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Differences Among HIV-1 Variants in Their Ability to Elicit Secretion of TNF-α

Kristen V. Khanna,* Xiao-Fang Yu,* David H. Ford,* Lee Ratner,† James K. Hildreth,‡ and Richard B. Markham2*

HIV-1 infection of human PBMC has been shown to elicit secretion of several different cytokines. TNF-α secretion induced by this virus has been of particular interest because it has been associated with the development of HIV-1 dementia and because TNF-α increases viral replication by enhancing NF-κB interaction with the viral promoter, the HIV-1 long terminal repeat. Thus, an autocrine pathway is potentially created in which HIV-1 stimulates its own replication. Conflicting reports exist, however, on the ability of HIV-1 to induce TNF-α secretion in vitro or in vivo. Using experimental protocols that controlled for potential bacterial endotoxin-induced TNF-α secretion, the current study demonstrates significant differences in TNF-α-eliciting properties among primary and laboratory obtained HIV-1. The relative TNF-α-inducing ability of different variants is conserved when tested using PBMC from different individuals. Elicitation of TNF-α secretion was not blocked by exposure of cells to zidovudine, indicating that viral integration was not required to induce secretion. Rather, the interaction between the virus and cell surface is critical for the ability of HIV-1 to induce TNF-α secretion in vitro or in vivo. Using experimental protocols that controlled for potential bacterial endotoxin-induced TNF-α secretion, the current study demonstrates significant differences in TNF-α-eliciting properties among primary and laboratory obtained HIV-1. The relative TNF-α-inducing ability of different variants is conserved when tested using PBMC from different individuals. Elicitation of TNF-α secretion was not blocked by exposure of cells to zidovudine, indicating that viral integration was not required to induce secretion. Rather, the interaction between the virus and cell surface is critical for the ability of HIV-1 to induce TNF-α secretion in vitro or in vivo.

Materials and Methods

TNF-α assay

Freshly obtained unstimulated PBMC from healthy HIV-seronegative volunteers were placed in a 96-well plate (Corning Glass, Cambridge, MA) at 4 × 10^5 well in 100 μl of RPMI 1640 tissue culture medium (Life Sciences, Grand Island, NY) containing 10% human AB serum. Different HIV-1 variants (500 μg of p24 Ag/well) or bacterial LPS (6.0 endotoxin units Salmonella typhimurium LPS; Sigma, St. Louis, MO) were added to the cultures in 100 μl of the same medium. Each virus was tested with and without 10 μg/ml bactericidal permeability-increasing protein (37, 38) (BPI;3 Xoma Pharmaceuticals, Berkeley, CA) present in the medium to ensure that observed TNF-α production was not attributable to contaminating bacterial endotoxin (39, 40). Sixteen hours later, 100 μl of the cell-free culture supernatant was assayed by ELISA (PerSeptive Diagnostics, Framingham, MA) for the presence of TNF-α. Each test condition was assayed in either duplicate or triplicate.

3 Abbreviations used in this paper: BPI, bactericidal permeability-increasing protein; AZT, zidovudine; SI, syncytia inducing.
Viruses and virus preparation

PBMC from HIV-1-seropositive individuals were cocultured with PBMC from healthy seronegative donors that had been placed in RPMI 1640 medium containing 10% human AB serum and stimulated with PHA (5 μg/ml; Life Sciences) for 2 days. Cocultures were subsequently maintained in media supplemented with IL-2 (2 U/ml; Boehringer Mannheim, Indianapolis, IN). Growth of virus from the cultures was determined by assay for viral p24 Ag (DuPont/NEN, Cambridge, MA) 10 days after the initiation of cultures. Culture supernatant fluids and control supernatant fluids from cultures containing no virus (referred to in the figures as "media controls") were centrifuged at 800 x g for 10 min to remove cellular debris and then centrifuged at 100,000 x g for 2 h over a 20% sucrose cushion. The sedimented material was resuspended in RPMI 1640 medium and passed through a 0.22-μm filter, tested for p24 concentration, and used at an appropriate dilution in the TNF-α assay.

The HIV-1 molecular clone p120 was originally derived from HIVNL-4-3, but contains an envelope derived from the molecular clone HIVHXB2, which was inserted between the Sall and BamHI sites, corresponding to positions 5785–8474 based on the nucleotide sequence of the prototype HIV-1 clone HXB2. The molecular clone p125 is identical to p120, but contains an insert from HIVADA, extending from positions 7039–7619 (based on HXB2), which includes the third hypervariable region of the virus envelope. This region is defined by two BglII sites at either end of the insert. The molecular clone p125MNcomp contains the envelope gene from HIVMN, amplified by nested PCR reactions, inserted between the Sall and BamHI sites of p125, extending between bases 5785–8474 of the HIV-1 envelope (based on HXB2). The external primers used for this amplification were 5′-AAACTGACAGGATCCATGGAACAGCC-3′ and 5′-TAAGCTATTGTTGTCAAGGACTGAGTTG-3′. The internal primers used were 5′-CAGAATTGTTGTCAAGTACCAGAATAGCAATTAATCC-3′ and 5′-TTGCTTTAAAGATCCCATGCACAATCAGCAGCGGAGTGTCTCCTGCTC-3′. The underlined portions of the primers contain the Sall and BamHI sites used for insertion into p125.

Conditions used for amplification for both first- and second-round PCR were 95°C for 2 min, followed by 10 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 2 min, followed by 20 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 2 min (including an additional 20 s for each cycle) and one final cycle at 72°C for 7 min.

To create the chimeric viruses, the amplified fragment from HIVMN was cloned into the cloning vector pGem11 (Promega, Madison, WI) by ligating each amplified Sall-BamHI fragment with T4 DNA ligase (Life Sciences) to the plasmid digested with the corresponding restriction enzymes. The DNA was transformed into Escherichia coli DH5α cells (Invitrogen, Carlsbad, CA) and then purified using standard procedures (41). The pGem11 construct was then digested with Sall and BamHI; p125 was also digested with the same enzymes independently. Upon agarose gel electrophoresis (purification), the complementarity fragments were ligated and prepared as described above. Transfection of virus into COS-7 cells was performed using the DEAE-dextran method, as previously described (42), with virus amplified through replication on PHA and IL-2-stimulated PBMC, which were added to the transfected COS-7 cells.

Cells and cell culture conditions

PBMC were obtained by centrifugation of leukopheresed blood from healthy HIV-seronegative donors on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) according to the manufacturer’s instructions. The RPMI 1640 medium, human AB serum, and all other culture supplements were purchased as minimal endotoxin reagents.

Abs

Human anti-CCR5 Abs were obtained from hybridoma 242.21.6 supernatant fluid and were used either without dilution or at 10-fold dilutions in RPMI 1640 medium with 10% human AB serum. Anti-CD4 Abs (RPA-T4; Pharmingen, San Diego, CA) were used at a concentration of 20 μg/ml. Isotype controls for the respective Abs were used at 20 μg/ml (murine IgG and murine IgG1; Pharmingen).

Statistical analysis

The significance of differences in TNF-α secretion between and among different groups was determined by a one-way ANOVA using the Stata statistical package (Stata, College Station, TX).

Results

HIV-1 isolates differ in their ability to elicit TNF-α

PBMC from three subjects were stimulated with low passage HIV-1 isolates, LPS, or tissue culture media, and TNF-α secretion was measured 16 h later (Fig. 1). Adherent cells, predominantly monocytes, secreted between 80 and 90% of the total TNF-α produced in response to LPS or viral stimuli (data not shown). Both LPS and virus 9 stimulated secretion of TNF-α, achieving concentrations of this cytokine that were significantly above those elicited by media alone (p < 0.001 for all subjects). None of the other viral isolates stimulated levels of TNF-α that were significantly greater than their respective media controls. The response of subject 1 to virus 11 compared with that subject’s response to tissue culture media approached, but did not achieve, significance (p = 0.054). Individual subjects differed in their TNF-α response to either LPS or virus 9, but the relative relationship of the responsiveness pattern to the different viruses was maintained among the subjects independent of the stimulus. This finding suggests that some subjects tend to secrete more TNF-α in response to an appropriate stimulus than others.

The virus 9 specimen used in this experiment was a low passage isolate. When passaged multiple times in tissue culture, it subsequently lost its ability to elicit TNF-α secretion, as did many of the primary isolates that initially elicited TNF-α secretion (data not shown).
The ability of LPS to stimulate TNF-α secretion is well established (39, 40) and endotoxin contamination must be considered when evaluating the ability of any agent to elicit TNF-α secretion. Therefore, the effect of BPI, an inhibitor of endotoxin activity, on the response to LPS and to all viral isolates was evaluated in each experiment and is shown for subject 2 from Fig. 1 (Fig. 2). As shown in Fig. 1, BPI effectively abrogated the ability of LPS to elicit TNF-α secretion (p < 0.001). It had, however, no significant effect on the ability of virus 9 to elicit TNF-α secretion (p = 1.0) from subject 2, indicating that contaminating LPS does not account for the ability of virus 9 to elicit this cytokine. For all subjects, the LPS response was significantly reduced by treatment with BPI (p < 0.001 for LPS response compared with LPS plus BPI response for all subjects, shown only for subject 2). However, none of the responses of the subjects to virus 9 was reduced by BPI treatment.

### Response to virus grown on autologous PBMC

Because of the demonstrated ability of HIV-1 to incorporate host cellular Ags into the virus particle during the budding process (43–47), the possibility was considered that the differential TNF-α response was attributable to reactivity against HLA or other host Ags which might stimulate a MLR. To evaluate this possibility, TNF-α responsiveness was assayed against virus 8, a primary clinical isolate, grown on either autologous or heterologous PBMC (Fig. 3). The mean response to virus grown on autologous cells was 3120 pg/ml of TNF-α compared with 2060 pg/ml of TNF-α produced in response to the same virus grown on heterologous cells. This difference was not statistically significant (p > 0.5).

### Reproducibility of results in the same subject challenged with the same viruses at different times

PBMC from a single subject were challenged with the same panel of viruses at two points separated by 5 wk (Fig. 4). The virus that elicited the greatest responses in the first experiment with the cells of this subject (virus 8) also did so in the second experiment. None of the responses to the same virus differed significantly (p > 0.05) at the two time points.

### Effect of a RT inhibitor on the ability of HIV-1 variants to elicit TNF-α secretion

To determine whether viral integration and/or protein synthesis were required for TNF-α secretion, TNF-α levels were measured in cultures to which a primary viral isolate and zidovudine (AZT, 50 μg/ml; Burroughs-Wellcome, Research Triangle Park, NC), an inhibitor of RT, were added. The TNF-α secreted in response to viral isolate 8203 was significantly greater than that secreted by PBMC alone (p = 0.008, Fig. 5). A dose of AZT which completely inhibited replication of this virus, as measured by p24 levels in culture supernatant fluid over a period of 2 wk (data not shown), had no significant effect on the ability of this virus to elicit TNF-α secretion (p = 1.0 when compared with virus alone, p = 0.03 when compared with cells and AZT alone). In the absence of virus, AZT also had no significant effect on TNF-α secretion (p = 1.0) compared with the level observed in cultures not exposed to virus.

### Abs to HIV-1 coreceptors block the induction of TNF-α

Because unintegrated HIV-1 was able to elicit TNF-α secretion, we hypothesized that secretion was stimulated by viral interactions at the cell surface. Binding of HIV-1 to the cell surface requires the interaction of the viral envelope with the host cell receptors CD4 and CCR5 or CXCR4 (48). To determine whether this binding event is critical for subsequent TNF-α secretion, PBMC from several subjects were cultured with anti-CD4 or anti-CCR5 Abs before the addition of p125, a CCR5-utilizing molecular clone of HIV-1 that routinely elicited high levels of TNF-α secretion. Both anti-CD4 and anti-CCR5 Abs significantly inhibited TNF-α induction by p125 (Fig.
Neither anti-CD4 nor anti-CCR5 Abs inhibited TNF-α synthesis induced by LPS. Isotype controls for the respective Abs also did not significantly alter TNF-α secretion.

**Mapping TNF-α-eliciting genes of HIV-1**

Since binding of HIV-1 with the coreceptors was necessary to elicit TNF-α from PBMC, we sought to further define the interaction of HIV-1 envelope with the cell surface that resulted in TNF-α secretion. We therefore examined the ability to elicit TNF-α secretion of chimeric viruses created by substituting parts of the envelope from different viruses into a molecular clone, p125. HIVMN failed to elicit TNF-α secretion (Fig. 7). A viral clone, p125MNenv, which contains the env region of HIVMN also failed to elicit TNF-α secretion. Clone p120, which is identical to p125, but...
contains the V3 loop region from the HIV-1 clone HXB2, also
failed to elicit TNF-\(\alpha\) secretion. Only p125 from this group of
chimeric viruses was able to elicit TNF-\(\alpha\) secretion at levels that
were significantly above those observed with the PBL controls
(\(p < 0.001\)). Levels of TNF-\(\alpha\) secreted after cultivation with
p125\(_{\text{MNenv}}\) were not significantly different from those observed
with HIV\(_{\text{MN}}\) (\(p > 0.5\)).

**Discussion**

These studies demonstrate that different HIV-1 variants have dif-
ferent capabilities to elicit secretion of TNF-\(\alpha\). The ability to elicit
TNF-\(\alpha\) appears to be intrinsic to certain viral variants and is main-
tained when those viruses are exposed to cells from different nor-
amal individuals. The response pattern of a given individual to a
panel of viral variants is generally conserved when observations
are made at different time points. Although different individuals
show variation in the amount of TNF-\(\alpha\) they produce in response
to the same virus, the relative stimulatory ability of different viral
variants is maintained among different individuals.

Since secretion of TNF-\(\alpha\) was observed even when viruses were
exposed to PBMC in the presence of inhibitory concentrations of
AZT, secretion was not dependent on viral integration.

In fact, the differences among viral variants in their ability to
elicit TNF-\(\alpha\) mapped in these studies to the viral envelope, spe-
cifically to the area around and including the V3 region. Previous
studies eliciting TNF-\(\alpha\) with purified HIV-1 envelope demon-
strated that secretion could be elicited with truncated envelope
proteins from many different regions within the viral envelope
(49). In the current studies with intact virus, analysis of the ability
of chimeric viruses to elicit TNF-\(\alpha\) suggested that the region
around the V3 loop of the envelope was a critical determinant of

**FIGURE 5.** Effect of AZT on the ability of HIV-1 variants to elicit TNF-\(\alpha\) secretion. AZT at a concentration of 50 \(\mu\text{m}\) was added at the time of culture
initiation. In a parallel culture using the same PBMC source and virus, AZT at a concentration of 50 \(\mu\text{m}\) completely inhibited HIV-1 replication, as measured by
the absence of increasing p24 concentrations over time (data not shown). Each group represents the mean \(\pm\) SD of TNF-\(\alpha\) concentrations obtained in two wells.

**FIGURE 6.** Anti-CD4 and anti-CCR5 Abs block TNF-\(\alpha\) induction by HIV-1. An-
ti-CD4 (20 \(\mu\text{g/ml}\)) and anti-CCR5 Abs (undiluted or 10-fold dilution) and isotype
controls (20 \(\mu\text{g/ml}\)) were added to human
PBMC in cRPMI-10% human serum. Im-
mmediately following the addition of Abs,
500 pg of p24 virus was added for a total
culture volume of 200 \(\mu\text{l}\). A companion
culture for each virus, Ab, or for LPS was
established in which 10 \(\mu\text{g/ml}\) of BPI was
added (data not shown). TNF-\(\alpha\) levels re-
fect the mean \(\pm\) SD of at least three mi-
crowells for each experimental group. Me-
dia control wells contained PBMC alone
(no virus, Ab, or LPS).
The TNF-α-eliciting ability. These studies do not exclude the possibility that other regions of the virus may also influence its ability to elicit TNF-α secretion.

The critical role of envelope in eliciting TNF-α secretion renders it likely that secretion results from interactions of virus and receptors on the cell surface. The current studies demonstrate that TNF-α induction is blocked by both anti-CD4 and anti-CCR5 Abs, which suggests that interactions with these receptors may influence the ability of different viral variants to elicit TNF-α secretion. That being the case, the induction of TNF-α by HIV-1 is not simply due to its interaction with CCR5 because not all viruses that bind CCR5 induce TNF-α. Previous studies have shown that binding of virus to CD4 results in TNF-α secretion (33). Because induction of TNF-α secretion in our studies mapped to the V3 envelope region, binding to the second HIV-1 receptor likely plays a role in this cytokine induction (13, 50), which it may do simply by altering the affinity with which the virus binds to CD4 (51, 52). Although large numbers of syncytia-inducing (SI) viruses were not studied in these experiments, no clinical or laboratory isolates known to contain only SI variants were able to elicit TNF-α secretion. Further- more, the chimeric viruses p120 and p125_MNenv, that lost TNF-α-eliciting ability had been switched from nonsyncytia inducing (NSI) to the SI phenotype in creating the chimera. Apart from the envelope change, the viruses were identical to the parental p125.

The role of TNF-α in the pathogenesis of HIV-1 infection has been controversial. The early demonstration that TNF-α could promote expression of virus from T cell lines carrying integrated proviral DNA (8) first suggested that virally induced inflammatory processes could indirectly accelerate the disease course by promoting increased viral expression.

A central role of TNF-α in AIDS pathogenesis has been proposed by others as well (53, 54). Careful analysis of the relationship between viral load and TNF-α levels in asymptomatic HIV-1-infected individuals showed a direct relationship between plasma TNF-α levels and viral load, although, in the same study, levels of TNF-α mRNA in PBMC did not correlate with viral load. These studies suggested that TNF-α in serum originated from other sites within the body, such as lymph nodes, where virus was actively replicating.

Other studies have called into question the role that TNF-α plays in disease progression. Li et al. (55) found no difference in TNF-α mRNA levels in tonsilar tissue from five normal individuals and similar tissue obtained from seven asymptomatic HIV-1-infected individuals. Furthermore, no correlation was found between the level of HIV-1 gene expression in these tissues and the level of TNF-α gene expression. These authors concluded that TNF-α is not critical for HIV-1 replication in lymph nodes. Recently, Lane et al. (56) reported that nanogram concentrations of TNF-α suppressed HIV-1 replication by inducing the CCR5 agonist RANTES. Macrophage inflammatory protein-1α and macrophage inflammatory protein-1β were also induced by TNF-α, but did not inhibit HIV-1 replication.

Foli et al. (57) challenged the entire notion that HIV-1 is capable of eliciting TNF-α secretion. Although acknowledging that TNF-α levels are elevated in HIV-1-infected individuals, these investigators demonstrated that high concentrations of two HIV-1 strains, HIV_Ba-L and HIV_LAI, did not elicit TNF-α secretion when care...
was taken to ensure that the virus preparations had minimal concentrations of contaminating endotoxin. These investigators concluded that the elevated levels of TNF-α observed in HIV-1-infected individuals was elicited by pathogens or processes other than HIV-1.

In the current studies, effects of potential endotoxin contamination were abrogated by inclusion of BPI, which inhibits LPS-induced TNF-α secretion (38). Most HIV-1 variants were incapable of eliciting TNF-α secretion. Neither HIV-BaL nor HIV-Bsub, which is closely related to HIV_LAI, could elicit TNF-α secretion (data not shown), as found by Foli et al. (57). However, several clinical isolates were capable of eliciting this cytokine, even in the presence of an LPS inhibitor.

The differences among HIV-1 strains in their ability to elicit TNF-α secretion introduce one more variable that might be associated with differences in disease course. These differences may be particularly relevant to understanding why some individuals with AIDS develop dementia whereas others with advanced disease do not. In a postmortem analysis of cytokine expression in the brains of demented and nondemented AIDS patients, Wesselhoff et al. (58, 59) demonstrated significantly increased levels of TNF-α mRNA produced by cells of the macrophage lineage in demented patients compared with nondemented controls. With the exception of IL-4 mRNA levels, which were diminished in demented individuals, TNF-α was the only cytokine for which levels differed significantly from nondemented control subjects. Since the development of dementia does not correlate simply with viral load (60), explaining differences among infected individuals in the development of the dementia syndrome may require phenotypic characterization of the dementia-associated viruses, particularly in terms of their TNF-α-eliciting ability.

Whether the appearance of TNF-α-eliciting HIV-1 variants affects other aspects of disease pathogenesis, including the rate of disease progression, remains to be determined. The knowledge that TNF-α secretion is viral-variant dependent should permit a more systematic approach to this question, with efforts to correlate disease progression with the TNF-α secretion associated with the viral variants present at a given point in the disease course.

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