Thymocyte-Thymic Epithelial Cell Interaction Leads to High-Level Replication of Human Immunodeficiency Virus Exclusively in Mature CD4\(^+\) CD8\(^-\) CD3\(^+\) Thymocytes: a Critical Role for Tumor Necrosis Factor and Interleukin-7

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This work aims at identifying the thymocyte subpopulation able to support human immunodeficiency virus (HIV) replication under the biological stimuli of the thymic microenvironment. In this report we demonstrate that interaction with thymic epithelial cells (TEC) induces a high-level replication of the T-tropic primary isolate HIV-1B-LAIp exclusively in the mature CD4\(^+\) CD8\(^-\) CD3\(^+\) thymocytes. Tumor necrosis factor (TNF) and interleukin-7 (IL-7), secreted during this interaction, are critical cytokines for HIV long terminal repeat transactivation through NF-κB-dependent activation. TNF is the major inducer of NF-κB and particularly of the p50-p65 complex, whereas IL-7 acts as a cofactor by sustaining the expression of the p75 TNF receptor. The requirement for TNF is further confirmed by the observation that the inability of the intermediate CD4\(^+\) CD8\(^-\) CD3\(^+\) thymocytes to replicate the virus is associated with a defect in TNF production during their interaction with TEC and correlates with the absence of nuclear NF-κB activity in these freshly isolated thymocytes. Addition of exogenous TNF to the intermediate thymocyte cultures induces NF-κB activity and is sufficient to promote HIV replication in the cocultures with TEC. The other major subpopulation expressing the CD4 receptor, namely, the double-positive (DP) CD4\(^+\) CD8\(^+\) CD3\(^+\) thymocytes, despite the entry of the virus, do not produce a significant level of virus, presumably because they are unresponsive to TNF and IL-7. Together, these data suggest that in vivo, despite an efficient entry of the virus in all the CD4\(^+\) subpopulations, a high viral load may be generated exclusively within the mature CD4\(^+\) CD8\(^-\) CD3\(^+\) subset of thymocytes. However, under conditions of inflammatory response after infection, TNF might also be present in the intermediate thymocyte compartment, leading to efficient HIV replication in these cells.

The progression of human immunodeficiency virus (HIV) infection is clearly associated with an increase in the viral load in plasma and a progressive depletion of CD4\(^+\) T cells. One explanation for this depletion is the exhaustion of T-cell turnover, which must occur at a very high rate to replace the CD4 lymphocytes permanently destroyed during HIV infection (15, 23, 59).

More recent studies on the kinetics of the increase in the number of CD4\(^+\) T cells after triple-combination therapies demonstrated that the mechanisms of T-cell depletion during HIV infection are more complex (36, 40). The depletion observed in the blood compartment may result from a combination of the trapping of these cells in lymph nodes and inflamed tissues (3, 40) and a failure of T-cell regeneration in primary lymphoid organs and particularly in the thymus (17, 36, 61). Although T-cell development can occur via extrathymic pathways, generation of the complete T-cell repertoire, required for immune system function, is dependent upon the thymus. This organ was shown to be still functional in adults, albeit less actively (17). HIV infection is accompanied by a decrease in thymic function (17), and antiviral therapies contribute to immune system reconstitution by a recovery of naïve T cells, probably through expansion of preexisting cells and thymic production of new cells (3, 37, 61, 65). However, this renewal occurs slowly (40), suggesting, in some cases, a severe impairment of T-cell progenitors, probably depending on the stage of the disease and the age of the patient. Indeed, observations of thymuses from HIV-infected pediatric patients, who underwent a rapid progression to AIDS, led to the conclusion that insult to this organ induces a severe thymocyte depletion associated with a profound disorganization of the epithelial network (27, 41, 44). Modification of the structure of the thymus and loss of thymocytes were also found in thymic tissues from infected macaques (39) and from SCID-hu mice (2, 48). This profound deterioration was also confirmed by the inability to completely restore thymopoiesis in infected thymuses from SCID-hu mice treated with highly active antiretroviral therapy (61).

For a better understanding of the immunopathogenesis associated with HIV infection of the thymus, we previously examined the factors and mechanisms controlling HIV replication in this organ (14, 45). The goal of the present work was the identification of thymocyte subpopulations that allow efficient HIV replication in the thymic environment. This relates to the general question of the control of spreading of the virus in this organ and its consequences for the peripheral viral load and thymopoiesis.

Using an autologous mixed culture with thymic epithelial cells (TEC) and the total population of thymocytes, we previously demonstrated that interaction of infected thymocytes with TEC is a prerequisite for high-level HIV replication (45).
Cytokines secreted during this TEC-thymocyte interaction were first identified as tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF), and a role for IL-7 was demonstrated later (14). Furthermore, we provided evidence that NF-κB activation is required for a high-level replication of HIV in thymocytes (14) by demonstrating that HIV provirus with its two κB sites deleted fails to replicate in thymocytes cocultured with TEC. NF-κB is composed of homo- and heterodimers of members of the Rel/NF-κB family (p65, c-Rel, Rel B, p50 and its precursor p105, and p52 and its precursor p100) (5, 35). These dimers are sequestered in the cytosol of unstimulated cells via interactions with a family of inhibitory proteins called IkBs (IκBα, IκBβ, and IκBe) (60). Following activation by various immune and inflammatory stimuli, IkB molecules are degraded through the ubiquitin-proteasome pathway, allowing nuclear translocation of NF-κB and activation of its target genes (32, 35). We previously demonstrated, using the total population of thymocytes, that TNF in association with IL-7 induced the p50-p65 activity observed in the nuclear extracts of these cells.

The data obtained in our coculture system with TEC and the total population of thymocytes did not indicate, however, whether all the subpopulations expressing the CD4 receptor were able to produce virus at a high level in this partially reconstituted thymic environment. This is an important question in view of the conflicting data found in the literature. A number of in vitro studies have shown that all the human CD4+ thymocytes are susceptible to infection (16, 20, 52, 55, 56). Controversial in vivo studies report a specific infection of TEC and activation of its target cells. The other CD4+ thymocyte subpopulations by negative selection, the following monoclonal antibodies were used: CD3 (X53), CD8 (B9.11), CD34 (QBEND-10), and CD34 (B6H12) and CD21/CD81 (B9.11) were used for positive selection of the DP thymocytes.

(ii) Antibodies used for immunostaining. To characterize thymic subpopulations and TEC, monoclonal antibodies conjugated with the fluorescent dyes CD3-ECD (Coulter, Hialeah, Fla.) or CD3-fluorescein isothiocyanate (FITC), CD4-phycocerythrin 7 (PE) (13B8.2), CDFC-FTIC (B9.11), CD3-P3 (HB15A) (Immunotech), and CD41-FITC (RMO52) (Becton Dickinson) were used, and for indirect staining, CD7 (SB1.1), CD10 (ALB 1), CD14 (QDB 10), Cytokeratin (KL1) (Immunotech), and Vimentin (vim3B4) (Biogenex, Westl. Mannheim, Meylan, France) were revealed with goat anti-mouse immunoglobulin G (1gG) (H + L) F(ab')2-FITC (Immunotech). Expression of the two TNF receptors, p55 and p75, were determined on CD4+ mature and intermediate thymocytes by using TNF-receptor 1 (RI)-FITC (p55) (168031) and TNF-R1-FITC (p75) (222535) antibodies (R&D Systems). Negative controls for these immunostaining reactions were performed with mouse IgG1 (MARK1) and IgG2a (L7.27) antibodies. Conjugated mouse IgG1-FITC (6791M7c), IgG1-FITC (6791M7c), goat anti-rabbit-FITC, and goat anti-mouse-FITC antibodies were purchased from Immunotech, and IgG1-ECDF was from Coulter Corp. Direct and indirect immunostaining was analyzed by cytofluorometry with an EPICS Profile II fluorometer (Coulter Corp.).

(iii) Cytokines. Human recombinant cytokines GM-CSF, IL-1β, and TNF-α were purchased from Genzyme Corp. (Cambridge, Mass.); IL-6 and IL-7 were purchased from R&D Systems (Minneapolis, Minn.). Cytokines were used at the following concentrations: IL-6, 10 ng/ml; TNF-α, 10 ng/ml; IL-1β, 10 ng/ml; IL-7, 0.5 ng/ml, GM-CSF, 20 ng/ml.

Cell culture conditions. (i) Preparation of TEC. Thymic fragments were obtained from HIV-1-seronegative children (aged 6 days to 2 years) undergoing elective cardiac surgery. TEC were obtained by the procedure of Rothe et al. (45). The dispersed TEC were seeded in a selective medium: McCoy’s 5A (GIBCO) containing 10% fetal calf serum (FCS), 1 mM l-glutamine, 50 μg of penicillin-streptomycin per ml, 100 μg of neomycin per ml, 5 × 10⁻³ M β-mercaptoethanol, 20 ng of epidermal growth factor (Sigma) per ml, 500 ng of hydrocortisone (Sigma) per ml, and 5 × 10⁻³ M cholera toxin (Interchim, Montluçon, France) (11). Prior to the coculture, TEC were maintained in the selective medium described above. An antikeratin antibody stained more than 95% of cells in the TEC-enriched population, confirming their epithelial characteristics (45).

(ii) Preparation of thymocyte subpopulations. Preparation of the total population of thymocytes has already been described (45). Except for the DP, most of the CD4-expressing subpopulations were obtained by negative selection. Mature CD4⁺ CD8⁻ CD3⁺ thymocytes were obtained after three depletion cycles with anti-CD8, anti-CD10, and anti-CD34 antibodies and anti-mouse IgG-coated magnetic beads (Immunotech). Immature CD4⁺ CD8⁻ CD3⁻ thymocytes were obtained after three depletion cycles with anti-CD3 and anti-CD8, anti-CD14, and anti-CD38 antibodies followed by anti-mouse IgG-coated magnetic beads. Anti-CD38 was used to remove dendritic cells. DP thymocytes were enriched by fluorescence-activated cell sorting with CD4 and CD8 antibodies in an Elite (Coulter) cell sorter. The antibodies that we used in this positive selection did not induce activation since no replication after activation of these cells. In addition, each subpopulation obtained by negative selection was further purified by depleting monocyte/macrophage and dendritic cells with CD14 and CD83 antibodies and anti-mouse IgG-coated magnetic beads (Immunotech).

Mixed-culture procedure. Preparation of TEC and infected thymocytes, either the total population or each of the enriched CD4⁺ subpopulations, was performed 3 days after thymus excision. A ratio of 4 × 10⁶ thymocytes/5 × 10⁵ TEC in 1 ml/well (24 wells/plate) was used in all the experiments. The coculture medium was McCoy’s 5A (GIBCO) containing 10% fetal calf serum (FCS), 1 mM l-glutamine, 50 μg of penicillin-streptomycin per ml, and 100 μg of neomycin per ml. In some experiments, cytokines were added at the start of the coculture.

Infection of thymocytes with HIV-1 Lai. All infections were performed with the primary isolate HIV-1 Lai (p is for primary isolate) (6). A total of 4 × 10⁶ (for most experiments) or 2 × 10⁶ (for the experiment in Fig. 4) thymocytes were infected at a multiplicity of infection of about 0.001 for 1 h at 37°C. The thymocytes were then washed three times with RPMI 1640 containing 10 mM HEPES, resuspended in culture medium, and cultured alone or with TEC in the presence or absence of cytokines. HIV-1 p24 antigen concentration was determined in culture supernatants by using a p24 antigen detection kit (Coulter HIV-1 p24 antigen assay) as specified by the manufacturer.

Electrophoretic mobility shift assay. Total-cell extracts were obtained as previously described (24). Briefly, 5 × 10⁶ thymocytes under different culture conditions (harvested and washed once) were incubated with 2 μl of lytic buffer for 15 min, and then centrifuged at 13,000 × g for 10 min. The protein concentration in the cell lysate was determined by using the Bradford reagent (Bio-Rad Laboratories, Ivory sur Seine, France).

The band shift assay, the procedure for the reaction mixture was prepared by adding, in the following order, the binding buffer (20 mM HEPES, 2 mM dithiothreitol, 60 mM KCl, 0.01% Nonidet P-40, 0.1 mg of bovine serum albumin, 4% Ficoll),
0.4 μg of sonicated salmon sperm, 10 μg of protein extract, and 30,000 cpm of \(^{32}\)P-labelled DNA probe (corresponding to 0.25 ng of probe). The sequence of the oligonucleotides used was

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5'\text{GACAGAGGGGGACTTTCCGAGAGG}\text{GTCTCCCCTGAAAGGCTCTCCCT}3'
\]

where the NF-κB consensus binding sequence is indicated in bold type.

Specific binding was controlled by competition with a 40-fold excess (10 ng) of the same nonlabeled oligonucleotide added to the protein extract before starting the binding reaction. To identify the subunits constituting NF-κB complexes, specific antibodies against p50, p65, and RelB were used. p50 and p65 antibodies were a kind gift from A. Israel (Pasteur Institute, Paris, France), and RelB antibody was provided by Santa Cruz Biotechnology (Santa Cruz, Calif.). Antibodies were added to the protein extract, and the mixture was incubated for 15 min at 4°C before further incubation with the radiolabeled probe.

**Statistical analysis.** The relative role of each cytokine in HIV replication within a given thymocyte subpopulation was statistically analyzed by the non-parametric test of Mann-Whitney. The level of significance was set at \(P < 0.05\).

**RESULTS**

**Enrichment of CD4\(^+\) thymocyte subpopulations.** To study whether the high-level replication observed in total thymocytes in coculture with TEC is a feature only of a specific thymocyte subset defined by the maturation state, we enriched the distinct thymocyte subpopulations expressing CD4 in the absence of cell activation (see Materials and Methods). Figure 1 represents the characterization by flow cytometry analysis of these enriched subpopulations according to their surface expression of CD4, CD8, and CD3.

Immature CD4\(^+\) thymocytes were enriched by negative selection of total thymocytes with antibodies against CD8 and CD3, to discard the mature CD4\(^+\) CD8\(^-\) CD3\(^+\) and the DP CD4\(^+\) CD8\(^+\) CD3\(^-\) thymocytes. As shown in Fig. 1B, this immature population was composed of two main subsets previously described in the human thymus (33): the immature triple-negative (TN) subset (CD4\(^-\) CD8\(^-\) CD3\(^-\)) and the intermediate subset (CD4\(^+\) CD8\(^+\) CD3\(^-\)), which in this experiment made up 51% (mean, 38% ± 10.6% for 13 thymuses) and 48% (mean, 57.3 ± 10.8% for 13 thymuses) of the thymocytes, respectively. Enrichment for DP thymocytes was performed by fluorescence-activated cell sorting with CD4 and CD8 antibodies. As shown in Fig. 1C, an enrichment of 94% was obtained. CD3 expression was observed in 55% of cells at an intermediate level and in 15% at a high level. However, this high level does not reach the maximum level observed in the mature population.

We developed a new technique for enrichment of the mature CD4\(^+\) CD8\(^-\) CD3\(^+\) thymocytes. We performed a negative selection with CD8, CD34, and CD10 antibodies. CD34 is expressed on very immature thymocytes (18), and CD10 is expressed early during thymocyte development but decreases with the increase of CD3 expression at the DP stage (54). The CD4\(^+\) mature population in the experiment in Fig. 1D was more than 94% (mean, 90.8 ± 2.9% for eight thymuses) CD3\(^+\) and more than 90% CD69\(^{hi}\) (data not shown). Only 6.6% (mean, 9.7 ± 4.8 for eight thymuses) of these cells had an undetectable level of CD4, as shown in Fig. 1D. The mature CD4 unstained thymocytes, could be depleted by using an antibody against CD4, suggesting that they also express CD4 but at a low level.
interaction by a mixture of cytokines (IL-1, IL-6, IL-7, GM-CSF, and TNF). HIV replication was determined by measuring the p24 gag protein concentration in the culture supernatants on days 3, 6, 10, and 14 postinfection. This pattern of response of the different subpopulations to the cytokines is representative of three experiments performed on four thymuses. Ag, antigen.

Interaction with TEC favors HIV replication exclusively in the mature CD4+ CD8- CD3+ thymocytes. We first tested whether the individually enriched CD4+ subpopulations of thymocytes were capable of producing HIV at a high level when cocultured with TEC. Total thymocytes as well as subpopulations containing predominantly DP CD4+ CD8+ CD3+, immature CD4+ CD8- CD3+, and mature CD4+ CD8- CD3+ cells were infected with the primary HIV-1_B-LAIp isolate and cultured alone or cocultured with autologous TEC. HIV-1 replication was evaluated by measuring the p24 gag protein concentration in the culture supernatants at various times postinfection. No HIV replication was detected when total thymocytes or any of the CD4+ subpopulations were cultured in the absence of TEC (data not shown). In contrast, an efficient HIV replication was observed in coculture of autologous TEC with total thymocytes and exclusively with the subset of mature CD4+ CD8- CD3+ thymocytes, as shown in Fig. 2. Under our coculture conditions, the observed replication level for mature thymocytes obtained from nine thymuses corresponded to 1.5 to 33 ng of p24 gag per ml. In each thymus, the level of replication observed in this specific subset was about 5- to 20-fold higher than that observed in total thymocytes. However, it is worth pointing out that virus entry was not impaired within the DP and immature thymocytes, as demonstrated by PCR analysis (data not shown).

CD4+ CD8- CD3+ as well as intermediate CD4+ CD8- CD3+ thymocytes sustain a high-level HIV replication in response to specific cytokines required for HIV replication. The cytokines mainly involved in HIV replication in total thymocytes during their interaction with TEC were previously determined as TNF, IL-1, IL-6, GM-CSF (45), and IL-7 (14). We first determined whether addition of these cytokines was sufficient to promote HIV replication in the mature CD4+ CD8- CD3+ subset. In parallel, we studied whether the absence of HIV replication in the DP and the immature subsets during their interaction with TEC was related to a possible unresponsiveness to these cytokines.

Therefore, total thymocytes, mature CD4+ CD8- CD3+, DP CD4+ CD8+ CD3+, and immature CD4+ CD8- CD3+ thymocytes were infected with HIV-1_B-LAIp and were cultured in absence of TEC but treated with a mixture of TNF, IL-1, IL-6, IL-7, and GM-CSF. As shown in Fig. 3A, the role of these cytokines in inducing HIV replication in total thymocytes was confirmed. As expected, a 5- to 20-fold-increased replication (between 5 and 130 ng for eight thymuses) was observed in the mature CD4+ CD8- CD3+ subset under these conditions (Fig. 3A). In the experiment represented in Fig. 3A, despite an especially high-level replication in total and mature thymocytes, no detectable replication was observed in the DP subset. In contrast, the immature pool of thymocytes, although unable to respond to specific cytokines required for HIV replication, responded to 1.5 to 33 ng of p24 gag per ml. In each thymus, the p24 gag concentration in the coculture supernatants on days 3, 6, 10, and 14 postinfection was confirmed. As expected, a 5- to 20-fold-increased replication (between 5 and 130 ng for eight thymuses) was observed in the mature CD4+ CD8- CD3+ subset under these conditions (Fig. 3A).
GM-CSF, between the different cytokine mixtures: for total thymocytes with all cytokines versus total thymocytes minus IL-7, the total population, the mature subset, and the immature thymocytes cultured at 2x10^6/well with either the mixture of all cytokines (IL-1, IL-6, IL-7, GM-CSF, and TNF) or with this mixture including all but one of these cytokines. HIV replication was determined by measuring the p24 gag concentration in the culture supernatants on various days postinfection. For each subpopulation, each graph represents three experiments performed with three different thymes. Statistical analysis by the nonparametric test of Mann-Whitney gave the following significant differences between the different cytokine mixtures: for total thymocytes with all cytokines versus total thymocytes minus IL-7, P < 0.006; for all cytokines versus minus GM-CSF, P < 0.006; and for all cytokines versus minus TNF, P < 0.006; for mature thymocytes with all cytokines versus minus IL-7, P < 0.02; for all cytokines versus minus IL-6, P < 0.02; and for all cytokines versus minus TNF, P < 0.02; for the immature thymocytes with all cytokines versus minus IL-7, P < 0.006; for all cytokines versus minus GM-CSF, P < 0.006; and for all cytokines versus minus TNF, P < 0.01.

FIG. 4. TNF and IL-7 play a crucial role in mature CD4+ CD8- CD3+ and in immature CD4+ CD8- CD3- thymocytes, whereas GM-CSF is a major factor only in immature thymocytes. Each population used in this experiment, i.e., total (A), mature CD4+ CD8- CD3+ (B), and immature CD4+ CD8- CD3- (C) was obtained from a different thymus. Thymocytes of each population were infected by HIV-1LAI, at a multiplicity of infection of 0.001, were seeded at 2 x 10^6 cells/ml, and cultured with the mixture of cytokines (IL-1, IL-6, IL-7, GM-CSF, and TNF) or with this mixture including all but one of these cytokines. HIV replication was determined by measuring the p24 gag concentration in the culture supernatants on various days postinfection. For each subpopulation, each graph represents three experiments performed with three different thymes. Statistical analysis by the nonparametric test of Mann-Whitney gave the following significant differences between the different cytokine mixtures: for total thymocytes with all cytokines versus total thymocytes minus IL-7, P < 0.006; for all cytokines versus minus GM-CSF, P < 0.006; and for all cytokines versus minus TNF, P < 0.006; for mature thymocytes with all cytokines versus minus IL-7, P < 0.02; for all cytokines versus minus IL-6, P < 0.02; and for all cytokines versus minus TNF, P < 0.02; for the immature thymocytes with all cytokines versus minus IL-7, P < 0.006; for all cytokines versus minus GM-CSF, P < 0.006; and for all cytokines versus minus TNF, P < 0.01.

to produce the virus in the presence of TEC, was able to do so when stimulated with these cytokines. Because the immature population consists of two different thymocyte subsets (as mentioned above), we determined whether this high-level replication took place either in the TN CD4+ CD8- CD3+ or in the intermediate CD4+ CD8- CD3- subset or in both. We considered the TN subset because the permissivity of these cells was previously reported in relation to low CD4 expression under conditions of stimulation by mitogen (57). The TN subset was isolated by depleting the immature subset from CD4+ cells by negative selection with CD4 antibodies. As shown in Fig. 3B, the high-level HIV replication observed in the immature subset when they were treated with cytokines was not observed in the TN subset and therefore must have been restricted to the intermediate CD4+ CD8- CD3- subset (Fig. 3B).

We then evaluated the relative importance of each of these cytokines in promoting HIV replication in total thymocytes and in the two responsive subsets, namely, the mature CD4+ CD8- CD3+ and the intermediate CD4+ CD8- CD3-. Due to the need for a large number of cells, each population used in this experiment was obtained from a different thymus. The total population, the mature subset, and the immature CD4+ CD8- CD3- pool (in which only the intermediate thymocytes replicate the virus) were infected by HIV-1LAI, and cultured at 2 x 10^6/well with either the mixture of all cytokines (TNF, IL-1, IL-6, IL-7, and GM-CSF) or with mixtures which each lacked one specific cytokine. As shown in Fig. 4A, we confirmed a crucial role of TNF and IL-7 in sustaining HIV replication in total thymocytes, since no detectable replication was observed in the absence of either one. The absence of GM-CSF resulted in a significant reduction of HIV replication. Comparison of Fig. 4B and C shows that TNF and IL-7 appear to play the same crucial role in mature and immature thymocytes whereas GM-CSF is an additional major factor only in immature thymocytes. The absence of IL-1 did not significantly affect HIV replication in either subpopulation, whereas the absence of IL-6 impaired the replication in the mature thymocytes exclusively.

TNF secreted during interaction of thymocytes with TEC is crucial in inducing NF-κB-dependent replication since its absence during the specific interaction between intermediate thymocytes and TEC is responsible for an impaired replication. Since the lack of a high-level HIV replication within the intermediate subset was not due to their unresponsiveness to the specific cytokines required for replication, we examined an alternative hypothesis, i.e., that of a defect in the secretion of one or more of these cytokines during intermediate thymocyte-TEC interaction. To test this hypothesis, we first performed experiments in which the cytokines exhibiting a crucial role in the immature subset (TNF, IL-7, and GM-CSF) for HIV replication were added, individually to cocultures of infected intermediate thymocytes with TEC. As shown in Fig. 5, of the three factors tested, only TNF was able to promote a detectable HIV replication in the coculture. These data suggest that TNF is not secreted or is secreted in insufficient amounts during immature thymocyte-TEC interaction.

In our previous report (14), we provided evidence that the activation of transcription factors of the Rel/NF-κB family is a requirement to promote efficient HIV replication in thymocytes. The prevalent NF-κB complex present in total freshly isolated thymocytes was shown to be the p50-p65 complex, whereas p50-p50 and p50-RelB complexes, although present, were less strongly represented. We also demonstrated that TNF, secreted during the coculture, is the cytokine mainly involved in the maintenance of NF-κB activity and that TNF signaling requires coactivation by IL-7. Here we first verified that the active p50-p65 complex actually resides in mature but not in immature thymocytes. NF-κB complexes were characterized, in mature CD4+ CD8- CD3+ and immature CD4+ CD8- CD3- thymocytes freshly isolated from the same thymus, by electrophoretic mobility shift assay with antibodies...
immature CD4

oligonucleotide representing the HIV long terminal repeat-derived

Immature CD4

thymocyte interaction correlates with an impairment of HIV replication in the

CD8

thymocytes were preincubated either with a preimmune serum (control) or with antibodies against RelB, p65, or p50 before incubation with a 32P-labeled

Fig. 6A, in the freshly isolated CD4

specific for the different members of this family. As shown in

Fig. 6A, in the freshly isolated CD4+CD8+CD3+ thymocytes, antibodies against p50 abolished the binding of most complexes, antibodies against p65 abolished the binding of the upper complex, and antibodies against RelB did not modify the binding of any complex. This suggests that the p50-p50 and the p50-p65 complexes are the main representatives in this subset. In contrast, as shown in Fig. 6B, in freshly isolated immature thymocytes, the major complex was the transcriptionally inactive p50-p50, while the p50-p65 complex was hardly visible (antibody upshifting confirmed this interpretation [data not shown]). We also confirmed that TNF maintained p50-p65 activity in the mature CD4+CD8−CD3+ thymocytes (Fig. 6A). As shown in Fig. 6B, this cytokine can induce p50-p65 activity also in the immature thymocytes cultured for 45 h, demonstrating again that the absence of p50-p65 complex in freshly isolated cells was not associated with a defect in responsiveness to this cytokine. In the two populations (mature and immature), IL-7 by itself does not induce p50-p65 but, rather, is a cofactor with TNF in the induction of p50-p65, as shown in Fig. 6. Therefore, convergent lines of evidence indicate that HIV replication cannot take place in intermediate thymocytes because of the absence of p50-p65 complex, essentially due to the impairment of TNF production during the interaction with TEC. In contrast, mature thymocytes fulfill the requirement for TNF secretion during coculture with TEC associated with an IL-7 secretion for an efficient NF-κB-dependent transcription of the viral genome.

IL-7 sustains expression of the p75 TNF receptor. The role of IL-7 as a cofactor of TNF in NF-κB activation was further investigated in the mature CD4+CD8−CD3+ thymocytes. We determined the level of expression of the two TNF receptors, p55 and p75 (12), by immunostaining and flow cytometry in mature thymocytes either freshly isolated or maintained in culture for 4 days in the presence or absence of IL-7. As shown in Fig. 7A, p75 was detected in the mature thymocytes just after their isolation. However, p75 expression was not maintained in culture and was no longer detectable at 4 days unless IL-7 was added to the culture. As shown in Fig. 7B, p55 expression, conversely to p75, persists in culture and IL-7 has no noticeable effect.


discussion

For a better evaluation of the consequences of HIV infection of the thymus, we investigated whether HIV replication was favored in thymocytes at certain stages of their differentiation. Our previous in vitro studies demonstrated the crucial role of interaction of thymocytes with TEC to produce a high-level virus replication. We pointed out the role of this interaction in creating a favorable cytokine microenvironment that was able to activate virus replication mainly through TNF,

FIG. 5. Defect in activation of TNF secretion during the TEC–immature-thymocyte interaction correlates with an impairment of HIV replication in the immature CD4+CD8−CD3+ thymocytes. Freshly isolated immature CD4+CD8−CD3+ thymocytes were infected by the HIV-1LAIp isolate at a multiplicity of infection of 0.001. They were cocultured with autologous TEC with no additional stimulation (control) or with TNF, IL-7, or GM-CSF. HIV replication was determined by measuring the p24 protein concentration in the culture supernatants on various days postinfection. This experiment is representative of three experiments performed on three different thymuses.

FIG. 6. Electrophoretic mobility shift assays. The p50-p65 complex is present in the freshly isolated mature CD4+CD8−CD3+ thymocytes (A) but not in the immature CD4+CD8−CD3+ ones (B). TNF and IL-7 coinduce this activity in both subsets. (A) Whole-cell extracts from freshly isolated mature CD4+CD8−CD3+ thymocytes were preincubated either with a preimmune serum (control) or with antibodies against RelB, p65, or p50 before incubation with a 32P-labeled oligonucleotide representing the HIV long terminal repeat-derived κB motif. The positions of the two specific bands, p50-p65 and p50-p50, are indicated. Mature CD4+CD8−CD3+ thymocytes were also cultured for 45 h either unstimulated (control) or stimulated with TNF, IL-7, or TNF plus IL-7, or cocultured with TEC as indicated. Whole-cell extracts from these various samples were incubated with a 32P-labeled oligonucleotide representing the HIV long terminal repeat-derived κB motif. The positions of the two specific bands, p50-p65 and p50-p50, are indicated. Immature CD4+CD8−CD3+ thymocytes were freshly isolated or cultured for 45 h either unstimulated (control) or stimulated with TNF or with IL-7 or with TNF plus IL-7. Whole-cell extracts from these various samples were incubated with a 32P-labeled oligonucleotide representing the HIV long terminal repeat-derived κB motif. This experiment is representative of five experiments performed on five different thymuses.
We first show that the population of mature CD4⁺ CD8⁻ CD3⁺ thymocytes is the only one able to exhibit a high-level virus replication when cocultured with TEC (Fig. 2). HIV replication in the mature subpopulation was about 5- to 20-fold higher than that observed in total thymocytes, whereas this subset represents 1/20 of the total thymocytes. Since infection progresses in an exponential manner, we might have expected a more marked difference. This result may also suggest that within the total thymocytes, the other subpopulations facilitated HIV replication within the mature subset. No replication was detectable in the other CD4⁺ CD3⁺ populations, either freshly isolated or cultured for 4 days without IL-7 or with IL-7 as indicated (Fig. 2). The presence of two TNF receptors, p75 (A) and p55 (B), was determined by flow cytometry on the mature CD4⁺ CD8⁻ CD3⁺ thymocytes, either freshly isolated or cultured for 4 days without IL-7 or with IL-7 as indicated. These previous data have been obtained by infecting the total population of thymocytes with various primary isolates or laboratory strains (14, 45). In this study, we identified, within this total population, the subpopulations of thymocytes in which this high-level replication readily occurs with the HIV-1_E_LAI primary isolate. We obtained the same results with the molecular clone NL-4-3 (data not shown). In addition, we specified the characteristics of these cells which permit such a virus production. The first step of this work consisted of enriching the CD4⁺ subpopulations susceptible to HIV infection. Therefore, the various CD4⁺ populations were isolated on the basis of their level of expression of CD4 but also of CD3, a criterion of cell maturity. We developed methods to enrich these different subpopulations, avoiding nonspecific activation. In most cases, except for the DP thymocytes, negative selection was used. However, no activation subsequent to CD4 and CD8 cell sorting of these cells was observed since no virus replication was detectable. Figure 1 indicates the rate of enrichment of these subpopulations and the level of expression of the different markers within each subset. In particular, mature CD4⁺ CD8⁻ CD3⁺ cells obtained by negative selection with CD8, CD10 and CD34 antibodies led to a subset of CD8⁻ thymocytes that have undergone positive selection, as also indicated by the expression of CD69 (data not shown), associated with a strong expression of CD3 (28, 54). A small part of this population was determined to express CD4 at a low level. These cells probably represent a mature population that has undergone positive selection and downregulated both CD4 and CD8 before differentiation to single-positive CD4⁺ or CD8⁺ cells (13, 58), or they might also represent thymocytes expressing the γδ T-cell receptor described that do not express detectable levels of CD4 (38).

FIG. 7. IL-7 maintains p75 TNF receptor expression on the mature CD4⁺ CD8⁻ CD3⁺ thymocytes. The presence of two TNF receptors, p75 (A) and p55 (B), was determined by flow cytometry on the mature CD4⁺ CD8⁻ CD3⁺ thymocytes, either freshly isolated or cultured for 4 days without IL-7 or with IL-7 as indicated. Thymocytes were stained with FITC anti-p75, FITC anti-p55, or FITC anti-IgG control. p75 and p55 fluorescence histograms are shown (solid line) in comparison with those obtained with the isotype-matched controls (broken line). This experiment is representative of three experiments performed on three different thymuses.
the intermediate subpopulation. It is very likely that in the presence of TNF added to the culture, the synergistic role of IL-1 is hardly visible, since both cytokines act on NF-κB activation and since TNF signaling has the major effect (14). In contrast, the effect of IL-6 on HIV replication is restricted to the mature population. This is in agreement with the fact that this cytokine was shown to specifically induce the proliferation of this thymocyte subset (50).

The requirement for IL-7 might explain the absence of viral production in the DP cells, since most of them do not express the IL-7 receptor (51). However, according to the murine model, a small fraction of the DP cells (around 5%) which have undergone positive selection have acquired this receptor. We cannot exclude replication in this small fraction of DP cells, which might be masked by the high rate of apoptosis of the remaining 95% of the DP cells which are not protected by the antiapoptotic effect of IL-7 in culture. Response to TNF might not also be efficient in this set of DP cells, since TNF receptors are present on only 3% of the total population of thymocytes (46).

We previously demonstrated with the total population of thymocytes (14, 45) that TNF and IL-7 act synergistically to enhance HIV transcription through NF-κB transcription factors, with p50-p65 being the prevalent complex observed. We demonstrate here that the p50-p65 activity observed in the total population is clearly located in the freshly isolated mature thymocytes and is maintained either by coculture with TEC or by the synergistic effect of TNF and IL-7 (Fig. 6A). The major role of TNF in HIV replication in thymocytes was further confirmed by the fact that the only barrier to virus production in intermediate thymocytes was a defect in TNF production during their interaction with TEC. Indeed, addition of exogenous TNF to the coculture was sufficient to induce replication (Fig. 5). The relevance of the absence of an efficient stimulation by TNF in the microenvironment of the intermediate thymocytes was supported in vivo by the fact that these thymocytes, freshly isolated, are devoid of nuclear p50-p65 activity. The p50-p50 complex was observed, but this complex is transcriptionally inactive (19) (Fig. 6B). When added to the culture medium of these thymocytes, TNF induces the activation of p50-p65, suggesting again the lack of TNF in the microenvironment.

The role of IL-7 as a cofactor with TNF in the activation of NF-κB was further investigated in the mature thymocytes. We showed that TNF receptors p55 and p75 (12) were both expressed when cells were freshly isolated from their thymic environment. Culture of these cells led to a decrease of p75 expression, whereas p55 expression was much more stable. However, IL-7 was able to maintain p75 expression in culture but has no effect on p55 expression. It is worth noting that IL-7, known for its antiapoptotic role, favors the expression of the p75 receptor, which leads exclusively to NF-κB activation, and not that of the p55 receptor, which also leads to an apoptotic pathway. This increase in NF-κB, by itself, might serve to protect against apoptosis induced by TNF signaling through p55 (47). Since p55 expression is constitutively stable, this receptor might be sufficient to induce the NF-κB activation necessary for HIV replication even in absence of IL-7, but the higher rate of cell death (observed in the culture in absence of IL-7) might impair this process. This interpretation is supported by the data in the literature showing that only agonists of the p75 receptor lead to thymocyte activation, as shown by their proliferation, whereas agonists of p55 receptor mediate cytotoxicity (53). Since GM-CSF plays an important role in HIV replication in the immature population, we also tested its effect on the expression of the two TNF receptors. However, since the viability of the immature thymocytes is highly dependent on IL-7, we tested GM-CSF in the presence of IL-7 and found that the levels of the two TNF receptors were identical to those observed with IL-7 alone.

Taken together, these data led us to establish the following model for the dynamics of HIV replication in the thymus. Our study argues for a preferential replication within the mature CD4+CD8+CD3 thymocytes as a result of their interaction with TEC by a process involving TNF and IL-7 secretion. The presence of a high viral load in this population might be responsible in vivo for an increase of the peripheral viral load, explaining a rapid progression toward AIDS. We can also speculate that the intermediate thymocytes may contribute to viral spreading in the presence of TNF, secreted in their microenvironment, by inflammatory cells in response to infection of the mature population. This sequence of events was actually observed in the thymus of juvenile cats infected by feline immunodeficiency virus: a high level of virus was observed within mature thymocytes early postinfection, whereas infection of the immature thymocytes was seen later and was associated with the inflammatory response and cortical atrophy (62). Furthermore, infection of this immature population is upregulated by GM-CSF. This cytokine might act by increasing thymocyte viability (23, 29) or, as suggested previously (21), by increasing IL-7 receptor expression on the thymocyte surface (21). We cannot exclude the possibility that GM-CSF with TNF favors the differentiation of immature CD34+ thymocytes into dendritic cells (34). Indeed, we observed that addition of GM-CSF to immature CD4+CD8+CD3+ cells cultured with IL-7 and TNF is associated with the appearance of cells with dendritic morphology (data not shown). Dendritic cells were shown to strongly enhance HIV replication in T cells (43), and thymic dendritic cells were also found to support HIV replication (8). It is possible that dendritic differentiation and infection, in the immature compartment, also contributes to the inflammatory response. Such an inflammatory response, by favoring virus spreading, might lead to direct destruction of these cells (49) and disruption of the cortical microenvironment and may contribute to an irreversible adverse effect on T-cell regeneration.

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