected patients. (C) Correlation between NKp44L expression in CD4+ T cells and viral load. (D) Time course of CD4 cell count (open symbols) and NKp44L expression (filled symbols) in three independent subjects (triangle, square, and circle) on highly active antiretroviral therapy.

![Figure 3](image2.png)  
**Fig. 3.** High sensitivity to NK lysis of CD4+ T cells expressing NKp44L. (A) Expression of NKp44 on CD3+ CD56+ NK cells from healthy donors and HIV-infected patients with a CD4 cells count >250 or <250 per mm3. The percentage of CD3+ CD56+ NK cells that expressed NKp44 was reported. ns, nonsignificant. Cytotoxic activity of NK92 NK cell line (B) and IL2-activated autologous NK cells (C) was analyzed against CD4+ T cells that did (square) or did not (circle) express NKp44L from three HIV-infected patients (patients BF, 250 CD4+ cells per mm3; DF, 182 CD4+ cells per mm3; MM, 213 CD4+ cells per mm3). Cytotoxic activity was analyzed after treatment with anti-NKp44 mAb. Filled circles, CD4+ NKp44L+ T cells plus IgM isotype; open circles, CD4+ NKp44L+ T cells plus anti-NKp44 mAb. Filled squares, CD4+ NKp44L+ cells plus IgM isotype; open squares, CD4+ NKp44L+ T cells plus anti-NKp44 mAb. (D) Inhibition of cytotoxicity of fresh autologous NK cells without IL2-activation by anti-NKp44 mAb.
A Highly Conserved HIV-1 gp41 Peptide Is Drastic in the Induction of Nkp44L

To identify the peptide motif of the gp41 protein involved in this increased NK lysis activity, we next examined the effect of pools of overlapping 15-mer peptides that cover the complete gp41 protein sequence. CD4$^+$ T cells were incubated with each peptide pool and then tested against activated autologous NK cells. NK lysis increased with only one peptide pool (data not shown). Each peptide in this pool was then tested individually. Only three continuous peptides (gp41-C145, gp41-C146, and gp41-C147) were involved in this effect, and they shared a common peptide motif: NH2-SWSNKS-COOH. Experiments with the gp41-C146 (WT) peptide and two mutated 15-mer peptides with mutations inside the motif enabled us to define its effect (Fig. 5C). The gp41-C146 (WT) peptide induced strong Nkp44L expression: 17.4% of CD4$^+$ T cells incubated with this peptide expressed this ligand. Conversely, neither untreated cells nor cells incubated with the mutated peptides expressed significant amounts of Nkp44L (<4%). (Fig. 5D). Concomitantly, NK cytotoxic activity increased markedly in the presence of the gp41-C146 (WT) peptide, but did not differ significantly between untreated CD4$^+$ T cells and those treated with the control peptides (Fig. 5C). In addition, anti-Nkp44L mAb drastically decreased the induction of cytotoxic lysis by the gp41-C146 (WT) peptide, close to the level obtained after incubation with the mutated peptides. Induction of NK lysis by this peptide therefore appears to be correlated with Nkp44L expression (Fig. 5D).

We then checked the frequency of each residue for each position in the SWSNKS motif among the 250 sequences of gp41 repertoires in the Los Alamos database (http://hiv-web.lanl.gov). This motif is highly conserved among diverse isolates of HIV-1, covered both R5 and X4 strains. More than 77% of HIV-1 strains express the SWSNKS motif. However, ~10% and 13% of HIV-1 strains expressed the SWSNRS and the SWSNKT motifs, respectively. These two other motifs induced levels of Nkp44L expression (≤5%) (Fig. 5B). Further support for these hypotheses come from experiments that raised a rabbit anti-gp41-C146 peptide polyclonal Ab and tested its inhibition of Nkp44L expression and sensitivity to NK lysis. CD4$^+$ T cells from HIV-1-infected patients were cultured for 2 days in presence of IL2 and then incubated overnight with
two highly HIV-1 infected patients (CG, 322 CD4

Vieillard et al. Critical role of the SWSNKS motif. (Fig. 5. An anti-gp41-C146 peptide polyclonal antibody. Purified CD4

B

C

D

E

F

Fig. 5. Critical role of the SWSNKS motif. (A) Sequences of gp41-C146 (WT) and two mutated control peptides (Ctrl1 and Ctrl2). (B) Induction of NKp44L expression. CD4+ T cells were treated with 5 µg/ml peptide and then stained with no. 7.1 anti-NKp44L mAb. The percentage of CD4+ T cells expressing NKp44L is noted for each panel. (C) Sensitivity of CD4+ T cells incubated with these peptides to IL2-activated autologous NK cells. (D) Inhibition of NK lysis by anti-NKp44L mAb. Effects of NKp44L were inhibited with an anti-gp41-C146 peptide polyclonal antibody. Purified CD4+ T cells from two highly HIV-1-infected patients (CG, 322 CD4+ cells per mm3 and 148,100 HIV copies per ml; and BT, 208 CD4+ cells per mm3 and 255,300 HIV copies per ml) were cultured 2 days in presence of 10 units/ml IL-2, and then treated overnight with several concentrations of anti-gp41-C146 Abs. (E) Inhibition of NKp44L expression in presence of anti-gp41-C146 Abs. (F) Inhibition of NK lysis in presence of anti-gp41-C146 Abs. Cytotoxic activity was performed by using IL2-activated autologous NK cells. Open circles, untreated cells; filled squares, triangles, and diamonds, CD4+ T cells treated with 1, 10, and 20 µg/ml of anti-gp41-C146 Abs, respectively. The results are representative of at least four independent experiments.

several concentrations of purified anti-gp41-C146 polyclonal Ab. As shown in Fig. 5E, incubation with at least 10 µg/ml of anti-gp41-C146 Ab induced a sharp decrease of NKp44L expression; indeed, <5.5% of anti-gp41-C146-treated cells expressed NKp44L, as compared to 18% and 22% of CD4+ T cells from CG and BT samples, respectively (Fig. 5E). This effect was confirmed by the drastic inhibition of NK activity in the presence of the anti-gp41-C146 polyclonal Ab (Fig. 5F). These results suggest that the SWSNKS motif plays a key role in inducing NKp44L expression during HIV infection and that the gp41 protein participates in the selective destruction of CD4+ T cells by activated NK cells.

Discussion

We report a mAb, 7.1, which recognizes a ligand of the NKp44 receptor (NKp44L). Phenotypic and functional analysis reveal that NKp44L plays a role in the cytotoxicity mediated by activated NK cells. We provide evidence that NKp44L is specifically induced during HIV infection, both in vitro and in vivo, whereas no expression of ligands for NKp30 and NKp46 was detected. Importantly, NKp44L expression on CD4+ T cells from HIV-1-infected patients was highly correlated to the absolute CD4+ T cell count and the viral load decrease and the viral load increase. The strong correlation between NKp44L expression on CD4+ T cells from HIV-infected patients and their depletion suggested a link in this process. This hypothesis was confirmed in patients on highly active antiretroviral therapy. This treatment leads to a significant increase of CD4+ T cells, which is associated with a significant decrease of NKp44L expression in CD4+ T cells. In HIV-infected patients with a CD4+ T cell count <250 per mm3, the level of NKp44 on NK cells remains closed to the background, as previously observed (17). In contrast, with a CD4+ T cell count <250 per mm3, NKp44 is highly expressed on NK cells, and this is correlated with up-regulation of the CD69 activation marker, suggesting that, during HIV infection, HIV directly stimulates NK cells, as seems with other viruses (18). De Maria et al. (19) have shown that NK cells from HIV-infected patients displayed a reduced ability to mediate NK cytotoxicity in correlation with the decrease of NKp44 expression on NK cells. However, a different experimental procedure may explain this discrepancy: they used NK cells cultivated in presence of IL2, whereas our experiments were performed with freshly isolated peripheral blood mononuclear cells.

The evolution of viruses has involved numerous mechanisms to destroy the human immune system. These mechanisms include direct killing of infected target cells after virus-gene expression and cytopathicity, alteration of the expression of the molecules regulating cellular apoptosis, and the killing of bystander cells by viral proteins released by infected cells (20, 21). In infected patients with a low CD4+ T cell count, the percentage of CD4+ T cells that express NKp44L is far higher than the percentage of infected cells expected (<1/1,000). This finding suggests that many uninfected CD4+ T cells express NKp44L and may therefore be a target for lysis by NK cells. Increasing evidence from other models indicates that NK cells can kill autologous cells that have neither been infected with virus nor transformed. For example, short-term activated NK cells can effectively destroy autologous dendritic cells (21) and oligodendrocytes (22). It remains uncertain whether a change in the expression of NK cell receptor ligands on target cells suffices to explain this NK cell-mediated killing. Several reports indicate that activating signals can override inhibitory signals and cause a loss of tolerance, which could have detrimental consequences. For example, the presence of KIR2DS2, an activating NK receptor, correlates with an increased risk for patients with rheumatoid arthritis to develop rheumatoid vasculitis, suggesting that NK cell activity can induce an autoimmune adverse effect (23). Hence, analysis of the expression of receptor ligands may
The expression of NKp44L seems critical in determining the sensitivity of CD4+ T cells to NK cell lysis. CD4+ T cell infection by HIV remains a rare phenomenon, and may not be sufficient to explain the sharp expression of NKp44L into HIV-infected CD4+ T cells. Circulating virions or their components may contribute to this process. Several studies have demonstrated that some HIV-encoded proteins, including Tat, Nef, Vpr, and Env, can partially activate T cells, induce anergy, or trigger apoptosis (20, 24). Indeed, the role of HIV particles on the NKp44L expression shown that gp41 plays a pivotal role in this phenomenon. This protein is a subunit of the Env complex protein, which is drastic in HIV entry into target cells. The interaction of the gp120 with the target cell surface induces conformational changes in the gp120-gp41 complex that expose the gp41 molecules (25). Similar structures have been proposed for several viruses (26–27). This conformational change of the gp41 subunit appears to promote fusion of the viral and cellular membranes (28). During this process, several motifs of the gp41 became accessible to the surface and could directly involve in the induction of NKp44L expression.

Our results from screening accessible overlapping synthetic peptides revealed that NK lysis strongly increases in presence of the gp41 SWSNKS motif. This motif is localized in position 626 (according to the position in gp160 of the BRU HIV-1 strain), between the N-terminal heptad repeat 1 (HR1) and the HR2. This region plays a key role in the formation of the six-helix bundled gp41 ectodomain core structure, and imposes several kinetic and steric constraints responsible for a high degree of motif preservation (28). These constraints may account for the remarkable conservation of the SWSNKS motif reported in >77% of the gp41 HIV-1 strains that have been analyzed, including both R5 and X4 HIV-1 strains. Only two other motifs have so far been observed in this position: SWSNRS and SWSNKT. These minimal variations in the motif, with no significant effect, certainly suggest its critical role. Several models propose that this region may have contact with the host cell membrane during the formation of the ectodomain core structure (29). NKp44L expression decreased strongly in the presence of a rabbit anti-gp41 peptide polyclonal Ab, suggesting that this specific gp41 motif may be transiently exposed outside. This inhibition of NKp44L expression by the anti-gp41-C146 Ab could also suggest a possible turnover of NKp44L cell surface expression. Further experiments are needed to study the recycling of this receptor and the gp41 triggering on CD4+ T cells.

Together, our data demonstrate that NKp44L, a cellular ligand for NKp44, is specifically induced on CD4+ T cells during HIV-1 infection. This finding suggests a harmful function for activated NK cells during HIV-1 infection. A gp41 motif highly conserved in HIV-1 isolates plays a critical role in the induction of this ligand, suggesting that HIV has acquired the ability to use NK cells to disarm the host immune system by selectively triggering CD4+ T cells. The blockade of NKp44L expression by an anti-gp41 peptide Ab indicates that immune strategies could be applied to counteract these deleterious effects.

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