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Infection Frequency of Dendritic Cells and CD4$^+$ T Lymphocytes in Spleens of Human Immunodeficiency Virus-Positive Patients

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Dendritic cells (DC) are specialized antigen-presenting leukocytes that are responsible for the activation of naive as well as memory T lymphocytes. If infected by human immunodeficiency virus (HIV), DC may transfer virus to CD4$^+$ lymphocytes. However, the question of whether DC are infected in vivo is controversial. As HIV infection is more active in secondary lymphoid organs than in blood, infection of splenic DC isolated from HIV-seropositive patients was investigated. Splenic DC were first enriched and characterized by flow cytometry from HIV$^-$ donors. After direct isolation, they were negative for monocyte and T- and B-lymphocyte markers, negative for CD1a, but positive for major histocompatibility complex class II and CD4. After in vitro maturation, major histocompatibility complex class II expression increased, while CD4 expression was lost. Extensive purification from the spleens of seven HIV$^+$ patients was performed by fluorescence-activated cell sorting. The frequency of cells harboring HIV DNA in purified populations was quantified by limiting-dilution PCR. Directly isolated DC (average, 1/3,000; range, 1/720 to 1/18,000) were in each patient 10 to 100 times less infected than CD4$^+$ T lymphocytes (average, 1/52; range, 1/17 to 1/190). On average, 1/1,450 (1/320 to 1/6,100) unseparated mononuclear splenocytes (containing 5% CD4$^+$ lymphocytes) harbored HIV DNA. In conclusion, in these HIV$^+$ patient spleens, DC seem to be infected, but HIV-DNA positive CD4$^+$ T lymphocytes accounted for the vast majority of infected mononuclear splenocytes.

Dendritic cells (DC) are professional antigen-presenting cells found in the blood, in the T-dependent areas of lymphoid organs, and in the skin and other epithelia in the form of Langerhans cells (28). They play an essential role in the stimulation of primary T-cell responses since, according to most reports, they are the only cells able to present antigen to naive T cells (13, 20).

By activating T lymphocytes, DC create optimal conditions for human immunodeficiency virus (HIV) replication. Therefore, the possibility that they play an important role in the clinical evolution of HIV infection has generated considerable interest. Consistent with this idea, it has been shown that DC or Langerhans cells exposed to HIV in vitro transmit infection to CD4$^+$ T lymphocytes very efficiently during the course of antigen presentation (4, 26). It has also been suggested that antigen presentation by DC is impaired early during HIV infection (15, 19, 39), which might help to explain the extensive T-cell dysfunction, particularly the loss of primary or secondary T-cell responses to soluble antigens, observed in HIV$^+$ individuals well before the onset of AIDS (7, 18).

Since both skin Langerhans cells and freshly isolated blood DC express CD4 (21, 36), they may be susceptible to HIV infection. Indeed, HIV infection of Langerhans cells in vivo (33, 38) and in vitro (6, 9) has been observed by several different groups. For blood DC, however, the question is less clear. In HIV-infected patients, some reports claimed infection in 3 to 21% of blood DC (19); others concluded that these cells are infected by HIV only rarely if at all (3, 14). After HIV infection of blood DC in vitro, production of virions or p24 antigen was found in some studies (16, 25), but in others, highly purified blood DC could not be infected by HIV (4).

This lack of consensus may be explained by the difficulty of obtaining pure populations of human blood DC; they do not express any known specific marker and are in a very low proportion among leukocytes (0.1 to 1% of peripheral blood mononuclear cells [12]), which are difficult to obtain in large amounts from HIV$^+$ patients. Furthermore, a large body of evidence points to the fact that HIV infection should be sought in lymphoid organs, in which viral replication is active at all stages of HIV disease and the proportion of infected CD4$^+$ lymphocytes is significantly higher than in peripheral blood (1, 2, 10, 24, 30).

For these reasons, the spleen was used as a source of DC in this study. Human splenic DC have not been previously investigated in detail. Methods of enrichment developed for human blood were adapted to the spleen, and splenic DC from normal organ donors were characterized by flow cytometry. Different cell populations from the spleens of HIV$^+$ patients who had undergone splenectomy (22) were purified extensively. The frequency of cells containing HIV DNA was determined by limiting-dilution PCR capable of detecting a single infected cell.

MATERIALS AND METHODS

Clinical samples. HIV$^-$ whole blood was obtained from the blood transfusion center of the Hôpital Pitié-Salpêtrière. Six HIV$^-$ spleens were taken from organ transplant donors at the Hôpital Pitié-Salpêtrière following national ethical

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TABLE 1. Clinical data for HIV$^+$ patients involved in this study

Patient	Clinical status	CDC stage	No. of CD4/mm^3 of blood[a]	% CD4 in spleen	Spleen wt (g)
6	Castleman's syndrome	IV D (Kaposi sarcoma)	125	NT[b]	2,400
7	ITP[c]	IV E	600	NT	150
8	ITP	IV A	140	6	400
10	Hemophagocytic syndrome	IV A	110	<1	1,100
11	Castleman's syndrome	IV E	200	5	330
12	ITP	IV E	94	2	820
13	ITP	IV E	312	7	352
14	ITP	IV E	202	6	180
16	Castleman's syndrome	IV D (Kaposi sarcoma)	95	2	715

[a] Before splenectomy.
[b] NT, not tested.
[c] ITP, idiopathic thrombopenic purpura.

guidelines regulating the use of human tissues. Spleens of nine patients seropositive for HIV-1 (Table 1) were obtained after splenectomy carried out as therapy for either idiopathic thrombopenic purpura (patients 7, 8, 12, 13, 14), Castleman's syndrome (patients 6, 11, and 16), or hemophagocytic syndrome (patient 10). HIV-1 seropositivity had been determined by enzyme-linked immunosorbent assay and Western blotting (immunoblotting). At the time of surgery, five of nine patients were in CDC stage IV E of HIV disease (with idiopathic thrombopenic purpura alone and no other symptoms of AIDS).

Enrichment of human splenic DC. Blocks of spleen, approximately 2 by 2 by 3 cm in size, were kept at 4°C in RPMI 1640 medium (ICN Flow, Irvine, Scotland) for the period between surgery and isolation of splenocytes, which varied from 1 to 2 h. Splenic DC were purified either directly or after 36 h of culture, using adaptations of previously published techniques for the isolation of peripheral blood DC (12, 21) (Fig. 1). Spleens were cut into small pieces, which were then crushed with the plunger of a sterile syringe. The suspension was transferred to a 50-ml tube and pipetted vigorously to detach cells from the connective tissue. Cells were then filtered through a sterile gauze and centrifuged over diatrizoate-Ficoll (density, 1.077 g/ml; Eurobio, Les Ulis, France). This mechanical dissociation was completed in some experiments with enzymatic dissociation of the remaining debris, using type VII collagenase (Sigma, St Quentin Fallavier, France) at 20 U/ml and DNase I (Sigma) at 40 U/ml in RPMI

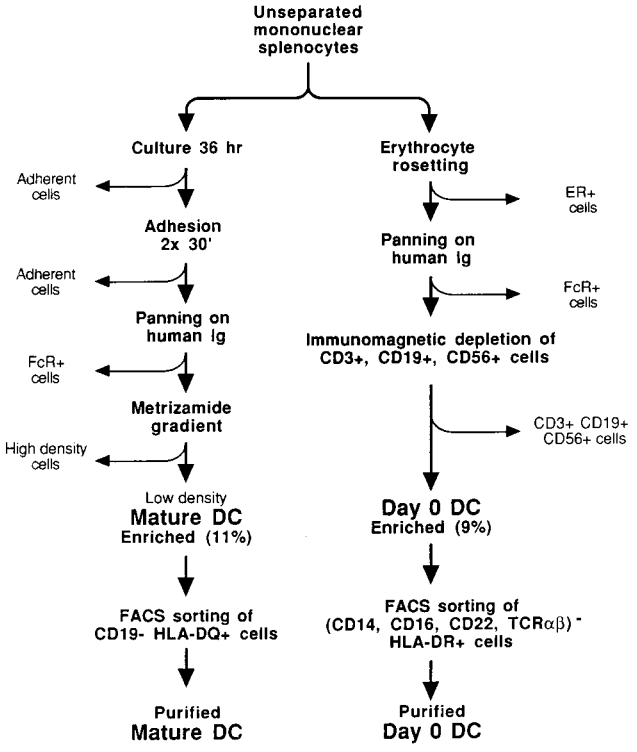

FIG. 1. Flow diagram of methods used for the enrichment of DC from spleen mononuclear cells. ER, erythrocyte rosette.

supplemented with 2% fetal calf serum for 30 min at room temperature. Cell aggregates were further dissociated by addition of EDTA to 10 mM and agitation for 5 min at room temperature as described previously (35). These conditions did not affect surface molecule expression.

For direct isolation, mononuclear cells were then depleted for T cells by erythrocyte rosetting overnight at 4°C with agitation. Rosette-negative cells were depleted of Fc receptor (FcR)-positive cells by panning for 60 min at 4°C on dishes coated with 10 μg of total human immunoglobulin (Ig; Biotransfusion, les Ulis, France) per ml. Nonadherent cells were incubated with monoclonal antibodies (Table 2) against CD3 (ascites fluid at 1/1,000), CD19 (5 μg/ml; Immunotech, Marseille, France), and CD56 (1/40; Immunotech) for 30 min at 4°C, washed twice, and then incubated with sheep anti-mouse IgG-coated magnetic beads (Dynal, Compiègne, France) for 60 min with agitation at 4°C. Cells which did not rosette with beads were collected and used for further analysis.

For isolation of mature DC, mononuclear cells were cultured at 5×10^6 to 10×10^6 cells per ml in R$^+$10 medium (RPMI supplemented with 10% fetal calf serum [Boehringer Mannheim, Meylan, France], 2 mM L-glutamine, 1 mM sodium pyruvate [Gibco, Paisley, Scotland], 1% minimal essential medium nonessential amino acids [Gibco], 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.25 μg of amphotericin B per ml at 37°C and 5% CO_2 in 225-cm^2 culture flasks (Costar, Cambridge Mass.). After 36 h, nonadherent cells were harvested and further depleted of macrophages by two rounds of plastic adhesion for 30 min at 37°C in cell culture petri dishes (Nunc, Roskilde, Denmark) or, for HIV$^+$ cells, ventilated tissue culture flasks (Costar). They were depleted of FcR$^+$ cells by panning on dishes coated with human Ig (Biotransfusion). This depleted population was then centrifuged at $1,000 \times g$ for 10 min at 20°C over 14.5 g of metrizamide (Sigma) per 100 ml in R$^+$ 10 culture medium. Low-density cells enriched for DC and high-density cells enriched for lymphocytes were harvested and used for further analysis.

Isolation of other cells from human spleen. CD4$^+$ T lymphocytes were isolated directly from spleen mononuclear cells by using anti-CD4 magnetic beads (Dynal). These beads preferentially selected CD4$^+$ T lymphocytes (>90% pure), as found from morphological and flow cytometry data, and not macrophages or DC, which could not be selected positively with this method in our hands, probably because of higher CD4 expression on lymphocytes. CD19$^+$ B lymphocytes were also purified to 90 to 95% (with undetectable levels of CD4 cells) by immunomagnetic separation using anti-CD19 monoclonal IgG (5 μg/ml) followed by sheep anti-mouse IgG-coated magnetic beads (Dynal). Adherent cells from the 36-h cultures were harvested by trypsinization after several washes to remove all nonadherent cells (0.25% trypsin in phosphate-buffered saline [PBS; Sigma]). May-Grünwald-Giemsa staining and flow cytometry confirmed that they were predominantly (50 to 70%) CD14$^+$ spread and vacuolated macrophages, with a minor population of small adherent lymphocytes (data not shown). Monocyte-conditioned medium was obtained after culturing of those cells for 48 h in R$^+$10 medium.

Flow cytometry. Cells were incubated for 30 min at 4°C in RPMI supplemented with 20% human AB serum and then stained for double or single immunofluorescence at 10^7 cells per ml with the antibodies listed below. All incubations were carried out in PBS containing 1% bovine serum albumin (BSA) for 30 min at 4°C, and cells were washed once in PBS-BSA between steps. After a final wash, cells were fixed in PBS containing 1% paraformaldehyde and then analyzed with a FACScan (Becton Dickinson, Pont-de-Claix, France) or sorted with a FACStar Plus (Becton Dickinson).

Antibodies (Table 2) were used at the following concentrations or dilutions: anti-CD3 conjugated to fluorescein isothiocyanate (FITC), 1/25; anti-CD4 (13B8.2)–FITC, 1/25; anti-CD8 conjugated to phycoerythrin (PE), 1/20; anti-CD16–FITC, 1/100; anti-CD22–FITC, 1/5; anti-HLA-DQ (Immunotech, Marseille, France), 4 μg/ml; anti-CD1a–PE, 5 μg/ml; anti-CD13–PE, 20 μg/ml; anti-CD14–PE, 5 μg/ml; anti-CD14–FITC, 1.25 μg/ml; anti-CD19 (B-4)–PE or –FITC, 10 μg/ml; anti-CD33 PE (Coulter, Margency, France), 20 μg/ml; anti-T-cell receptor (TCR) αβ chain–FITC, 1/5; anti-B7, 20 μg/ml; anti-CD3 (Leu4)–

TABLE 2. Antibodies used for selection and phenotyping of human splenic dendritic cells[a]

Antibody clone	Molecule recognized	Major specificity(ies)
T6	CD1a	LC, thymocytes
UCHT1	CD3 (complex associated with TCR)	T lymphocytes
SK3	CD4 (MHC class II ligand)	T-lymphocyte subset, monocytes, macrophages, immature DC, LC
B9.11	CD8 (MHC class I ligand)	T-lymphocyte subset
My-7	CD13	Monocytes, granulocytes
My-4	CD14 (LPS receptor)	Monocytes, macrophages, LC
3G8	CD16 (FcγRIII)	NK cells, granulocytes, monocytes, macrophages
B-4	CD19 (associated with B-cell receptor complex)	B lymphocytes
J4.119	CD19	B lymphocytes
SJ10.1H11	CD22	B lymphocytes
My-9	CD33	Monocytes, macrophages, LC, DC subset
T199	CD56	NK cells, T-lymphocyte subset
B7-BB1	CD80 (ligand for T-cell costimulatory molecules CD28 and CTLA-4)	Activated B lymphocytes, DC
WT31	TCRαβ	T lymphocytes
L243	HLA-DR (MHC class II molecule)	B lymphocytes, macrophages, DC
SPVL3	HLA-DQ (MHC class II molecule)	B lymphocytes, macrophages, DC

[a] The concentrations at which antibodies were used are listed in Materials and Methods. LC, Langerhans cells; MHC, major histocompatibility complex; LPS, lipopolysaccharide; NK, natural killer.

FITC/anti-CD19 (Leu12)–PE, 1/10; polyclonal mouse IgG1 and IgG2 conjugated to FITC and PE (Becton Dickinson), 1/10. The second-step reagents for unconjugated antibodies were PE- or FITC-conjugated polyclonal goat anti-mouse IgG (Immunotech) used at 10 and 5 μg/ml, respectively.

Mixed lymphocyte reaction. Stimulating cells were irradiated and added in decreasing numbers (from 2×10^6 to 100 cells per well) to 4×10^6 mononuclear cells from another donor in a final volume of 1 ml of R^+10 culture medium in 24-well plates (29). Three days later, the cells from each well were centrifuged, divided in 200-μl aliquots in flat-bottom 96-well plates, cultured overnight with 1 μCi of [^{3}H]thymidine (Amersham, Les Ulis, France), and then harvested, and DNA associated radioactivity was counted on a β counter (Beckman, Gagny, France). The stimulation index was calculated as (average of cpm obtained in triplicate)/(average of cpm obtained in the six control wells cultured without stimulating cells).

Measurement of the frequency of HIV infection in cell populations. The frequency of HIV-infected cells was measured by a limiting-dilution PCR method (8). Splenocyte subpopulations were counted, adjusted to an exact concentration, and then diluted carefully in series in a suspension of Daudi cells (a noninfected B-cell line) in PBS. At each dilution, 10 replicates of 10 μl were added to 40 μl of lysis buffer containing 0.5% Tween 20, 0.5% Nonidet P-40, 200 μg of proteinase K (Sigma) per ml, and 1× Biotaq reaction buffer (Bioprobe Sys, Montreuil sous Bois, France). Cells were lysed for 30 min at 56°C, then heated for 5 min at 94°C to inactivate proteinase K, and finally stocked at −20°C. Cell lysates were transferred into PCR tubes, and HIV proviral DNA was amplified under hot start conditions, using the *env*-specific LV13-LV15 primer pair (8). After 30 amplification cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 10 min), 5 μl of the product of this reaction was reamplified (35 cycles; 94°C for 30 s, 55°C for 30 s, and 72°C for 10 min), using the nested SK122-SK123 primer pair (8) under hot start conditions. The primers map within the conserved C1 and C2 regions of HIV-1 *env* (8). In both amplifications, the final concentrations of reagents were as follows: 200 μM each deoxynucleoside triphosphate, 1 μM each primer, 0.025 U of Biotaq DNA polymerase (Bioprobe Sys), per μl, and 2.5 mM $MgCl_2$. Nested PCR products were electrophoresed through a 2% agarose gel, and the number of positive replicates was scored for each dilution. By limiting dilution, the primers were shown to detect a single provirus (data not shown). Hybridization of the nested PCR products with a C1-specific probe failed to reveal any more positive samples.

Statistical analysis. Since a single infected U1 cell diluted in 10^4 Daudi cells could be detected (data not shown), a single-hit Poisson model could be applied. The fraction of negative replicates among all replicates was plotted as a function of the number of cells per replicate. According to the Poisson law (27), the probability of obtaining a certain fraction of negative replicates in a given dilution is $y = e^{-\mu}$ ($\mu^\eta/\eta!$), where η is the number of infected cells in the replicate and μ is the average number of infected cells ($\mu = x/a$, where x is the number of cells per replicate and a is the inverse of the infected cell frequency). In this case, we look for the probability of finding no infected cells in the replicate (i.e., $\eta = 0$) for a given μ, and then y reduces to $e^{-\mu}$. The number a of cells among which one infected cell could be found was thus determined by fitting $e^{-x/a}$ to the data with a weighted least-squares method (see Fig. 6). This method provides an error estimate σ for a. The weighted mean ($\bar{a}$) of the results obtained in all the patients for a given cell population was calculated by using $1/\sigma$ as a weight. These results are given in the text as frequency of infection = $1/a$, with the range of a obtained in all patients. They were represented as $f = 1/\bar{a}$ in Fig. 7 so that the most infected cell populations would appear with the highest bars.

RESULTS

Phenotype of mature splenic DC isolated after 36 h of culture in vitro. Mature DC from normal spleens were enriched from organ transplant donors by the method summarized in Fig. 1, adapted from published techniques for the isolation of mature peripheral blood DC (12). After 36 h of culture to allow maturation of DC from mononuclear splenocytes, the vast majority of recovered cells were nonadherent. They were further depleted of adherent FcR^+ $CD14^+$ macrophages (as checked by fluorescence-activated cell sorting [FACS] analysis) by adhesion and panning. Nonadherent FcR^- cells were then separated by centrifugation over metrizamide. As expected, high-density cells were almost exclusively lymphocytes, and the low-density fraction was enriched for DC (mean, 11%; range, 2 to 36%; $n = 5$). After May-Grünwald-Giemsa staining, mature splenic DC were identified as large cells displaying a typical dendritic morphology and frequently containing small vacuoles and a cloverleaf-shaped nucleus. Consistent with their T-cell-stimulating function, they expressed major histocompatibility complex class II molecules (HLA-DR and HLA-DQ) and the costimulatory molecule B7.1 (CD80) (Fig. 2). They were negative for B-cell (CD19), T-cell (CD3), and monocyte (CD14) markers but stained weakly positive for the myeloid lineage markers CD33 and CD13. Further experiments showed that mature splenic DC did not express CD4, FcγRII (CD32), or CD1a, which are expressed by Langerhans cells. In terms of morphology, forward and side scatter parameters, and surface phenotype, these cells shared many similarities with previously described blood DC (12, 32) and were distinct from all other cell types depleted during the isolation procedure.

In the mature DC-enriched population, occasional residual macrophages were present, as well as some residual T cells, which could be excluded by scatter gating because of their small size, but B lymphocytes were by far the major contaminant. Two-color CD19/HLA-DQ staining was therefore used to determine the yield of mature DC and to purify them by FACS (Fig. 3A).

Phenotype of directly isolated human splenic DC (day 0 DC). An alternative method which avoided the initial 36-h culture step was sought (21). Total mononuclear splenocytes were sequentially depleted of T cells by erythrocyte rosetting, of FcR^+ cells by panning, and then of B ($CD19^+$), T ($CD3^+$), and natural killer ($CD56^+$) cells by immunomagnetic separa-

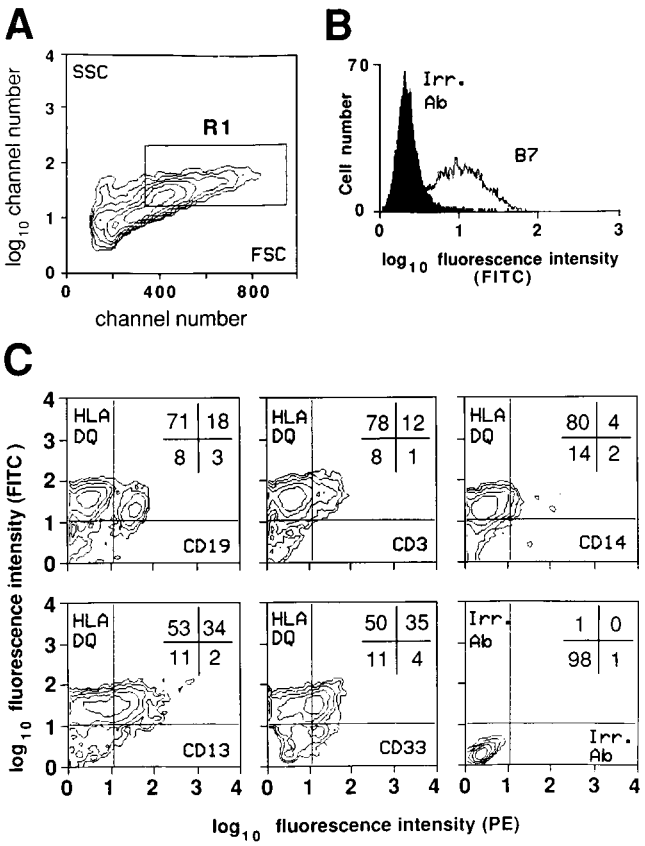

FIG. 2. Flow cytometric analysis of mature splenic DC. (A) Scatter gating of large cells in enriched mature DC stained for different surface molecules. SSC, side scatter; FSC, forward scatter. (B) Single-color analysis of expression of B7 (BB1) on large cells. Control staining with an irrelevant antibody (Irr. Ab) then FITC-conjugated secondary antibody is shown in black. (C) Two-color analysis of large cells. Bottom right, control staining with an FITC- and PE-conjugated irrelevant antibody; remaining panels, staining with anti HLA-DQ followed by FITC-conjugated goat anti-mouse IgG and, finally, different PE-conjugated monoclonal antibodies as indicated. The percentage of gated cells in each quadrant is shown in the top right-hand corner of each plot.

tion (Fig. 1). The remaining population was principally composed of $CD14^+$ macrophages and small $CD22^+$ B lymphocytes (data not shown) and was enriched for dendritic cells (9%; range, 5 to 12%; $n = 6$). Day 0 splenic DC stained negative with a cocktail of antibodies to T-cell (TCR$\alpha\beta$), B-cell (CD22), NK cell (CD16), and monocyte (CD14) markers but positive with antibody to major histocompatibility complex class II molecules (HLA-DR) (Fig. 4). Like day 2 DC, day 0 DC were negative for CD1a, and a variable proportion also expressed CD33. However, all day 0 DC were positive for CD4, with levels of expression of this molecule higher on DC than on the cocktail-positive macrophages also contained within this population. The surface phenotype of directly isolated splenic DC is therefore virtually identical to that reported for peripheral blood DC precursors (21). The cocktail-negative, HLA-DR^+ phenotype was used to determine the yield of day 0 dendritic cells and to purify them by FACS (Fig. 3B). The cocktail-positive, HLA-DR^+ population present in the same scatter gate as DC (large cells) was sorted as a control. Additional staining showed this population to be mostly composed of $CD14^+$ $CD33^+$ macrophages (Fig. 4 and data not shown).

In two experiments, day 0 DC were cultured in the presence of monocyte-conditioned medium to ensure that they could acquire the characteristics of mature DC isolated by a different method and to test their infection before and after culture. After 48 h, fully activated DC with typical dendritic morphology could be identified on May-Grünwald-Giemsa-stained cytospins, although such cells were not initially present in this population (not shown). This change in morphology was accompanied by an increase in the expression of HLA-DR by DC, which remained cocktail negative (Fig. 3B). Therefore, cells with the phenotype of mature DC were obtained after culture of DC precursors isolated directly (day 0 DC).

The results presented above were obtained from spleen mononuclear cells dissociated mechanically. In two experiments (one HIV^- and one HIV^+ spleen), enzymatic dissociation was performed on the remaining spleen debris to analyze the cells which could not be dissociated mechanically. The DC isolation protocol was then performed in parallel from the two spleen mononuclear cells populations (mechanical dissociation versus enzymatic dissociation of debris). Day 0 DC obtained from the remaining spleen debris (i.e., most likely to be all resident DC) had the same phenotype as day 0 DC obtained from the mechanically dissociated fraction (data not shown).

Yield. Typically, $2{,}500 \times 10^6$ mononuclear splenocytes yielded 27×10^6 mature DC-enriched cells with 11% $CD19^-$ HLA-DQ^{++} dendritic cells or 76×10^6 day 0 DC-enriched cells with 9% large cocktail-negative, HLA-DR^+ DC. The yields of mature DC enrichment from HIV^+ and HIV^- mono-

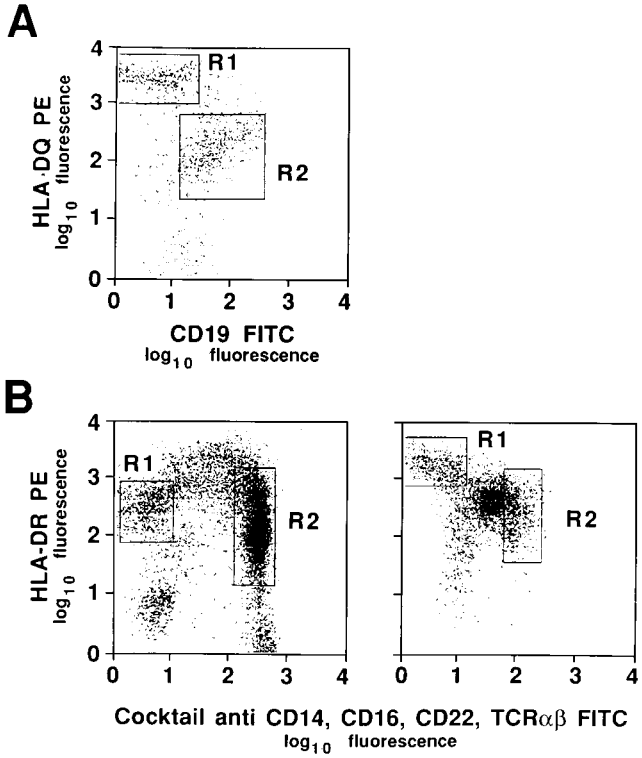

FIG. 3. FACS sorting of splenic DC. (A) Sorting of mature DC enriched from spleen mononuclear cells of patient 8. Large cells were gated and DC sorted as HLA-DQ^+ $CD19^-$ cells (R1). HLA-DQ^+ $CD19^+$ cells were also sorted (R2) as controls. (B) Sorting of day 0 DC enriched from spleen mononuclear cells of patient 13. Enriched day 0 DC were stained before (left) or after (right) 48 h in culture with a cocktail of FITC-conjugated antibodies (anti-CD14, anti-CD16, anti-CD22, and anti-TCR$\alpha\beta$) and PE-conjugated anti-HLA-DR. Large cells were gated, and DC were sorted as HLA-DR^+, cocktail-negative cells (R1) and macrophages were sorted as HLA-DR^+, cocktail-positive cells (R2) as explained in Results. After culture, HLA-DR expression increased on all cells and expression of CD14 on macrophages became less intense.

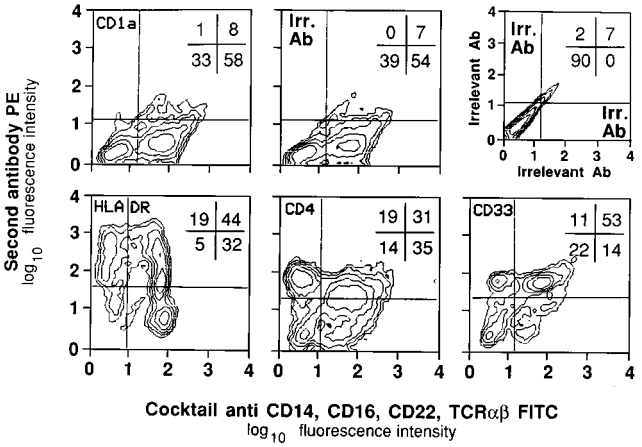

FIG. 4. Flow cytometric analysis of day 0 spleen DC. Contour plots show the expression of surface markers on large cells. Different PE-conjugated antibodies (as indicated in each plot) and an irrelevant antibody (Irr. Ab) were used in conjunction with a cocktail of FITC-conjugated antibodies. Top right, control staining with an FITC- and PE-conjugated irrelevant antibody. The percentage of gated cells in each quadrant is shown in the top right-hand corner of each plot.

nuclear splenocytes were compared. Mature DC represented 0.03 to 0.18% (mean, 0.08%, standard deviation [SD] 0.05%; $n = 6$) of unseparated mononuclear splenocytes from HIV^- patients and 0.03 to 0.11% (mean, 0.07%; SD, 0.03%; $n = 5$) of unseparated mononuclear splenocytes from HIV^+ patients. Thus, there was no significant difference between the yields of splenic DC from HIV^- and HIV^+ donors. The yield of directly isolated splenic DC from HIV^+ spleens (cocktail-negative, HLA-DR^+ large cells after immunomagnetic depletion of $CD3^+$ $CD19^+$ $CD56^+$ cells) was variable but seemed to be higher than that of mature DC, ranging from 0.09 to 0.49% of unseparated mononuclear splenocytes (mean, 0.25%, SD, 0.16%; $n = 6$).

Mixed lymphocyte reaction. A mixed lymphocyte reaction using mature DC-enriched stimulatory cells from patient 7 was performed (Fig. 5). Low-density cells from the metrizamide gradient, which were enriched for dendritic cells at 9%, stimulated the proliferation of allogeneic peripheral blood leukocytes from a different donor with a stimulation index of 12. The number of cells necessary to obtain half of this maximal effect was 2×10^4 low-density cells (i.e., 200 times less than the number of effector cells), compared with 2×10^5 adherent cells (70% $CD14^+$ macrophages). High-density cells from the same gradient, which contained a majority of small lymphocytes, reached a stimulation index of only 8 with 2×10^6 cells. This result shows a normal T-cell stimulatory function in a DC-enriched population for this patient.

Frequencies of infection of splenic DC and other cell types. The proportion of cells containing HIV proviral DNA in different populations from the spleens of eight HIV^+ patients was determined by limiting-dilution PCR. Results from a representative patient (patient 13) are shown in Fig. 6, and the results for all patients are shown in Fig. 7. For all cell populations apart from immunomagnetically selected $CD19^+$ cells, HIV DNA was always detected in some replicates at 10^4 cells per replicate. The frequency of positive replicates always decreased with the number of cells per replicate, proving that the HIV DNA detected originated from the cell population under analysis and not from extraneous contamination. Length polymorphism in the V1V2 region of *env* sometimes gave rise to products differing from the 320 bp predicted from the HIV-LAI sequence (not shown) as was seen in previous quasispecies studies (8). In some such cases, two bands were present in replicates containing many infected cells, whereas a single band, corresponding to one of the two, was amplified at lower cell concentrations. Although these bands were not extracted and sequenced, this observation indicates that in clinical samples, as well as in control experiments, DNA from a single infected cell was detected with this technique.

(i) Spleen mononuclear cells. Among the unseparated mononuclear cells (Fig. 7A), an average of 1/1,450 were positive for HIV provirus (range, 1/320 to 1/6,100; frequency of infected cells [f] = 3.2×10^{-3} to 1.6×10^{-4}).

(ii) $CD4^+$ lymphocytes. Of the cell populations analyzed, $CD4^+$ lymphocytes were by far the most heavily infected cell type, with an average frequency of 1/52 (range, 1/17 to 1/190, $f = 5.9 \times 10^{-2}$ to 5.4×10^{-3}; Fig. 7B and C). When multiplied by the proportion of $CD4^+$ lymphocytes in the spleen (Table 1), this frequency accounted for the infection in the total spleen mononuclear population.

(iii) Macrophages. Surprisingly, $CD14^+$ splenic macrophages sorted by FACS as controls for day 0 DC (Fig. 7B) were very rarely infected, with frequencies too low to be quantified with accuracy (1/22,000 to 1/130,000; $f = 4.5 \times 10^{-5}$ to 7.6×10^{-6}, weighted mean, 1/33,000).

(iv) DC. In sorted day 0 DC (Fig. 7B), HIV DNA was present in 1/3,000 cells (range, 1/760 to 1/18,000; $f = 1.3 \times 10^{-3}$ to 5.5×10^{-5}). This result was found in DC purified after mechanical dissociation of the spleen. For patient 16, a similar frequency of infection was found in day 0 DC obtained after enzymatic dissociation of the debris (1/6,500) as in day 0 DC obtained after mechanical dissociation (1/3,900). After culture for 48 h, the infection frequency of day 0 DC did not change significantly (Fig. 3 and 7E). However, HIV proviral DNA was present in 1/720 to 1/26,000 (weighted mean, 1/972; $f = 1.4 \times 10^{-3}$ to 3.8×10^{-5}) sorted mature (day 2) DC (Fig. 7C). These values were higher (Fig. 7D) and, as discussed below, less accurate than the frequencies seen in day 0 DC.

Were DC really infected or only contaminated with infected $CD4^+$ T lymphocytes? It is possible for $CD4^+$ T lymphocytes to coenrich with DC during FACS, since DC as well as Langerhans cells may form clusters with T cells (26). In day 0 DC,

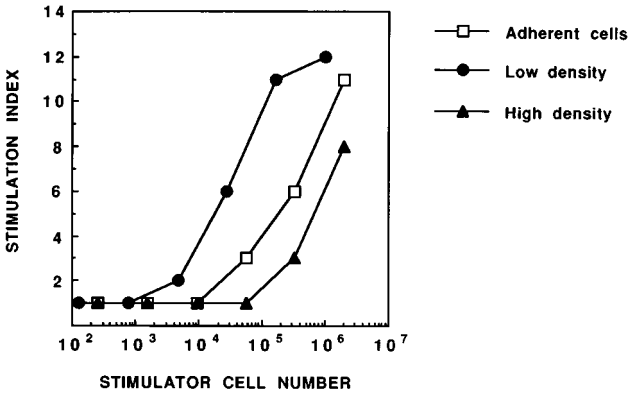

FIG. 5. Mixed lymphocyte reaction. Peripheral blood mononuclear cells from a normal donor were cultured for 4 days in the presence of irradiated cell populations from HIV^+ patient 7. Low-density cells from the metrizamide gradient were enriched for mature DC at 9%. High-density cells from the same gradient were used as controls. Adherent cells were enriched for $CD14^+$ macrophages at 70%. [^{3}H]thymidine incorporation was measured. Results are presented as stimulation indices (average cpm in tested wells/average cpm in six control wells cultured without stimulating cells, the value for which was 1,366 ± 210 cpm).

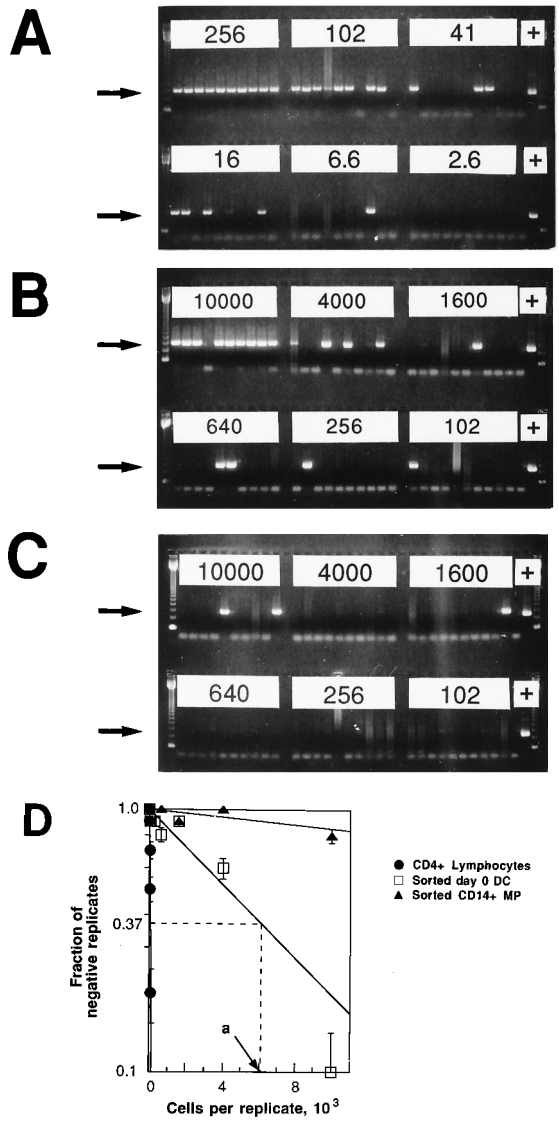

FIG. 6. Comparison of HIV infection frequencies in purified cell populations from patient 13. Migration of amplification products from serial dilutions of purified $CD4^+$ lymphocytes (A), FACS-sorted day 0 DC (B) and FACS-sorted day 0 $CD14^+$ macrophages (C). Purified cells were diluted in a suspension of uninfected Daudi cells. Each cell dilution was divided into 10 replicates, which were lysed in proteinase K lysis buffer. After heat inactivation of proteinase, HIV DNA present in the lysate was amplified under hot start conditions using HIV-1 *env* specific primers. Five microliters of the product of this reaction was reamplified by using nested primers, and the products from the second amplification were allowed to migrate through a 2% agarose gel. Labels show the number of cells per well in the replicates from which HIV *env* DNA was amplified. +, positive control (DNA isolated from U1 cells). Migration of amplification products from uninfected cells alone (negative control) is shown in the lane to the left of the positive control. Arrows indicate the positions of HIV *env*-specific amplification products. (D) Limiting-dilution analysis of the results shown in panels A to C. Calculations were performed and graphs were prepared as indicated in Materials and Methods. The number of day 0 DC among which one infected cell is found (*a*) is indicated. Infection frequencies (1/*a*) were 1/67 for $CD4^+$ T lymphocytes, 1/6,100 for day 0 DC, and 1/62,000 for $CD14^+$ macrophages (MP).

these clusters should be efficiently depleted by the erythrocyte rosetting and the anti-CD3 magnetic beads. Any such clusters remaining in the presort population would stain cocktail positive (with the anti-TCRαβ monoclonal antibody) and would therefore not be sorted with cocktail-negative day 0 DC. Moreover, day 0 DC had a round shape without dendrites, in contrast to mature DC. This question was examined (Table 3) by calculating the maximal theoretical infection frequencies that might be due to $CD4^+$ T-cell contamination in the sorted day 0 DC populations in all experiments for which enough data were available. The percentage of $CD4^+$ T lymphocytes in the presort population was calculated as the percentage of TCR^+ cells in the presort population × CD4/CD8 ratio in the spleen mononuclear cells (from $TCR\alpha\beta^+$, $CD4^+$, and $CD8^+$ lymphocyte analysis by FACS in the respective populations). It was assumed that $CD4^+$ T cells were present in sorted day 0 DC in the same proportion as in the presort population, even though $TCR\alpha\beta^+$ cells had been excluded by sorting. Given this assumption, contaminating infected $CD4^+$ T cells might account for the frequency of infection found in day 0 DC in patient 11 but not in patients 12 and 16. Indeed, the maximal calculated infection frequency due to $CD4^+$ T cells was in the order of 10^{-5} in these two experiments, whereas the DC infection frequencies were 1/18,000 and 1/3,700, respectively. Therefore, it must be concluded that at least in these two patients, DC were infected.

Conversely, in mature DC, it was impossible to rule out a $CD4^+$ lymphocyte contamination. The purification procedure excluded lymphocytes during the metrizamide gradient separation, but 0 to 12% small $CD4^+$ cells were still found in the low-density cells. Sorting excluded small cells; up to 15% of the $CD19^-$ $HLA\text{-}DQ^+$ cells that had been sorted as single FACS events had a small, round cell entrapped in their dendritic processes. Contamination of mature dendritic cells by $CD4^+$ lymphocytes is best exemplified in patient 11 (Fig. 7B and C). This patient had 1/11,000 infected day 0 DC and a high infection frequency of $CD4^+$ T lymphocytes (1/17), which could account for the infection apparently found in the sorted mature DC population (frequency as high as 1/720), considering that the presort population contained 12% $CD4^+$ T lymphocytes. This was confirmed by an apparent infection frequency of 1/1,300 of the control $CD19^+$ population sorted at the same time as $CD19^-$ dendritic cells, while as expected, no HIV DNA could be detected in immunomagnetically purified $CD19^+$ B lymphocytes from two other patients (data not shown).

Compared with the other purified cell populations, splenic DC were in each patient 8 to 650 times less infected than $CD4^+$ lymphocytes (see the legend to Fig. 7) but were apparently more infected than control $CD14^+$ macrophages. The frequency of infection of $CD4^+$ lymphocytes (1/53) accounted for most of the mononuclear splenocyte infection (1/1,300, with 4.5% $CD4^+$ lymphocytes).

DISCUSSION

The data presented above constitute the first flow cytometric characterization of human splenic DC, isolated from the spleens of normal organ donors either directly (day 0 DC) or after 36 h of mononuclear cell culture (mature DC or day 2 DC). Day 0 splenic DC displayed a phenotype very similar to that of blood DC precursors (21). They expressed CD4, as previously noted in directly isolated blood DC, Langerhans cells (36), and thymic DC (35). Day 2 splenic DC were found to closely resemble previously described blood DC, with a more mature phenotype than day 0 DC, including higher expression of HLA class II antigens and of CD80 (one of the ligands for the costimulatory molecule CD28 on T lymphocytes) and the loss of CD4 expression (12, 21). Neither day 0 nor mature splenic DC expressed the CD1a surface molecule characteristic of epidermal Langerhans cells.

DC were enriched from HIV^+ patient spleens. In agreement

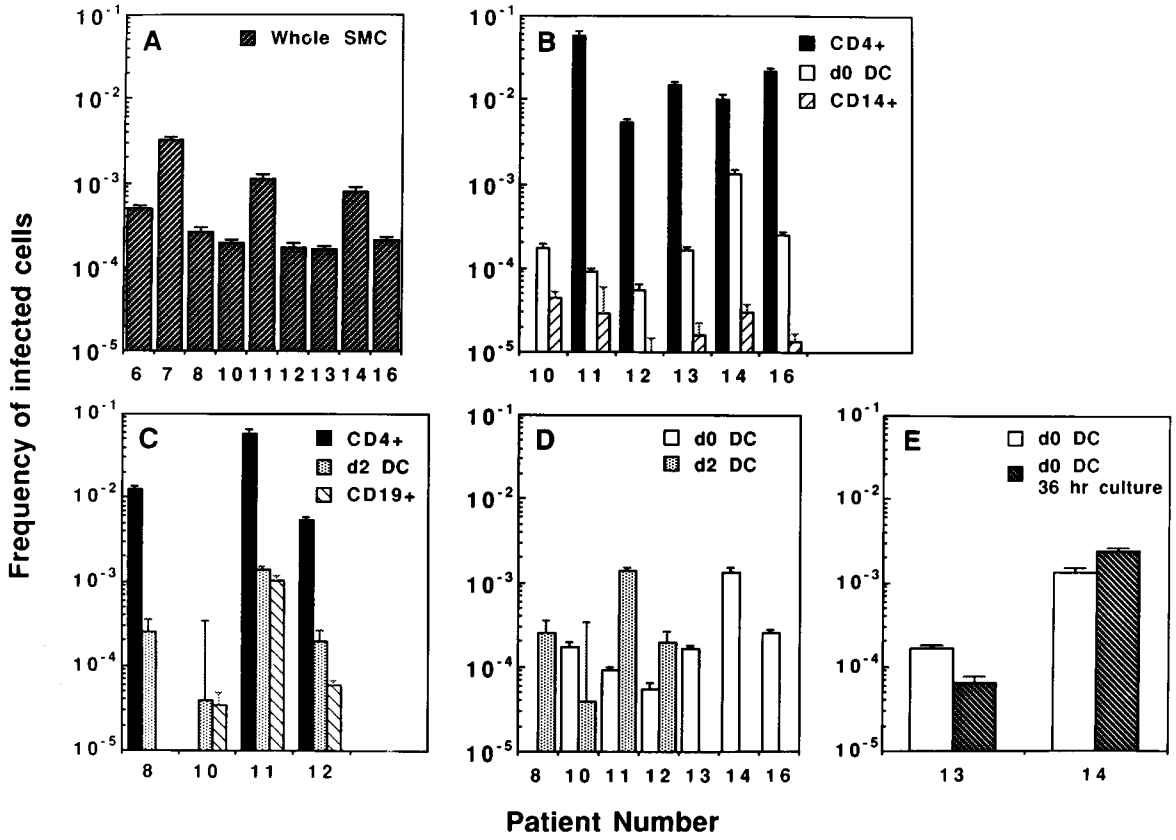

FIG. 7. Frequencies of HIV-infected cells in different cell populations from the spleens of HIV$^+$ patients. DC sorted after direct isolation (day 0 [d0] DC) or mature DC sorted after 2 days in culture (day 2 [d2] DC) were compared with CD4$^+$ T lymphocytes isolated from spleen mononuclear cells on day 0 and with control populations sorted as indicated in Fig. 3. Calculations were performed and graphs were prepared as indicated in Materials and Methods. (A) Unseparated spleen mononuclear cells (SMC). (B) FACS-sorted day 0 DC (d0 DC), CD4$^+$ lymphocytes, and HLA-DR$^+$, cocktail-positive control sorted cells (mostly CD14$^+$ macrophages). Ratios of infected day 0 DC to infected CD4 lymphocytes were 1/654, 1/97, 1/91, 1/8, and 1/86 for patients 11, 12, 13, 14, and 16, respectively. (C) FACS-sorted mature DC (d2 DC), CD4$^+$ lymphocytes, and HLA-DQ$^+$ CD19$^+$ control sorted cells. Ratios of infected mature DC to infected CD4$^+$ lymphocytes were 1/50, 1/42, and 1/27 for patients 8, 11, and 12, respectively. (D) Comparison of FACS-sorted mature DC (d2 DC) and day 0 DC. (E) Day 0 DC before and after culture. DC were enriched directly from the spleens of patients 13 and 14. Enriched populations were then stained, fixed, and sorted by FACS either immediately or after 48 h culture in the presence of monocyte conditioned medium (see figure 3B).

with results obtained in blood DC (3, 14), there was no evidence for a selective depletion of splenic DC in HIV$^+$ compared with HIV$^-$ donors, as far as the yields could show (0.07 $\pm$ 0.03 versus 0.08 $\pm$ 0.05).

Infection was studied by limiting-dilution PCR in cell populations further purified by FACS. The frequency of infected splenic DC ranged from 1/760 to 1/18,000 (mean, 1/3,000). These results were obtained from directly isolated DC, in which contaminating CD4$^+$ lymphocytes were virtually eliminated, as discussed in Results. Conversely, in mature DC, higher infection frequencies (1/720 to 1/26,000; mean, 1/970) were related to contamination with infected CD4$^+$ lymphocytes. After 2 days in culture with a monocyte culture supernatant, day 0 DC acquired the same characteristics as mature DC, and the proportion of infected cells did not change. The data presented in this report are consistent with other results showing that cell populations enriched for blood DC contain very few infected cells (3, 14). Here the limiting-dilution method, with 10 replicates at each cell dilution, yields more accurate values and leads to the conclusion that DC can be infected, but at most at an average frequency of 1/3,000 in the spleens of these HIV-infected patients.

TABLE 3. Calculation of maximal infection frequency from potential CD4$^+$ T-cell contamination in sorted day 0 DC

Patient	Infection frequency		% TCR$^+$ cells in presort population	% CD4$^+$ T cells in SMC[a]	% CD8$^+$ T cells in SMC	CD4/CD8 in SMC	% CD4$^+$ T cells in presort population (calculated)	Maximal infection frequency from potential CD4$^+$ T-cell contamination in sorted day 0 DC (calculated)
	Day 0 DC	CD4$^+$ T cells						
10	1/5,900	ND[b]	0.5	<1	32	<1/30		
11	1/11,000	1/17	1	6	22	1/4	0.25	1/6,800
12	1/18,000	1/190	2.5	2	42	1/20	0.12	1/152,000
16	1/3,700	1/36	0.5	2	28	1/14	0.036	1/100,800

[a] SMC, spleen mononuclear cells.
[b] ND, not determined.

The methods that were used for DC isolation may have selected against some types of DC. The major concern was that some resident DC might have been left in the debris after mechanical dissociation of the spleens. However, parallel enzymatic dissociation of the debris followed by enrichment gave DC that had an entirely comparable infection frequency (and the same phenotype) as the DC obtained by mechanical dissociation. Another concern was the depletion of FcR^+ cells by panning (32). However, in one experiment, FcR^+ cells further isolated in parallel with FcR^- cells by using similar procedures were rather less infected than FcR^- DC (data not shown). Heterogeneity of human DC in terms of morphology, phenotype, and HIV infection was not found or investigated thoroughly in the present work, although it has been noted in other studies in the peripheral blood (25, 34). Langerhans cells isolated from the skins of patients with AIDS were found to contain 100 to 4,000 HIV proviral copies per 10^5 cells (6). Assuming one provirus copy per cell, the frequency of infected Langerhans cells would be of the order of 1/25 to 1/1,000. This study (6) confined itself to postmortem material from patients with AIDS, while in the present work, cells from patients with less advanced disease were analyzed, and the cells were diluted before DNA extraction to determine the frequency of infected cells within a population. While the comparison is imperfect, it would suggest that splenic DC are less infected than Langerhans cells despite the fact that the two types of cells are of the same lineage.

In some ways, the most surprising result in this series of experiments was the finding that at least a subpopulation of splenic $CD14^+$ macrophages, sorted by FACS as a control for DC, were less frequently infected (1/33,000) than DC. More data on the infection frequency of splenic macrophages are currently being collected.

Unseparated spleen mononuclear cells carried HIV proviral DNA with a mean frequency of 1/1,300 (range, 1/320 to 1/6,100). These numbers highlight once again the elevated virus load present in the lymphoid organs of HIV^+ patients (1, 2, 5, 10, 11, 17, 23, 31). A normal spleen can easily contain 10^{10} mononuclear cells, of which, from these results, 10^7 can be expected to be infected. Although it is not certain that these numbers are representative of those found in all HIV^+ patients, the spleens studied here came from patients with different syndromes (Table 1), and similar infection frequencies were found in all cases, indicating that they were not related to one particular syndrome.

As in the blood (37) and other lymphoid organs (23), practically all of the viral load in these spleens was contained within $CD4^+$ T lymphocytes. Indeed, this cell type was the most frequently infected in all the patients, with a mean of 1/53 (range, 1/17 to 1/190) harboring HIV provirus. When the $CD4^+$ lymphocyte infection frequencies were multiplied by the percentages of $CD4^+$ lymphocytes in spleen mononuclear cells, calculated values for the infection of total spleen mononuclear cells were at least as high as the observed values.

In conclusion, $CD4^+$ T-lymphocyte infection accounted for most of the HIV DNA in mononuclear splenocytes. DC seemed to harbor HIV, but at least 10 to 100 times less frequently than $CD4^+$ T lymphocytes. They are unlikely to serve as a major source of HIV infection in lymphoid organs.

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