

# Culturing of human peripheral blood cells reveals unsuspected lymphocyte responses relevant to HIV disease

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Recombinant HIV-Tat (Tat) induces extensive apoptosis in peripheral blood mononuclear cells (PBMCs) cultured in typical CO<sub>2</sub> incubators, which are equilibrated with air (21% O<sub>2</sub>). However, as we show here, Tat apoptosis induction fails in PBMCs cultured at physiological oxygen levels (5% O<sub>2</sub>). Under these conditions, Tat induces PBMCs to divide, efficiently primes them for HIV infection, and supports virus production by the infected cells. Furthermore, Tat takes only 2 h to prime PBMCs under these conditions. In contrast, PHA/IL-2, which is widely used to prime cells for HIV infection, takes 2–3 days. These findings strongly recommend culturing primary cells at physiological oxygen levels. In addition, they suggest HIV-Tat as a key regulator of HIV disease progression.

cell culture | HIV infection | HIV-Tat | PBMC

The studies presented here reveal a core problem that potentially poisons interpretations of data from virtually all mammalian cell culture studies. In the overwhelming majority of such studies today, cells are cultured in incubators that are gassed with a mixture of CO<sub>2</sub> and air, which maintains the internal oxygen level essentially at atmospheric oxygen (atmosO<sub>2</sub>) levels (atmosO<sub>2</sub>, 20–21% O<sub>2</sub>). The biomedical community (or at least some portion thereof) has, of course, been aware that these oxygen levels are 2- to 4-fold greater than the oxygen levels that lymphocytes and most other cells encounter *in vivo* (Fig. 1). However, the implied caution has largely been ignored, keeping the entrenched culture methods in use despite the rapidly growing recognition that all cell types sense and respond to even small shifts in oxygen levels (1–6).

Findings presented here signal an end to this age of innocence. In essence, we demonstrate dramatic differences in the way that human peripheral blood mononuclear cells (PBMCs) respond to a well studied HIV protein depending on whether the cells are cultured at atmosO<sub>2</sub> or at physiologically relevant oxygen (physO<sub>2</sub>) levels that are typical of those lymphocytes encounter *in vivo* (physO<sub>2</sub>, 5% O<sub>2</sub>). The striking qualitative differences in these responses, which shift from the previously demonstrated apoptosis induction at atmosO<sub>2</sub> to the stimulation of cell division and support for HIV infection at physO<sub>2</sub>, suggest that interpretation of other *in vitro* HIV findings may similarly require evaluation under more physiological culture conditions.

These considerations apply to conclusions from essentially all current culture studies with mammalian cells. However, they may be particularly relevant to those conducted with cell lines (including stem cell lines) because long-term growth at atmosO<sub>2</sub> is likely to induce functionally significant mutations, in addition to altering cell functions relative to those the cells perform *in vivo*.

Important for understanding the dynamics of HIV disease progression, the findings we present here introduce an extracellular role for HIV-Transactivator of transcription (Tat), a multifunctional viral protein named originally for its intracellular role as a transactivator of transcription. Although previously shown to induce T cell apoptosis *in vitro* (at atmosO<sub>2</sub>) (7, 8) and proposed to

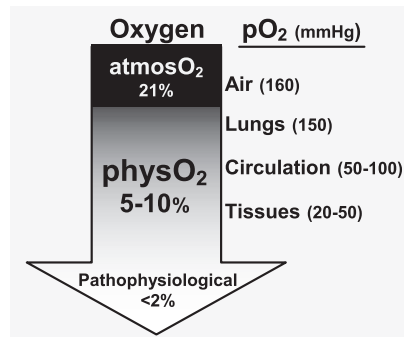


Fig. 1. Oxygen levels that cells encounter *in vivo* are lower than oxygen levels in air.

play a similar role *in vivo* (8–10), we show here that, instead of inducing apoptosis, Tat induces cell division in PBMCs cultured at physO<sub>2</sub> as efficiently as the mitogen/cytokine combination (PHA/IL-2) widely used for this purpose. Furthermore, we show that, although PHA/IL-2 stimulation is typically used to prime for and support HIV infection in PBMCs (at atmosO<sub>2</sub>), Tat at physO<sub>2</sub> is substantially more effective. PHA/IL-2 stimulation requires 2–3 days to prime PBMCs for productive HIV infection. In contrast, Tat requires only 2 h to enter and prime significant numbers (perhaps all) of the cultured cells that can host the virus.

At a minimum, these findings introduce recombinant Tat as an effective replacement for the more artificial stimuli commonly used for *in vitro* HIV-infection studies with primary cells. However, based on findings presented here, Tat also can be envisioned as playing a key role in HIV disease economy. Tat is well known to be released from HIV-infected cells *in vitro*. The evidence for Tat release *in vivo* is of necessity indirect because, as we confirm here, Tat is taken up by neighboring cells too rapidly to be reliably detected *in vivo*. Nevertheless, several studies indicate that Tat is indeed released *in vivo* (11–15). Our findings support these arguments by suggesting that local Tat release and uptake by neighboring cells may be central to the curious kinetics of HIV disease, which begins with an intense viral storm that only abates after depleting a high percentage of the memory T cells (16) in lymph nodes and other crowded lymphoid sites.

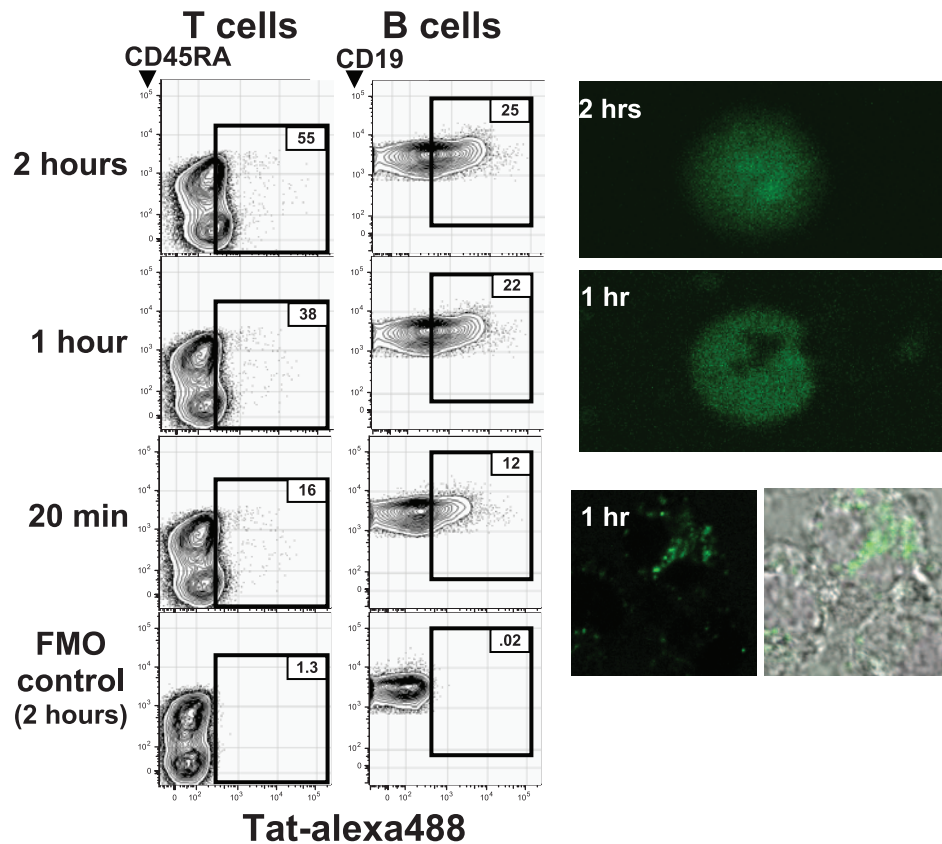
Overall, the studies presented here show that important responses of primary lymphocytes have been masked by studying

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**Fig. 2.** HIV-Tat rapidly enters PBMCs. Human PBMCs mixed with AlexaFluor488-labeled Tat (Tat-alexa) were incubated at 37°C at physO<sub>2</sub> or kept on ice for the indicated times. (Left) Cell-associated fluorescence in the Tat-alexa channel is shown for viable T and B cells in FACS analyses gated to reveal these subsets. Fluorescence-minus-one (FMO) controls show fluorescence detected in the Tat channel when cells were incubated with all stains but Tat. (Right) Intracellular Tat-alexa in PBMCs was revealed by a confocal microscope (LSM 510; Carl Zeiss) in cells incubated with Tat for the indicated times.

the behavior of primary lymphocytes at oxygen levels that the cells are highly unlikely to encounter *in vivo*.

## Results

**Tat Rapidly Enters Cultured PBMCs.** Consistent with a large body of evidence (13, 17–21), FACS and fluorescence microscopy studies with the recombinant Tat used here show that fluorochrome-labeled Tat efficiently enters all cells in PBMC cultures maintained at physO<sub>2</sub> (Fig. 2). After 1 h at 37°C, the Tat is detectable in cytoplasm; by 2 h, it is detectable in both the cytoplasm and nuclei of the cells. In contrast, Tat is not detectable in cells cultured at 0°C for this period (data not shown).

Flow-cytometry analyses of PBMCs incubated with Tat coupled to Alexa488 for up to 2 h at physO<sub>2</sub> similarly demonstrate that Tat enters cells in all lymphocyte subsets in the PBMC culture (Fig. 2 Left). On average, B cells take up more Tat than T cells, and memory T cells take up more Tat than naïve T cells. However, there is substantial overlap among the populations, and there are many cells in all populations that take up little or no Tat, at least as measured by uptake of the fluorochrome-coupled Tat used here.

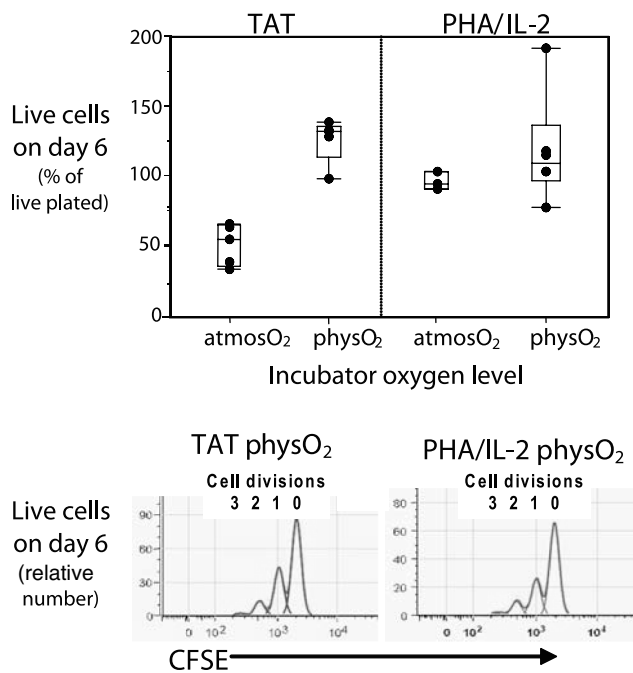
**Tat Induces Apoptosis in PBMCs Cultured at AtmosO<sub>2</sub> levels.** Tat is well known to induce extensive apoptosis in PBMCs cultured in standard CO<sub>2</sub> incubators maintained at atmosO<sub>2</sub>. Data in Fig. 3 confirm these findings by showing that only about half the PBMCs put into culture with recombinant Tat at atmosO<sub>2</sub> are detectable 6 days later and that a significant proportion of the remaining cells are apoptotic, as indicated by Annexin-V staining, or are necrotic. In contrast, consistent with previous findings, apoptosis induction is

minimal (2–5%) in cultures stimulated at atmosO<sub>2</sub> with phytohemagglutinin (PHA) supplemented with IL-2 (PHA/IL-2), a mitogen/cytokine combination used widely to induce cell division and prime PBMCs for HIV infection in *in vitro* studies. Furthermore, it is minimal in cultures stimulated with Tat at physO<sub>2</sub> levels (Fig. 3 Upper).

**Tat Induces Cell Division in PBMCs Cultured at PhysO<sub>2</sub> levels.** In contrast to the extensive apoptosis induction and cell loss that occurs when PBMCs are cultured with Tat at atmosO<sub>2</sub>, the number of cells recovered when PBMCs are cultured with Tat at physO<sub>2</sub> for 6 days is 1- to 1.5-fold higher than the number of cells plated (Fig. 3 Upper). Furthermore, very few (2–5%) of the recovered cells are apoptotic.

The increased cell yields in the Tat-stimulated physO<sub>2</sub> cultures reflect the induction of cell division at levels and rates equivalent to those obtained in PBMC cultures stimulated with PHA/IL-2 (Fig. 3 Lower). Consistent with previous findings (1, 2), responses to PHA/IL-2 shown in Fig. 3 are equivalent at atmosO<sub>2</sub> and physO<sub>2</sub>. Responses to Tat, however, differ markedly and, in this sense, are more like responses to anti-CD3/CD28 and other physiological stimuli, which we also have shown to differ at physO<sub>2</sub> and atmosO<sub>2</sub> (1, 2). In any event, at physO<sub>2</sub>, PBMC responses to Tat approximate PBMC responses to PHA/IL-2. Thus, when cells are maintained at oxygen levels similar to those encountered *in vivo*, Tat emerges as a potent inducer of cell division and, hence, as a candidate primer for HIV infection.

**Tat Primes for HIV Infection and Supports Viral Production in PBMCs Cultured at PhysO<sub>2</sub>.** PHA and IL-2 are typically used to prime cells for HIV infection and to support viral production by the infected



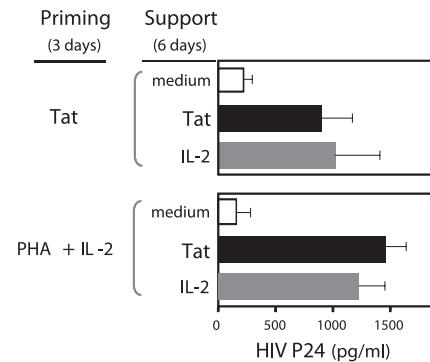
**Fig. 3.** HIV-Tat stimulates proliferation and induces minimal cell death in PBMCs at  $\text{physO}_2$  levels. PBMCs were cultured with the indicated stimuli (5  $\mu\text{g}/\text{ml}$  recombinant HIV-Tat, 2.5  $\mu\text{g}/\text{ml}$  PHA, and 50 units/ml IL-2) at  $\text{physO}_2$  or  $\text{atmosO}_2$ . Viable cell counts were determined at the beginning and end of the culture period by flow cytometry (see *Materials and Methods*). (Upper) Live cell recoveries at the end of the culture period, expressed as the percentage of live cells plated initially. (Lower) The number of cell divisions occurring during the culture period, computed from the distribution of CFDA-SE (CFSE) staining for live cells recovered from PBMCs that were preincubated with 0.5  $\mu\text{M}$  CFDA for 10 min at  $37^\circ\text{C}$  before culturing. Typically, the peak with the highest CFSE staining reflects the frequency of cells that have not divided and the successive peaks with lower fluorescence represent cells that have undergone one, two, or three cell divisions. CFSE data are not shown for PBMCs cultured with Tat at  $\text{atmosO}_2$  because low cell yields and viability problems prevented accurate data collection and analysis.

cells. Because Tat mimics PHA/IL-2 in stimulating cell division at  $\text{physO}_2$ , it may efficiently replace this mitogen/cytokine combination in providing priming and support for HIV-infection assays. Evidence presented here, in which Tat is used in place of PHA/IL-2 at various points in the standard three-stage protocol for *in vitro* HIV-infection protocol, confirms this hypothesis (Figs. 4 and 5).

For these studies, all cultures were maintained at  $\text{physO}_2$ . PBMCs were primed with Tat or PHA/IL-2 for 3 days, washed to remove free priming agent, incubated for 3 h with HIV (LAI) to allow infection to proceed, washed to remove free virus, and finally cultured for 6 days with Tat or IL-2 as indicated to allow viral production to proceed. HIV p24 in culture supernatants, an index of viral production, was measured by ELISA at the end of the 6-day period (see *Materials and Methods* for further details).

Replacing PHA/IL-2 with Tat during the priming stage of the protocol demonstrates that Tat and PHA/IL-2 stimulation are equivalent with respect to priming for HIV infection because viral yields obtained from cells primed with Tat match those obtained with the standard PHA/IL-2 protocol (Fig. 4). Viral yields (p24) are maximal when Tat is added at 5  $\mu\text{g}/\text{ml}$  and decrease as a function of the amount of Tat added to the culture (data not shown). Tat was not tested at  $>5 \mu\text{g}/\text{ml}$  because it tends to induce some apoptosis (10–20%) at higher levels, even at  $\text{physO}_2$ .

Replacing IL-2 with Tat during the support phase of the

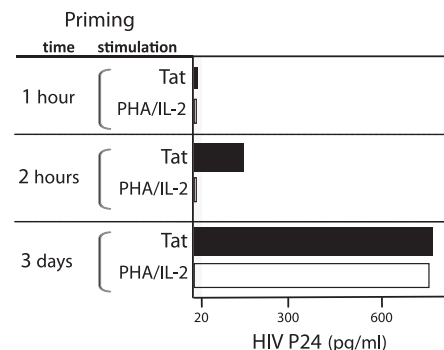


**Fig. 4.** Tat efficiently primes PBMCs for HIV infection and supports viral production by the infected cells. PHA/IL-2 and Tat are equivalent for this purpose. In this mix-and-match assay, all cultures were maintained at  $\text{physO}_2$ , and Tat was substituted for PHA/IL-2 during the priming and/or support phases of the standard three-stage *in vitro* HIV-infection protocol. Thus, PBMCs cultured at  $\text{physO}_2$  were primed with Tat or PHA/IL-2 as indicated for 3 days, washed to remove free priming agent, incubated for 3 h with HIV (LAI) to allow infection to proceed, washed to remove free virus, and finally cultured for 6 days with Tat or IL-2 as indicated to allow viral production to proceed. Tat and PHA/IL-2 were used at the concentrations indicated in *Materials and Methods*. HIV-core p24 was measured by ELISA. Viral yields, expressed as pg/ml, were computed from a p24 standard curve. Response values shown in the figure are averages of responses obtained for five subjects. The relative response levels of PBMCs from individual subjects were consistent under the various stimulatory conditions tested. Five to 15 subjects were tested for each stimulatory condition.

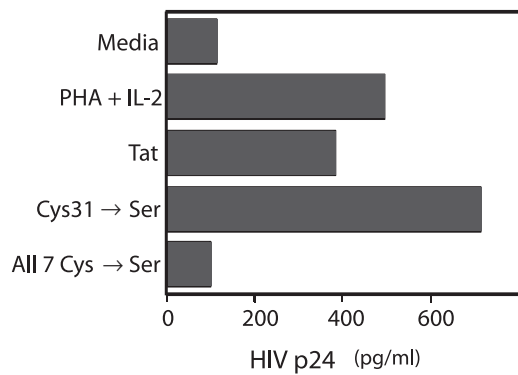
infection protocol shows that Tat provides efficient support for viral production by the primed and infected cells regardless of whether Tat or PHA/IL-2 was used for priming. Substituting Tat for PHA/IL-2 added during the 3-day priming phase of the assay and/or for the IL-2 added during the 6-day support phase results in viral yields comparable to those obtained with the standard PHA/IL-2 protocol in all cases (1,000–1,500 pg/ml of p24) (Fig. 4). Thus, Tat can fully substitute for PHA/IL-2 in the standard HIV infection assay.

Neither the Tat support for HIV production nor the Tat priming for HIV infection is explained by Tat induction of IL-2 production because  $<0.05$  units/ml IL-2 is detectable in supernatants from Tat-primed or Tat-supported cultures assayed with a commercial ELISA kit (Biosource/Invitrogen).

**Tat Primes Cells for HIV Infection Much More Rapidly than PHA/IL-2.** Priming PBMCs for just 2 h with Tat (at  $\text{physO}_2$ ) is sufficient to enable significant HIV infection and viral production by the



**Fig. 5.** Culturing with Tat for 2 h primes PBMCs for HIV infection. PBMCs were primed for the indicated period with Tat or PHA plus IL-2 before infection. After infection, HIV production by the Tat- and PHA/IL-2-primed cells was supported, respectively, with Tat and IL-2. See Fig. 4 legend for additional experiment details. Average responses for two subjects are shown in the figure. Data are representative of two experiments.



**Fig. 6.** Mutating the seven Cys residues to Ser in Tat destroys priming for HIV infection. Three Tat proteins were tested for the ability to prime for HIV infection: native Tat and two mutated forms (Cys31Ser and Cys22Ser, Cys25Ser, Cys27Ser, Cys30Ser, Cys31Ser, Cys34Ser, Cys37Ser). IL-2 was used for the support phase in the assay.

primed cells, whereas priming with PHA/IL-2 for 2 h does not result in detectable infection (Fig. 5). In fact, Tat priming for 2 h results in viral yields that are roughly one quarter the yield obtained from cells primed with either Tat or IL-2 for 3 days.

These findings suggest that Tat priming for 2 h may be sufficient to enable infection of all of the PBMCs capable of hosting the virus because such cells are likely to be activatable and, hence, among the cells that Tat (or PHA/IL-2) stimulates to proliferate during the 3-day culture period (Fig. 3). In essence, the 4-fold increase in viral yield that is obtained after 3 days of priming may reflect the  $\approx$ 4-fold increase in cell number that occurs during this period. In any event, the high viral production obtained after the infection of PBMCs that were primed with Tat for only 2 h demonstrates that HIV infection of PBMCs does not require cell division and requires events that can occur within 2 h.

**Only Certain Cysteine (Cys) Residues in Tat Are Required for Tat Priming and Support of HIV Infection.** Tat's ability to prime and support HIV infection in PBMCs is abolished when the codons encoding all seven of the Cys residues in the protein are mutated to Serine (Ser) residues (see Fig. 6). However, mutating Cys 31 to Ser does not affect this priming and support capability. Thus, at least one Cys other than Cys 31 is required for priming and support of HIV infection.

In addition to providing initial structure/function insights, these findings sharply decrease the likelihood that a contaminant in the recombinant Tat preparation used here is responsible for the Tat activities we have detected. The recombinant Tat used here was prepared by a method that excludes endotoxin (prepared by J.R. and E.R.) and was shown to have  $<8$  units/ml and is not known to contain other active contaminants. Therefore, the failure to obtain priming and/or support with mutated Tat in which Ser residues replace the 7 Tat Cys residues adds confidence that a functional Tat protein is required for this activity.

## Discussion

The findings presented here, which echo and extend results from previous studies (1, 2), raise questions about interpretations of data from virtually all cell culture studies conducted with primary cells or with cell lines developed in  $\text{atmosO}_2$  incubators. Basically, we show here that key responses to stimulation can differ dramatically even in short-term cultures maintained at  $\text{physO}_2$  versus  $\text{atmosO}_2$ . Because adaptation to long-term growth performance requires changes/mutations that alter function, adding the need to adapt to growth at  $\text{atmosO}_2$  can be expected to have introduced substantial deviation from the *in vivo* behav-

ior of cells (although creating cell lines in this way may have been the only practical alternative in years gone by). This consideration may be particularly important for the development of stem cell lines, where minimal mutation is clearly a desirable end. In any event, findings presented here suggest that, although results from studies at  $\text{atmosO}_2$  and  $\text{physO}_2$  both reveal aspects of the biology of the cells, artifacts potentially introduced by culturing cells at oxygen levels well above those encountered *in vivo* may cloud the interpretation of the biomedical significance of findings obtained with cells cultured at the higher oxygen level.

There is, of course, room for argument as to whether culturing cells at  $\text{atmosO}_2$  or  $\text{physO}_2$  is more useful in different systems. We maintain that it is best to culture cells under conditions that approximate the physiological environment as closely as possible. Other investigators may prefer to continue with current culturing methods because so much work already has been done with cells cultured at  $\text{atmosO}_2$  and because virtually all cell lines under study today have been developed in  $\text{atmosO}_2$  incubators. Furthermore, as the PHA/IL-2 data presented here show clearly, some responses are equivalent at the two oxygen levels. Nevertheless, the striking differences we demonstrate between PBMC responses to Tat at  $\text{atmosO}_2$  versus  $\text{physO}_2$  strongly recommend a shift toward culturing cells at  $\text{physO}_2$  whenever findings predictive of *in vivo* responses are desirable.

Results from the Tat studies described here also show a new perspective on the role(s) that Tat plays in HIV disease. The oxidative stress that develops in HIV-infected subjects as the disease progresses leaves room for Tat to act, as previously proposed (7, 8, 22, 23), to exacerbate oxidative stress and to induce apoptosis in late-stage HIV disease. However, our studies demonstrate that Tat can enter all lymphocytes and can rapidly and efficiently prime cells that can host HIV to render these cells infectable by the virus. Furthermore, our studies demonstrate that Tat can support viral production by the infected cells. Together, these surprising findings open a route through which Tat may influence the course of HIV disease.

Specifically, our findings suggest that Tat released by HIV-infected cells *in vivo* may act locally to prime neighboring cells and thus to amplify the infection. This Tat release could be particularly important during the early stages of the infection, when HIV-infected cells are seeded into regions that contain a high proportion of uninfected cells capable of hosting the virus. Under these conditions, the Tat that is released would be highly likely to be taken up by HIV-susceptible neighboring cells, which would then be readied within hours for infection by locally released viral particles.

Repeated cycles of Tat priming, HIV infection, and Tat release would drive viral expression up until cells capable of being primed by Tat and infected by the virus become scarce. At this point, the Tat-mediated spread of the infection would be likely to subside because lack of local targets and locally produced Tat would likely be picked up by neighboring cells that cannot be infected by HIV. Thus, Tat may be most important in propagating the infection early in the disease.

This scenario suggests that Tat priming and support for HIV propagation could be central to the propagation of what has been referred to as the viral storm that rages during the acute phase of the infection, when 30–60% of CD4 memory T cells are infected and lost (16, 24, 25). This massive T cell destruction, which heralds subsequent immunodeficiency, occurs to the same extent in many tissues (16, 24–26) and has been recognized in both simian and human HIV disease. Its striking rapidity and short-term voracity argue for a specialized mechanism that can operate locally to increase HIV-infection rates and viral production but will wane as T cells are lost, enabling its replacement with a chronic infection that slowly degrades the immune system, but allow survival of the infected host and thereby optimize the opportunity for passage of the virus to new hosts. Our findings nominate Tat as a key player in this specialized mechanism.

Gallo, Ensoli, and Zagury (11, 27–29), recognizing that anti-Tat antibody production is associated with improved survival in HIV disease, argue that Tat immunization may be effective for preventing or attenuating HIV infection. The unique HIV priming and support capabilities that our studies now demonstrate for Tat also support the idea that stimulating high-titer humoral immunity to Tat may be an important addition to vaccine strategies.

In any event, regardless of whether Tat proves important in the economy of the HIV infection, studies presented here introduce Tat as a potential replacement for PHA/IL-2 for priming PBMCs for HIV infection and supporting viral production by the infected cells. The use of Tat, which requires that the PBMCs be cultured at oxygen levels that approximate those encountered by lymphocytes *in vivo*, offers the potential for studying HIV infection under conditions that may more closely reflect the *in vivo* infection conditions and, hence, is likely to provide a more informative environment for mechanism and drug-susceptibility studies.

## Materials and Methods

**Culture Conditions and Oxygen Level.** Cell stimulation and short-term culture were carried out at two oxygen levels. Cells were maintained at physO<sub>2</sub> levels by culturing in a Sanyo "Tri-Gas" MCO-175M O<sub>2</sub>/CO<sub>2</sub> incubator (Sanyo Scientific) in which gas-phase oxygen tension is maintained at 5% oxygen by continuous injection of appropriate amounts of medical-grade N<sub>2</sub>. Cells maintained at atmosO<sub>2</sub> levels (20% O<sub>2</sub>) by culturing in a standard incubator equilibrated with air (no nitrogen injection). CO<sub>2</sub> levels were maintained at 5% in both incubators.

**PBMCs.** PBMCs from healthy individuals were isolated from blood drawn immediately before initiation of the experiment (informed consent was obtained before the draw). The blood was drawn into heparin-containing tubes (Vacutainer; Becton Dickinson). PBMCs were isolated by gradient centrifugation on Ficoll-Hypaque (Amersham Pharmacia). RPMI 1640 medium used to isolate and resuspend PBMCs at all stages was equilibrated for >12 h at the oxygen level at which the PBMCs were ultimately cultured.

**Recombinant HIV-1 Tat.** Recombinant HIV-1 Tat protein and the Cys to Ser mutant Tat proteins were obtained from J.R. Endotoxin levels were shown to be <8 units/ml.

**AlexaFluor-Conjugated Tat Protein.** Tat protein was conjugated to the AlexaFluor-488 carboxylic-acid succinimidyl ester (Molecular Probes/Invitrogen) according to the manufacturer's protocol for conjugation of Alexa dyes to proteins. Slight modifications were made to adjust for the low molecular weight of the protein. The conjugated Tat was separated from the unconjugated dye by centrifugation through a separation filter with a molecular mass cutoff of 2 kDa (Millipore).

**p24 ELISA Kit.** HIV-1 p24 levels in the culture supernatant were determined by using a Beckman Coulter kit for quantitative measurement of HIV-1 p24 antigen (EIA assay) according to the manufacturer's instructions. Briefly, the supernatants from cultures were serially diluted in 2-fold steps, as were the negative controls and the positive (purified p24 antigen) provided by the manufacturer. The samples were then added to a 96-well plate precoated with anti-p24, and the plate was sealed and incubated for 60 min at 37°C. After washing, biotin-labeled anti-p24 antibodies were added, followed by enzyme-coupled Streptavidin and the enzyme substrate (with appropriate washing between each step). Absor-

bance was measured at 450 nm in a Labsystem Multiskan RC (Fisher). A standard curve was constructed from the values obtained for dilutions of the purified p24 antigen and was used to translate supernatant p24 per ml levels from OD values to pg/ml.

**High-Dimensional (Hi-D) FACS Staining and Analysis.** Antibodies detecting cell-surface markers (CD3, CD4, CD8, CD45RA, CD45RO, CD62L, and CD11a) that distinguish T cell, B cells, and memory and naïve T cell subsets were produced in our laboratory or obtained from BD Pharmingen or Invitrogen. Fluorochrome conjugates were obtained as such from the manufacturer or conjugated according to the protocols available at [www.drmr.com/abcon/index.html](http://www.drmr.com/abcon/index.html). Briefly, PBMCs were washed and resuspended in media containing probenecid (Sigma-Aldrich) and FCS (pH 7.4) and then stained with monochlorobimane (MCB; Molecular Probes/Invitrogen) for 20 min at room temperature. Cells were then washed, chilled, and stained with a mixture of fluorophore-conjugated antibodies against cell-subtype markers for 15 min. Propidium iodide (Invitrogen) exclusion was used for dead cell exclusion. Finally, cells were resuspended in staining media with 0.4% formaldehyde and analyzed with the Stanford Shared FACS Facility Flasher-II Hi-D FACS, which uses Becton Dickinson FACS DiVa electronics and software for data collection. Data were analyzed with FlowJo software (Tree Star).

**Carboxyfluorescein Diacetate Succinimidyl-Ester (CFDA-SE) Proliferation Assay.** Human PBMCs were stained with CFDA-SE (referred to as CFSE) following a previously described procedure (30) with some modifications. Briefly, PBMCs were suspended at 10<sup>6</sup> cells per ml in serum-free RPMI medium 1640 and stained with 0.5 μM CFDA-SE for 10 min at 37°C. The reaction was terminated by the addition of a 3-fold excess volume of RPMI medium 1640 with 10% FCS. After two washes, the cells were resuspended at 10<sup>6</sup> cells per ml in RPMI 1640 with 10% FCS and cultured as indicated. CFSE associated with the cells at the end of the culture period was determined by FACS.

**Cell Counts and Identification of Viable Cells.** The numbers of cells in the cultures were determined by FACS using the BD TruCount beads according to the manufacturer's instructions. Cell viability was determined either by MCB stain or Invitrogen live/dead marker.

**In Vitro HIV Infection.** PBMCs isolated immediately after blood draw were cultured with PHA/IL-2 (2.5 μg/ml and 50 units/ml IL-2, respectively) or 5 μg/ml Tat protein for the period and at the indicated oxygen levels. For infection, the cultured cells were harvested, washed, resuspended, and cultured with 1,000 IC-50 of the HIV (LAI-1) for 3 h. They were then washed, resuspended, and cultured with Tat or IL-2 as indicated. After 6 days, culture supernatants were obtained, and the accumulated p24 was measured by ELISA with a kit obtained from Beckman Coulter.

**Statistical Analysis.** Analyses of FACS data, including calculations of absolute cell numbers, cell division, and cell proliferation indices, were performed by using the FlowJo software (Tree Star). Statistical analyses were performed with the JMP statistical software package (SAS Institute).

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