VCAM-1 Expression on CD8+ Cells Correlates with Enhanced Anti-HIV Suppressing Activity

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CD8 cells from HIV-infected individuals showing the CD8 cell noncytotoxic antiviral response unexpectedly revealed mRNA for VCAM-1, a cell surface molecule found on endothelial cells. Uninfected subjects had undetectable levels of VCAM-1 mRNA in their CD8 cells. Flow cytometry analysis showed that up to 12% of the CD8+ cells from HIV-positive individuals expressed VCAM-1 compared with 0.8% of the CD8+ cells of HIV-negative individuals. Enrichment of the CD8+VCAM-1+ cell population and subsequent coculture with CD4+ cells acutely infected with HIV-1 showed that the VCAM-1+ CD8+ cells were able to suppress viral replication with 50% less input cells than the unseparated CD8+ cell population. This study demonstrates, for the first time, the expression of VCAM-1 on CD8+ cells. Moreover, the CD8+VCAM-1+ cells show enhanced CD8+ cell noncytotoxic antiviral response activity that could have clinical importance in HIV infection. *The Journal of Immunology*, 2005, 174: 1574–1579.

**Materials and Methods**

**Subjects**

Peripheral blood samples were obtained by venipuncture in Vacutainer tubes containing sodium heparin from clinically healthy seronegative subjects (provided by Blood Centers of the Pacific, San Francisco, CA) and from HIV-1-seropositive subjects followed in our laboratory. The study was approved by the University of California, San Francisco, Committee for Human Research, and all participants signed an informed consent form before the study.

**Recovery of peripheral blood cells**

PBMC were obtained by Ficoll-Hypaque (Sigma-Aldrich) gradient centrifugation. The cells were stimulated with PHA (3 μg/ml; Sigma-Aldrich) for 3 days in RPMI 1640 medium containing 10% heat-inactivated (56°C, 30 min) FBS (BioWhittaker) supplemented with 2 mM glutamine (Bio-Whittaker), 100 μg/ml streptomycin (BioWhittaker), 100 U/ml penicillin (BioWhittaker), and 100 U/ml natural IL-2 (Roche). CD4+ cells and CD8+ cells from the activated PBMC were subsequently obtained by positive selection using immunomagnetic (IM) beads bearing anti-CD4 or anti-CD8 mAbs (Miltenyi Biotec). Purity of the cell subset population was confirmed by flow cytometry which by >97% of the cells were CD4+ or CD8+ cells, respectively (10). Enrichment for CD8+VCAM-1+ (CD106) population was obtained by labeling the double-positive cells in a purified CD8+ cell population with PE-conjugated anti-CD106 Ab and subsequent positive selection using IM beads bearing anti-PE mAbs (Miltenyi Biotec).

**Acute infection assay**

Acute virus suppression assays were conducted as previously described (4). Purified CD4+ cells from seronegative donors were first acutely infected with 10,000 50% tissue culture-infective dose of the β-chemokine-insensitive HIV-1_L9623 strain (11). After 1 h, the cells were washed and then cultured in the RPMI 1640 growth medium containing 100 U/ml rIL-2 (generously provided by Glaxo Wellcome) alone or in the presence of CD8+ cells at a 1:1 cell input ratio unless otherwise indicated. After 5–7
days of coculture, fluids were collected from the culture for virus detection by the reverse transcriptase (RT) assay (12), and the CD8+ cells were isolated from the coculture with IM beads (4).

Acute infection assays to evaluate the role of the CD8+ Vcam-1+ cells were performed using CD8+ cells from HIV+ asymptomatic individuals and acutely infected CD4+ cells from seronegative blood donors as described above. Culture fluids were removed every 2 days and monitored for RT activity. In some experiments, the cocultures were incubated with Abs to Vcam-1 (R&D Systems) at 100 ng/ml, or IL-15 (Cell Sciences) at 100 ng/ml, or TNF-α (Cell Sciences) at 100 ng/ml, or IL-15 (Cell Sciences) at 100 ng/ml, or TNF-α (Cell Sciences) at 100 ng/ml. In some cases, a Transwell culture insert device (Costar; Corning) was used to physically separate the CD8+ cells from the CD4+ cells while still allowing culture medium to pass freely through the 0.4-um pore size membrane.

All acute infection assays were performed in triplicate, and the results were averaged. The percentage of suppression at the peak level of HIV replication (usually day 5 or 7) was determined by comparing the RT activity in the culture fluids of the CD8+ and CD4+ cell cocultures with that of the infected CD8+ cells cultured alone.

Real-time RT-PCR

The total RNA concentration was determined by spectrophotometer and adjusted to a concentration of 200 ng/μl. A total of 500 ng of total RNA was reverse transcribed using 250 U of Moloney murine leukemia virus-RT (InVitrogen Life Technologies) in 1× AmpLTaq Buffer (Applied Biosystems) supplemented with 7.5 mM MgCl2 with 5 μM random hexamers (InVitrogen Life Technologies), 1 mM each of dNTPs, and 40 U of RNase inhibitor (Promega). The reaction mixture was incubated at 25°C for 10 min, 48°C for 40 min, and 95°C for 5 min. A volume of 5 μl of a 1/10 dilution of the RT reaction was subjected to PCR using the GeneAmp Polymerase Chain Reaction System, SYBR Green PCR Master Mix, and 250 nM primers in a 25-μl reaction. Cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. Primer sequences for detection of Vcam-1 mRNA were as follows: 5′-GGGACCATCTACGCTGACA for the forward primer and 5′-CGGTGGTGTCTGTCATCCAGAAA for the reverse primer.

Flow cytometry and Vcam-1 detection

Standard flow cytometry was performed to identify different cell subsets. The percentage of CD8+ cells expressing Vcam-1 (CD106) was determined by staining PBMCs or purified CD8+ cells from seronegative and seropositive donors with PE-conjugated anti-CD106 Ab (BD Biosciences) and FITC-conjugated anti-CD8 Ab (BD Biosciences) for CD8+ cell expression. Fluorochrome-conjugated isotype-specific Igs were used as negative controls. Flow cytometry was performed on the FACSort (BD Biosciences) using the CellQuest program for analysis.

For Vcam-1 detection, cell culture fluids from coculture experiments were collected and centrifuged at 12,000 x g to pellet viral particles, and supernatants were stored at −70°C. sVcam-1 concentrations were determined using a sVcam-1 ELISA kit (R&D Systems) according to the manufacturer’s instructions.

PBMC or CD8+ cells isolated with IM beads from seronegative donors were treated with TNF-α (BioSource) at 100 ng/ml, HIV-1 Tat (AIDS Research and Reference Reagent Program) at 100 ng/ml, or IL-15 (Cell Sciences) at 10 ng/ml. Vcam-1 expression on the surface of CD8+ cells was monitored by flow cytometry as described above.

Statistical methods

Statistical analysis was done by the R software package ((http://lib.stat.cmu.edu/R/CRAN)). The exact Wilcoxon rank-sum test was used to evaluate the significance of mRNA expression differences between the infected and uninfected groups for each gene tested.

Results

Expression of Vcam-1 mRNA in CD8+ cells from HIV+ individuals

CD8+ cells from a pair of discordant HIV+ identical twins were evaluated for their ability to suppress HIV replication when added to HIV-1-acutely infected CD4+ cells. In these studies, the HIV+ twin suppressed virus replication by >90%, whereas the uninfected twin showed no antiviral activity. During the study of the genes potentially involved in the suppression of HIV replication, Vcam-1 mRNA was found overexpressed in the CD8+ cells of the infected twin.

Based on this observation, kinetic RT-PCR was used to study Vcam-1 mRNA expression in the CD8+ cells from a set of HIV-infected subjects and uninfected individuals. CD8+ cells were cultured at a 1:1 CD8:CD4 cell ratio for 5 days in an acute infection assay. The mRNA was then isolated from the CD8+ cells from HIV-positive subjects with either moderate or high suppressing ability as well as from uninfected individuals. In these studies, the CD8+ cells from the high suppressors (n = 7) showed >90% suppression of viral replication in the acute infection assay. CD8+ cells from moderate suppressors (n = 7) showed 20–60% suppression. CD8+ cells from seronegatives (n = 7) gave negligible viral suppression in the acute infection assay.

The relative levels of Vcam-1 mRNA in these samples confirmed that the HIV-positive individuals had significantly higher expression of the molecule in these CD8+ cells (p = 0.004) (Fig. 1A) than the CD8+ cells from uninfected subjects. The moderate suppressors also showed the presence of Vcam-1 mRNA but in lower amounts when compared with the CD8+ cells from suppressors. This difference was not statistically significant; however, the trend of lower expression in the moderate suppressors suggests a correlation between the expression Vcam-1 mRNA and suppression of HIV replication.

![FIGURE 1. Vcam-1 mRNA expression levels determined by kinetic RT-PCR. A, mRNA expression in CD8+ cells subjected to the coculture assay from seven HIV-positive subjects with high CNAR activity (>90%) (●, suppressors), seven HIV-positive subjects with low CNAR (40–60%) (○, moderate suppressors), and seven HIV-negative subjects (●, nonsuppressors). Values of p calculated using the Wilcoxon rank-sum test are as follows: suppressors vs nonsuppressors, p = 0.004; suppressors vs moderate suppressors, p = 0.240; moderate suppressors vs nonsuppressors, p = 0.015. B, mRNA expression in unstimulated CD8+ cells from suppressors (●, n = 4), moderate suppressors (○, n = 6), and HIV-negative subject (●, n = 6). Average mRNA expression per sample set is denoted by a black line. mRNA levels were standardized by the level of porphobilinogen deaminase and reported in arbitrary units. Gene-specific primers were subjected to PCR once, and each RNA sample was run in triplicate as described in Materials and Methods.](http://www.jimmunol.org/doi/abs/10.4049/jimmunol.20011575)
Expression of VCAM-1 on the surface of CD8⁺ cells from HIV-positive individuals

Flow cytometry analysis using a mAb against VCAM-1 showed that CD8⁺ cells from HIV-positive individuals expressed low amounts of the molecule on their surface (Fig. 2A). The percentage of CD8⁺ cells in the HIV-positive subjects expressing VCAM-1 ranged from 1 to 12% (Fig. 2B). In contrast, the majority of CD8⁺ cells from uninfected individuals showed very low or no expression of the protein.

Studies have also suggested that sVCAM-1 is present in the plasma of HIV-positive individuals (9). Using an ELISA designed to detect sVCAM-1, we found that culture fluids from cultured CD8⁺ cells did not contain measurable quantities of the soluble form of the molecule (data not shown). Therefore, it appears that CD8⁺ cells may not contribute to the levels of sVCAM-1 noted in plasma of HIV-infected individuals.

Role of cell activation in VCAM-1 expression

Because the CD8⁺ cells used in the coculture were PHA-stimulated, which could artificially induce the expression of VCAM-1, we also looked at the levels of mRNA in unstimulated CD8⁺ cells from HIV⁺ and HIV⁻ subjects (Fig. 1B). The results were similar to those found with the PHA-stimulated CD8⁺ cells. The unstimulated CD8⁺ cells from the peripheral blood of HIV⁺ individuals expressed VCAM-1 mRNA, whereas CD8⁺ cells from uninfected subjects did not. Moreover, PHA-stimulated CD4⁺ cells from either HIV-positive or HIV-negative individuals did not express significant levels of the VCAM-1 mRNA (data not shown).

Activation markers of CD8⁺ cells were also analyzed to determine whether VCAM-1⁺ cells had a higher level of cell activation than the unseparated CD8⁺ cell population. PHA-stimulated CD8⁺ cells from HIV-positive individuals were evaluated (Fig. 3). The levels of CD25 and HLA-DR as well as CD28 were proportionally equal in the total CD8⁺ cell population when compared with the VCAM-1⁺ cell subpopulation. Thus, the level of cell activation did not appear to affect VCAM-1 expression.

Enrichment for CD8⁺ VCAM-1⁺ cells increases CNAR

To investigate the potential role of VCAM-1 in CNAR, we positively selected the CD8⁺ VCAM-1⁺ subpopulation, which was substantially enriched 40–70% (Fig. 4A). In subsequent acute infection assays, these VCAM-1⁺ CD8⁺ cells were added to acutely infected CD4⁺ cells at 1:1 and 0.5:1 CD8⁺ cell:CD4⁺ cell input ratios. These results indicated that the VCAM-1-enriched population suppressed HIV replication with much fewer cells than the CD8⁺ cell population (Fig. 4B). At a 0.5:1 ratio, the VCAM-1-positive cells suppressed viral replication by 69%, whereas the total CD8⁺ population only suppressed virus by 19%. Essentially, the levels of suppression at the lower input were comparable to the levels obtained with double the number of unseparated CD8⁺ cells. Moreover, the HIV-positive subject’s CD8⁺ cells were able to suppress viral replication by 73% when added at a 1:1 ratio with infected CD4⁺ cells, whereas the VCAM-1⁺ subpopulation still showed greater suppression at 89%. Furthermore, the VCAM-1-depleted CD8⁺ cell population showed lower suppression at all CD8:CD4 cell ratios than the unseparated CD8⁺ cells and the VCAM-1-enriched cells. To substantiate that the viral suppression in the VCAM-1-enriched cells is due to a nontoxic mechanism, we removed the CD8⁺ cells from the coculture after 5 days of incubation. A sharp increase in RT activity from the HIV-infected
CD4+ cells was observed upon the removal of the CD8+ cells when compared with the cocultures in which the CD8+ cells were not removed (Fig. 5). The results indicate that virus-infected cells were present in the cocultures and not killed by the CD8+ cells.

Because VCAM-1 is an adhesion molecule, and our studies were performed using a contact coculture assay, we also wanted to assess whether cell-to-cell interaction was necessary for the enhanced CNAR activity observed with the VCAM-1-enriched population. Experiments were performed using a Transwell device in which fluid is allowed to pass through a filter but the cells remain separated. This study showed a pattern of suppression similar to that observed in the contact coculture assay (Fig. 6). Thus, the CD8+ VCAM-1 cells appear to produce the soluble factor CAF, which shows antiviral activity.

Abs against VCAM-1 added to the coculture assays did not inhibit CNAR activity in either the unseparated CD8+ cell population or the VCAM-1-enriched population (Fig. 7). Therefore, we conclude that VCAM-1 itself does not mediate the anti-HIV effect.

HIV infection and IL-15 can induce VCAM-1 expression in CD8+ cells obtained from acutely infected seronegative PBMC

To evaluate the possible reason for VCAM-1 expression on CD8+ cells, several approaches were taken using PBMC from seronegative individuals. First, PHA-stimulated PBMC were cultured in the presence of IL-2, and no appreciable VCAM-1 expression was induced (Fig. 8A). Second, infection of the PBMC with HIV-1 did not lead to expression of VCAM-1 in CD8+ cells above the levels seen with CD8+ cells from uninfected individuals (Fig. 8B).

In the HUVEC cell line, TNF-α up-regulates the expression of VCAM-1 on the surface of the cell and purified HIV Tat protein.
can have the same effect (8). However, we found that neither TNF-α nor purified Tat induced the expression of VCAM-1 on normal CD8⁺ cells (data not shown). We also attempted to induce VCAM-1 expression by culturing PBMC with fluids from plasmacytoid dendritic cells, which had been induced to produce IFN-α by exposure to HSV (13). Addition of ~200 ng of IFN-α to unstimulated as well as PHA-stimulated PBMC had no effect on VCAM-1 expression on CD8⁺ cells.

IL-15 has been reported to enhance CNAR by CD8⁺ cells (14). We therefore evaluated the potential effect of IL-15 on infected and uninfected PBMC obtained from seronegative donors. As noted above, neither PHA stimulation of PBMC nor HIV infection of those cells led to an appreciable amount of VCAM-1 surface expression on CD8⁺ cells. The addition of IL-15 to the infected PBMC, however, gave a notable increase in expression (~4%), doubling what was observed with PHA stimulation alone (Fig. 8C). The addition of IL-15 alone to the uninfected PHA-stimulated PBMC also induced expression of VCAM-1 in CD8⁺ cells but below the levels seen in CD8⁺ cells obtained from HIV-infected PBMC (data not shown).

Discussion
Disease states are associated with VCAM-1 expression on the surface of endothelial cells. With lymphocytes expressing VCAM-1 ligand VLA-4 (7, 15), an interaction can take place, leading to trafficking of immune cells to sites of infection. Some immune cells, such as mature dendritic cells, have also been found to express low levels of VCAM-1 on their surface (16).

In our gene expression studies, we unexpectedly found that CD8⁺ cells from HIV-positive individuals consistently express higher levels of VCAM-1 mRNA than CD8⁺ cells from HIV-negative subjects (Fig. 1). Furthermore, flow cytometry analysis of CD8⁺ cells from both HIV-seronegative and -seropositive individuals showed that this difference is also reflected on the cell surface expression of VCAM-1 (Fig. 2). CD4⁻ cells from HIV-positive individuals had undetectable levels of expression of both VCAM-1 mRNA and cell surface protein, suggesting that this phenomenon is specific to CD8⁺ cells rather than a general response of T lymphocytes.

These studies of CD8⁺ cells were undertaken to evaluate the gene expression in CD8⁺ cells showing the CNAR. It was therefore noteworthy that the CD8⁺VCAM-1⁺ cells were found to have higher levels of CNAR than the unseparated CD8⁺ cell population. Because of the small number of VCAM-1⁺ cells in the CD8⁺ cells, we were unable to obtain a highly purified cell population but were able to increase the proportion of VCAM⁺ cells from <10% to 40–70% (Fig. 4A). This enrichment of CD8⁺VCAM-1⁺ cells was sufficient to observe a higher level of CNAR when these cells were used in coculture experiments with acutely infected CD4⁺ cells. The VCAM-1⁺ cell-enriched population showed double the amount of CNAR activity than the unseparated CD8⁺ cell population (Fig. 4B). Experiments in which the cells in coculture were separated by a semipermeable filter, also showed higher suppression with the VCAM-1⁺ enriched cell population (Fig. 6). These findings suggest that the antiviral activity of VCAM-1⁺ CD8⁺ cells is mediated by the anti-HIV soluble factor, CAF. However, VCAM-1 is not this factor, because Abs against the adhesion properties of VCAM-1 had no effect on CNAR in the CD8⁺ cell whole population as well as the CD8⁺ VCAM-1⁺ population (Fig. 7).
An activated CD8+ cell state has been previously associated with CNAR (17), and CD8+ cells coexpressing CD28 and HLA-DR show the highest level of CNAR. In the present studies, the level of activation was similar for the unseparated CD8+ and CD8- VCAM-1+ cell populations (Fig. 3). Moreover, two subjects with an acute respiratory virus syndrome and an HIV-negative individual with Kaposi’s sarcoma and human herpesvirus-8 infection did not show VCAM-1 expression on their CD8+ cells. Thus, VCAM-1 on CD8+ cells appears to serve as an additional marker for a subpopulation of cells found in HIV-infected subjects with a specific function, such as CNAR activity.

We attempted to induce this expression of VCAM-1 on CD8+ cells from HIV-seronegative subjects by exposing PBMC to different culture conditions. It has been reported that HIV infection, in the case of astrocytes, or exposure to purified Tat protein, in the case of endothelial cells, induces the expression of VCAM-1 (8, 18). However, PHA activation, acute infection of PBMC with HIV, and a variety of cytokines (e.g., IL-2, TNF-α, IFN) were not sufficient to induce the expression of this molecule in CD8+ cells. However, the addition of IL-15 to HIV-infected PBMC showed a substantial induction of VCAM-1 on the surface of CD8+ cells (Fig. 8). The levels noted were close to those seen with CD8+ cells from HIV-infected subjects.

IL-15 has T cell-stimulatory activity and has been reported to enhance the function of HIV-specific CD8+ cells. Its activity on lymphocytes has been likened to that of IL-2, which is necessary for the generation of CNAR by CD8+ cells (19). Moreover, IL-15 can enhance anti-HIV responses of CD8+ cells from patients with decreased or low CNAR activity (14). Although the present study does not address other characteristics of CD8+VCAM-1+ cells besides their CNAR activity, the results provide another link between IL-15 and increased CD8 cell anti-HIV activity. Further functional and phenotypic characterization of CD8+VCAM-1+ cells and their induction by IL-15 could lead to therapeutic strategies that can restore a natural antiviral immune response.

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References


