cervical dysplasia or cancer in the presence of HPV-16 [34]. Thus, it is possible that co-infections with HPV58 and HPV16 or 18 are more likely to cause AIN compared with infection by a single HPV type. On the other hand, HPV types 16 and 18 in MSM were detected in 29% and 11%, respectively, which are in agreement with other studies of Asian HIV-positive MSM. Specifically, the rates for types 16 and 18 in previous studies were 16% and 11% in Beijing, China; 34% and 14% in Beijing and Tianjin, China; and 10% and 8% in Taiwan, respectively. For a given HPV type in the bivalent HPV vaccine, 35% of MSM were infected with the same HPV type in our study. Because the HPV vaccine appears to be safe in HIV-positive men[35], a significant proportion of HIV-positive MSM in Japan may potentially benefit from the vaccine, which might prevent HPV-associated AIN.

Co-infection with multiple oncogenic HPV types has been associated with persistent infection and infection with a longer duration, and it thus represents an important risk factor for anal cancer[2,4,5]. We found that infection with multiple oncogenic HPV types was detected in 54.6% of MSM. The prevalence rates of infection with multiple HPV types in HIV-positive men have been reported to range widely, namely, between 6% and 61%. The difference in rates was thought to be due to the different number of HPV types that are detected by different HPV sample kits. In agreement with two studies that showed 5–10% of HIV-positive heterosexual men were infected with multiple HPV types in the anal canal[11,29], our study revealed that 9% had multiple oncogenic HPV infection.

Previous studies have shown specific risk factors for anal HPV infection including younger age[36,37], HBV infection[38], and positive *C. trachomatis* serology[39]. These findings suggested that sexual transmission has an important role in anal HPV infection. Consistent with these findings, multivariate analysis in our study showed that younger age, MSM, and having $\geq$2 STIs were independently associated with infection by any oncogenic HPV type and multiple oncogenic HPV types. In addition, we found that anal infection with oncogenic and multiple oncogenic HPV types was significantly associated with decreased CD4 counts, high HIV RNA, and short HAART duration, and CD4 $<$100 was an independent risk factor. Some [9,11,37–40], but not all[9,11,38], studies found increased anal HPV infection among HIV-positive men with decreased CD4 counts. Nishijima et al showed that HPV types 16 or 18, low CD4 cell count, and current smoking are associated with an increased risk of anorectal condyloma in HIV-infected patients [41]. These findings and our data indicate that immunosuppression is an important risk factor for anal HPV infection.

There are several limitations to this study. Although we collected information on syphilis, HBV, *C. trachomatis*, and *E. histolytica* infection, information on the number of sexual partners, receptive anal intercourse, condom use, and *Neisseria gonorrheae* infection was not collected. In addition, the number of subjects in this study was relatively large (n = 421), but the study population was a convenience sample of patients attending the Department of Gastroenterology; therefore, it remains unknown if this sample population is representative of the general HIV-positive community.

In conclusion, this prospective, cross-sectional study of Japanese HIV-infected patients demonstrated that approximately two-thirds of MSM and one-fifth of heterosexual men and women had anal oncogenic HPV infection. In the risk analysis, younger age, MSM, CD4 $<$100, and $\geq$2 STIs were independently associated with oncogenic HPV infection and multiple oncogenic HPV types. Our findings support the need for establishing an anal cancer screening system and regular follow-up strategies, especially in HPV high-risk HIV-positive patients, for preventing HPV transmission and detection of early anal neoplasia in Asia as well as Western countries.

Acknowledgments

The authors thank clinical research coordinators Hisae Kawashiro, Sawako Iijima, Yoko Tanigawa, Aiko Gotanda, and Yaeko Sawada for assistance with data collection.

Author Contributions

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Fibrocytes Differ from Macrophages but Can Be Infected with HIV-1

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Fibrocytes (fibroblastic leukocytes) are recently identified as unique hematopoietic cells with features of both macrophages and fibroblasts. Fibrocytes are known to contribute to the remodeling or fibrosis of various injured tissues. However, their role in viral infection is not fully understood. In this study, we show that differentiated fibrocytes are phenotypically distinguishable from macrophages but can be infected with HIV-1. Importantly, fibrocytes exhibited persistently infected cell-like phenotypes, the degree of which was more apparent than macrophages. The infected fibrocytes produced replication-competent HIV-1, but expressed HIV-1 mRNA at low levels and strongly resisted HIV-1–induced cell death, which enabled them to support an extremely long-term HIV-1 production at low but steady levels. More importantly, our results suggested that fibrocytes were susceptible to HIV-1 regardless of their differentiation state, in contrast to the fact that monocytes become susceptible to HIV-1 after the differentiation into macrophages. Our findings indicate that fibrocytes are the previously unreported HIV-1 host cells, and they suggest the importance of considering fibrocytes as one of the long-lived persistently infected cells for curing HIV-1. *The Journal of Immunology*, **2015, 195: 4341–4350.**

Human immunodeficiency virus-1 persists in latent reservoirs despite optimal antiretroviral therapy (ART), which is the major barrier to curing HIV-1. Resting memory CD4$^+$ T cells are the best characterized HIV-1 reservoir (1). However, there is increasing evidence that HIV-1 exists in cells other than the resting memory CD4$^+$ T cells (2–7), which may include CD4$^+$ T memory stem cells (8), monocytes/macrophages (9), bone marrow CD34$^+$ hematopoietic progenitor cells (10, 11), or as yet unrecognized cells. The establishment of HIV-1 infection in bone marrow CD34$^+$ cells is controversial, as recent studies failed to detect infected bone marrow CD34$^+$ cells in patients (12, 13). Interestingly, it was demonstrated that peripheral blood CD34$^+$ cells could be infected with HIV-1 both in vitro (14, 15) and in vivo (16). In the latter study, HIV-1 proviral DNA was frequently detected in the peripheral blood CD34$^+$ cells of HIV-1–infected patients, and in most patients, the number of proviral copies of the peripheral blood CD34$^+$ cell fraction was significantly higher than that of total PBMCs (16). Thus, peripheral blood CD34$^+$ cells are also candidates for latently or persistently infected cells in patients. However, the peripheral blood CD34$^+$ cells are heterogeneous and it is unclear which populations of peripheral blood CD34$^+$ cells are infected with HIV-1.

Interestingly, the newly discovered cells called fibrocytes are present in the CD34$^+$ fraction of peripheral blood (17–20). Fibrocytes are fibroblast-like peripheral blood cells (fibroblastic leukocytes) that are generally defined as CD45$^+$CD34$^+$collagen I$^+$ cells and are shown to migrate to injured tissues as a consequence of the local release of chemokines such as CXCR4 or CCR7 ligand (21–24). Fibrocytes migrated to tissues are distinguishable from fibroblasts, as they express CD45, the pan-hematopoietic marker (17–20). Tissue fibrocytes are also distinguishable from classical macrophages, as they were negative for nonspecific esterase (21). Although fibrocytes comprise a small fraction of peripheral blood cells under normal conditions, accumulating evidence demonstrates elevated levels of the number of circulating fibrocytes in diverse forms of tissue remodeling and fibrosis, as well as chronic inflammation (17–20). Indeed, various mouse models have demonstrated

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Received for publication April 24, 2015. Accepted for publication August 31, 2015.

This work was supported by the grants from the Takeda Science Foundation and the Senshin Medical Research Foundation (to S.S.). E.W.A.E.-W. was supported by Science and Technology Development Fund, Egypt Grant 6385.

The microarray data presented in this article have been submitted to the National Center for Biotechnology Information's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE71290.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ART, antiretroviral therapy; dpi, day postinfection.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1500955

that fibrocytes produce extracellular matrix proteins including collagens, and they thereby contribute to the remodeling and/or fibrosis of injured tissues, including the lungs (25–28), heart (29), bone marrow (30), liver (31), and kidneys (32, 33).

Fibrocytes are unique hematopoietic cells with features of fibroblasts (17–20). However, there is an overlap in the gene expression profiles between fibrocytes and macrophages (34). It has been reported that fibrocytes possess Ag-presenting functions and are potent stimulators of $CD4^+$ and $CD8^+$ T cells (35, 36), suggesting that fibrocytes play a role in the initiation of adaptive immune response. It was also reported that fibrocytes efficiently respond to TLR2, TLR4, and TLR7 ligands as well as poly(I:C) (37), suggesting that they also play a role in the innate immune response. Thus, although detailed lineage tracing studies of fibrocytes have not been carried out, it appears that fibrocytes are cells of myeloid origin. However, the functional relationship between fibrocytes and macrophages has not been fully explored. Moreover, the role of fibrocytes in viral infection is not fully understood.

In this study, given the two HIV-1–related features of fibrocytes, that is, they are present in the $CD34^+$ cell fraction of peripheral blood and there is an overlap in the gene expression profiles with macrophages, we attempted to clarify whether HIV-1 infects fibrocytes and how they respond to HIV-1 infection.

Materials and Methods

Human peripheral blood–derived fibrocytes and macrophages

Peripheral blood was collected from healthy donors, which was approved by the Ethics Committee of Kumamoto University. Written informed consent was obtained from all subjects according to the Declaration of Helsinki. Fibrocytes were prepared according to the previously reported method (21, 22, 38, 39). Briefly, PBMCs were suspended in DMEM/20% inactivated FCS (1×10^6 cells/ml) and seeded in dishes. In this culture method, $CD4^+$ T cells are essential during the initial period as the feeder cells or source of cytokines that induce the differentiation of peripheral blood fibrocytes into mature fibrocytes (22, 33). The culture media were replaced with fresh complete media after extensive wash with PBS to remove nonadherent cells at day 6, and the adherent cells were further cultured. M-CSF–derived macrophages were also prepared as a reference because they potently support HIV-1 replication as described previously (40). Macrophages were also prepared using 10 ng/ml recombinant human GM-CSF (Miltenyi Biotec). In selected experiments, PBMCs were exposed to HIV-1 for 2 h, washed extensively with PBS to remove unbound viruses, and then cultured under conditions that support the differentiation into either fibrocytes or macrophages. Unless otherwise stated, fibrocytes and macrophages were analyzed at days 11 and 5, respectively. In quantitative analyses including HIV-1 replication, cell survival, and cytokine/chemokine production, fibrocytes were detached using trypsin, reseeded so that their number was equivalent to that of macrophages, and cultured for 2 or 3 d.

Flow cytometry

The expression of surface molecules on macrophages and fibrocytes, which were detached from dishes using enzyme-free cell dissociation buffer (Life Technologies), was determined by flow cytometry on an LSR II (BD Biosciences) or FACSVerse (BD Biosciences) using FlowJo software (Tree Star) as described previously (40). The following Abs were used: allophycocyanin-labeled anti–HLA-A/B/C (no. W6/32; BioLegend), PE-labeled anti–HLA-DR (no. LN3; eBioscience), PE-labeled anti–M-CSF receptor (no. 3-4A4; Santa Cruz Biotechnology), FITC-labeled anti–GM-CSF receptor α-chain (no. 4H1; eBioscience), allophycocyanin-labeled anti-CD14 (no. M5E2; BD Biosciences), FITC-labeled anti-CD14 (no. 61D3; eBioscience), PE-labeled anti-CD163 (no. GHI/61; BioLegend), allophycocyanin-labeled anti-CD80 (no. 2D10; BioLegend), PE-labeled anti-CD86 (no. IT2.2; eBioscience), and PE-labeled anti-SLAMF7 (no. 162.1; BioLegend). The phagocytic activity of fibrocytes and macrophages was determined by measuring their uptake of fluorescent microspheres (Fluoresbrite carboxylate microspheres, 0.7 μm in diameter; Polysciences) as described previously (41).

Western blotting

Western blotting was performed as described previously (40). The following Abs were used: anti-Hck (no. 18; BD Transduction), anti-Lyn (no. 42; BD Transduction), anti-phosphotyrosine (no. PY99; Santa Cruz Biotechnology), anti–HIV-1 Gag (no. 65-004; BioAcademia, Japan), and anti-actin (no. C-2; Santa Cruz Biotechnology). The intensity of the bands was quantified using the ImageQuant TL software (GE Healthcare) using actin blot as a loading control.

Cell survival

Cell survival was assessed using MTT reagent as described previously (40). The absorbance of the wells was measured at 595 nm. Liposomal clodronate was purchased from FormuMax Scientific and added to cultures at 1:300 dilution.

Microarray

Total RNA prepared from macrophages or fibrocytes was biotin labeled using a GeneChip 3′ IVT express kit (Affymetrix), and microarray analysis was performed at TaKaRa Bio using high-density oligonucleotide array (Human Genome U133 Plus 2.0) and GeneSpring 12.5 software (Agilent Technologies). Microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GSE71290; http://www.ncbi.nlm.nih.gov/geo).

Cytokine/chemokine production

The relative levels of various cytokines and chemokines in media conditioned by macrophages or fibrocytes were analyzed using a human cytokine array (R&D Systems) according to the manufacturer's instructions. The intensity of the spots was quantified using the ImageQuant TL software as described previously (41).

HIV-1 infection, Gag detection, and viral infectivity assay

Primary viruses were expanded as described previously (42). Recombinant viruses such as JRFL, AD8, and NL(AD8) were prepared using HEK293 cells as viral producer cells (43). HIV-1 replication assay with fibrocytes was performed as described for macrophages (40). Fibrocytes and macrophages were incubated with HIV-1 (100 ng/ml Gag unless otherwise stated) for 2 h at 37°C, washed with PBS to remove unbound viruses, and cultured in complete media. One half of the media was replaced with fresh media every 3 d. The supernatants were analyzed for Gag concentrations by ELISA (ZeptoMetrix or Medical and Biological Laboratories, Japan). The cell lysates were analyzed for Gag by Western blotting. The viral infectivity was assessed using TZM-bl cells as described previously (43).

Cell sorting

Fibrocytes in a live cell gate were sorted using a FACSAria (BD Biosciences). The $CD14^{low}SLAMF7^{high}$ cells were sorted into 96-well plates (1×10^5 cells/well) as the purified fibrocyte fraction, and subjected to HIV-1 replication assay. The fraction containing the $CD14^{high}SLAMF7^{low}$ macrophages was also sorted as a control.

HIV-1 genome integration

A modified Alu-PCR method (44) was employed to quantify proviral DNA. The semiquantitative assay was also performed as described previously (40). NL(AD8) viral stock was treated with Benzonase (Novagen) to digest proviral plasmids. The genomic DNA was isolated using DNeasy blood/tissue kit (Qiagen). In first PCR, two Alu primers that annealed within the conserved regions of Alu element were used with an LTR primer L-M667. In the second real-time PCR, a lambda-specific primer lambda T was used as a sense primer to detect fragments amplified in the first PCR, and a TaqMan probe and an antisense primer were selected from the set for R/U5 DNA detection (45). Genomic DNA of infected U937 cells was used as a standard (46).

HIV-1 mRNA expression

HIV-1 mRNA was quantified according to a recently reported method (47). Total RNA was used for cDNA synthesis with random hexamer. Primers used were P9501 (5′-CAGATGCTGCATATAAGCAGCTG-3′) and 5T25 (5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTGAAG-3′), or the internal control 18S rRNA forward (5′-GTAACCCGTTGAACCCCATT-3′) and 18S rRNA reverse (5′-CCATCCAATCGGTAGTAGCG-3′). The level of HIV-1 mRNA was calculated by the ΔΔCt method and normalized to the number of integrated proviral copies (48).

Immunofluorescence

Immunofluorescence was performed as described previously (49). Cells were stained with anti-Gag Abs (Kal-1; Dako), and nuclei were stained

with DAPI. Signals were visualized with an LSM 700 (Carl Zeiss) or an FV1200 confocal laser-scanning microscope (Olympus). Image processing was performed using LSM ZEN 2009 (Carl Zeiss) or FV Viewer 4.1 software (Olympus).

Rate zonal gradient analysis of viruses

The particle size of viruses produced was analyzed according to a previously reported method (50). The viruses in the culture supernatants were pelleted by centrifugation. Each concentrated sample was layered onto 10–30% sucrose and ultracentrifuged. Fractions were collected from each gradient and analyzed for Gag concentrations by ELISA.

Patients, cell sorting, and PCR

Chronically HIV-1–infected Japanese patients were recruited, which was approved by the Ethics Committees of Kumamoto University and the National Center for Global Health and Medicine, Japan. PBMCs of infected patients kept in liquid nitrogen were stained with the following Abs: allophycocyanin–anti-CD34 (no. 581; BioLegend), FITC–anti-CD14 (no. 61D3; eBioscience), Pacific Blue–anti-CD16 (no. 3G8; BioLegend), and PE–anti-CD3 (no. OKT3; BioLegend). The cells in live cell gates were sorted using a FACSAria. The $CD3^-CD14^+CD16^-CD34^+$ fibrocyte-enriched fraction, $CD3^-CD14^+CD16^-CD34^-$ monocytes, and $CD3^+$ lymphocytes were sorted into 96-well plates (2000 cells/well). The higher numbers of $CD3^+$ cells (20,000 cells/well) were also sorted as a positive control. Genomic DNA was prepared using a QIAamp DNA micro kit (Qiagen). Two-step PCR to amplify Pol or Gag regions was performed as described previously (40) with eight different primer pairs (sequences are available upon request). The first PCR was performed using genome DNA (100 or 1000 cells/reaction, 38 cycles), and the second PCR was performed using an aliquot of the first PCR (1/2500 dilution, 38 cycles). G3PDH was amplified (35 cycles) as a loading control with a primer pair 5′-CCACCCTGTTGCTGTAGCCAAATTCG-3′ and 5′-TCCGGGAAAC TGTGGCGTGATGG-3′.

Statistical analysis

The statistical significance of the intersample differences was determined using the paired Student *t* test. A p value <0.05 was considered significant.

Results

Fibrocytes are phenotypically distinguishable from macrophages

Fibrocytes prepared in this study were phenotypically similar to those described in previous studies (17–22). Unlike macrophages, fibrocytes often emerged as expanding colonies (Supplemental Fig. 1A, 1B). The number of fibrocytes obtained was significantly lower than that of macrophages (Supplemental Fig. 1C). Fibrocytes produced a larger amount of collagens than did macrophages on a per cell basis (Supplemental Fig. 1D, 1E), and they showed the fibroblast-like spindle-shaped morphology (Supplemental Fig. 1F). Fibrocytes expressed CD45 (hematopoietic cell marker), but their expression level of CD14 (macrophage marker) was much lower than that of macrophages (Supplemental Fig. 1G). These differentiated fibrocytes minimally expressed CD34 (Supplemental Fig. 1G), presumably due to its decline during the cultures (20, 23).

We initially attempted to further reveal the phenotypic characteristics of fibrocytes and confirm that fibrocytes and macrophages share several phenotypes but are distinct cell types. The expression of Src family kinases is often cell lineage specific (51), and both macrophages and fibrocytes expressed Hck and Lyn among Src kinases, albeit slightly weakly in fibrocytes (Fig. 1A). Fibrocytes also expressed HLA-A/B/C and HLA-DR (Fig. 1B). The phagocytic activity of fibrocytes was detectable but significantly weaker than that of macrophages (Fig. 1C). Liposomal clodronate is widely used to deplete macrophages because macrophages efficiently ingest liposomal clodronate using their strong phagocytic activity (52). Indeed, liposomal clodronate markedly reduced the number of macrophages (Fig. 1D, *left*). However, such inhibitory effect of liposomal clodronate was not observed in the fibrocyte cultures (Fig. 1D, *right*), which was consistent with the

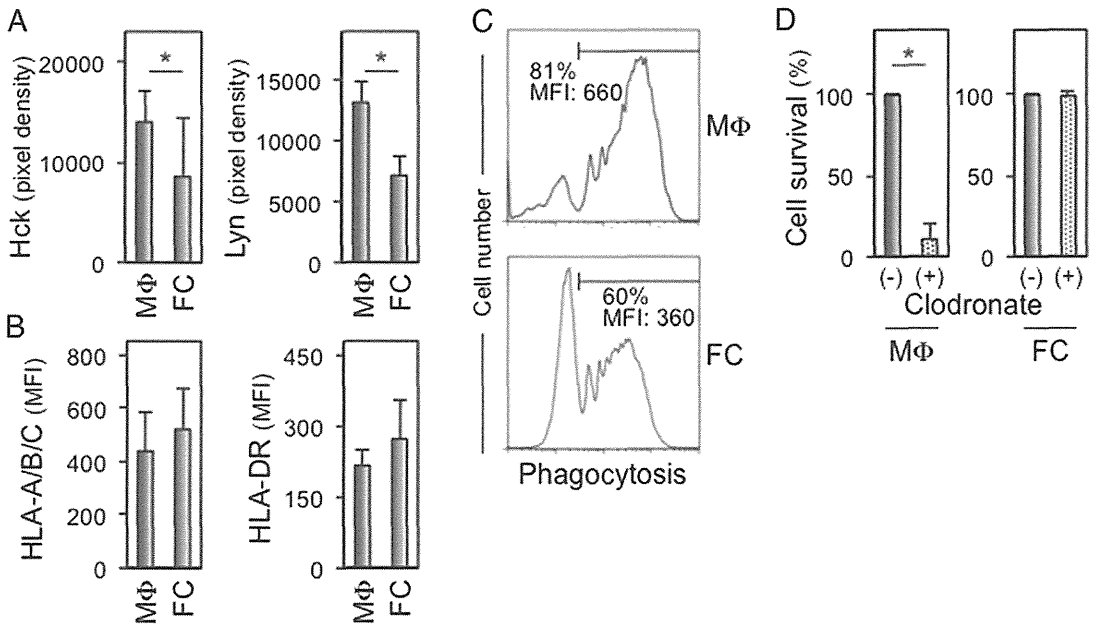

FIGURE 1. Myeloid-related phenotypes of fibrocytes. (**A**) The expression of Src family kinases (Hck and Lyn) in fibrocytes and macrophages was analyzed by Western blotting followed by the densitometric analysis ($n = 5$). (**B**) The expression of HLA-A/B/C or HLA-DR on the surface of fibrocytes and macrophages was analyzed by flow cytometry, and their mean fluorescence intensities are shown ($n = 3$). (**C**) The phagocytic activity of fibrocytes and macrophages was analyzed by flow cytometry. The percentage of phagocytic cells and their mean fluorescence intensities are shown. The data shown are representative of six donors with similar results. (**D**) Fibrocytes and macrophages were cultured for 4 d in the absence or presence of liposomal clodronate, and their survival was assessed by MTT assay. The results are expressed as percentages of the value for the liposomal clodronate-free cultures ($n = 6$). $*p <$ 0.05. FC, fibrocyte; MΦ, macrophage; MFI, mean fluorescence intensity.

weaker phagocytic activity of fibrocytes when compared with macrophages.

We next compared the cytokine and chemokine production profiles between fibrocytes and macrophages. For instance, the levels of GRO-α, IL-1ra, and MCP-1 were lower in fibrocytes; conversely, the levels of IL-8, I-309, RANTES, MIP-1α, MIP-1β, and IL-27 were higher in fibrocytes (Fig. 2A). However, as a whole, fibrocytes and macrophages shared the similar profile of cytokine/chemokine production. Interestingly, fibrocytes only weakly expressed receptors for cytokines such as M-CSF and GM-CSF (Fig. 2B). M-CSF and GM-CSF promote the survival of macrophages, and M-CSF upregulates CD14 and CD163 whereas GM-CSF upregulates CD80 and CD86 in macrophages (40, 41, 53, 54). However, neither of them affected the survival (Fig. 2C) or the expression of those surface molecules of fibrocytes (Fig. 2D, 2E). Moreover, we did not observe any activation of M-CSF receptor upon the stimulation with M-CSF in fibrocytes (Fig. 2F). Thus, it is highly likely that fibrocytes are cells of myeloid origin but distinct from macrophages.

Fibrocytes can be infected with HIV-1, and they produce HIV-1 at a lower rate but for a longer time than do macrophages

In this study, we found that fibrocytes expressed HIV-1 receptor CD4 and coreceptors CCR5 and CXCR4 (Supplemental Fig. 2A). More importantly, we found that fibrocytes supported the replication of primary (Fig. 3A) and recombinant HIV-1 viruses (Fig. 3B). Anti–HIV-1 drugs inhibited the viral replication in fibrocytes (Supplemental Fig. 2B). Interestingly, there were several differ-

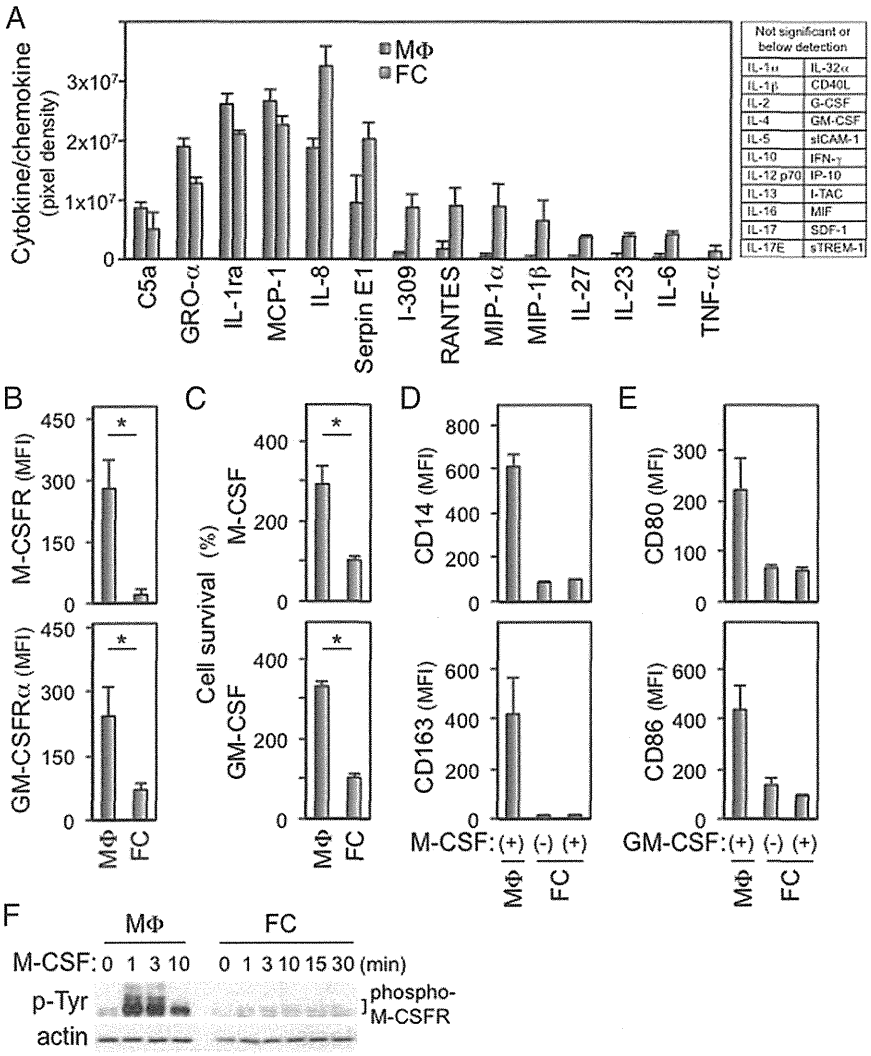

FIGURE 2. Cytokine production and response to cytokines of fibrocytes. (**A**) The relative levels of various cytokines and chemokines in media conditioned by fibrocytes or macrophages were analyzed by the Ab array. Fibrocytes were reseeded so that their number was equivalent to that of macrophages. Fibrocytes and macrophages were cultured for 2 d after changing media. The data shown were obtained by densitometric analysis ($n = 4$). (**B**) The expression of M-CSF receptor and GM-CSF receptor α-chain on the surface of fibrocytes and macrophages was analyzed by flow cytometry, and their mean fluorescence intensities are shown ($n = 5$). (**C**) Fibrocytes and macrophages were cultured for 4 d in the absence or presence of M-CSF (*upper*) or GM-CSF (*lower*), and their survival was assessed by MTT assay. The results are expressed as percentages of the value for the cytokine-free cultures ($n = 6$). (**D**) Fibrocytes were cultured in the absence or presence of M-CSF for 4 d and analyzed for their expression of CD14 and CD163 by flow cytometry ($n = 3$). M-CSF macrophages were the positive control. (**E**) Fibrocytes were cultured in the absence or presence of GM-CSF for 4 d and analyzed for their expression of CD80 and CD86 by flow cytometry ($n = 3$). GM-CSF macrophages were the positive control. (**F**) Macrophages and fibrocytes were stimulated with M-CSF for the indicated periods, and their total cell lysates were analyzed for tyrosine-phosphorylated proteins by Western blotting. Data shown are representative of three independent experiments with similar results. $*p < 0.05$. FC, fibrocyte; MΦ, macrophage; MFI, mean fluorescence intensity; phospho-M-CSFR, tyrosine-phosphorylated M-CSF receptor; p-Tyr, tyrosine-phosphorylated.

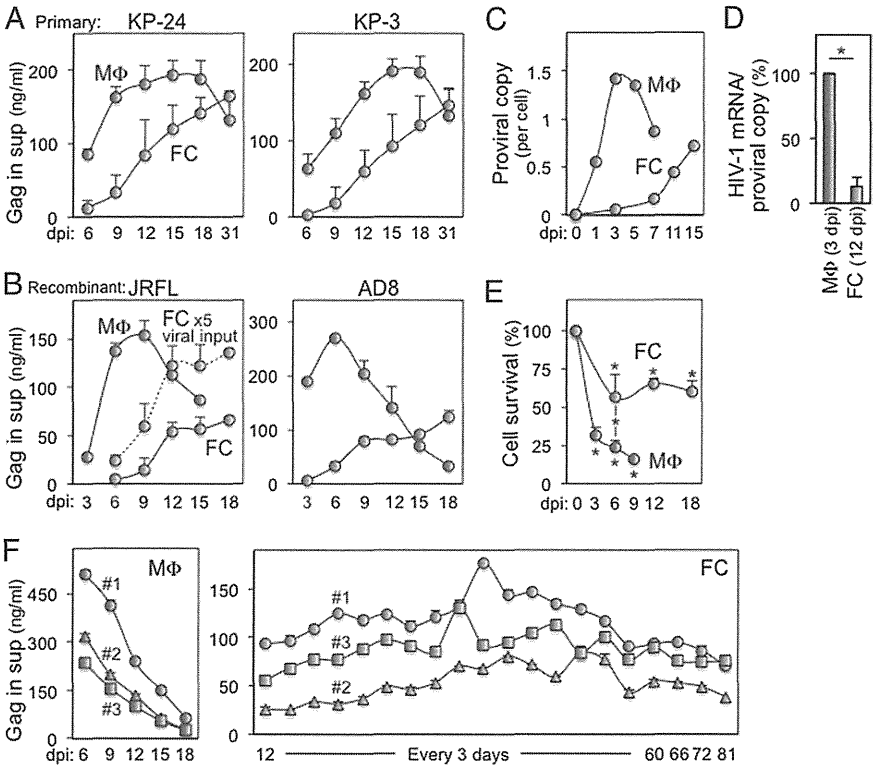

FIGURE 3. HIV-1 replication in fibrocytes. (**A–F**) Fibrocytes were reseeded so that their number was equivalent to that of macrophages, and they were cultured for 2 or 3 d prior to HIV-1 infection. (A) Cells were infected with the primary HIV-1 viruses (KP-24 and KP-3) and analyzed for the concentration of Gag in the supernatants by ELISA. The results obtained from three donors are summarized. (B) Cells were infected with the recombinant HIV-1 viruses (JRFL and AD8) and analyzed as in (A). In the *left panel*, fibrocytes were also infected with 5-fold higher input amount of JRFL (×5 viral input). The data shown are representative of five donors with similar results. (C) Cells were infected with NL(AD8) and analyzed for the integrated proviral DNA (the number of copies per cell) by quantitative PCR. The data shown are representative of three donors with similar results. (D) Cells were infected with NL (AD8) and analyzed for HIV-1 mRNA expression by quantitative PCR followed by normalization to the number of integrated proviral DNA copies, and the results are expressed as percentages of the value for macrophages. The results obtained from three donors are summarized. (E) Cells were infected with AD8 and analyzed for their survival by MTT assay. The results are expressed as percentages of the value seen at the beginning (day 0) of the assay ($n = 5$). (F) Cells were infected with AD8 and analyzed for the concentration of Gag in the supernatants by ELISA (three donors). The data shown are representative of three independent experiments with similar results. $*p < 0.05$. FC, fibrocyte; MΦ, macrophage; sup; supernatant.

ences in HIV-1 replication between fibrocytes and macrophages. First, the viral replication in fibrocytes was slower than that in macrophages even when fibrocytes were infected with 5-fold higher input viral amount (Fig. 3B, *left*, see ×5 viral input). This was well correlated with the slow proviral DNA integration into fibrocytes (Fig. 3C), which was likely due to the lower amount of viruses bound to their surface (Supplemental Fig. 2C) or their lower intracellular dNTP level (Supplemental Fig. 2D). Second, the peak of viral production of fibrocytes was lower than that of macrophages (see Fig. 3B, *right*). This was consistent with the finding that the expression level of HIV-1 mRNA of fibrocytes was significantly lower than that of macrophages even when normalized to the number of proviral copies (Fig. 3D). Indeed, although the percentage of intracellular Gag^+ cells was not different between fibrocytes and macrophages in the flow cytometric analysis (Supplemental Fig. 2E), the intensity of Gag^+ fibrocytes was significantly lower than that of macrophages (Supplemental Fig. 2F). Third, and most importantly, the viral production of macrophages gradually declined after the peak, whereas that of fibrocytes was relatively stable (see Fig. 3B). Consistent with this, infected fibrocytes survived for a longer time than infected macrophages (Fig. 3E), although infected fibrocytes formed giant cells as seen with infected macrophages (Supplemental Fig. 3A). The longer survival of infected fibrocytes was still observed under the conditions in which we made the viral replication slow in macrophages by decreasing the input viral amount (1:10) for macrophages (Supplemental Fig. 3B). Because macrophages could survive for a long time under the uninfected cultures (Supplemental Fig. 3C), fibrocytes appeared to be more resistant to cytolytic effects of HIV-1 than did macrophages, although we did not find obvious differences in expression levels of host genes involved in the regulation of HIV-1 proviral transcription or apoptosis-related genes between infected fibrocytes and infected macrophages in the microarray analysis (Supplemental Fig. 3D). As a consequence, unlike macrophages, fibrocytes supported an extremely long-term HIV-1 production at low but steady levels (Fig. 3F).

HIV-1 production in fibrocyte cultures is not due to contaminating macrophages

We extensively washed the fibrocyte cultures with PBS prior to HIV-1 replication assay. Because the percentage of $CD3^+$ lymphocytes was usually <0.1% under the conditions (Supplemental Fig. 4A), it was unlikely that the observed HIV-1 production was due to contaminating $CD4^+$ T cells. Indeed, we did not detect the replication of T cell–tropic HIV-1 viruses such as NL43 and IIIB in the fibrocyte cultures (Supplemental Fig. 4B).

Macrophages can be removed from the fibrocyte cultures using CD14 as the marker (35, 38). To ensure the removal of macrophages, we attempted to identify another marker through microarray analysis. We found that fibrocytes expressed a cell surface

molecule SLAMF7 (also known as CS1), which is often highly expressed in multiple myeloma cells (55), at a higher level than did macrophages (Fig. 4A). The costaining of CD14 and SLAMF7 allowed us to distinguish fibrocytes from macrophages in flow cytometric analysis (Fig. 4B), and the percentage of the $CD14^{high}$ $SLAMF7^{low}$ macrophages in the fibrocyte cultures was estimated as 2–5%. Importantly, the $CD14^{high}SLAMF7^{low}$ macrophage-free fibrocyte fraction prepared by cell sorting supported HIV-1 production at a similar level to that observed with the whole cells of the fibrocyte cultures (Fig. 4C), strongly suggesting that the observed HIV-1 production in the fibrocyte cultures was not due to contaminating macrophages. Indeed, as shown in Supplemental Fig. 2E, the maximal percentage of Gag^+ cells of the fibrocyte cultures was not statistically different from that of the macrophage cultures. The following results further supported the conclusion that fibrocytes were infected with HIV-1. 1) The pretreatment with liposomal clodronate, which efficiently depletes macrophages (see Fig. 1D), did not reduce HIV-1 production in the fibrocyte cultures (Fig. 4D). 2) M-CSF, which potently stimulates HIV-1 replication in macrophages at multiple steps (40), did not stimulate HIV-1 production in the fibrocyte cultures (Fig. 4E). This was consistent with the weak expression of M-CSF receptor in fibrocytes (see Fig. 2B, *upper*). 3) Gag^+ spindle fibrocyte-like cells were readily detected by immunofluorescence (Fig. 4F). 4) Fibrocytes prepared by an alternative method, that is, the serum-free culture (56), formed visible syncytia and produced HIV-1 into the culture media at a detectable level when exposed to HIV-1 (Supplemental Fig. 4C).

Fibrocytes produce replication-competent HIV-1 viruses

We next investigated whether fibrocytes produce replication-competent HIV-1 viruses. To this end, we initially analyzed the particle size of produced viruses. The macrophage-produced viruses were collected at the peak of viral replication, that is, day 6 (see Fig. 3B, *right*). As indicated by the shift toward light fractions (Fig. 5A, see fractions 3 and 4), the fibrocyte-produced viruses were smaller in the particle size when compared with the macrophage-produced viruses. Indeed, when assessed by the single-round replication assay (Fig. 5B), the peak of the infectivity of the fibrocyte-produced viruses was slightly lower than that of the macrophage-produced viruses (see 6 days postinfection [dpi]). However, as observed with the viral replication (see Fig. 3F), the infectivity of the macrophage-produced viruses gradually declined after the peak whereas that of the fibrocyte-produced viruses was relatively stable (Fig. 5B). Importantly, when added to macrophages, the fibrocyte-produced viruses replicated slowly due to their low infectivity, but they subsequently reached the level of input

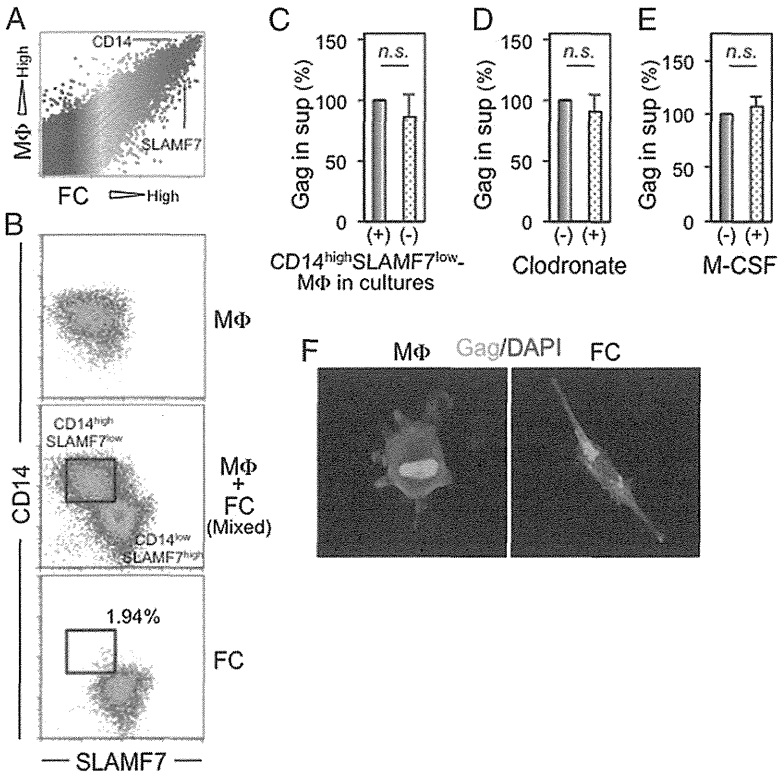

FIGURE 4. HIV-1 replication in fibrocyte cultures is not due to contaminating macrophages. (**A**) Microarray analysis showing the differential expression of SLAMF7 and CD14 between fibrocytes and macrophages. (**B**) Macrophages (*top*), fibrocytes (*bottom*), and their mixtures (*middle*) were costained with anti-SLAMF7 and anti-CD14 Abs and analyzed by flow cytometry. The percentage of $CD14^{high}SLAMF7^{low}$ macrophages in the fibrocyte cultures is shown (*bottom*). The data shown are representative of six donors with similar results. (**C**) $CD14^{high}SLAMF7^{low}$ macrophages in the fibrocyte cultures (see B) were removed by cell sorting (right bar). The fraction containing $CD14^{high}SLAMF7^{low}$ macrophages was also sorted as a reference (left bar). These sorted cells (1×10^5 cells/well) were infected with AD8, cultured for 18 d, and analyzed for the concentration of Gag in the supernatants by ELISA. The results are expressed as percentages of the value for the macrophage-containing fraction ($n = 6$). (**D**) Fibrocytes were cultured in the absence or presence of liposomal clodronate for 4 d, infected with JRFL, cultured for 18 d, and analyzed for the concentration of Gag in the supernatants by ELISA. The results are expressed as percentages of the value for the liposomal clodronate-free cultures ($n = 6$). (**E**) Fibrocytes were cultured for 4 d in the absence or presence of M-CSF, infected with AD8, cultured for 18 d in the absence or presence of M-CSF, and analyzed for the concentration of Gag in the supernatants by ELISA. The results are expressed as percentages of the value for the M-CSF–free cultures ($n = 6$). (**F**) Fibrocytes and macrophages were infected with AD8, cultured for 3 d, and costained with anti-Gag Abs (green) and DAPI (blue). Original magnification ×60. The data shown are representative of six donors with similar results. FC, fibrocyte; MΦ, macrophage; sup; supernatant.

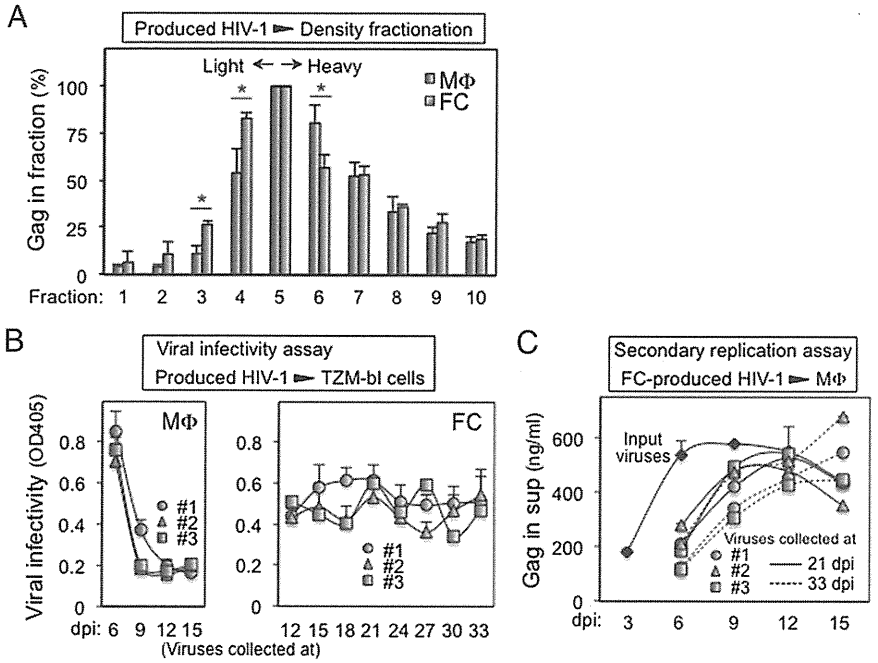

FIGURE 5. Production of replication-competent HIV-1 viruses by fibrocytes. (**A**) The particle size of AD8 viruses in the supernatants of fibrocytes (20 dpi) or macrophages (6 dpi) was analyzed by density fractionation followed by Gag ELISA ($n = 3$). The results are expressed as percentages of the value for the peak fraction (no. 5). *$p < 0.05$. (**B**) The infectivity of AD8 viruses in the supernatants of fibrocytes or macrophages was analyzed by the single-round infection assay with TZM-bl cells (three donors). The supernatants analyzed were collected at different days as indicated. The data shown are representative of three independent experiments with similar results. (**C**) The ability of the replication of AD8 viruses in the supernatants of fibrocytes was assessed using macrophages as the secondary target cells. The AD8 viruses, which were used in the first replication assay with fibrocytes, were also included as a reference (see Input viruses). The supernatants of infected fibrocytes analyzed were collected from three donors and at different days (21 and 33 dpi). The data shown are representative of three independent experiments with similar results. FC, fibrocyte; MΦ, macrophage.

(founder) viruses (Fig. 5C), indicating that fibrocytes produced the replication-competent HIV-1 viruses.

Peripheral blood fibrocytes are more susceptible to HIV-1 than are monocytes, but they express HIV-1 proteins at low levels

It is well known that monocytes are infrequently infected, but they become susceptible to HIV-1 as they differentiate into mature macrophages (57, 58). Thus, we next attempted to investigate the susceptibility of circulating (undifferentiated) fibrocytes to HIV-1. To this end, PBMCs containing circulating fibrocytes and monocytes were briefly (for 2 h) exposed to HIV-1, washed extensively to remove unbound viruses, and then cultured under the conditions that support the differentiation into either macrophages or fibrocytes (Fig. 6A). As expected, the number of integrated proviral DNA increased slowly in the macrophage-inducing cultures (Fig. 6B, 6C), which was in contrast to the rapid proviral integration into the differentiated macrophages (see Fig. 3C). However, we unexpectedly found the sufficient proviral integration in the fibrocyte-inducing cultures even at 7 dpi, and its degree was significantly higher than that in the macrophage-inducing cultures (Fig. 6B, 6C). The strong PCR signal detected was not due to contaminating $CD4^+$ T cells in the fibrocyte-inducing cultures because the nonadherent lymphocyte fraction in the same cultures showed only a weak signal (Fig. 6B, see Lymphocytes in FC cultures). More importantly, despite this sufficient viral integration, the amount of Gag of the fibrocyte-inducing cultures was much lower than that of the macrophage-inducing cultures in both the supernatants (Fig. 6D) and the cell fraction (Fig. 6E), which was consistent with the low HIV-1 mRNA expression in fibrocytes that were exposed to HIV-1 after their differentiation (see Fig. 3D). It is therefore likely that fibrocytes are susceptible to HIV-1 regardless of their differentiation state unlike the monocyte-to-macrophage differentiation, but they exhibit persistently infected cell-like phenotypes, the degree of which was more apparent than for monocytes/macrophages.

Peripheral blood fibrocyte-enriched fraction of HIV-1–infected patients harbors proviral DNA more frequently than in monocytes

We finally investigated whether fibrocytes could be infected with HIV-1 in vivo. Circulating fibrocytes are generally defined as $CD45^+CD34^+$collagen I^+ cells (17–20). Interestingly, several studies implied that circulating fibrocytes could be enriched in $CD14^+$ cells, for instance, in the $CD45^+CD34^+CD14^+CD16^-$ fraction (17–22, 59). We therefore collected PBMCs of ART untreated chronically HIV-1–infected patients, sorted the fibrocyte-enriched fraction by adding CD3 as a negative marker to exclude $CD4^+$ T cells (Fig. 7A), and analyzed the HIV-1 proviral DNA by two-step PCR with different primer pairs (Fig. 7B). As a result (Fig. 7C), we detected proviral DNA in the $CD3^-CD14^+CD16^-CD34^+$ fibrocyte-enriched fraction of all six patients tested, the frequency of which was higher than that of $CD3^+$ lymphocytes or $CD3^-CD34^-CD14^+CD16^-$ monocytes under the same conditions (100 cells/PCR reaction). These results indicated that the proviral DNA detected in the fibrocyte-enriched fraction was not due to the contamination of lymphocytes, and they raised the possibility that peripheral blood fibrocytes were infected with HIV-1 more frequently than monocytes in patients, as observed in the culture systems (see Fig. 6).

Discussion

It has been reported that fibrocytes are susceptible to circovirus (60) and rhinovirus (61). In this study, we demonstrated that fibrocytes are also susceptible to HIV-1. The previous studies revealed that

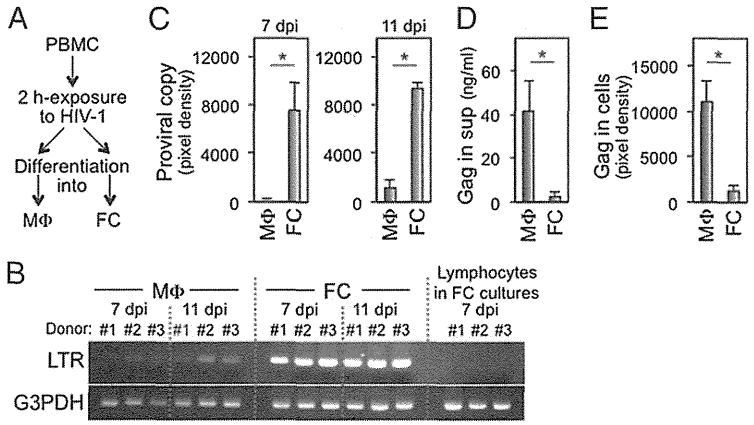

FIGURE 6. Susceptibility of circulating fibrocytes to HIV-1. (**A**) The experimental procedures for (**B**)–(**E**) are shown. PBMCs were exposed to AD8 for 2 h, washed extensively with PBS to remove unbound viruses, and cultured under the conditions that support the differentiation into either macrophages or fibrocytes for 7 or 11 d. (B) The integrated proviral DNA was analyzed by Alu-LTR two-step PCR (three donors). G3PDH PCR [*lower panel in* (**B**)] is the loading control. The nonadherent lymphocyte fraction in the fibrocyte cultures collected at 7 dpi (Lymphocytes in FC cultures) was also analyzed as a reference. The data shown are representative of two independent experiments with similar results. (C) The results of the proviral DNA PCR (see B) were quantified by the densitometric analysis ($n = 6$). $^*p < 0.05$. (D and E) Cells (11 dpi) were analyzed for their expression of Gag in the supernatants by ELISA or in the cell fraction by Western blotting followed by the densitometric analysis ($n = 5$). $^*p < 0.05$. FC, fibrocyte; MΦ, macrophage.

peripheral blood $CD34^+$ cells could be infected with HIV-1 in vitro (14, 15) and in vivo (16). However, it is unclear which populations of peripheral blood $CD34^+$ cells are infected with HIV-1. Our finding suggests that fibrocytes are one of the HIV-1–susceptible peripheral blood $CD34^+$ populations (Figs. 6, 7).

Despite its potency to suppress HIV-1 replication, ART does not eliminate HIV-1 reservoirs, and it needs lifelong adherence to regimens that are associated with toxic effects. Although resting memory $CD4^+$ T cells are the important HIV-1 reservoir (1), there is increasing evidence that HIV-1 exists also in other cells (2–7). A clear demonstration that macrophages are an HIV-1 reservoir is lacking because of the difficulty in obtaining tissue samples (62). However, given their characteristics, including the long-term HIV-1 production and resistance to HIV-1–induced cell death, macrophages could serve as one of HIV-1 reservoirs (9). In this study, we demonstrated that fibrocytes exhibited persistently infected cell-like phenotypes, the degree of which was more apparent than macrophages: fibrocytes produced replication-competent HIV-1, but expressed HIV-1 mRNA at low levels and strongly resisted HIV-1–induced cell death, which enabled them to support an extremely long-term HIV-1 production at low but steady levels (Figs. 3–5). A recent study implies that tissue fibrocytes have a relatively long (>6 mo) lifespan (63). Thus, our findings suggest the importance of considering fibrocytes as one of the long-lived persistently infected cells for curing HIV-1.

In this study, we further confirmed that differentiated fibrocytes and macrophages share several phenotypes but not identically (Figs. 1, 2), for instance, in their expression of M-CSF receptor (Fig. 2B). It is therefore likely that fibrocytes are not necessarily differentiated from classical monocytes. Interestingly, our result suggests that circulating fibrocytes are more susceptible to HIV-1 than are classical monocytes: although the viral production of the fibrocyte-inducing cultures was lower than that of the macrophage-inducing cultures, the viral integration of the former was much higher than that of the latter (Fig. 6). The finding further supports the idea that circulating fibrocytes are cells other than

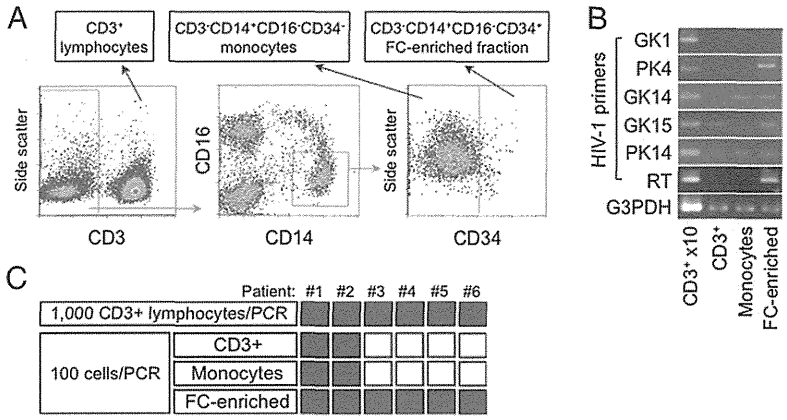

FIGURE 7. HIV-1 proviral DNA in peripheral blood fibrocyte fraction of chronically infected patients. (**A**) Sorting strategy for PBMCs of ART-untreated chronically HIV-1–infected patients. (**B** and **C**) The $CD3^-CD14^+CD16^-CD34^+$ fibrocyte-enriched fraction, $CD3^-CD14^+CD16^-CD34^-$ monocytes, and $CD3^+$ lymphocytes were sorted from PBMCs of six chronically HIV-1–infected ART-untreated patients (CD4 T cell count, 237–536/μl; plasma viral load, 3,000–130,000 copies/ml). Genomic DNA was prepared and subjected to two-step PCR to amplify the Gag or Pol regions using eight different primer pairs (100 cells/reaction). PCR reaction containing genomic DNA from 10-fold higher number of $CD3^+$ lymphocytes (1000 cells/reaction) was also performed as a positive control (*top*). In (B), an example of genomic PCR [patient no. 1 in (C)] is shown. In (C), the cell fractions, which showed the positive PCR with one or more primer pairs, were indicated by red. FC, fibrocyte.

classical monocytes, or a subpopulation of monocytes (17–20). Circulating fibrocytes comprise a small fraction in peripheral blood. However, because it appears that circulating fibrocytes are more susceptible to HIV-1 than are classical monocytes, the cells of fibrocyte lineage may play a role in HIV-1 infection different from monocytes/macrophages.

The differentiated fibrocytes support the extremely long-term HIV-1 production at low but steady levels (Fig. 3F), suggesting that HIV-1 is better adapted to reside in fibrocytes than in macrophages. The molecular mechanism that determines these unique features of HIV-1–infected fibrocytes remains to be determined. Although we did not find obvious differences in expression levels of host genes involved in the regulation of HIV-1 proviral transcription or apoptosis-related genes between infected fibrocytes and infected macrophages in the preliminary microarray analysis (Supplemental Fig. 3D), more careful time course analysis will be needed. It will also be necessary to compare how different viral inocula, including lower inoculum, induce the viral replication and cellular survival in macrophages and fibrocytes in more detail.

We detected HIV-1 proviral DNA in the $CD3^-CD14^+CD16^-CD34^+$ peripheral blood fibrocyte-enriched fraction of ART-untreated chronically HIV-1–infected patients, the frequency of which was higher than that of monocytes (Fig. 7C). The result was well consistent with the in vitro findings (Fig. 6). However, the in vivo HIV-1 infection of fibrocytes must await further investigation because circulating fibrocytes are not purified yet, which is reflected by the heterogeneity in the side scatter of the $CD3^-CD14^+CD16^-CD34^+$ fraction (Fig. 7A). It was also reported that circulating fibrocytes could be enriched in the $CD34^+CD45^+CD11b^+CD13^+HLA\text{-}DR^+$ fraction (61, 64). HIV-1 persists in latent reservoirs despite optimal ART, which is the major barrier to curing HIV-1. Thus, it will be also important to investigate whether fibrocytes could serve as one of HIV-1 reservoirs by assessing HIV-1 proviral DNA in fibrocytes of ART-treated patients. Because fibrocytes showed elevated levels in diverse forms of tissue remodeling and fibrosis, as well as in chronic inflammation (17–20), it will be worth investigating whether fibrocytes are augmented in acute and chronic infections of HIV-1 in tissues such as lymph nodes. These analyses are required to understand to what extent fibrocytes, the frequency of which is much lower than $CD4^+$ T cells and monocytes, contribute to the total HIV-1 reservoirs.

In summary, we confirmed that fibrocytes differed from macrophages. More importantly, both in vitro and in vivo results strongly suggested that fibrocytes could be infected with HIV-1. Furthermore, the in vitro results suggested that fibrocytes were susceptible to HIV-1 regardless of their differentiation state and exhibited persistently infected cell-like phenotypes. Despite the unresolved important questions mentioned above, the identification of fibrocytes as the possible HIV-1 host cells will expand our knowledge of the pathogenesis of HIV-1.

Acknowledgments

We thank S. Harada, E. Nakayama, T. Shioda, T. Kuwata, S. Matsushita, and M. Kuroda for helpful discussions. We also thank A. Fukuda, Y. Hiyoshi-Yoshidomi, and N. Tokushige for quantitative PCR, fibrocyte preparation, and secretarial assistance, respectively.

Disclosures

The authors have no financial conflicts of interest.

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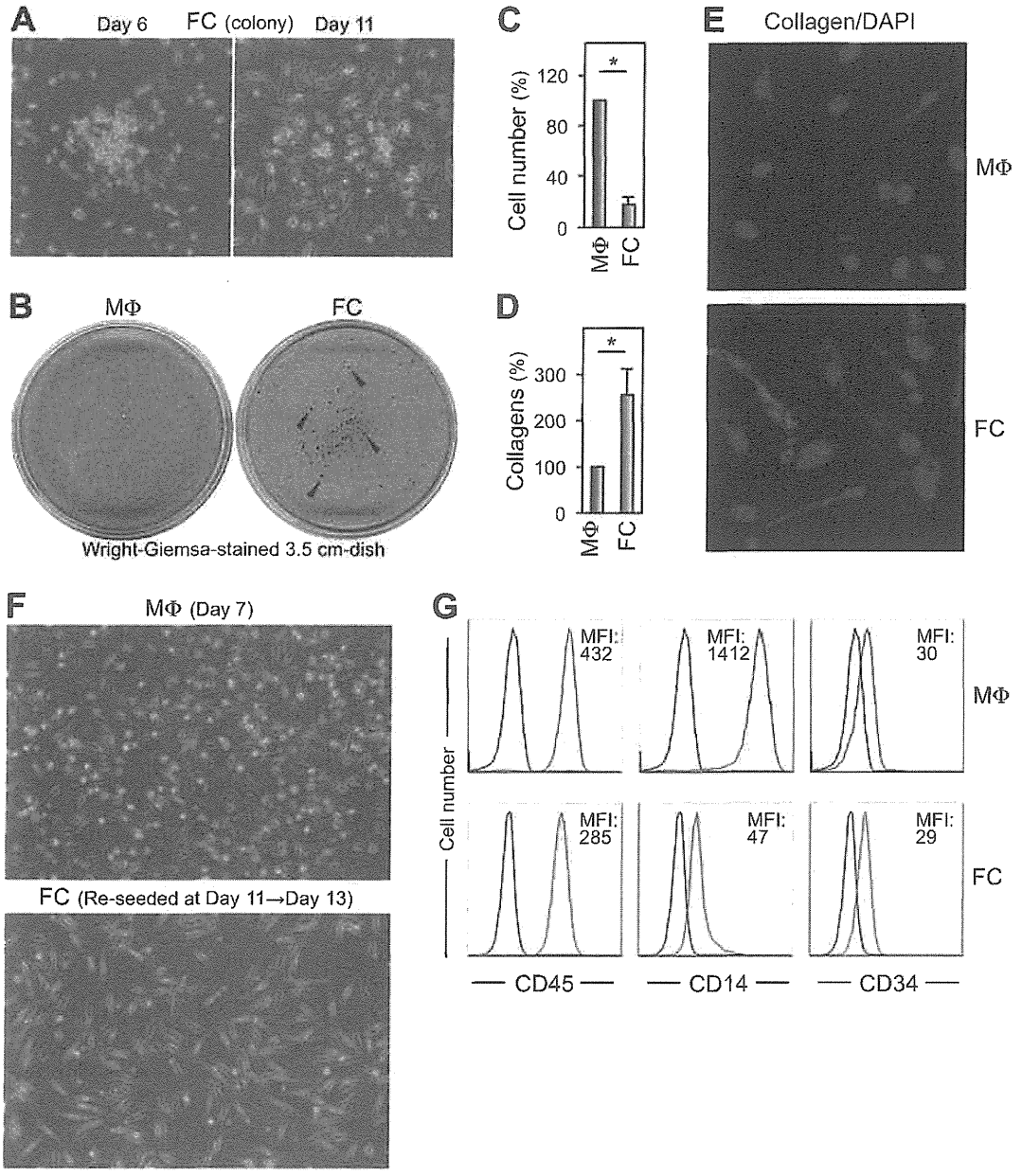
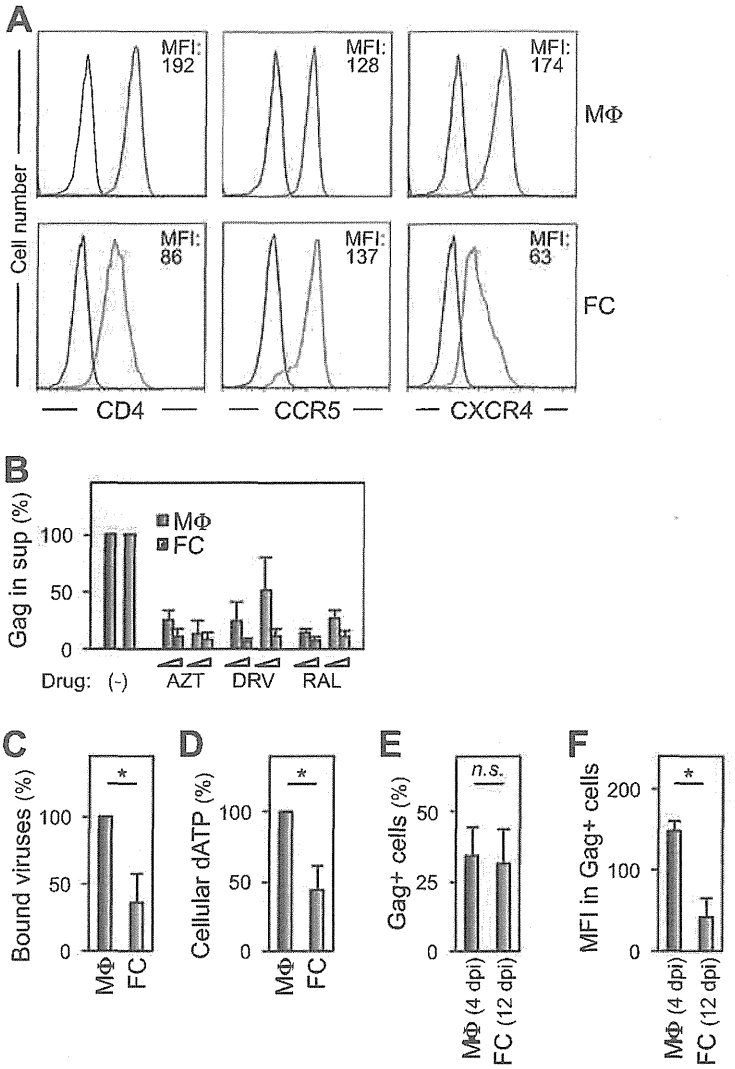


Supplemental Figure 1. Phenotypes of fibrocytes prepared in this study. (A) The typical colony in the fibrocyte cultures is shown. The same field was photographed at day 6 or 11. (B) Wright-Giemsa stained 3.5 cm dishes are shown (macrophages at day 5 and fibrocytes at day 11, respectively). The arrowheads indicate fibrocyte colonies. (C) PBMC were seeded in 3.5 cm dishes ($1x10^6$ cells/ml), and cultured under the conditions that support the differentiation into either macrophages (6 days) or fibrocytes (11 days). The number of adherent cells was enumerated, and the results are expressed as percentages of the value for the macrophage cultures (n=6). $^*p<0.05$. (D) The amounts of collagens in the extracellular matrix were analyzed by the colorimetric assay (sircol collagen assay; Biocolor), in which equivalent number of fibrocytes and macrophages were cultured for 5 days after re-seeding. The results are expressed as percentages of the value for the macrophage cultures (n=6). $^*p<0.05$. (E) Fibrocytes and macrophages prepared as in D were fixed, permeabilized, and stained with anti-collagen type I antibody (600-401-103; ROCKLAND, Gilbertsville, PA) followed by anti-rabbit IgG-AlexaFluor568 (Molecular Probes). Nuclei were also stained with DAPI. The data shown are representative of 4 donors with similar results. (F) The typical morphologies of macrophages (day 7) and fibrocytes (collected at day 11, re-seeded to enrich, and cultured for another 2 days) that were obtained from the same donor are shown. (G) The expression levels of CD45, CD14, and CD34 on the surface of fibrocytes (day 11) and macrophages (day 5) were analyzed by flow cytometry, and their MFI are shown. The data shown are representative of 6 donors with similar results.



Supplemental Figure 2. HIV-1-related phenotypes of fibrocytes.

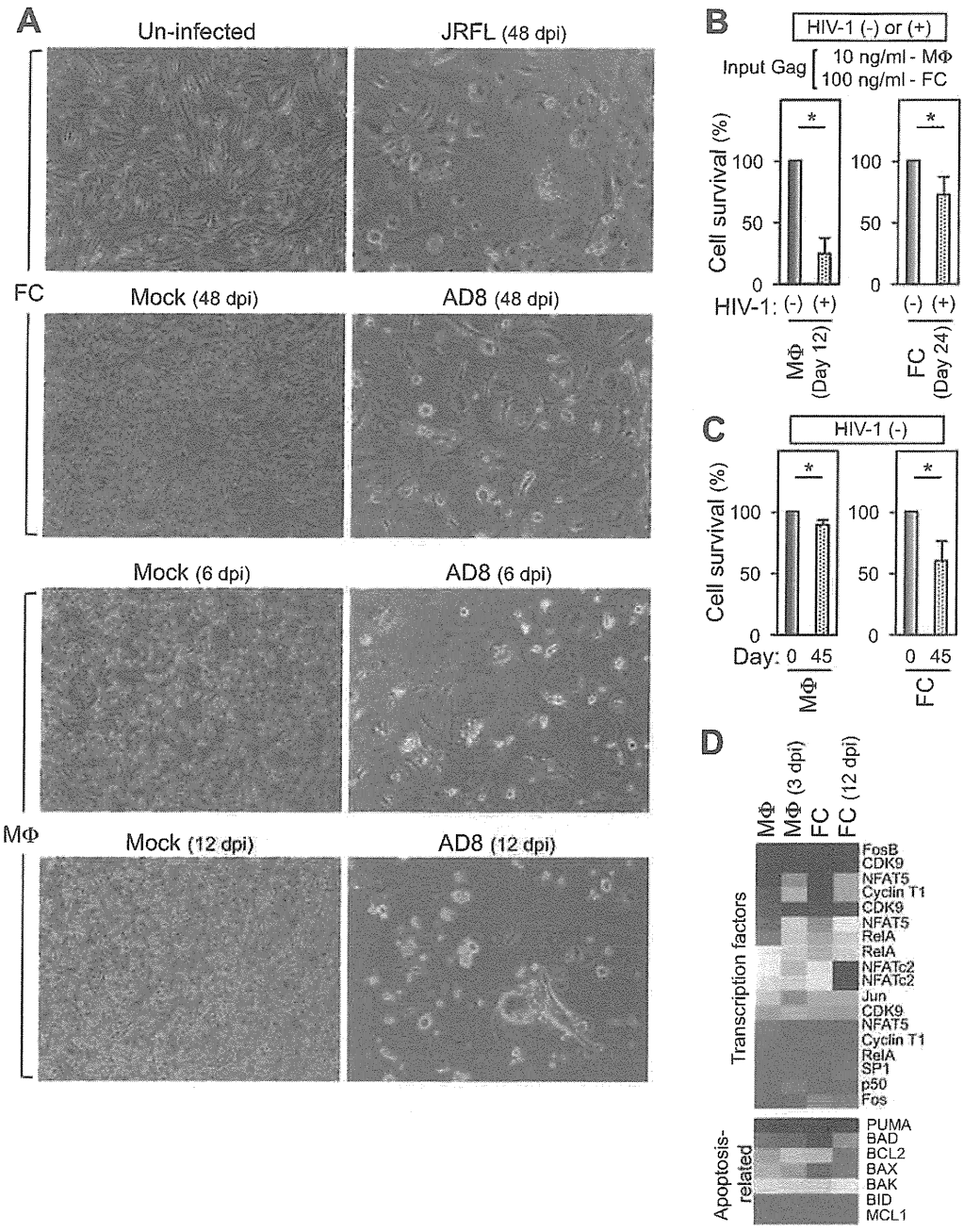
(**A**) The expression levels of CD4, CCR5 and CXCR4 on the surface of fibrocytes and macrophages were analyzed by flow cytometry, and their MFI are shown. The data shown are representative of 5 donors with similar results.

(**B**) The cells were infected with AD8 in the absence or presence of AZT (40 or 100 nM), darunavir (DRV; 20 or 50 nM), or raltegravir (RAL; 20 or 50 nM). The supernatants of macrophages and fibrocytes were collected at day 3 and 6, respectively, and the p24 Gag concentration of each cell supernatant was determined by ELISA (n=3). The results are expressed as percentages of the value for the drug-free cultures.

(**C**) The amounts of viruses bound to the surface of fibrocytes or macrophages were quantified according to a previously reported method (Gobeil, L.A., R. Lodge, and M.J. Tremblay. 2012. Differential HIV-1 endocytosis and susceptibility to virus infection in human macrophages correlate with cell activation status. *J. Virol.* 86: 10399-10407), in which equivalent number of fibrocytes and macrophages were exposed to AD8 for 2 h at 4°C (n=4). The results are expressed as percentages of the value for macrophages. $^{*}p<0.05$.

(**D**) Fibrocytes and macrophages were analyzed for their intracellular dATP level per cell basis (n=3), according to a previously reported method (Ferraro, P., E. Franzolin, G. Pontarin, P. Reichard, and V. Bianchi. 2010. Quantitation of cellular deoxynucleoside triphosphates. *Nucleic Acids Res.* 38: e85). Although the dATP levels of both cell types were lower than those seen in other cell types such as HeLa cells (data not shown), the dATP level of fibrocytes was generally 2-fold lower than that of macrophages. The results are expressed as percentages of the value for macrophages. $^{*}p<0.05$.

(**E** and **F**) Fibrocytes and macrophages were infected with AD8, cultured for the indicated periods, and analyzed for the intracellular Gag expression by flow cytometry (n=5), as described previously (40). The percentages of Gag^{+} cells are shown in **E**. The MFI in the Gag^{+} cells are shown in **F**. *n.s.*; not significant. $^{*}p<0.05$.



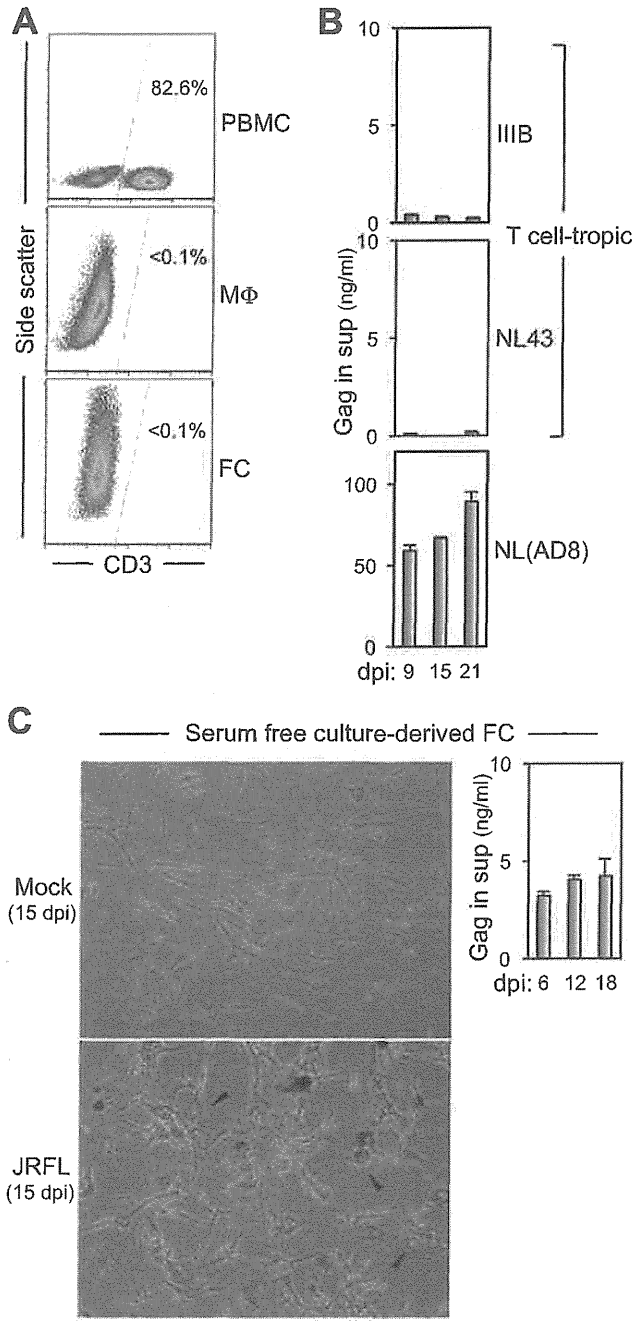
Supplemental Figure 3. Survival of HIV-1-infected fibrocytes.

(**A**) The morphology of the infected cultured fibrocytes. In the upper panels, the morphologies of the un-infected, mock-infected (48 dpi), JRFL-infected (48 dpi), and AD8-infected (48 dpi) fibrocytes are shown. In the lower panels, the morphologies of the un-infected, mock-infected (12 dpi), and AD8-infected macrophages (6 or 12 dpi) are shown.

(**B**) Fibrocytes and macrophages were left un-infected, or infected with the indicated amounts of AD8, and analyzed for their survival by MTT assay (macrophages at day 12 and fibrocytes at day 24, respectively). The results are expressed as percentages of the value for the un-infected control cells (n=6). $^{*}p<0.05$.

(**C**) Macrophages and fibrocytes were left un-infected, and analyzed for their survival by MTT assay at day 45. The results are expressed as percentages of the value seen at the beginning (day 0) of the assay (n=6).

(**D**) Heat maps generated form microarray analysis of un-infected macrophages (MΦ), macrophages infected with AD8 and cultured for 3 days (MΦ 3dpi), un-infected cultured fibrocytes (FC), and the fibrocytes infected with AD8 and cultured for 12 days (FC 12 dpi).



Supplemental Figure 4. HIV-1 replication in fibrocytes including serum-free culture-derived fibrocytes.
(**A**) The cultures of fibrocytes and macrophages were analyzed for the presence of $CD3^+$ lymphocytes by flow cytometry. PBMC were the positive control. The data shown are representative of 3 donors with similar results.
(**B**) Fibrocytes were infected with T cell-tropic HIV-1 viruses (IIIB and NL43), and analyzed for the concentration of Gag in the supernatants by ELISA. NL(AD8), in which Env of NL43 was replaced with that of AD8, was used as a positive control. The data shown are representative of 3 donors with similar results.
(**C**) Fibrocytes were prepared using the serum-free culture method (56). In the left panels, fibrocytes were left un-infected or infected with JRFL, and cultured for 15 days. The arrowheads indicate a typical syncytium formation. In the right panel, fibrocytes were infected with AD8, and analyzed for the concentration of Gag in the supernatants by ELISA. The data shown are representative of 3 donors with similar results.

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RESEARCH ARTICLE

A 21-Day of Adjunctive Corticosteroid Use May Not Be Necessary for HIV-1-Infected Pneumocystis Pneumonia with Moderate and Severe Disease

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Citation: Shibata S, Nishijima T, Aoki T, Tanabe Y, Teruya K, Kikuchi Y, et al. (2015) A 21-Day of Adjunctive Corticosteroid Use May Not Be Necessary for HIV-1-Infected Pneumocystis Pneumonia with Moderate and Severe Disease. PLoS ONE 10(9): e0138926. doi:10.1371/journal.pone.0138926

Editor: Sarah L George, Saint Louis University Division of Infectious Diseases and Immunology, UNITED STATES

Received: April 29, 2015

Accepted: September 4, 2015

Published: September 22, 2015

Data Availability Statement: All relevant data are within the paper.

Funding: This work was supported by Grant-in Aids for AIDS research from the Japanese Ministry of Health, Labour, and Welfare (H23-AIDS-001; H24-AIDS-003). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Background

The current guidelines recommend 21-day adjunctive corticosteroid therapy for HIV-1-infected pneumocystis pneumonia patients (HIV-PCP) with moderate-to-severe disease. Whether shorter adjunctive corticosteroid therapy is feasible in such patients is unknown.

Methods

We conducted a retrospective study to elucidate the proportion of patients with moderate and severe HIV-PCP who required adjunctive corticosteroid therapy for 21 days. The enrollment criteria included HIV-PCP that fulfilled the current criteria for 21-day corticosteroid therapy; PaO_2 on room air of <70mmHg or A-aDO_2 ≥35 mmHg.

Results

The median duration of corticosteroid therapy in the 73 study patients was 13 days (IQR 9–21). Adjunctive corticosteroid therapy was effective and discontinued within 10 and 14 days in 30% and 60% of the patients, respectively. Only 9% of the patients with moderate HIV-PCP (n = 22, A-aDO_2 35–45 mmHg) received steroids for >14 days, whereas 35% of the patients with severe HIV-PCP (n = 51, A-aDO_2 ≥45 mmHg) required corticosteroid therapy for ≥21 days. Four (13%) of the severe cases died, whereas no patient with moderate disease died. Among patients with severe HIV-PCP, discontinuation of corticosteroid therapy within 14 days correlated significantly with higher baseline CD4 (p = 0.049).

Conclusion

Shorter adjunctive corticosteroid therapy was clinically effective and adjunctive corticosteroid could be discontinued within 14 days in 60% of moderate-to-severe HIV-PCP and 90% of moderate cases.

Introduction

Although combination antiretroviral therapy (cART) has substantially improved the prognosis of patients with HIV-1 infection, a large number of patients are still diagnosed with HIV-1 infection at a late stage, often with concurrent opportunistic infections [1]. Pneumocystis pneumonia (PCP) is one of the most common opportunistic infections in patients with HIV-1-infection [2,3], and it is important to provide appropriate management of PCP. The American CDC Guidelines recommend 21 days of adjunctive systemic corticosteroid therapy for moderate-to-severe PCP associated with HIV-1 infection [defined as PCP with room air alveolar-arterial O_2 gradient (A-aDO_2) $\geq$35 mmHg or partial pressure of atrial oxygen (PaO_2) <70 mmHg] [4]. However, corticosteroid therapy may cause deterioration of cell-mediated immunity and enhance the development of other opportunistic infections, especially in those patients with poor immunity [5–8]. Moreover, although the American CDC guidelines further categorized moderate-to-severe PCP into two categories; severe (defined as A-aDO_2 $\geq$45 mm Hg) and moderate (A-aDO_2 $\geq$35 mmHg and <45 mmHg, or PaO_2 <70 mmHg), they recommend the same duration (21 days) of adjunctive steroid therapy for both moderate and severe PCP cases [4]. At this point, whether 21 days of concurrent corticosteroid therapy is necessary for moderate-to-severe PCP in HIV-1-infected patients, especially for moderate cases, is unknown.

Based on above background, this study was designed *1)* to elucidate the proportion of HIV-1-infected patients with moderate and severe PCP who fulfilled the criteria for use of adjunctive corticosteroid for 21 days who actually needed corticosteroid use for 21 days from retrospective chart review, and *2)* to investigate the factors associated with shorter (<21 days) duration of steroid use among such patients. In this retrospective review, the CDC guidelines were not always followed but instead use of adjunctive corticosteroids was modified based on the hospital protocol and treating physician's judgment. Thus, this study discussed what has been done in actual clinical practice and was both a discussion of actual clinical practice and patient outcomes.

Patients and Methods

Study design

We performed a single-center retrospective chart review of HIV-1-infected patients using the medical records at the National Center for Global Health and Medicine, Tokyo, Japan [9]. The study population was HIV-1-infected patients, aged 18 years and older, who was diagnosed with PCP between January 2004 and December 2012. The diagnosis of PCP was required to fulfill either one of following two criteria; confirmed PCP based on *1)* history of shortness of breath, dyspnea on exertion, and cough; and *2)* histological or cytological evidence of *Pneumocystis jirovecii* in bronchoalveolar lavage fluid, or probable PCP based on *1)* history of shortness of breath, dyspnea on exertion, and cough and *2)* abnormal CT scan findings compatible with PCP, and *3)* initiation of specific anti-pneumocystis therapy [10]. Both confirmed and probable