

Innovative, Intuitive, Flexible.

Luminex Flow Cytometry Solutions
with Guava® and Amnis® Systems

Learn More >



Luminex
complexity simplified.



This information is current as
of February 26, 2022.

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

Michihiro Hashimoto, Hesham Nasser, Farzana Bhuyan, Nozomi Kuse, Yorifumi Satou, Shigeyoshi Harada, Kazuhisa Yoshimura, Jun-ichi Sakuragi, Kazuaki Monde, Yosuke Maeda, Sarah Welbourn, Klaus Strebel, Ekram W. Abd El-Wahab, Mitsue Miyazaki, Shinichiro Hattori, Nopporn Chutiwitoonchai, Masateru Hiyoshi, Shinichi Oka, Masafumi Takiguchi and Shinya Suzu

J Immunol 2015; 195:4341-4350; Prepublished online 28 September 2015;

doi: 10.4049/jimmunol.1500955

<http://www.jimmunol.org/content/195/9/4341>

Supplementary Material <http://www.jimmunol.org/content/suppl/2015/09/28/jimmunol.1500955.DCSupplemental>

References This article **cites 64 articles**, 23 of which you can access for free at:
<http://www.jimmunol.org/content/195/9/4341.full#ref-list-1>

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2015 by The American Association of
Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



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

Michihiro Hashimoto,^{*,†,1} Hesham Nasser,^{*,†,1} Farzana Bhuyan,^{*,†} Nozomi Kuse,^{*} Yorifumi Satou,^{*,†} Shigeyoshi Harada,[‡] Kazuhisa Yoshimura,[‡] Jun-ichi Sakuragi,[§] Kazuaki Monde,[¶] Yosuke Maeda,[¶] Sarah Welbourn,^{||} Klaus Strebel,^{||} Ekram W. Abd El-Wahab,^{*,‡,2} Mitsue Miyazaki,^{*,†} Shinichiro Hattori,^{*} Nopporn Chutiwitoonchai,^{*,3} Masateru Hiyoshi,^{*,4} Shinichi Oka,[#] Masafumi Takiguchi,^{*,†,5} and Shinya Suzu^{*,†,5}

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

^{*}Center for AIDS Research, Kumamoto University, Kumamoto 860-0811, Japan; [†]International Research Center for Medical Sciences, Kumamoto University, Kumamoto 860-0811, Japan; [‡]AIDS Research Center, National Institute of Infectious Diseases, Tokyo 162-8640, Japan; [§]Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan; [¶]Department of Medical Virology, Kumamoto University, Kumamoto 860-8556, Japan; ^{||}National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; and [#]AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo 162-0052, Japan

¹M. Hashimoto and H.N. contributed equally to this work.

²Current address: High Institute of Public Health, Alexandria University, Alexandria, Egypt.

³Current address: Viral Infectious Diseases Unit, RIKEN, Wako, Saitama, Japan.

⁴Current address: Department of Safety Research on Blood and Biologics, National Institute of Infectious Diseases, Tokyo, Japan.

⁵M.T. and S.S. contributed equally to this work.

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.

Address correspondence and reprint requests to Prof. Shinya Suzu, Center for AIDS Research, International Research Center for Medical Sciences, Kumamoto University, Honjo 2-2-1, Kumamoto 860-0811, Japan. E-mail address: ssuzu06@kumamoto-u.ac.jp

The online version of this article contains supplemental material.

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

Copyright © 2015 by The American Association of Immunologists, Inc. 0022-1767/15/\$25.00

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 potentially 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. GH1/61; 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 μ m 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'-TTTTTTTTTTTTTTTTTTTTTTTTTTTGAAG-3'), or the internal control 18S rRNA forward (5'-GTAACCCGTTGAACCCATT-3') and 18S rRNA reverse (5'-CCATCCAATCGGTAGTAGCG-3'). The level of HIV-1 mRNA was calculated by the $\Delta\Delta$ Ct 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

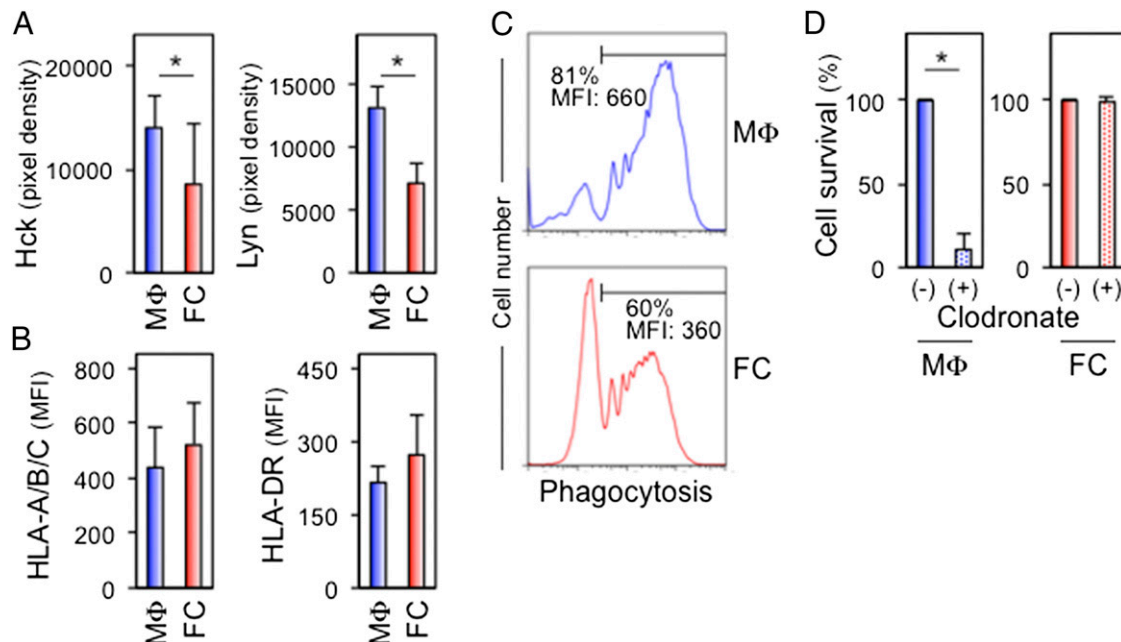


FIGURE 1. Myeloid-related phenotypes of fibrocytes. **(A)** The expression of Src family kinases (Hck and Lyn) in fibrocytes and macrophages was analyzed by Western blotting followed by the densitometric analysis ($n = 5$). **(B)** The expression of HLA-A/B/C or HLA-DR on the surface of fibrocytes and 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-1 α , and MCP-1 were lower in fibrocytes; conversely, the levels of IL-8, I-309, RANTES, MIP-1 α , MIP-1 β , 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-

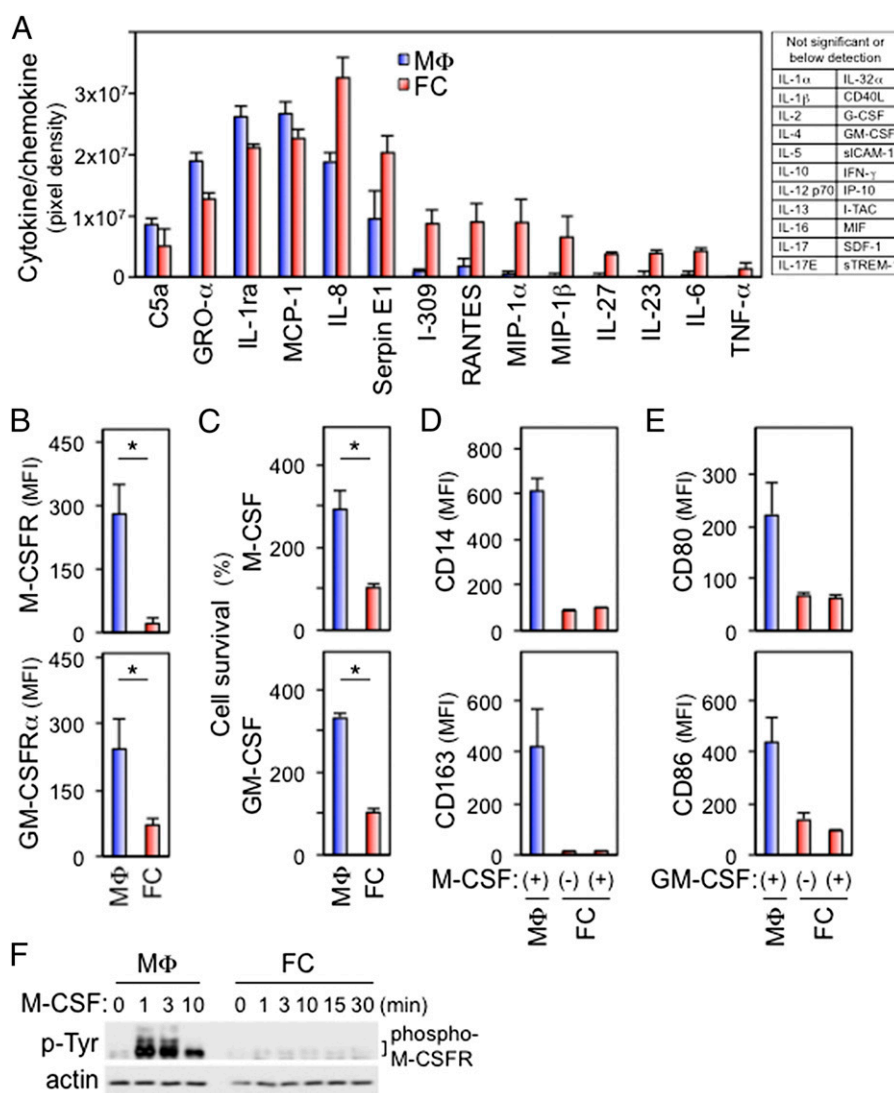


FIGURE 2. Cytokine production and response to cytokines of fibrocytes. **(A)** The relative levels of various cytokines and chemokines in media conditioned by fibrocytes or macrophages were analyzed by the Ab array. Fibrocytes were reseeded so that their number was equivalent to that of macrophages. 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.

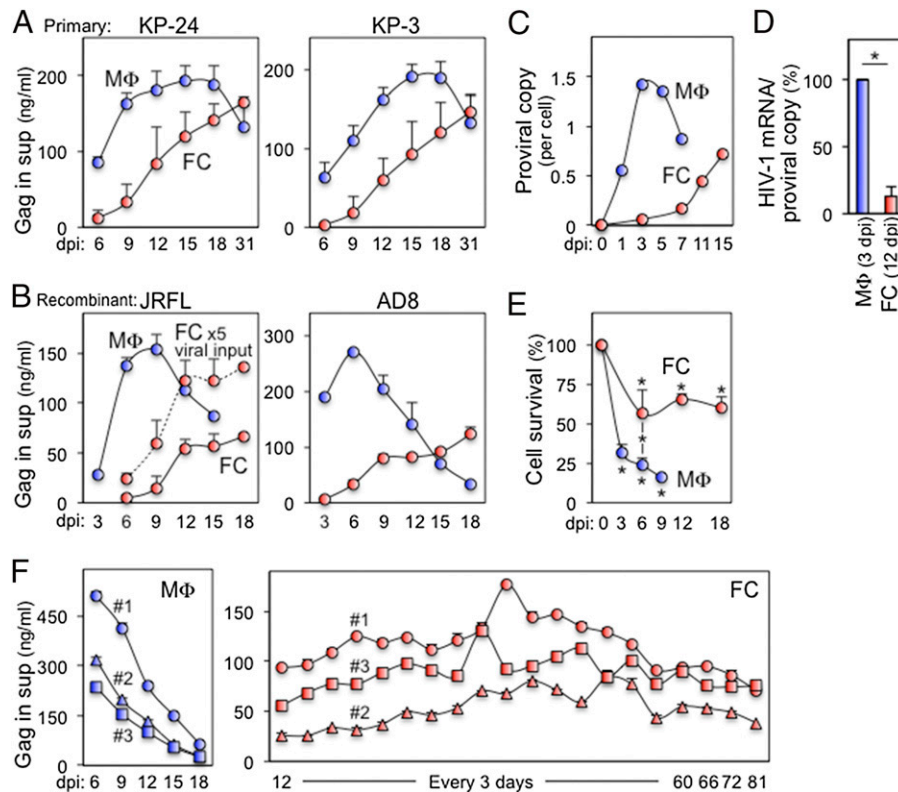


FIGURE 3. HIV-1 replication in fibrocytes. (A–F) Fibrocytes were reseeded so that their number was equivalent to that of macrophages, and they were cultured for 2 or 3 d prior to HIV-1 infection. (A) Cells were infected with the primary HIV-1 viruses (KP-24 and KP-3) and analyzed for the concentration 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 ($\times 5$ 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 $\times 5$ 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 mac-

rophages 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 IIBB 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

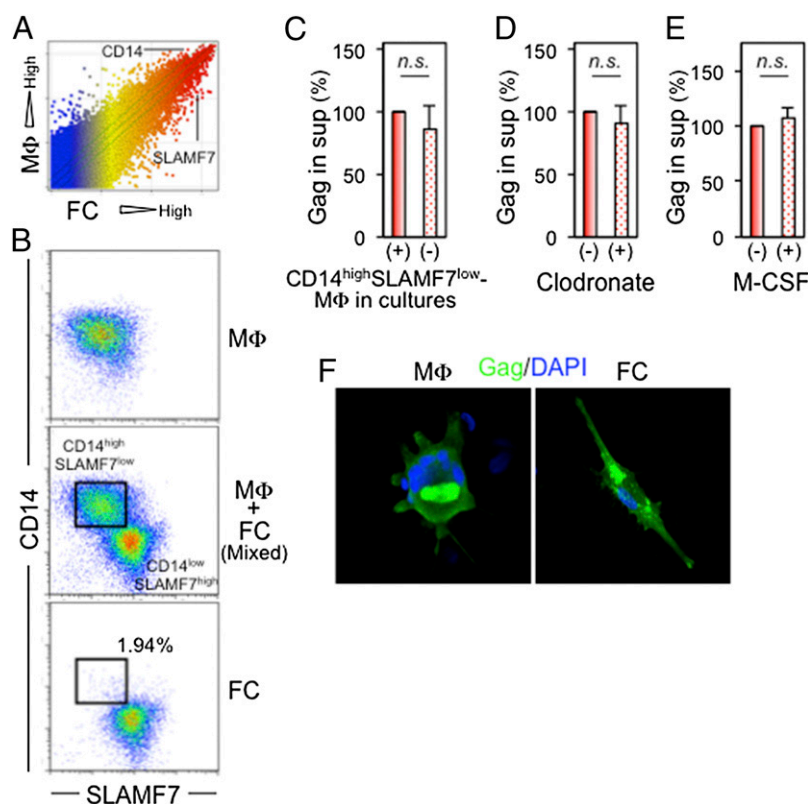


FIGURE 4. HIV-1 replication in fibrocyte cultures is not due to contaminating macrophages. (A) Microarray analysis showing the differential expression of SLAMF7 and CD14 between fibrocytes and macrophages. (B) Macrophages (top), fibrocytes (bottom), and their mixtures (middle) were costained with anti-SLAMF7 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 $\times 60$. The data shown are representative of six donors with similar results. FC, fibrocyte; MΦ, macrophage; sup, supernatant.

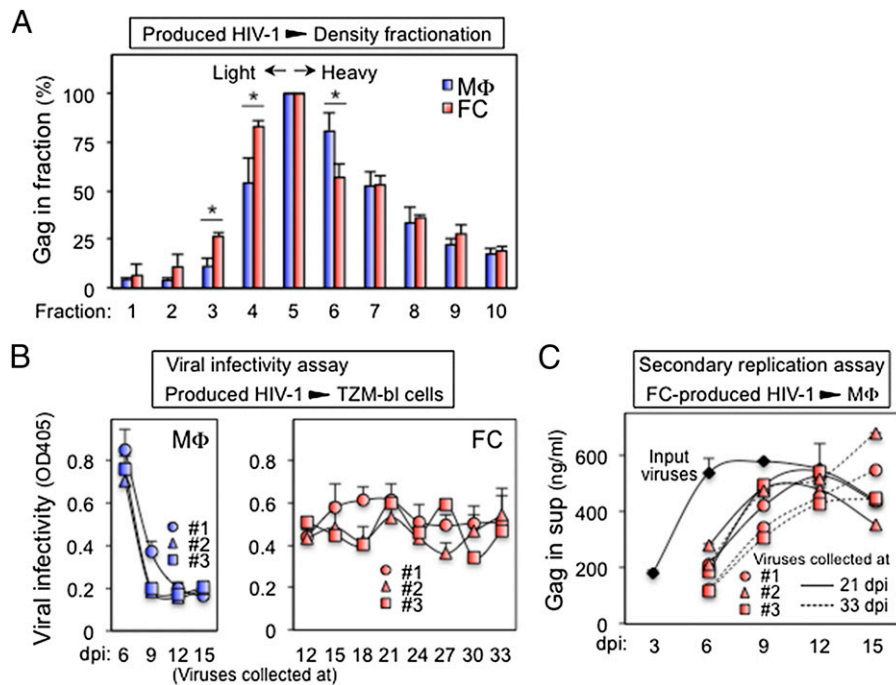


FIGURE 5. Production of replication-competent HIV-1 viruses by fibrocytes. **(A)** The particle size of AD8 viruses in the supernatants of fibrocytes (20 dpi) or macrophages (6 dpi) was analyzed by density fractionation followed by Gag ELISA ($n = 3$). The results are expressed as percentages of the 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

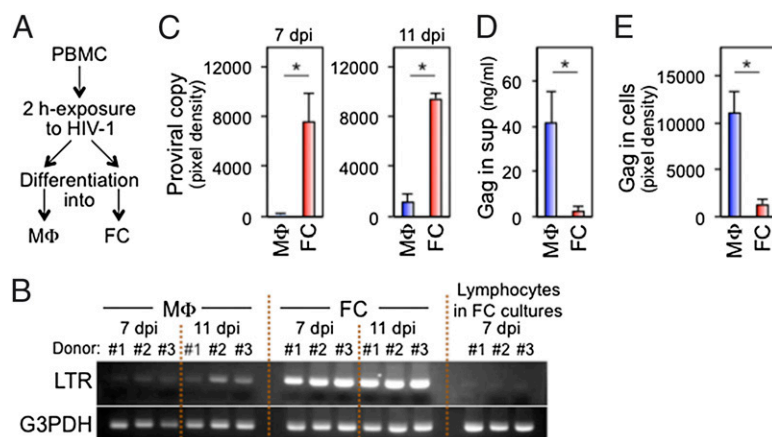


FIGURE 6. Susceptibility of circulating fibrocytes to HIV-1. **(A)** The experimental procedures for **(B)–(E)** are shown. PBMCs were exposed to AD8 for 2 h, washed extensively with PBS to remove unbound viruses, and cultured under the conditions that support the differentiation into either macrophages 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

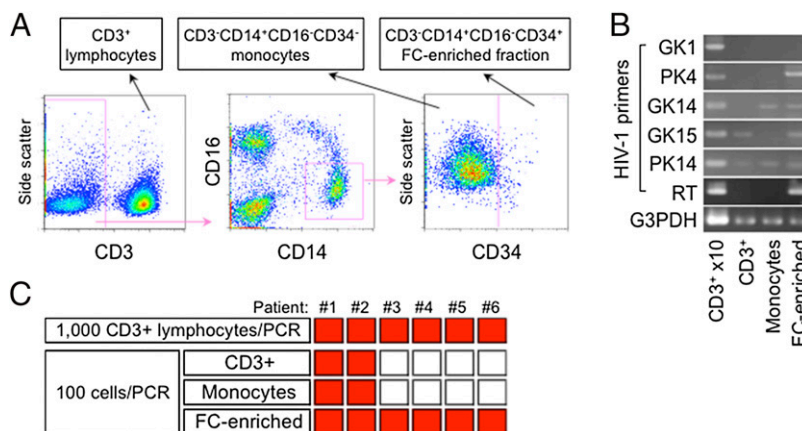


FIGURE 7. HIV-1 proviral DNA in peripheral blood fibrocyte fraction of chronically infected patients. **(A)** Sorting strategy for PBMCs of ART-untreated chronically HIV-1-infected patients. **(B and C)** The CD3[−]CD14⁺CD16[−]CD34⁺ fibrocyte-enriched fraction, CD3[−]CD14⁺CD16[−]CD34[−] monocytes, and 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-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.

References

- Eisele, E., and R. F. Siliciano. 2012. Redefining the viral reservoirs that prevent HIV-1 eradication. *Immunity* 37: 377–388.
- Chun, T. W., R. T. Davey, Jr., M. Ostrowski, J. Shawn Justement, D. Engel, J. I. Mullins, and A. S. Fauci. 2000. Relationship between pre-existing viral reservoirs and the re-emergence of plasma viremia after discontinuation of highly active anti-retroviral therapy. *Nat. Med.* 6: 757–761.
- Bailey, J. R., A. R. Sedaghat, T. Kieffer, T. Brennan, P. K. Lee, M. Wind-Rotolo, C. M. Haggerty, A. R. Kamireddi, Y. Liu, J. Lee, et al. 2006. Residual human immunodeficiency virus type 1 viremia in some patients on antiretroviral therapy is dominated by a small number of invariant clones rarely found in circulating CD4⁺ T cells. *J. Virol.* 80: 6441–6457.
- Brennan, T. P., J. O. Woods, A. R. Sedaghat, J. D. Siliciano, R. F. Siliciano, and C. O. Wilke. 2009. Analysis of human immunodeficiency virus type 1 viremia and provirus in resting CD4⁺ T cells reveals a novel source of residual viremia in patients on antiretroviral therapy. *J. Virol.* 83: 8470–8481.
- Sahu, G. K., D. Paar, S. D. Frost, M. M. Smith, S. Weaver, and M. W. Cloyd. 2009. Low-level plasma HIVs in patients on prolonged suppressive highly active antiretroviral therapy are produced mostly by cells other than CD4 T-cells. *J. Med. Virol.* 81: 9–15.
- Yukl, S. A., A. K. Shergill, T. Ho, M. Killian, V. Girling, L. Epling, P. Li, L. K. Wong, P. Crouch, S. G. Deeks, et al. 2013. The distribution of HIV DNA and RNA in cell subsets differs in gut and blood of HIV-positive patients on ART: implications for viral persistence. *J. Infect. Dis.* 208: 1212–1220.
- Yukl, S. A., E. Sinclair, M. Somsouk, P. W. Hunt, L. Epling, M. Killian, V. Girling, P. Li, D. V. Havlir, S. G. Deeks, et al. 2014. A comparison of methods for measuring rectal HIV levels suggests that HIV DNA resides in cells other than CD4⁺ T cells, including myeloid cells. *AIDS* 28: 439–442.
- Buzon, M. J., H. Sun, C. Li, A. Shaw, K. Seiss, Z. Ouyang, E. Martin-Gayo, J. Leng, T. J. Henrich, J. Z. Li, et al. 2014. HIV-1 persistence in CD4⁺ T cells with stem cell-like properties. *Nat. Med.* 20: 139–142.
- Campbell, J. H., A. C. Hearps, G. E. Martin, K. C. Williams, and S. M. Crowe. 2014. The importance of monocytes and macrophages in HIV pathogenesis, treatment, and cure. *AIDS* 28: 2175–2187.
- Carter, C. C., A. Onafuwa-Nuga, L. A. McNamara, J. Riddell, IV, D. Bixby, M. R. Savona, and K. L. Collins. 2010. HIV-1 infects multipotent progenitor cells causing cell death and establishing latent cellular reservoirs. *Nat. Med.* 16: 446–451.
- Carter, C. C., L. A. McNamara, A. Onafuwa-Nuga, M. Shackleton, J. Riddell, IV, D. Bixby, M. R. Savona, S. J. Morrison, and K. L. Collins. 2011. HIV-1 utilizes the CXCR4 chemokine receptor to infect multipotent hematopoietic stem and progenitor cells. *Cell Host Microbe* 9: 223–234.
- Durand, C. M., G. Ghiaur, J. D. Siliciano, S. A. Rabi, E. E. Eisele, M. Salgado, L. Shan, J. F. Lai, H. Zhang, J. Margolick, et al. 2012. HIV-1 DNA is detected in bone marrow populations containing CD4⁺ T cells but is not found in purified CD34⁺ hematopoietic progenitor cells in most patients on antiretroviral therapy. *J. Infect. Dis.* 205: 1014–1018.
- Josefsson, L., S. Eriksson, E. Sinclair, T. Ho, M. Killian, L. Epling, W. Shao, B. Lewis, P. Bacchetti, L. Loeb, et al. 2012. Hematopoietic precursor cells isolated from patients on long-term suppressive HIV therapy did not contain HIV-1 DNA. *J. Infect. Dis.* 206: 28–34.
- Chelucci, C., H. J. Hassan, C. Locardi, D. Bulgarini, E. Pelosi, G. Mariani, U. Testa, M. Federico, M. Valtieri, and C. Peschle. 1995. In vitro human immunodeficiency virus-1 infection of purified hematopoietic progenitors in single-cell culture. *Blood* 85: 1181–1187.
- Ruiz, M. E., C. Cicala, J. Arthos, A. Kinter, A. T. Catanzaro, J. Adelsberger, K. L. Holmes, O. J. Cohen, and A. S. Fauci. 1998. Peripheral blood-derived CD34⁺ progenitor cells: CXCR chemokine receptor 4 and CC chemokine receptor 5 expression and infection by HIV. *J. Immunol.* 161: 4169–4176.
- Redd, A. D., A. Avalos, and M. Essex. 2007. Infection of hematopoietic progenitor cells by HIV-1 subtype C, and its association with anemia in southern Africa. *Blood* 110: 3143–3149.
- Reilkoff, R. A., R. Bucala, and E. L. Herzog. 2011. Fibrocytes: emerging effector cells in chronic inflammation. *Nat. Rev. Immunol.* 11: 427–435.
- Bellini, A., and S. Mattoli. 2007. The role of the fibrocyte, a bone marrow-derived mesenchymal progenitor, in reactive and reparative fibrosis. *Lab. Invest.* 87: 858–870.
- Galligan, C. L., and E. N. Fish. 2013. The role of circulating fibrocytes in inflammation and autoimmunity. *J. Leukoc. Biol.* 93: 45–50.
- Strieter, R. M., E. C. Keeley, M. A. Hughes, M. D. Burdick, and B. Mehrad. 2009. The role of circulating mesenchymal progenitor cells (fibrocytes) in the pathogenesis of pulmonary fibrosis. *J. Leukoc. Biol.* 86: 1111–1118.
- Bucala, R., L. A. Spiegel, J. Chesney, M. Hogan, and A. Cerami. 1994. Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. *Mol. Med.* 1: 71–81.
- Abe, R., S. C. Donnelly, T. Peng, R. Bucala, and C. N. Metz. 2001. Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. *J. Immunol.* 166: 7556–7562.
- Schmidt, M., G. Sun, M. A. Stacey, L. Mori, and S. Mattoli. 2003. Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma. *J. Immunol.* 171: 380–389.
- Phillips, R. J., M. D. Burdick, K. Hong, M. A. Lutz, L. A. Murray, Y. Y. Xue, J. A. Belperio, M. P. Keane, and R. M. Strieter. 2004. Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. *J. Clin. Invest.* 114: 438–446.
- Hashimoto, N., H. Jin, T. Liu, S. W. Chensue, and S. H. Phan. 2004. Bone marrow-derived progenitor cells in pulmonary fibrosis. *J. Clin. Invest.* 113: 243–252.
- van Deventer, H. W., Q. P. Wu, D. T. Bergstralh, B. K. Davis, B. P. O'Connor, J. P. Ting, and J. S. Serody. 2008. C-C chemokine receptor 5 on pulmonary fibrocytes facilitates migration and promotes metastasis via matrix metalloproteinase 9. *Am. J. Pathol.* 173: 253–264.

27. Wynn, T. A. 2011. Integrating mechanisms of pulmonary fibrosis. *J. Exp. Med.* 208: 1339–1350.
28. van Deventer, H. W., D. A. Palmieri, Q. P. Wu, E. C. McCook, and J. S. Serody. 2013. Circulating fibrocytes prepare the lung for cancer metastasis by recruiting Ly-6C⁺ monocytes via CCL2. *J. Immunol.* 190: 4861–4867.
29. Haudek, S. B., Y. Xia, P. Huebner, J. M. Lee, S. Carlson, J. R. Crawford, D. Pilling, R. H. Gomer, J. Trial, N. G. Frangogiannis, and M. L. Entman. 2006. Bone marrow-derived fibroblast precursors mediate ischemic cardiomyopathy in mice. *Proc. Natl. Acad. Sci. USA* 103: 18284–18289.
30. Ohishi, M., W. Ono, N. Ono, R. Khatiri, M. Marzia, E. K. Baker, S. H. Root, T. L. Wilson, Y. Iwamoto, H. M. Kronenberg, et al. 2012. A novel population of cells expressing both hematopoietic and mesenchymal markers is present in the normal adult bone marrow and is augmented in a murine model of marrow fibrosis. *Am. J. Pathol.* 180: 811–818.
31. Scholten, D., D. Reichart, Y. H. Paik, J. Lindert, J. Bhattacharya, C. K. Glass, D. A. Brenner, and T. Kisseleva. 2011. Migration of fibrocytes in fibrogenic liver injury. *Am. J. Pathol.* 179: 189–198.
32. Sakai, N., T. Wada, H. Yokoyama, M. Lipp, S. Ueha, K. Matsushima, and S. Kaneko. 2006. Secondary lymphoid tissue chemokine (SLC/CCL21)/CCR7 signaling regulates fibrocytes in renal fibrosis. *Proc. Natl. Acad. Sci. USA* 103: 14098–14103.
33. Niedermeier, M., B. Reich, M. Rodríguez Gomez, A. Denzel, K. Schmidbauer, N. Göbel, Y. Talke, F. Schweda, and M. Mack. 2009. CD4⁺ T cells control the differentiation of Gr1⁺ monocytes into fibrocytes. *Proc. Natl. Acad. Sci. USA* 106: 17892–17897.
34. Pilling, D., T. Fan, D. Huang, B. Kaul, and R. H. Gomer. 2009. Identification of markers that distinguish monocyte-derived fibrocytes from monocytes, macrophages, and fibroblasts. *PLoS One* 4: e7475.
35. Chesney, J., M. Bacher, A. Bender, and R. Bucala. 1997. The peripheral blood fibrocyte is a potent antigen-presenting cell capable of priming naive T cells in situ. *Proc. Natl. Acad. Sci. USA* 94: 6307–6312.
36. Balmelli, C., N. Ruggli, K. McCullough, and A. Summerfield. 2005. Fibrocytes are potent stimulators of anti-virus cytotoxic T cells. *J. Leukoc. Biol.* 77: 923–933.
37. Balmelli, C., M. P. Alves, E. Steiner, D. Zingg, N. Peduto, N. Ruggli, H. Gerber, K. McCullough, and A. Summerfield. 2007. Responsiveness of fibrocytes to Toll-like receptor danger signals. *Immunobiology* 212: 693–699.
38. Chesney, J., C. Metz, A. B. Stavitsky, M. Bacher, and R. Bucala. 1998. Regulated production of type I collagen and inflammatory cytokines by peripheral blood fibrocytes. *J. Immunol.* 160: 419–425.
39. Curnow, S. J., M. Fairclough, C. Schmutz, S. Kissane, A. K. Denniston, K. Nash, C. D. Buckley, J. M. Lord, and M. Salmon. 2010. Distinct types of fibrocyte can differentiate from mononuclear cells in the presence and absence of serum. *PLoS One* 5: e9730.
40. Osman, A., F. Bhuyan, M. Hashimoto, H. Nasser, T. Maekawa, and S. Suzu. 2014. M-CSF inhibits anti-HIV-1 activity of IL-32, but they enhance M2-like phenotypes of macrophages. *J. Immunol.* 192: 5083–5089.
41. Chihara, T., M. Hashimoto, A. Osman, Y. Hiyoshi-Yoshidomi, I. Suzu, N. Chutiwitoonchai, M. Hiyoshi, S. Okada, and S. Suzu. 2012. HIV-1 proteins preferentially activate anti-inflammatory M2-type macrophages. *J. Immunol.* 188: 3620–3627.
42. Yoshimura, K., S. Harada, S. Boonchawalit, Y. Kawanami, and S. Matsushita. 2014. Impact of maraviroc-resistant and low-CCR5-adapted mutations induced by in vitro passage on sensitivity to anti-envelope neutralizing antibodies. *J. Gen. Virol.* 95: 1816–1826.
43. Chutiwitoonchai, N., M. Hiyoshi, P. Mwimanz, T. Ueno, A. Adachi, H. Ode, H. Sato, O. T. Fackler, S. Okada, and S. Suzu. 2011. The identification of a small molecule compound that reduces HIV-1 Nef-mediated viral infectivity enhancement. *PLoS One* 6: e27696.
44. Brussel, A., and P. Sonigo. 2003. Analysis of early human immunodeficiency virus type 1 DNA synthesis by use of a new sensitive assay for quantifying integrated provirus. *J. Virol.* 77: 10119–10124.
45. Julias, J. G., A. L. Ferris, P. L. Boyer, and S. H. Hughes. 2001. Replication of phenotypically mixed human immunodeficiency virus type 1 virions containing catalytically active and catalytically inactive reverse transcriptase. *J. Virol.* 75: 6537–6546.
46. Ohishi, M., T. Shioda, and J. Sakuragi. 2007. Retro-transduction by virus pseudotyped with glycoprotein of vesicular stomatitis virus. *Virology* 362: 131–138.
47. Shan, L., S. A. Rabi, G. M. Laird, E. E. Eisele, H. Zhang, J. B. Margolick, and R. F. Siliciano. 2013. A novel PCR assay for quantification of HIV-1 RNA. *J. Virol.* 87: 6521–6525.
48. Douek, D. C., J. M. Brenchley, M. R. Betts, D. R. Ambrozak, B. J. Hill, Y. Okamoto, J. P. Casazza, J. Kuruppu, K. Kunstman, S. Wolinsky, et al. 2002. HIV preferentially infects HIV-specific CD4⁺ T cells. *Nature* 417: 95–98.
49. Hiyoshi, M., S. Suzu, Y. Yoshidomi, R. Hassan, H. Harada, N. Sakashita, H. Akari, K. Motoyoshi, and S. Okada. 2008. Interaction between Hck and HIV-1 Nef negatively regulates cell surface expression of M-CSF receptor. *Blood* 111: 243–250.
50. Garnier, L., L. J. Parent, B. Rovinski, S. X. Cao, and J. W. Wills. 1999. Identification of retroviral late domains as determinants of particle size. *J. Virol.* 73: 2309–2320.
51. Berton, G., A. Mócsai, and C. A. Lowell. 2005. Src and Syk kinases: key regulators of phagocytic cell activation. *Trends Immunol.* 26: 208–214.
52. Van Rooijen, N., and A. Sanders. 1994. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J. Immunol. Methods* 174: 83–93.
53. Hashimoto, M., H. Nasser, T. Chihara, and S. Suzu. 2014. Macropinocytosis and TAK1 mediate anti-inflammatory to pro-inflammatory macrophage differentiation by HIV-1 Nef. *Cell Death Dis.* 5: e1267.
54. Hamilton, J. A., and A. Achuthan. 2013. Colony stimulating factors and myeloid cell biology in health and disease. *Trends Immunol.* 34: 81–89.
55. Veillette, A., and H. Guo. 2013. CS1, a SLAM family receptor involved in immune regulation, is a therapeutic target in multiple myeloma. *Crit. Rev. Oncol. Hematol.* 88: 168–177.
56. Pilling, D., Z. Zheng, V. Vakil, and R. H. Gomer. 2014. Fibroblasts secrete Slit2 to inhibit fibrocyte differentiation and fibrosis. *Proc. Natl. Acad. Sci. USA* 111: 18291–18296.
57. Koyanagi, Y., W. A. O'Brien, J. Q. Zhao, D. W. Golde, J. C. Gasson, and I. S. Chen. 1988. Cytokines alter production of HIV-1 from primary mononuclear phagocytes. *Science* 241: 1673–1675.
58. Kedzierska, K., S. M. Crowe, S. Turville, and A. L. Cunningham. 2003. The influence of cytokines, chemokines and their receptors on HIV-1 replication in monocytes and macrophages. *Rev. Med. Virol.* 13: 39–56.
59. Mathai, S. K., M. Gulati, X. Peng, T. R. Russell, A. C. Shaw, A. N. Rubinowitz, L. A. Murray, J. M. Siner, D. E. Antin-Ozerkis, R. R. Montgomery, et al. 2010. Circulating monocytes from systemic sclerosis patients with interstitial lung disease show an enhanced profibrotic phenotype. *Lab. Invest.* 90: 812–823.
60. Steiner, E., C. Balmelli, B. Herrmann, A. Summerfield, and K. McCullough. 2008. Porcine circovirus type 2 displays pluripotency in cell targeting. *Virology* 378: 311–322.
61. Isgrò, M., L. Bianchetti, M. A. Marini, and S. Mattoli. 2013. Involvement of fibrocytes in allergen-induced T cell responses and rhinovirus infections in asthma. *Biochem. Biophys. Res. Commun.* 437: 446–451.
62. Archin, N. M., J. M. Sung, C. Garrido, N. Soriano-Sarabia, and D. M. Margolis. 2014. Eradicating HIV-1 infection: seeking to clear a persistent pathogen. *Nat. Rev. Microbiol.* 12: 750–764.
63. Xu, L., S. Xiong, R. Guo, Z. Yang, Q. Wang, F. Xiao, H. Wang, X. Pan, and M. Zhu. 2014. Transforming growth factor β 3 attenuates the development of radiation-induced pulmonary fibrosis in mice by decreasing fibrocyte recruitment and regulating IFN- γ /IL-4 balance. *Immunol. Lett.* 162(1 Pt A): 27–33.
64. Isgrò, M., L. Bianchetti, M. A. Marini, A. Bellini, M. Schmidt, and S. Mattoli. 2013. The C-C motif chemokine ligands CCL5, CCL11, and CCL24 induce the migration of circulating fibrocytes from patients with severe asthma. *Mucosal Immunol.* 6: 718–727.