Available online at www.sciencedirect.com

SCIENCE DIRECT

Virology 328 (2004) 1–6

VIROLOGY

www.elsevier.com/locate/yviro

Rapid Communication

Depletion of CD4 T lymphocytes in human lymphoid tissue infected ex vivo with doxycycline-dependent HIV-1

Yana Kiselyeva[a], Yoshinori Ito[a], Rosangela G. Lima[a,1], Jean-Charles Grivel[a], Atze T. Das[b], Ben Berkhout[b,*], Leonid B. Margolis[a,*]

[a]*Laboratory of Molecular and Cellular Biophysics, National Institute of Child Health and Human Development, Bethesda, MD 20892, USA*
[b]*Department of Human Retrovirology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands*

Received 15 April 2004; returned to author for revision 17 May 2004; accepted 16 July 2004
Available online 21 August 2004

Abstract

We investigated whether $CD4^+$ T cells that do not produce HIV-1 are killed in HIV-infected human lymphoid tissue. Tissue blocks were inoculated with high amount of doxycycline-dependent HIV-rtTA. Doxycycline triggered productive infection and loss of $CD4^+$ T cells in these tissues, whereas without doxycycline, neither productive infection nor $CD4^+$ T cell depletion was detected in spite of the massive presence of virions in the tissue and of viral DNA in the cells. Thus, HIV-1 alone is sufficient to deplete productively infected $CD4^+$ T cells but is not sufficient to cause the death of uninfected or latently infected $CD4^+$ T cells.
Published by Elsevier Inc.

Keywords: $CD4^+$ T cell; Human lymphoid tissue; HIV-1

Introduction

The hallmark of HIV infection is the depletion of $CD4^+$ T lymphocytes. It is well established that HIV productive infection kills $CD4^+$ T cells (reviewed in (Pantaleo et al., 1997)). However, $CD4^+$ T cells that do not produce virus, but which reside in infected tissues, are killed as well. Such cells may be infected latently or may not be infected at all ("bystanders") (Badley et al., 1996; Banda et al., 1992; Gandhi et al., 1998; Herbein et al., 1998; Laurent-Crawford et al., 1995; Oyaizu et al., 1997). The mechanisms of cell killing by HIV-1 in general and of bystander cells in particular are not fully understood. While some published data suggest that cell interactions with viral particles or their components are sufficient to kill uninfected cells without productive infection, others suggest that general activation of the immune system is required for uninfected cells to become apoptotic (Badley et al., 2000; Casella and Finkel, 1997; Gougeon and Montagnier, 1993; Grossman et al., 2002; Mahlknecht and Herbein, 2001; Meyaard et al., 1992; Yang and Ashwell, 2001). Critical events of HIV disease occur in lymphoid tissue (Pantaleo and Fauci, 1995). Therefore, we addressed the problems of $CD4^+$ T cell death in human lymphoid tissues infected ex vivo with HIV-1 (Glushakova et al., 1997). This system supports productive HIV-1 infection without exogenous stimulation, and such an infection results in a loss of $CD4^+$ T cells (Glushakova et al., 1997). In the study we report here, we used a newly developed virus construct, HIV-rtTA, that can infect cells and establish an integrated provirus in the absence of doxycycline but whose transcription and replication is dependent on the presence of the antibiotic doxycycline ("dox-dependent HIV-rtTA") (Berkhout et al., 2002; Das et al., 2002; Verhoef et al., 2001).

* Corresponding authors. Ben Berkhout is to be contacted at Department of Human Retrovirology, University of Amsterdam, Academic Medical Center K3-110. Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands. Fax: +31 20 691 6531. Leonid Margolis, Laboratory of Molecular and Cellular Biophysics, National Institute of Child Health and Human Development, NIH, Building 10, Room 9D58, 10 Center Drive, Bethesda, MD 20892. Fax: +1 301 480 0857.

E-mail addresses: b.berkhout@amc.uva.nl (B. Berkhout), margolis@helix.nih.gov (L.B. Margolis).

[1] Current address: Department of Immunology, Oswaldo Cruz Foundation, Rio de Janeiro-RJ, Brazil.

0042-6822/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.virol.2004.07.014

We used this construct to test whether uninfected cells that have interacted with virions, or cells that are non-productively infected, are depleted in the context of human lymphoid tissue. We found that in the presence of high amounts of virions and intracellular viral DNA, no depletion of $CD4^+$ T cells occurs in lymphoid tissue unless productive replication is triggered by doxycycline. Thus, in the context of human lymphoid tissue ex vivo, HIV infection kills productively infected cells but is not sufficient to cause the death of uninfected or latently infected $CD4^+$ T cells.

Results and discussion

In the work that we report here, we (i) tested HIV-rtTA dox-dependence in the context of human lymphoid tissue and (ii) evaluated the effects of latent and productive HIV-rtTA infection on $CD4^+$ T cell depletion in human lymphoid tissue ex vivo.

Dox dependence of HIV-rtTA in ex vivo-infected human lymphoid tissue

To evaluate this dependence, we inoculated blocks of human lymphoid tissue with HIV-rtTA and simultaneously added doxycycline. The culture medium was changed every 3 days, and doxycycline was supplied with each medium change. In the presence of 0.5 to 1.5 μg/ml doxycycline, a vigorous productive infection was detected in tissues infected, and a dose as low as 0.1 μg/ml triggered notable viral replication (data not shown). For experiments described hereafter, we chose the concentration of doxycycline of 0.5 μg/ml. This dose supports efficient tissue productive infection by HIV-rtTA (although 4 times lower compared to the wild-type virus) as evaluated from the increase of CA-p24 in the medium (Fig. 1a). A less efficient replication of HIV-rtTA compared to the wild-type virus was observed also in T cell lines (Das et al., 2004) and may be due to the usage of different regulatory systems by HIV-rtTA and wild-type viruses. As was reported earlier for various HIV-1 variants, viral replication became detectable after day 6 post-inoculation and continued to increase to the end of experiment on day 12–15. Without adding doxycycline, no productive infection was detected in inoculated tissue blocks (Fig. 1a). The amount of CA-p24 on day 3 (before the first medium change) roughly corresponded to the amount of inoculum. These amounts were similar in cultures not treated with doxycycline and in cultures to which doxycycline was added at the time of virus inoculation.

Next, we studied whether HIV replication could be turned on in HIV-rtTA-inoculated tissues when doxycycline addition was delayed relative to viral inoculation. Tissue blocks were inoculated with the virus on day 0 and cultured without doxycycline for another 6 days. The amount of provirus was evaluated by real-time PCR, and the HIV copy number was normalized to the number of copies of the GAPDH gene. This assay revealed that in tissues inoculated without doxycycline, there were $1.4 \times 10^{-4} \pm 0.8 \times 10^{-4}$ genome equivalent of viral DNA. The cultures were washed and the medium was

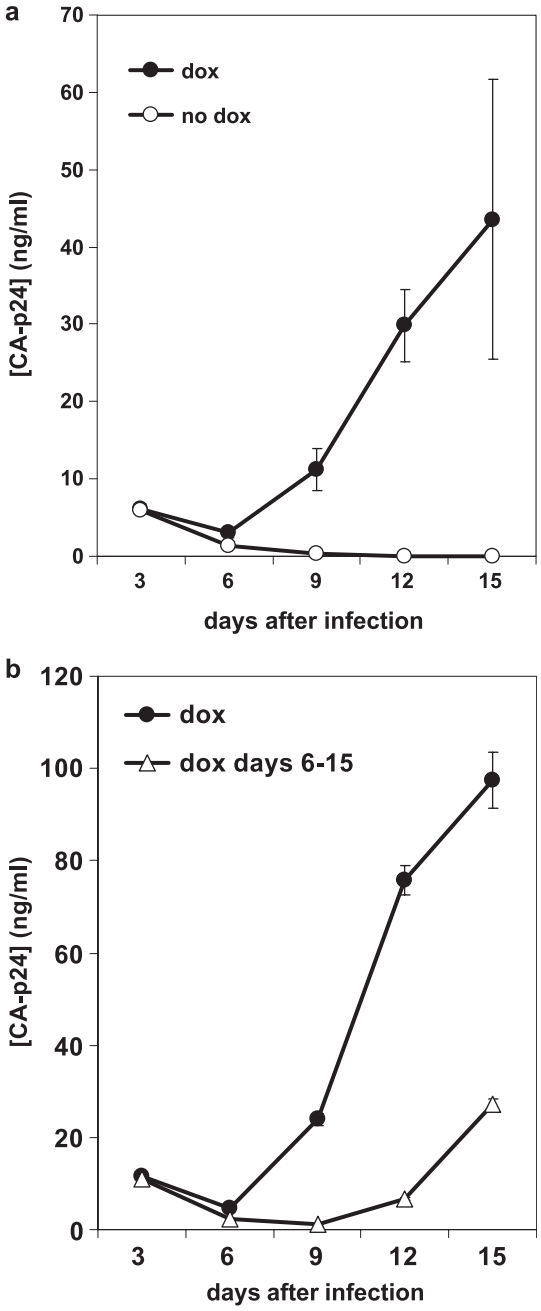

Fig. 1. Doxycycline dependence of HIV-rtTA in ex vivo human lymphoid tissue infected ex vivo. (a) Tissue blocks were inoculated with HIV-rtTA. Doxycycline at the concentration of 0.5 μg/ml was added to some of the cultures at different time points after virus inoculation and supplemented thereafter with each medium change every 3 days. For each donor tissue, 27 tissue blocks were inoculated and the medium was pooled for CA-p24 measurements. Each data point represents the average (±SEM). Tissue blocks were inoculated with virus and cultured in the absence or presence of doxycycline. Each bar represents the average (±SEM) of tissues from 13 donors. (b) Tissue blocks were inoculated with virus and doxycycline was administered immediately or 6 days after inoculation. The mean values and estimated SEM of triplicate measurements of pooled samples are presented.

changed (for a fresh virus-free one) on day 3 and day 6 of culture. Doxycycline was then added, and was replenished with each subsequent medium change for another 9 days up to day 15 post-inoculation. In a control experiment, we inoculated matched tissues with HIV-rtTA on day 0 and simultaneously added doxycycline, which was constantly present until the end of experiment on day 15. In both the control and the experimental sets of blocks, viral replication became noticeable 6–9 days after addition of doxycycline (days 6–9 post-inoculation for control blocks and days 12–15 post-inoculation for experimental blocks). The kinetics of viral replication after doxycycline was added to the medium were similar in both cases (Fig. 1b), and the amount of viral DNA was increased to $1.1 \times 10^{-1} \pm 0.2 \times 10^{-1}$ genome equivalent of viral DNA.

To prove that in these experiments viral production came from latently infected cells containing a silent provirus, rather than from de novo infection by 1.5 ng of CA-p24 of cell-free virus that remained in culture, we inoculated tissues with this amount of virus in the presence of doxycycline. No replication was detected in this condition for at least 2 weeks of culture. Thus, it seems that, in the above-described experiments, viral production came from viral DNA harbored in cells that were latently infected before the addition of doxycycline. This DNA was likely to be integrated in host DNA because episomal HIV-1 DNA could not serve as an efficient source for viral transcription (Jeang et al., 1993), and most likely is quickly lost (Zack et al., 1990).

$CD4^+$ T cell depletion in human lymphoid tissue ex vivo inoculated with HIV-rtTA

We compared $CD4^+$ T cell depletion in tissues inoculated with HIV-rtTA in the presence and absence of doxycycline. Because almost the entire pool of T ($CD3^+$) cells express CD4 or CD8 and CD4 is down-regulated upon HIV infection, we assessed $CD4^+$ T cell depletion by evaluating the $CD3^+CD8^-/CD3^+CD8^+$ ratio among lymphocytes, as determined with flow cytometry of cells recovered mechanically from tissue blocks (Glushakova et al., 1997; Grivel and Margolis, 1999). In the productively infected tissues (i.e., those inoculated with HIV-rtTA and cultured in the constant presence of 0.5 μg/ml doxycycline), 36 ± 5% ($P < 0.001$, $n = 10$) of $CD4^+$ T cells remained in the tissue after 12 days of culture relative to matched uninfected control (Fig. 2). In contrast, no significant $CD4^+$ T cell depletion was observed in matched tissues that were similarly inoculated but cultured in the absence of doxycycline (and thus without viral replication): the $CD3^+CD8^-/CD3^+CD8^+$ lymphocyte ratio was 104 ± 5% ($P = 0.21$, $n = 6$) of that of matched uninfected control (Fig. 2).

This lack of detectable depletion may indicate that productive infection rather than latent infection or some kind of interactions between lymphocytes and viral particles is necessary for $CD4^+$ T cells to be killed by HIV. However,

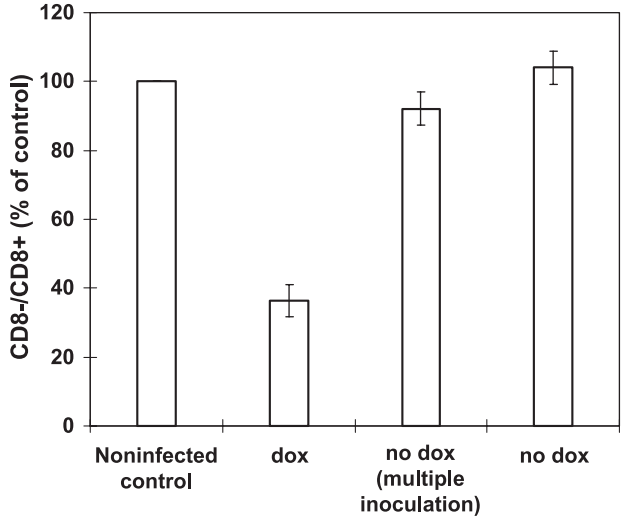

Fig. 2. $CD4^+$ T cell depletion in human lymphoid tissue infected ex vivo with HIV-rtTA. Tissues inoculated once (on day 0) or multiple times (every 3 days) were cultured with or without doxycycline (0.5 μg/ml). On day 12, cells were mechanically isolated, stained with Ab to surface markers, and analyzed by means of flow cytometry. $CD4^+$ T cell depletion was evaluated by measuring the $CD3^+CD8^-/CD3^+CD8^+$ lymphocyte ratio and expressed as percent of this ratio in matched uninfected tissues. For each tissue donor and each condition, cells from 27 tissue blocks were pooled together. Each bar represents the average ± SEM of tissues from seven to nine donors.

in the above-described experiments, we compared cultures in which there was dramatic difference in the number of virions and the length of their interaction with cells, because in one case tissues were singly inoculated with HIV-1 (and no viral replication was triggered by doxycycline) and in the other case there were multiple rounds of productive infection in the presence of doxycycline. To make the comparison more adequate, we matched the amount of virus present in productively infected (doxycycline-treated) cultures by multiple inoculation of tissue (13 times over the 9 days) with increasing amounts of HIV-rtTA in the absence of doxycycline (Fig. 3). In spite of the presence of a similar amount of virus, there was no significant depletion of $CD4^+$ T cells (the $CD8^-/CD8^+$ T cell ratio was 92 ± 5% of matched uninfected control tissue, $n = 8$, $P = 0.08$) in the absence of doxycycline (and productive infection) (Fig. 2). Although the difference between the numbers of $CD4^+$ T cells in control and experiment did not reach the statistically significant level, this number was lower in tissues inoculated with nonreplicating virus. This may indicate that although the main depletion occurs in productively infected $CD4^+$ T cell subset, we cannot exclude that in productively infected tissues some $CD4^+$ T cells that do not produce virus die as well.

As shown above, when tissues were inoculated in the absence of doxycycline, some of the cells become infected nonproductively. Because there was no detectable general $CD4^+$ T cell depletion without doxycycline (Fig. 2) even when the amount of virus was similar to that in productively infected culture, it seems that nonproductive infection does

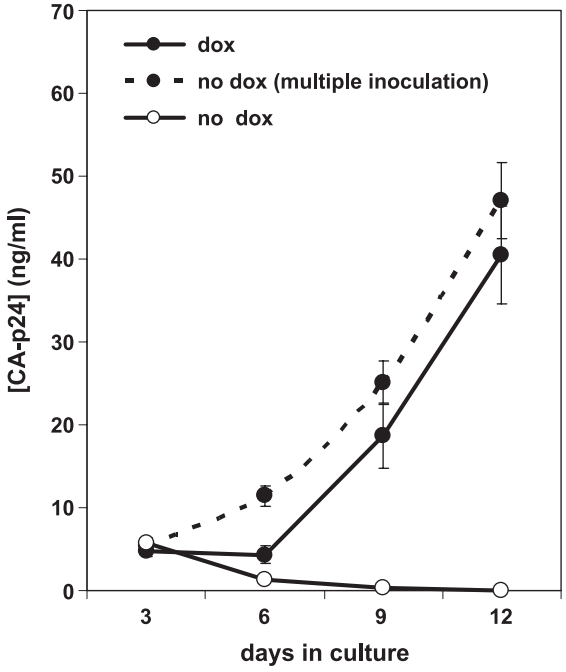

Fig. 3. Multiple inoculation of tissue with HIV-rtTA. Tissue blocks from every donor were divided into three fractions. Tissue blocks from the first fraction were inoculated once (on day 0) and cultured without doxycycline; the second was also inoculated once (on day 0) and cultured with doxycycline (0.5 μg/ml) as described in the legend for Fig. 1; the third was inoculated every 3 days with an increasing amount of virus and no doxycycline was added. Twenty-seven tissue blocks from every donor were inoculated for each condition and pooled culture medium was assayed for CA-p24. Each data point represents average (±SEM) for tissues from three to five donors.

not contribute significantly to the death of $CD4^+$ T cells as well. To control whether we would detect the depletion of latently infected cells if they are depleted, we inoculated tissues blocks with 2 ng of viral stock per block 11 times over the period of 3 days in the absence of doxycycline; then, we triggered viral replication by doxycycline and prevented the spread of infection to new cells by adding an inhibitory dose of a non-nucleoside reverse transcriptase inhibitor Nevirapine 1.5 days before doxycycline treatment (Grivel et al., 2003). Triggering viral production in these infected cells resulted in a significant depletion of $CD8^-$ T cells (the $CD8^-/CD8^+$ T cell ratio was 80.6% of that of matched uninfected control, $P = 0.05$, Fig. 4). Thus, in the absence of doxycycline, cells that are (nonproductively) infected seem not to be depleted in human lymphoid tissues inoculated with HIV-1. However, these cells are depleted after doxycycline triggers their productive infection.

Thus, in ex vivo-infected human lymphoid tissue, neither a massive presence of HIV-1 virions nor viral entry into the cells and reverse transcription trigger significant depletion of $CD4^+$ T lymphocytes. However, when HIV-1 genome transcription was triggered and the virus replicated, a significant death of $CD4^+$ T lymphocytes concomitantly occurred. This is consistent with our published finding that $CD4^+$ T cells that are not productively infected survive in productively infected tissues (Grivel et al., 2003). Also, consistent with the present results, aldrathiol-treated virions (which retain their conformation, but are not capable of performing reverse transcription, and thus do not establish productive infection) do not induce $CD4^+$ T cell depletion in inoculated human lymphoid tissues (Sylwester et al., 1998). In contrast to the aldrathiol-treated virions, the construct used in the study we report on here was able to reverse transcribe. Therefore, using HIV-rtTA, we were able to demonstrate that not only extracellular viral particles but also viral DNA in cells is insufficient to induce significant $CD4^+$ T cell depletion in the absence of productive infection. Furthermore, our results are in general agreement with the data published by the Sodroski group (LaBonte et al., 2000), which has shown that gp120-mediated killing in constitutively activated cell lines requires expression of this protein on the cell surface, and thus gp120-mediated cell death should not affect uninfected cells. Other HIV-related cell alterations, such as down-regulation of CD4, are also restricted to T cells productively infected with HIV-1 (Doms and Trono, 2000; Garcia and Miller, 1991; Hoxie et al., 1986).

Further studies are needed to investigate if all $CD4^+$ T cells that die as the result of productive HIV infection of tissues ex vivo produce virus. Alternatively, a minor fraction of uninfected or latently infected cells could die as well. It was suggested that in vivo general immunostimulation contributes to lymphocyte death in HIV-infected individuals (Muro-Cacho et al., 1995). Unlike lymphoid tissue in vivo, which responds to HIV infection by general immunoactivation and proliferation, blocks of lymphoid tissue ex vivo

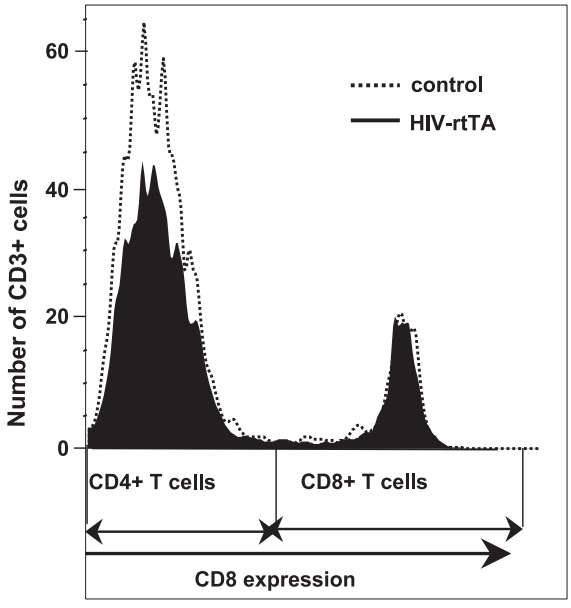

Fig. 4. Depletion of $CD4^+$ T cells by HIV-rtTA in the presence of Nevirapine. Tissue blocks were inoculated with HIV-rtTA 11 times over the period of 3 days in the absence of doxycycline. Nevirapine (5 μM) was added on day 3 and continuously supplied till day 12 post HIV-rtTA inoculation. Doxycycline (0.5 μg/ml) was added on day 4 and continuously present till day 12. $CD4^+$ T cells (19.6%) were depleted as compared to the number of $CD4^+$ T cells in matched uninfected Nevirapine-treated control.

are not activated upon infection (Malkevitch et al., 2001), and cell proliferation in these blocks remains negligible through the entire course of ex vivo infection (Grivel et al., 2000). Thus, our ex vivo tissue allows to evaluate the contribution of viral infection to the $CD4^+$ T cell death in the absence of tissue immunostimulation.

In conclusion, HIV is sufficient to deplete tissue of productively infected $CD4^+$ T cells, but is not sufficient to cause a significant death of uninfected or latently infected $CD4^+$ T cells in the context of human lymphoid tissue, and thus additional factors seem to contribute to bystander cell death in vivo. Because, unlike in vivo, ex vivo-infected tissues do not respond to HIV infection by general immunostimulation (Grivel et al., 2003); such an immunostimulation may be the main additional factor for $CD4^+$ T cell depletion in HIV-infected individuals (Grossman et al., 2002; Muro-Cacho et al., 1995).

Materials and methods

Dox-dependent HIV-rtTA was designed and described earlier (Berkhout et al., 2002; Verhoef et al., 2001). Briefly, in the full-length, infectious HIV-1 molecular clone pLAI the TAR-Tat transcriptional axis was inactivated (by mutation of multiple nucleotides in the single-stranded bulge and loop domains of TAR, the binding sites for Tat and cyclin T, respectively, and also by introduction of the Tyr26Ala point mutation in Tat protein) and replaced by the tetracycline-inducible tetO–rtTA system (Berkhout et al., 2002; Das et al., 2002; Verhoef et al., 2001). The activity of rtTA is critically dependent on doxycycline, a tetracycline analogue. In this study, we used the HIV-$rtTA_{F86Y\ A209T}$ variant with optimized tetO DNA binding sites and a more doxycycline-sensitive and more potent rtTA variant that was obtained by virus evolution (Das et al., 2004). For the production of virus stocks, SupT1 cells were transfected with the HIV-$rtTA_{F86Y\ A209T}$ molecular clone by electroporation. Briefly, 5×10^6 cells were grown at 37°C and 5% CO_2 in RPMI1640 medium containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 units/ml streptomycin, washed in RPMI1640 with 20% FBS, and mixed with 5 μg of DNA in 250 μl RPMI1640 with 20% FBS. Cells were electroporated in 0.4-cm cuvettes at 250 V and 960 μF, and subsequently resuspended in 5 ml RPMI1640 with 10% FBS and 1 μg/ml doxycycline. Transfected cells were mixed with 20 ml RPMI1640 with 10% FBS and 1 μg/ml doxycycline containing 8×10^6 untransfected SupT1 cells at 3 days after transfection, and cultured until massive syncytium was observed (7 days). Cells were removed by centrifugation at $400 \times g$ for 10 min. The virus containing supernatant was filtered (0.2-μm filter) and stored in aliquots at −80°C. HIV-1 CA-p24 antigen was measured by ELISA (Beckman-Coulter, Miami, FL).

HIV infection of human lymphoid tissue ex vivo was performed as described earlier (Glushakova et al., 1997). Briefly, human tonsils removed during routine tonsillectomy were received within several hours of excision, washed thoroughly with medium, and dissected into 2- to 3-mm blocks. These tissue blocks were placed on top of collagen sponge gels in culture medium at the air–liquid interface (9 blocks per well with 3 ml of culture medium) and, after overnight incubation, were inoculated with dox-dependent HIV-rtTA. In a typical experiment, 3–6 μl of clarified medium containing 2–4 ng of CA-p24 were applied to the top of each tissue block. Tissue was cultured in either the presence or the absence of doxycycline. We assessed productive HIV infection by measuring CA-p24 in the culture medium, using p24 ELISA (Beckman-Coulter). Specifically, the concentration of CA-p24 accumulated in 3 ml of culture medium bathing nine tissue blocks during the 3 days between successive medium changes was used as a measure of virus replication.

Flow cytometry was performed on cells mechanically isolated from control and infected tissue blocks on day 12 after infection. Cells were stained with anti-CD3-fluorescein isothiocyanate (FITC), anti-CD8-TriColor, and anti-CD4-allophicocyanin (APC). Data were acquired and analyzed with CellQuest software. To normalize for differences in tissue block size and cellularity, $CD4^+$ T cell depletion was expressed as the ratio of the number of $CD3^+CD8^-$ T cells to the number of $CD3^+CD8^+$ T cells (Glushakova et al., 1997; Grivel et al., 2003).

Real-time PCR assay was used to evaluate dox-dependent HIV-rtTA DNA. Twenty-seven tissue blocks infected with dox-dependent HIV-rtTA and 27 matched uninfected control blocks were lysed in ATL buffer containing 10% proteinase K (Qiagen Inc., USA) (100 μl per 10 mg block, 2 h at 55°C). We extracted viral DNA using the DNeasy Tissue kit (Qiagen) according to the manufacture's instructions. Extracted DNA (total volume of 200 μl) was diluted 10-fold. For the real-time PCR assay, the following gag-specific primer set was used: Gag forward primer, 5′-ATAATCCACCTATCCCAGTAGGAGAAAT-3′; Gag reverse primer, 5′-TTGGTCCTTGTCTTATGTCCAGAAT-3′. The reaction was performed in a 50 μl mixture of SYBR Green PCR master mix (Applied Biosystems, Foster City, CA), each primer at 300 nM, and 10 μl of DNA. Following activation of the Taq polymerase for 10 min at 95°C, 45 cycles, each cycle consisting of 15 s at 95°C followed by 1 min at 60°C were carried out by the ABI Prism 7000 Sequence Detection System (Applied Biosystems). To normalize the HIV DNA copy number, we also measured GAPDH, using the following specific primer set: GAPDH forward primer: 5′-GAAGGTGAAGGTCGGAGTAGTC-3′; GAPDH reverse primer: 5′-GAAGATGGTGATGGGATTTC-3′. Serially diluted plasmids that contained the target sequence were used as a standard. Real-time fluorescent measurements were analyzed by the attached software using the values obtained from serially diluted positive control plasmids that contained the target gene. Both control plasmids of Gag and those GAPDH produced wide linear ranges of values from 10 to

10^6 copies per reaction mixture. The absolute copy numbers we detected in the present study was in the middle of this range. We also analyzed the dissociation curve for each amplification to confirm that there were no nonspecific PCR products. Each sample was tested in triplicate and the mean of the three values was used as the copy number of the sample.

Acknowledgments

We thank Monique Vink and Bep Klaver for their help with the experiments. This research was sponsored in part by the Technology Foundation STW (applied science division of NWO and the technology program of the Ministry of Economic Affairs, Utrecht, the Netherlands). The work of Y.K. was sponsored, in part, by the HHS Biotechnology Engagement Program.

References

Badley, A.D., McElhinny, J.A., Leibson, P.J., Lynch, D.H., Alderson, M.R., Paya, C.V., 1996. Upregulation of Fas ligand expression by human immunodeficiency virus in human macrophages mediates apoptosis of uninfected T lymphocytes. J. Virol. 70 (1), 199–206.

Badley, A.D., Pilon, A.A., Landay, A., Lynch, D.H., 2000. Mechanisms of HIV-associated lymphocyte apoptosis. Blood 96 (9), 2951–2964.

Banda, N.K., Bernier, J., Kurahara, D.K., Kurrle, R., Haigwood, N., Sekaly, R.P., Finkel, T.H., 1992. Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. J. Exp. Med. 176 (4), 1099–1106.

Berkhout, B., Verhoef, K., Marzio, G., Klaver, B., Vink, M., Zhou, X., Das, A.T., 2002. Conditional virus replication as an approach to a safe live attenuated human immunodeficiency virus vaccine. J. NeuroVirol. 8 (Suppl. 2), 134–137.

Casella, C.R., Finkel, T.H., 1997. Mechanisms of lymphocyte killing by HIV. Curr. Opin. Hematol. 4 (1), 24–31.

Das, A.T., Zhou, X., Vink, M., Klaver, B., Berkhout, B., 2002. Conditional live virus as a novel approach towards a safe live attenuated HIV vaccine. Exp. Rev. Vaccines 1 (3), 293–301.

Das, A.T., Zhou, X., Monique, V., Klaver, B., Verhoef, K., Marzio, G., Berkhout, B., 2004. Viral evolution as a tool to improve the tetracycline-regulated gene expression system. J. Biol. Chem. 279 (18), 1877–1882.

Doms, R.W., Trono, D., 2000. The plasma membrane as a combat zone in the HIV battlefield. Genes Dev. 14 (21), 2677–2688.

Gandhi, R.T., Chen, B.K., Straus, S.E., Dale, J.K., Lenardo, M.J., Baltimore, D., 1998. HIV-1 directly kills $CD4^+$ T cells by a Fas-independent mechanism. J. Exp. Med. 187 (7), 1113–1122.

Garcia, J.V., Miller, A.D., 1991. Serine phosphorylation-independent down-regulation of cell-surface CD4 by nef. Nature 350 (6318), 508–511.

Glushakova, S., Baibakov, B., Zimmerberg, J., Margolis, L.B., 1997. Experimental HIV infection of human lymphoid tissue: correlation of $CD4^+$ T cell depletion and virus syncytium-inducing/non-syncytium-inducing phenotype in histocultures inoculated with laboratory strains and patient isolates of HIV type 1. AIDS Res. Hum. Retroviruses 13 (6), 461–471.

Gougeon, M.L., Montagnier, L., 1993. Apoptosis in AIDS. Science 260 (5112), 1269–1270.

Grivel, J.C., Margolis, L.B., 1999. CCR5- and CXCR4-tropic HIV-1 are equally cytopathic for their T-cell targets in human lymphoid tissue. Nat. Med. 5 (3), 344–346.

Grivel, J.C., Malkevitch, N., Margolis, L., 2000. Human immunodeficiency virus type 1 induces apoptosis in CD4(+) but not in CD8(+) T cells in Ex vivo-infected human lymphoid tissue [In Process Citation]. J. Virol. 74 (17), 8077–8084.

Grivel, J.C., Biancotto, A., Ito, Y., Lima, R.G., Margolis, L.B., 2003. Bystander $CD4^+$ T lymphocytes survive in HIV-infected human lymphoid tissue. AIDS Res. Hum. Retroviruses 19 (3), 211–216.

Grossman, Z., Meier-Schellersheim, M., Sousa, A.E., Victorino, R.M., Paul, W.E., 2002. $CD4^+$ T-cell depletion in HIV infection: are we closer to understanding the cause? Nat. Med. 8 (4), 319–323.

Herbein, G., Van Lint, C., Lovett, J.L., Verdin, E., 1998. Distinct mechanisms trigger apoptosis in human immunodeficiency virus type 1-infected and in uninfected bystander T lymphocytes. J. Virol. 72 (1), 660–670.

Hoxie, J.A., Alpers, J.D., Rackowski, J.L., Huebner, K., Haggarty, B.S., Cedarbaum, A.J., Reed, J.C., 1986. Alterations in T4 (CD4) protein and mRNA synthesis in cells infected with HIV. Science 234 (4780), 1123–1127.

Jeang, K.T., Berkhout, B., Dropulic, B., 1993. Effects of integration and replication on transcription of the HIV-1 long terminal repeat. J. Biol. Chem. 268 (33), 24940–24949.

LaBonte, J.A., Patel, T., Hofmann, W., Sodroski, J., 2000. Importance of membrane fusion mediated by human immunodeficiency virus envelope glycoproteins for lysis of primary CD4-positive T cells. J. Virol. 74 (22), 10690–10698.

Laurent-Crawford, A.G., Coccia, E., Krust, B., Hovanessian, A.G., 1995. Membrane-expressed HIV envelope glycoprotein heterodimer is a powerful inducer of cell death in uninfected $CD4^+$ target cells. Res. Virol. 146 (1), 5–17.

Mahlknecht, U., Herbein, G., 2001. Macrophages and T-cell apoptosis in HIV infection: a leading role for accessory cells? Trends Immunol. 22 (5), 256–260.

Malkevitch, N., McDermott, D.H., Yi, Y., Grivel, J.C., Schols, D., De Clercq, E., Murphy, P.M., Glushakova, S., Collman, R.G., Margolis, L., 2001. Coreceptor choice and T cell depletion by R5, X4, and R5X4 HIV-1 variants in CCR5-deficient (CCR5delta32) and normal human lymphoid tissue. Virology 281 (2), 239–247.

Meyaard, L., Otto, S.A., Jonker, R.R., Mijnster, M.J., Keet, R.P., Miedema, F., 1992. Programmed death of T cells in HIV-1 infection. Science 257 (5067), 217–219.

Muro-Cacho, C.A., Pantaleo, G., Fauci, A.S., 1995. Analysis of apoptosis in lymph nodes of HIV-infected persons. Intensity of apoptosis correlates with the general state of activation of the lymphoid tissue and not with stage of disease or viral burden. J. Immunol. 154 (10), 5555–5566.

Oyaizu, N., Adachi, Y., Hashimoto, F., McCloskey, T.W., Hosaka, N., Kayagaki, N., Yagita, H., Pahwa, S., 1997. Monocytes express Fas ligand upon CD4 cross-linking and induce $CD4^+$ T cells apoptosis: a possible mechanism of bystander cell death in HIV infection. J. Immunol. 158 (5), 2456–2463.

Pantaleo, G., Fauci, A.S., 1995. New concepts in the immunopathogenesis of HIV infection. Annu. Rev. Immunol. 13, 487–512.

Pantaleo, G., Graziosi, C., Fauci, A.S., 1997. Virologic and immunologic events in primary HIV infection. Springer Semin. Immunopathol. 18 (3), 257–266.

Sylwester, A.W., Grivel, J.C., Fitzgerald, W., Rossio, J.L., Lifson, J.D., Margolis, L.B., 1998. CD4(+) T-lymphocyte depletion in human lymphoid tissue ex vivo is not induced by noninfectious human immunodeficiency virus type 1 virions. J. Virol. 72 (11), 9345–9347.

Verhoef, K., Marzio, G., Hillen, W., Bujard, H., Berkhout, B., 2001. Strict control of human immunodeficiency virus type 1 replication by a genetic switch: Tet for Tat. J. Virol. 75 (2), 979–987.

Yang, Y., Ashwell, J.D., 2001. Exploiting the apoptotic process for management of HIV: are we there yet? Apoptosis 6 (1–2), 139–146.

Zack, J.A., Arrigo, S.J., Weitsman, S.R., Go, A.S., Haislip, A., Chen, I.S., 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell 61 (2), 213–222.