Impact of bone marrow hematopoiesis failure on T-cell generation during pathogenic simian immunodeficiency virus infection in macaques

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Experimental infection of macaques with pathogenic strains of simian immunodeficiency virus (SIV) represents one of the most relevant animal models for studying HIV pathogenesis. In this study, we demonstrated a significant decrease in the generation of CD4$^+$ T cells from bone marrow (BM) CD34$^+$ progenitors in macaques infected with SIVmac251. This decrease appears to result from changes in the clonogenic potential of BM progenitors of both the myeloid and lymphoid lineages. We also demonstrated a significant decrease in the numbers of the most immature long-term culture-initiating cells (LTC-ICs). Hematopoietic failure occurred as early as primary infection, in the absence of CD34$^+$ BM cell infection and was not related to plasma viral load. No major change was observed in the phenotype of BM CD34$^+$ cells from infected macaques, including apoptosis markers such as annexin V staining and BcL-2 expression, but a significantly higher that normal proportion of cells were in the G_0/G_1 phase. This is the first demonstration that failure of BM hematopoiesis results in impaired T-cell production, which may contribute to the disruption of T-lymphocyte homeostasis characteristic of pathogenic lentiviral infections in primates. (Blood. 2005;105:2403-2409)

Introduction

HIV type 1 (HIV-1) infection typically involves a progressive loss of blood CD4$^+$ T lymphocytes, associated with functional T-cell abnormalities and immunodeficiency. There may be several reasons for the decrease in CD4$^+$ T-cell counts, including direct and indirect virus-mediated cell destruction and defective homeostasis.[1,2] The half-life of peripheral blood T cells, which is approximately 82 days in healthy individuals, is more than 50 days shorter in HIV-infected patients[3] and T-cell numbers appear to be maintained at high levels principally by an increase in the production of CD8$^+$ T cells.

Different mechanisms have been proposed for explaining defective production of T lymphocytes, including deregulated thymus activity, infection, and depletion of immature thymocytes expressing CD4 and alteration of early progenitors including at the level of bone marrow (BM) CD34$^+$ precursors. To our knowledge, this last point has never been clearly addressed and was therefore the purpose of our work.

Defective de novo production of T lymphocytes by the thymus may make a significant contribution to CD4$^+$ T-cell lymphopenia. In HIV infection, a low proportion of recent thymic emigrants, with episomal DNA formed by rearrangement of the T-cell receptor genes (TREC),[4] is correlated with disease progression, although the value of quantitative TREC measurement in HIV-seropositive patients remains unclear.[5,6] Highly active antiretroviral therapy (HAART) helps to increase the number of CD4$^+$ T cells in the periphery, probably due to both the expansion and redistribution of cell populations present in lymphoid tissues and the de novo generation of T lymphocytes by the thymus.[7-9] T-cell counts are restored to high levels in a biphasic manner following the initiation of HAART. There is an initial rapid increase in the numbers of CD4$^+$ and CD8$^+$ T cells of the memory phenotype (CD45RO$^+$), and then the proportion of naive T cells (CD45RA$^+$CD62L$^+$) increases.[7] Changes in thymus volume and an increase in TREC levels in the periphery[10] also provide evidence that successful HAART increases thymus activity.[11] However, CD4$^+$ T-cell counts remain below normal values in treated patients, suggesting that thymic production is not completely restored and is not necessarily associated with an increase in lymphocyte half-life[3] and the complete restoration of efficient antiviral immunity.

Changes in thymus function in patients infected with HIV-1 result directly from degeneration of the stroma, infection of immature thymocytes, and dysregulation of thymocyte/stromal cell interactions.[12-14] Decreases in the pool of early progenitor stem cells, including the most primitive CD34$^+$ hematopoietic BM cells, may also limit T-cell regeneration.[15,16] Indeed hematologic abnormalities are frequently observed during HIV infection and BM

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dysfunction probably contributes to the observed cytopenia.[17] Intercurrent infections, antiviral drugs,[18] and the antibiotics commonly used in patients with AIDS may all affect the production of blood cells. However, although hematopoietic cells do not seem to undergo direct infection with HIV, they may be directly damaged due to the inhibitory effect of HIV-related proteins[19] or proinflammatory cytokines and chemokines, the production of which is dysregulated in response to HIV infection.[20-22] It remains unclear whether BM failure in infected individuals affects T-cell lymphopoiesis. However, a few studies have provided evidence supporting this hypothesis. (1) In severe combined immunodeficiency (SCID) mice with human tissue grafts, HIV-1 infection interrupts multilineage hematopoiesis, resulting in the depletion of early hematopoietic progenitors even though these cells are not infected.[15,23-25] (2) Decreases in the capacity of adult peripheral blood $CD34^+$ progenitors from patients with HIV to develop T cells have been observed in murine fetal thymus organ culture (FTOC) assays.[26]

Macaques infected with pathogenic strains of simian immunodeficiency virus (SIV) or related chimeras expressing the envelope of HIV-1 (SHIV), constitute a powerful model for studies of the fundamental mechanisms of HIV pathogenesis. Indeed, SIV/SHIV and HIV have similar biologic properties and the infection of macaques with pathogenic SIV/SHIV isolates reproducibly induces an immunodeficiency strikingly similar to that observed in human AIDS.[27] As in HIV-seropositive patients, hematologic disorders are commonly observed in macaques infected with SIV[28,29] and T-lymphocyte turnover also appears to be rapid.[30] Virus DNA is detected in the thymus and significant changes in this tissue are observed during primary infection.[31,32] Finally, $CD3^-CD4^+CD8^-$ intermediate single-positive (ISP) thymocytes were found to be functionally impaired in murine FTOC assays after in vivo infection with SIVmac251.[33] We and others demonstrated that BM hematopoiesis is severely damaged in primate models of AIDS.[28,34] In the work presented here, we try to address in SIVmac251-infected macaques the relationship between alteration of early BM progenitors and de novo production of T cells.

Materials and methods

Animals and virus

Adult male cynomolgus macaques (*Macaca fascicularis*) weighing 3.5 to 6 kg were imported from Mauritius and housed in single cages within level 3 biosafety facilities, in accordance with national guidelines. Animals were handled under ketamine chlorohydrate anesthesia (Rhone-Mérieux, Lyon, France). All experimental procedures were conducted in accordance with European guidelines for animal care (Journal Officiel des Communautés Européennes, L358, 18 décembre 1986). The pathogenic SIVmac251 was kindly provided by Dr A. M. Aubertin (Laboratoire de Virologie, Université Louis Pasteur, Strasbourg, France). The virus was isolated from macaque peripheral blood mononuclear cells (PBMCs) cocultured with a spleen homogenate from a rhesus macaque infected with SIVmac251 (provided by Dr R. C. Desrosiers, New England Regional Primate Center, Southborough, MA). The cell-free stock was obtained by a second passage of the isolate in macaque PBMCs. The in vivo titer of the resulting stock after intravenous inoculation was 40 000 AID_{50} (50% animal infectious dose) per milliliter. We injected 50 AID_{50} into the saphenous vein of the monkeys.

Culture of BM hematopoietic myeloid cells

BM mononuclear cells were obtained from the iliac crest by aspiration and isolated by standard Ficoll density-gradient centrifugation (MSL, Eurobio, Les Ulis, France). Cells were washed once in 3% fetal calf serum (FCS; Boehringer-Mannheim, Mannheim, Germany) in phosphate-buffered saline (PBS) and resuspended in PBS for subsequent staining. T cells were eliminated by an immunomagnetic sorting system (MACS; Miltenyi Biotec, Paris, France) according to the manufacturer's instructions, using anti-CD2 (clone MT910; Dako, Trappes, France) and anti-CD3ϵ (clone SP34; BD PharMingen, San Diego, CA) monoclonal antibodies (mAbs). The resulting fraction, depleted of $CD3^+$ and $CD2^+$ cells, was washed twice in Dulbecco modified Eagle medium (DMEM) Glutamax supplemented with 10% FCS (Roche, Meylan, France) and 1% commercial antibiotic solution (penicillin-streptomycin-neomycin sulfate [PSN]; Gibco, Courbevoie, France). The fraction was then enriched in $CD34^+$ cells by positive immunomagnetic selection (Dynal, Compiègne, France), using an anti-CD34 mAb (clone 563, BD PharMingen), accordingly to the manufacturer's instructions.

$CD34^+$ mononuclear BM cells (5×10^4) were then suspended in 1 mL Methocult HF4434 medium (StemCell Technologies, Meylan, France) in 35-mm Petri dishes. Cells were incubated at 37°C for 14 days and scored by examination under an inverted microscope for granulocyte-macrophage colony-forming units (GM-CFUs), granulocyte colony-forming units (G-CFUs), macrophage colony-forming units (M-CFUs), and erythrocyte burst-forming units (BFU-Es).

Long-term culture-initiating cells (LTC-ICs) were obtained as follows. Feeder MS-5 stromal cells were cultured in 96-well plates at a density of 6×10^3 cells/well, in 100 μL long-term culture medium per well (Myelocult GF4434; StemCell Technologies) to which 10^{-6} M hydrocortisone hemisuccinate (StemCell Technologies) had been added. MS-5 cultures were incubated for 24 hours at 37°C under an atmosphere containing 5% CO_2 and were then placed in coculture with $CD34^+$ BM cells. A limiting-dilution coculture method was used; $CD34^+$ cells were added to MS-5 cultures, with 200, 100, 50, or 20 cells/well added to 20 wells for each dilution point. Cocultures were initiated in long-term Myelocult H5100 medium (StemCell Technologies) supplemented with 10^{-6} M hydrocortisone hemisuccinate. We replaced half the medium each week and cultures were maintained for 5 weeks. The entire content of each well was treated with trypsin (Eurobio) for 5 minutes and then plated in 1 mL Methocult GF4434 medium in 35-mm Petri dishes. Cells were incubated at 37°C in an atmosphere containing 5% CO_2 for 14 days and for the number of CFUs present was then determined under an inverted microscope.

Coculture of thymic stromal cells and purified $CD34^+$ progenitors

Human thymus was obtained from newborns aged 2 to 15 days who had undergone cardiac surgery; thymic cells were isolated as previously described.[35] Briefly, the thymus was ground into small fragments and subjected to 8 rounds of digestion as follows: 45 minutes at 37°C with gentle shaking, in serum-free DMEM (Gibco) supplemented with 500 μg/mL collagenase (Sigma Chemicals, St Louis, MO) and 1 μg/mL DNAse (Sigma Chemicals). During the first 4 rounds of digestion, the thymic cells were nonadherent. The last 4 digestion mixtures were enriched in adherent stromal cells. These cells were washed 3 times in DMEM Glutamax (Gibco) supplemented with 10% FCS (Roche), and 1% commercial antibiotic solution (PSN; Gibco). After digestion, each thymus provided approximately 0.3×10^8 to 1×10^9 cells, which were stored frozen at −135°C in 10% dimethyl sulfoxide FCS (Roche).

For culture experiments, cryopreserved stromal cells were thawed and washed in DMEM Glutamax medium (Gibco) supplemented with 10% FCS (Roche) and 1% PSN (Gibco). Viable cells were purified by Ficoll density-gradient centrifugation (MSL2000; Eurobio). Selective depletions were performed to eliminate contaminating thymic progenitors, together with immature and mature thymocytes. $CD34^+$ cells were removed with immunomagnetic beads (Dynal) coupled to the 563-A2 mAb (Dynal); thymocytes were then removed with anti-CD7 (BD PharMingen), anti-CD3ϵ (clone SP34; BD PharMingen), and anti-CD3 (clone FN18; Biosource, Montrouge, France) mAbs and a commercial immunosorting system (MACS; Miltenyi Biotec). The negative fraction, depleted of $CD34^+$, $CD7^+$, and $CD3^+$ cells, was resuspended at a concentration of 2.5×10^6 cells/mL in 100 μL DMEM Glutamax (Gibco) supplemented with 10% FCS (Roche) and 1% PSN (Roche), and cultured at 37°C in 96-well plates (Becton Dickinson, Erembodegem, Belgium) in a humidified atmosphere containing 5% CO_2. On day 1, culture wells were washed with

complete medium to remove nonadherent cells. On day 2, the culture medium was replaced with fresh medium supplemented with 5 μg/mL mycophenolic acid (MPA; Sigma Chemicals). MPA was maintained in the medium for 4 days to eliminate residual proliferating progenitors and thymocytes, as previously described.[36] A confluent monolayer of thymic stromal cells was obtained after 8 to 10 days of culture. This cell monolayer was then used to study the differentiation of exogenous simian $CD34^+$ progenitors into T cells. We validated this culture approach by checking that differentiated thymocytes were not generated by the replication of cell populations contaminating the purified $CD34^+$ progenitor fraction. Simian BM mononuclear cell preparations depleted of $CD2^+$ and $CD3^+$ and enriched in $CD34^+$ progenitors by means of an immunomagnetic method consistently displayed a high level of purity; the mean proportion of $CD34^+$ cells obtained in 13 independent experiments was 97.52% ± 1.23%. Thus, 2×10^5 $CD34^+$-enriched cells were seeded in each culture well, and approximately 400 $CD34^-$ BM cells contaminated each coculture. We therefore checked that cocultures containing 400 cells/well of the $CD34^-CD3^-CD2^-$ fraction after BM cell purification did not generate a significant proportion of thymocytes after 16 days of culture (0.50% ± 0.30 of $CD3^+$ cells).

For coculture assays, highly purified $CD34^+$ simian BM cells were resuspended at a concentration of 10^5 cells/mL in DMEM Glutamax (Gibco) supplemented with 10% FCS (Roche), 1% PSN (Roche), and growth factors supporting lymphopoiesis: 10 ng/mL interleukin 7 (IL-7; StemCell Technologies), 10 ng/mL stem cell factor (SCF; StemCell Technologies), and 10 ng/mL Flt3-L (StemCell Technologies). We added 200 μL (2×10^4 $CD34^+$ cells) of this suspension to each of 72 wells of a 96-well plate containing a monolayer of thymic stromal cells at confluence. The remaining 24 wells were used as controls and contained stromal cells alone. We replaced half the culture medium each week, without shaking the culture. Every 4 days, we used the contents of 2 of the wells for the phenotyping of thymocyte populations. We harvested the nonadherent and adherent cell fractions. Adherent cells were detached with a commercial dissociation solution (Sigma).

Phenotype determination and characterization of apoptotic cells

The cells harvested from the coculture were washed twice, resuspended in 100 μL 1% FCS (Roche) in PBS (Gibco, France) and then incubated for 30 minutes at 4°C with 10 μL selected mAbs for 2- or 3-color membrane staining. The following antibodies were used: phycoerythrin (PE)–conjugated anti-IgG1 (clone 679.1Mc7, Beckman Coulter, Villepente, France), fluorescein isothiocyanate (FITC)–conjugated IgG1 (clone 679.1Mc7, Beckman Coulter), or allophycocyanin (APC)–conjugated total IgG (Beckman Coulter) as isotypic controls and PE-conjugated anti-CD2 (clone MT910; Dako), PE-conjugated anti-CD3ϵ (clone SP34; BD PharMingen), a simian-specific PE-conjugated anti-CD3 (FN18; Biosource), APC-conjugated anti-CD4 (clone MT310; Dako), FITC-conjugated anti-CD7 (clone 563-A2; PharMingen), FITC-anti-CD8 (clone DK25; Dako), APC-conjugated anti-CD4 (clone MT310; Dako), FITC-conjugated anti-CD8 (clone DK25; Dako), FITC-conjugated anti-CD11b (clone ICRF44; BD PharMingen), PE-conjugated anti-CD14 (clone M5E2; BD PharMingen), PE-conjugated anti-CD34 (clone 563-A2; BD PharMingen), FITC-conjugated anti-CD38 (clone OKT10; Ortho Diagnostic Systems, Raritan, NJ), FITC-conjugated anti-CD90 (Beckman Coulter, Hialeah, FL), anti-CD1a (MT102 clone; BD PharMingen), anti–HLA-DR (G46-6 clone; BD PharMingen), anti-CXCR4 (12G5 clone; BD PharMingen), and anti-CCR5 (3A9 clone; BD PharMingen). Cells were washed twice and fixed in CellFix solution (Becton Dickinson) for 3 days before analysis in a Becton Dickinson fluorescence-activated cell sorting (FACS) apparatus (Becton Dickinson), with CellQuest software (Becton Dickinson).

Bcl-2 expression was studied by flow cytometry. $CD34^+$ cells were fixed and permeabilized, using a commercial assay (Ortho Permeafix; Ortho Diagnostic Systems). Permeabilized cells were stained with a monoclonal FITC-conjugated anti–Bcl-2 antibody (clone 124; Dako Diagnostic, Zug, Switzerland). Annexin V conjugated to FITC and propidium iodide (PI)–conjugated PE (Annexin-V Kit, BD PharMingen) were used to quantify apoptotic $CD34^+$ cells. A commercial PI-based assay (0.1% Triton X-100, 50 μg/mL PI; Sigma) was used for cell cycle analysis by flow cytometry (LSR; Becton Dickinson), using CellQuest Software (Becton Dickinson).

Detection of viral DNA in mononuclear cells

We extracted cellular DNA with the High Pure PCR Template Preparation Kit according to the manufacturer's instructions (Boehringer Mannheim). DNA was quantified by measuring optical density (Pharmacia Biotech, Science Park, Cambridge, United Kingdom). This method involved carrying out a primary polymerase chain reaction (PCR) amplification with primers specific for the *gag* gene (1386N 5′: GAAACTATGCCAAAAACAAGT and 2129 5′: TAATCTAGCCTTCTGTCCTGG). We used an automated thermocycler (Crocodile III, Appligene, Illkirch, France) and the following conditions: 3 minutes at 94°C, followed by 40 cycles of 45 seconds at 94°C, 2 minutes at 56°C and 1 minute 30 seconds at 72°C, and a final heating at 72°C for 10 minutes. The reaction mixture consisted of 1 μg test DNA, 1 U *Taq* polymerase (Appligene), 10 μl *Taq* polymerase buffer (10 mM Tris [tris(hydroxymethyl)aminomethane)–HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100), 5 mM deoxyribonucleotides (dNTPs), and 30 pmol of each primer in a total volume of 100 μL. A second, nested PCR amplification was performed with 3 μL amplimer inner primers 1731N5′: CCGTCAGGATCAGATATTGCAGGAA and 2042C; 3′: CACTAGCTGCAATCTGGGTT, in the following conditions: denaturation for 3 minutes at 94°C, followed by 25 cycles of denaturation for 45 seconds at 94°C, annealing for 1 minute 30 seconds at 56°C and extension for 1 minute at 72°C, and a final heating at 72°C for 10 minutes. The PCR products were visualized by electrophoresis in a 1.5% agarose gel containing ethidium bromide. The complete PCR was run on limiting dilutions of the initial stock of DNA. Copy numbers were determined by comparison with a standard stock of DNA from CEMX174 cells containing a known number of copies of the integrated SIVmac251 DNA. The sensitivity of the method was estimated at one copy of viral DNA in 10^5 cells.

Plasma viral load

Plasma viral load was determined as we previously described.[34] Briefly, viral RNA was extracted with a kit (High Pure Viral RNA Kit; Roche, Mannheim, Germany) from 200 μL plasma collected into EDTA (ethylenediaminetetraacetic acid), according to the manufacturer's instructions. The extracted RNA was stored frozen at −80°C. We subjected 10 μL of the extracted material to reverse transcription (RT) and PCR for amplification of a region of *gag*. The reaction mixture contained 25 IU MuLv reverse transcriptase, 0.2 mM each of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), 0.19 mM dTTP, 0.01 mM digoxigenin-11-deoxyuridine triphosphate (dUTP) (PCR DIG labeling mix; Roche), 2 IU ribonuclease inhibitor (RNasin, Promega, Madison, WI), 0.5 IU *Taq* DNA polymerase (Roche), 20 nM of each primer (5′-GTGCTGTTGGTCTACTTGTTTTTG-3′ and 5′-ATGTAGTATGGGCAAACGAAT-3′), with H_2O making the final volume up to 50 μL. Amplification was performed in a Crocodile III thermocycler (Appligene): 42°C for 25 minutes, 94°C for 5 minute, 35 cycles of 94°C for 35 seconds, 60°C for 45 seconds and 72°C for 1 minute, then 5 minutes at 60°C, and a final 5 minutes at 72°C.

We used 10 μL of the amplification product for quantification by anti-DIG enzyme-linked immunosorbent assay (PCR ELISA DIG detection; Roche) according to the manufacturer's instructions, using the following probe, at a concentration of 20 nM: 5′-CATTTGGATTAGCAGAAAGCCTGTTGGAGAACAAAGAAGGATGTCAA-3′. Samples were tested in duplicate and two 10-fold dilutions were tested for each sample. Positive controls consisted of a plasma sample previously quantified by 2 different approaches (RT-PCR and bDNA; Bayer, Mannheim, Germany). In the standard was a culture supernatant containing free SIVmac251 that had been aliquoted and mixed with negative EDTA-plasma from macaques. Plasma virus concentration was estimated by 2 different approaches (quantitative RT-PCR with limiting dilutions and the bDNA assay; Bayer).

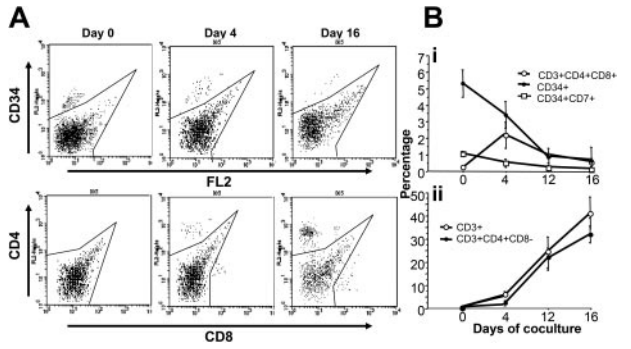

Figure 1. Changes in thymocyte populations in cocultures of CD34$^+$ simian BM cells from healthy and uninfected macaques and human thymic stromal cells. (A) Flow cytometry diagrams of a representative coculture for CD34 or CD4 and CD8 expression. On day 0 of coculture, 2×10^4 purified BM CD34$^+$ cells were seeded on a stromal monolayer. At each time point, all of the cells present in the culture well were used for flow cytometry. (Bi) Changes in CD34$^+$ (●), CD34$^+$CD7$^+$ (□), or CD3$^+$CD4$^+$CD8$^+$ (○) populations in 4 independent experiments using BM CD34$^+$ cells obtained from 4 different cynomolgus macaques. (ii) Changes in CD3$^+$ (○) or CD3$^+$CD4$^+$CD8$^-$ (●) in the same 4 cultures; simian BM CD34$^+$ cells were seeded on day 0.

Statistical analysis

Paired and unpaired comparisons were made, using the nonparametric Wilcoxon rank test and the Mann-Whitney test, respectively, both of which are suitable for the analysis of small samples. Correlations were analyzed with the Spearman rank correlation test. All statistical analyses were carried out with StatView software (SAS Institute, Cary, NC).

Results

Defective de novo generation of CD4$^+$ T cells from CD34$^+$ BM progenitors from SIV-infected macaques

We demonstrate here, for the first time, that the clonogenic potential of BM hematopoietic progenitors to develop into mature CD4$^+$ T lymphocytes is affected during lentiviral infection in primates. We developed an in vitro model of thymocyte differentiation from fractions of cynomolgus macaque BM enriched in CD34$^+$ cells cocultured with thymic stromal cells of human origin. This system can be used for the qualitative and quantitative follow-up of thymocyte differentiation in vitro, which is more difficult to achieve with organ culture-based techniques. In our study, cultures of progenitors from healthy, uninfected macaques (Figure 1A) generated few double-positive CD4$^+$CD8$^+$ cells (0.53% ± 0.26%). However, significant numbers of CD3$^+$ and CD3$^+$CD4$^+$CD8$^-$ T lymphocytes were obtained (40.64% ± 6.99% and 31.46% ± 3.56%, respectively) at the end of the coculture period (Figure 1A-Bii). Concomitantly, seeded BM CD34$^+$ cells (Figure 1Bi) rapidly disappeared and were only barely detectable after day 16 (mean ± SD: 5.31% ± 0.81% to 0.66% ± 0.83%). These results are consistent with previously reported data obtained in similar culture systems.[37] We compared the differentiation patterns of BM CD34$^+$ cells obtained from 6 uninfected macaques with those obtained from 8 SIVmac251-infected macaques. As expected, SIV infection in these animals resulted in significant lymphopenia (1482 ± 662 cells/μL versus 3281 ± 845 before infection; $P = .0003$) and a decrease in the number of circulating CD4$^+$ T lymphocytes (197 ± 158 cells/μL versus 564 ± 299 before infection; $P = .0013$) as early as 4 months after infection.

At 6 months after infection, levels of production of CD3$^+$CD7$^+$ cells ($P = .0126$) and single-positive (SP) CD3$^+$CD4$^+$CD8$^-$ mature thymocytes ($P = .0015$) were significantly lower in cultures seeded with progenitor cells from SIV-infected macaques than in those seeded with progenitor cells from uninfected macaques (Figure 2A). This decrease in lymphocyte production was associated with a slower decrease in the number of CD34$^+$ cells in culture ($P = .014$), which may be attributed to a significant delay in BM progenitor differentiation. Indeed, after 1 week of coculture, larger than normal numbers of undifferentiated CD34$^+$CD7$^+$ cells ($P = .0209$), which have been considered to be potential T-cell precursors,[35] mature CD34$^+$CD38$^+$ progenitors ($P = .0209$) or the most immature BM CD34$^+$CD90$^+$ ($P = 0.0357$) cells persisted in cocultures seeded with BM CD34$^+$ cells from SIV-infected macaques (Figure 2B). Although the decrease in SP CD4$^+$ T-cell production was associated with the infection of macaques, no correlation was found with plasma viral load (294 195 ± 586 415 copies of viral RNA/mL; $P = .82$) or cell-associated viral load (1344 ± 1994 copies of viral DNA/10^6 PBMCs; $P = .13$).

In 3 of these animals, we characterized the T-cell differentiation potential of BM CD34$^+$ cells at various time points after infection. Decreases in CD4$^+$ T-lymphocyte production were demonstrated as early as 3 weeks after the intravenous infection of macaques with pathogenic SIVmac251. At 6 months after infection, the production of CD3$^+$CD4$^+$CD8$^-$ T cells had decreased by 43% (Figure 2C). This

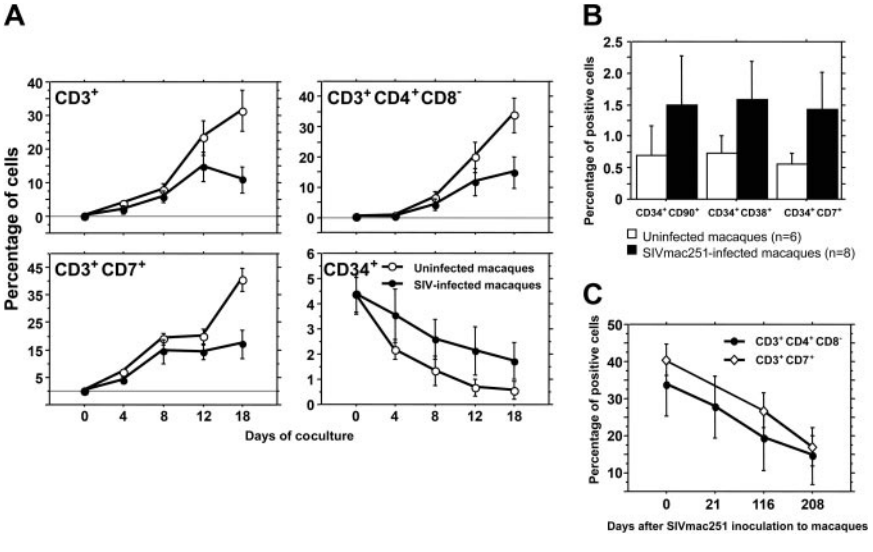

Figure 2. Generation of T cells from BM CD34$^+$ cells obtained from SIV-infected macaques. (A) Differentiation of simian CD34$^+$ BM after 16 days of coculture with human thymic stromal cells; ○, mean value (± SD) of cells from 6 uninfected cynomolgus macaques; ●, mean values (± SD) of cells of 8 macaques infected with the primary SIVmac251 isolate. BM cells were obtained 6 months after infection. (B) Proportion of BM progenitors of various phenotypes after 8 days of coculture with human thymic stromal cells; □, mean ± SD of cells from 6 uninfected cynomolgus macaques; ■, mean ± SD of cells of 8 cynomolgus macaques infected with SIVmac251, 6 months after intravenous inoculation. (C) Changes in the T-cell differentiation potential of CD34$^+$ BM cells obtained from 3 cynomolgus macaques (mean ± SD) at various time points after the infection of macaques with SIVmac251.

decrease in the production of T lymphocytes was not associated with the infection of early progenitors. Indeed, viral DNA was not detected by PCR in highly purified BM CD34$^+$ cells obtained from SIV-infected macaques (data not shown). However, despite the absence of detectable infection in purified CD34$^+$ cells seeded in the cocultures, we also checked that SIV *gag*-nested PCR performed on DNA extracted from cultured cells at the end of the culture period was negative (data not shown). We were therefore unable to attribute the decrease in lymphocyte numbers to an increase in viral replication from a minority of contaminating infected cells.

Altered de novo T-cell generation is associated with BM hematopoiesis failure in macaques infected with SIV

We demonstrated that generation of T cells from BM precursors is affected in macaques infected with SIVmac251 and that seems to result from altered differentiation potential of CD34$^+$ precursors. To better understand mechanisms involved, we tried to further characterized BM hematopoiesis in theses animals. The total number of CFUs had already decreased by 21 days after infection (Figure 3), and this decrease became significant 4 months after infection ($P = .0048$). At 6 months after infection (day 208), total CFUs were more than 40% lower than that before infection ($P = .0020$). This early and persistent decrease in the number of CFUs was accompanied by decrease in the numbers of GM-CFUs (59% on day 208 after infection; $P = .0003$) and BFU-Es (43% on day 208 after infection; $P = .0052$; Figure 3). However, as we previously reported in the SHIV model,[38] no significant changes were observed in the number of M-CFUs ($P = .7345$ on day 208 after infection). In the meantime, the number of BM-derived immature cells detected by LTC-IC also decreased in all animals, by a mean of 71% with respect to preinfection values ($P = .020$; Figure 3). Thus, BM myelopoiesis is also affected in SIVmac251-infected macaques and, therefore, altered generation of CD4$^+$ T cells may be related to a more general deregulation of BM hematopoiesis rather than a specific failure of lymphoid precursors.

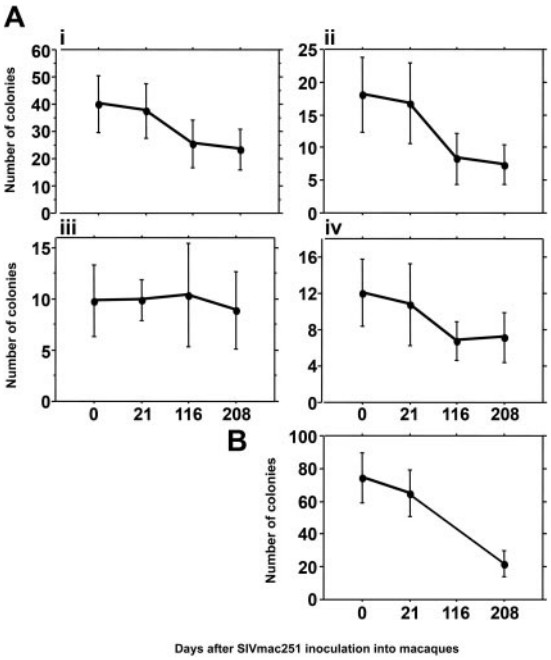

Figure 3. Myeloid differentiation of CD34$^+$ BM. Number of colonies obtained after 14 days of culture in semisolid methyl cellulose medium (mean ± SD of measures for the cultures of cells from 8 macaques). (Ai) Total number of CFUs; (ii) GM-CFUs; (iii) M-CFUs; (iv) BFU-Es. (B) LTC-ICs identified after 5 weeks of coculture with the MS-5 stromal cell line, followed by 14 days of culture in semisolid medium (mean ± SD of measures for cultures of cells from 4 macaques).

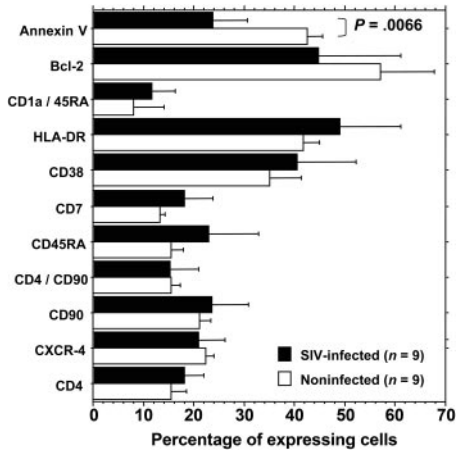

Figure 4. Phenotype and apoptosis markers of CD34$^+$ BM cells. □ indicates mean ± SD for 6 uninfected cynomolgus macaques; ■ indicates mean ± SD for 9 cynomolgus macaques infected with SIVmac251, 6 months after intravenous inoculation.

Phenotypic characterization of BM CD34$^+$ cells in SIV-infected macaques

Changes in the distribution of the BM CD34$^+$ subpopulation may account for the observed changes in hematopoiesis in SIV-infected macaques. However, in our study, the characterization of BM CD34$^+$ cell differentiation markers expression revealed no significant difference that could be used to distinguish cells from uninfected and SIVmac251-infected monkeys (Figure 4). In particular, no difference was observed in the expression of CD4 and CXCR-4. The proportions of cells expressing HLD-DR and CD38 seemed to be slightly higher in infected monkeys. Although this difference did not reach statistical significance, this may reflect the tendency of SIV-infected macaques to accumulate mature progenitors in vivo that may be blocked during differentiation. No differences in the most immature CD34$^+$CD90$^+$ BM cells were observed between infected and uninfected macaques.

Apoptosis and cell cycle arrest at the G_0/G_1 stage have been suggested as possible mechanisms underlying the failure of CD34$^+$ BM hemopoietic cells to differentiate during HIV infection.[19] We therefore evaluated the level of apoptosis in BM cells from infected macaques by flow cytometry, using annexin V to stain the cell surface membrane phosphatidyl serine.[39] The proportion of annexin V-binding cells was significantly lower ($P = .0066$) in CD34$^+$ cells purified from the BM of SIVmac251-infected macaques than in cells from uninfected macaques (Figures 4-5). Bcl-2 is known to increase the survival of progenitor cells.[40] We therefore investigated Bcl-2 levels by immunohistochemical staining. There is a clear tendency to lower percentage of Bcl-2$^+$ CD34$^+$ cells or in the levels of Bcl-2 expression in BM of infected macaques although this not reach statistical significance ($P = .20$, $P = .08$ for mean fluorescence intensity, respectively; Figures 4-5), Thus, apoptosis may play a role in the impaired differentiation of myeloid and lymphoid lineages we observed during SIV infection.

Finally, because we observed that undifferentiated cells my accumulate during differentiation of BM progenitors in T cells (Figure 2B), we investigated cell cycle in CD34$^+$ BM cells freshly isolated from infected macaques. As previously reported for normal human hematopoietic cells exposed to HIV-1 virions or gp120[19] in

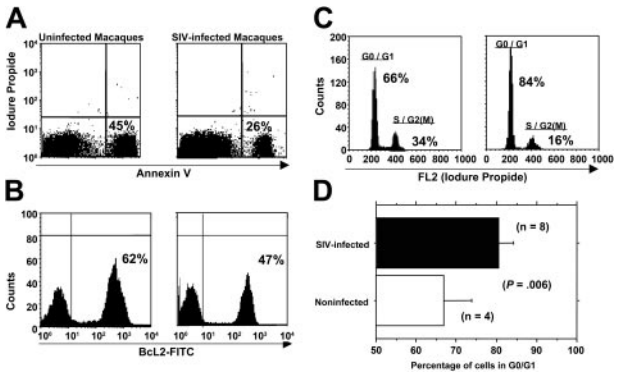

Figure 5. Apoptosis in CD34$^+$ BM cells obtained from SIV infected macaques. (A) Representative flow cytometry diagrams for annexin V staining of BM CD34$^+$ cells obtained from healthy (left panel) and SIV-infected (right panel) macaques. (B) Representative flow cytometry diagrams of intracellular BcL2 staining of BM CD34$^+$ cells obtained from healthy (left panel) and SIV-infected (right panel) macaques. (C) Representative flow cytometry diagrams of intracellular cell cycle analysis (PI staining) of BM CD34$^+$ cells obtained from healthy (left panel) and SIV-infected (right panel) macaques. (D) Percentages of cells in G_0/G_1 in healthy macaques (□) or SIV-infected macaques (■).

vitro, the proportion of cells in the G_0/G_1 stage was significantly higher ($P = .0066$) among CD34$^+$ BM cells obtained from SIV-infected macaques (81% ± 4%) than among those obtained from uninfected macaques (67% ± 7%), as shown by PI staining (Figure 5C-D).

Discussion

To conclude, this study demonstrates that the infection of primates with pathogenic lentiviruses such as SIVmac251 results in rapid and major changes in BM hematopoiesis that affect both myeloid and lymphoid lineages. Changes in the potential of BM cells to differentiate into cells of the lymphoid lineage may be a key element in the disruption of T-lymphocyte homeostasis typical of HIV/SIV infections.

As we and others previously reported, impaired hematopoiesis does not appear to be associated with infection of CD34$^+$ hematopoietic cells, although we cannot exclude that a minority of early precursors are directly affected by the virus.[21,23,34,38,41] We recently reported that the infection of cynomolgus macaques with a pathogenic chimeric HIV/SIV (SHIV) expressing the envelope of the 89.6 HIV-1 primary isolate (SHIV 89.6P) results in a 50% decrease in the number of colony-forming cell progenitors and a more than 70% decrease in the clonogenic potential of the most primitive BM CD34$^+$ LTC-ICs. This reduction was not associated with BM CD34$^+$ cell infection and was not correlated with classical parameters of infection such as plasma viremia and peripheral CD4$^+$ T-cell counts.[34] However, SHIV 89.6P is a dual tropic virus, with CXCR-4 as its principal coreceptor, and the infection of macaques with SHIV 89.6P results in an atypical pathogenic profile, with rapid and persistent depletion of circulating CD4$^+$ T cells. This may render the study of BM hematopoiesis in this model irrelevant to the situation in humans. The infection of macaques with a primary isolate of SIVmac251 induces a pathogenic profile much more similar to that observed in human HIV-1 infection. Like primary HIV isolates and as in early infection stages, SIV uses CCR5 as the coreceptor for entry into target cells. Interestingly, in this study SIVmac251 affects BM myelopoiesis to a similar extent than we previously observed in SHIV-infected animals. The effects of lentiviral infection on macaque hematopoiesis, therefore, do not depend on the viral strain used, the acute pathogenic profile of SHIV89.6P, or the use of CXCR4 or CCR5 as a coreceptor.

The characterization of BM CD34$^+$ cell differentiation marker expression did not reveal dramatic changes in cells from SIVmac251-infected monkeys except a reduced expression of apoptosis marker, suggesting a role for apoptosis in the T-cell homeostasis and an increased proportion of cells in cell cycle arrest. This observation is consistent with the persistence of undifferentiated CD34$^+$ cells we observed in thymic stromal layers seeded with BM progenitors from SIV-infected macaques. The increase in the proportion of cells in the G_0/G_1 phase of the cell cycle, in the absence of an increase in the expression of apoptosis markers, suggests the involvement of soluble factors, including viral proteins or cytokines (or both) released in the medullar microenvironment of infected macaques and which are known to inhibit hematopoiesis. Recombinant viral Tat protein has been reported to induce the production, in normal BM macrophages from enriched preparations, of factors inhibiting the growth of immature hematopoietic progenitors in vitro. Transforming growth factor β1 (TGF-β1) is thought to be the main factor responsible for this inhibition.[42] This observation may have relevance to the situation in vivo because TGF-β1 levels are known to be high in individuals with HIV-1 infection[43] and in culture supernatants of PBMCs from HIV-infected donors.

However, other factors, the production of which is also dysregulated by HIV infection, must also be considered. The chemokine macrophage inflammatory protein 1α (MIP-1α) has been reported to inhibit erythropoiesis specifically[44,45] and several of the cytokines overproduced during the course of HIV infection (IL-1, interferons [IFNs] including IFN-α,[46] tumor necrosis factor α [TNF-α]) may adversely affect hematopoiesis. Consistent with our observation that hematopoiesis is affected early in SIV infection, the inhibitory effect of soluble factors may occur very early on, during primary infection. Indeed, studies in primate models of AIDS have shown that the early phase of pathogenic SIV infection is associated with major inflammation, resulting in the overproduction of cytokines such as TNF-α and IFN-α.[47-49]

Characterization of molecular events that result in cell cycle arrest of BM hematopoietic cells and identification of factors affecting hematopoiesis in the BM microenvironment are therefore of key importance if we are to understand the pathogenesis of HIV infection and to develop therapeutic interventions that may improve the reconstitution of normal immune functions.

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