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Signaling through Toll-Like Receptors Triggers HIV-1 Replication in Latently Infected Mast Cells

J. Bruce Sundstrom,*‡ Dawn M. Little,* Francois Villinger,* Jane E. Ellis,† and Aftab A. Ansari*‡

Evidence that human progenitor mast cells are susceptible to infection with CCR5-tropic strains of HIV-1 and that circulating HIV-1-infected FcεRIα+ cells with a similar progenitor phenotype have been isolated from AIDS patients has led to speculation that mast cells may serve as a potential reservoir for infectious HIV-1. In this study, progenitor mast cells, developed in vitro from CD34+ cord blood stem cells, were experimentally infected with the CCR5-tropic strain HIV-1Bal after 28 days in culture as they reached their HIV-1-susceptible progenitor stage. HIV-1 p24 Ag levels were readily detectable by day 7 postinfection (PI), peaked at 2–3 wk PI as mature (tryptase/chymase-positive) HIV-1 infection-resistant mast cells emerged, and then steadily declined to below detectable limits by 10 wk PI, at which point integrated HIV-1 proviral DNA was confirmed by PCR quantitation in (~34%) of latently infected mast cells. Stimulation by ligands for Toll-like receptor (TLR) 2, TLR4, or TLR9 significantly enhanced viral replication in a dose- and time-dependent manner in both HIV-1-infected progenitor and latently infected mature mast cells, without promoting degranulation, apoptosis, cellular proliferation, or dysregulation of TLR agonist-induced cytokine production in infected mast cells. Limiting dilution analysis of TLR activated, latently infected mature mast cells indicated that one in four was capable of establishing productive infections in A301 sentinel cells. Taken together, these results indicate that mast cells may serve both as a viral reservoir and as a model for studying mechanisms of postintegration latency in HIV infection. The Journal of Immunology, 2004, 172: 4391–4401.

The advent and administration of highly active antiretroviral therapy (HAART) for AIDS patients has revealed that HIV-1 infection can persist even when plasma levels of viral RNA have dropped below detectable limits (1). Viral persistence is maintained either in specific anatomical compartments, where limited drug penetration allows viral replication to continue even in the presence of HAART, or in viral reservoirs composed of latently infected long-lived nonreplicating cells in which integrated proviral DNA is transcriptionally silent (2). The several cell lineages that are susceptible to infection with HIV-1 include T cells, macrophages, dendritic cells (3), astrocytes, endothelial cells (4), and microglial cells (5). Of these lineages, the CD4 memory T cells have to date been considered to have the greatest potential for fulfilling the essential requirements for serving as a viral reservoir for HIV-1 (6). According to the prevailing model (2, 7, 8), latency is established during the waning phase of an inflammatory response when HIV-1-susceptible proliferating effector T cells become infected because they are in the process of differentiating into long-lived resting memory T cells. Within the stable cellular environment of nondividing memory T cells, latent infection persists as long as integrated proviral DNA remains transcriptionally silent. However, as latently infected memory T cells encounter their recall Ags, ensuing proliferative responses trigger the replication and release of HIV-1 archival forms of viral sequences that have been stably preserved within the reservoir. Strategies such as drug holidays from HAART, sometimes in combination with the use of proinflammatory cytokines, have been developed in efforts to exploit this model of latency to expose reservoirs of infected memory T cells and to flush out and deplete ephemeral (t1/2 = 1 day) infected proliferating lymphoblasts in AIDS patients (9). Nevertheless, the effectiveness of these strategies is limited to viral reservoirs in anatomical sites that are exposed to HAART and to when viral replication occurs in productively infected cells that are actively proliferating. Recent reports that human progenitor mast cells (MCs) are susceptible to infection with HIV-1 and remain productively infected into maturity with a life span of months to years suggest that this new and unique reservoir HIV latency should also be considered.

Mast cells and monocytes appear to evolve from a common CD34+CD13+c-kit+ progenitor (10); however, unlike monocyte MCs, they are able to mature into effector cells with a very long life span. Mast cells develop along diverse ontological pathways in vivo, influenced by environmental signals that dictate homing patterns of precursor progenitor MCs and their ultimate mature functional phenotype. Furthermore, MCs recruit and interact with both T cells and dendritic cells in tissue sites of MC activation (11). Thus, in vivo, mature MCs are positioned in a variety of tissue spaces near blood vessels and in mucosal sites where they are exposed to environmental stimuli and function at the interface between innate and adaptive immunity (12, 13). Human cord blood-derived progenitor MCs (CB-MCs) cultured in vitro become susceptible to CCR5-tropic strains of...
HIV-1 as they transiently express CD4 and CCR5 HIV coreceptors (14, 15). As virally infected progenitors mature, they remain productively infected even after they lose their expression of CD4 and CCR5 along with their susceptibility to infection with HIV. Thus, these findings led us to speculate that MCs may serve as a potential reservoir for persistent HIV infection, and we present three important questions to address this issue: 1) can a latent (nonproductive) infection be established in mature MCs; 2) if so, can postintegration latency be reversed, leading to productive infections and is this associated with MC apoptosis or necrosis; and 3) what are the biologically relevant signals that trigger reinitiation of viral replication in latently infected MCs.

In this report, we show that HIV-infected human progenitor MCs, when cultured in vitro, lead to the emergence of latently infected tryptase/chymase mature MCs after 12–14 wk in culture. Postintegration viral latency was confirmed by PCR analysis for integrated provirus, quantitation of HIV-1 p24 Ag concentrations in culture supernatants by ELISA, and limiting dilution analysis. Stimulation with agonists signaling through Toll-like receptor (TLR)2, TLR4, or TLR9 induced reinitiation of HIV replication in one of four latently infected cells without inducing degranulation or apoptosis. Taken together, these results indicate that MCs may be both an important viral reservoir and a model for studying mechanisms of postintegration latency in HIV infection.

Materials and Methods

Culture and infection of human CBMCs

Mononuclear cells were isolated by density gradient centrifugation from whole heparinized human umbilical cord blood collected at term at Grady Memorial Hospital in Atlanta, GA in accordance with Emory University Institutional Review Board-approved protocols. CD34+ cells were then isolated from cord blood mononuclear populations by EasySep human CD34 isolation protocol (Stem Cell Technologies, Vancouver, BC, Canada) and then placed in culture with MC growth medium consisting of StemSpan Medium (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 100 ng/ml recombinant human (rec-hu) stem cell factor (SCF), 50 ng/ml IL-6, 10 ng/ml IL-10 (R&D Systems, Minneapolis, MN), 10% (v/v) FBS, 2 mM l-glutamine, and 50 µg/ml gentamicin. The human CD34+ cord blood stem cells were cultured in a humidified environment at 37°C with 7% CO2, with weekly replacement of medium. After 4 wk in culture, CD14+ cells were removed by positive selection using anti-human (anti-hu) CD14 immunomagnetic beads (Dynal, Lake Success, NY) and aliquots of the resulting enriched cultures of CD14+ CD34- progenitor MCs were incubated overnight with the R5-tropic HIV-1 strain HIV-1Bal in complete medium containing HIV-1Bal at an multiplicity of infection of 0.01 and were then washed and cultured in MC growth medium as described above.

Immunophenotyping by flow microfluorometry (FMF) and confocal microscopy

FMF was performed routinely on aliquots of CD14-depleted 4-wk-old cultured CD34+ cord blood stem cells. The cell suspension was incubated in individual 12-× 75-mm plastic tubes with 1 µg of PE-conjugated anti-human anti-hu CD14 (clone WM15), CD14 (clone M5E2), CD117 (clone YB5.B8), or -Ig isotype control (BD Pharmingen, Pleasantville, CA) and with FITC-conjugated anti-hu-TLR4 (Stressgen, Victoria, British Columbia, Canada) and with rabbit anti-human FcR ßIIa (described below) in medium for 30 min on ice and then washed twice with Dulbecco’s PBS, pH 7.4. Because these progenitor MCs had been recently purified by positive selection with mouse monoclonal anti-CD14 Abs conjugated to immunomagnetic beads, an additional cell sample was immunostained with (goat) anti-mouse PE as a second control to confirm the absence of residual cell associated anti-CD14 Ab. The cells were then immediately analyzed by FMF with a FACScan using Cellquest software (BD Biosciences, San Jose, CA).

For confocal microscopy, HIV-infected progenitor MCs were immunostained by incubation for 30 min at 4°C with PE-anti-CD117 and (rabbit) anti-human FcR ßIIa antiserum (Upstate Biotechnology, Lake Placid, NY). The cells were washed and then immunostained by incubation for 30 min at 4°C with a secondary Alexa Fluor 594 (goat) anti-rabbit IgG (H + L) conjugate (Molecular Probes, Eugene, OR). These cells were then washed again and resuspended in 200 µl of Cytofix permix (BD Pharmingen, San Diego, CA) and were then incubated at 4°C for 20 min. The fixed and permeabilized cells were then resuspended in an equal volume of PermWash containing 5 µl of FITC mouse anti-HIV-1 core Ag (clone KC57; Coulter Immunology, Miami, FL) and incubated and washed as described. Immunostained cells were mounted on spot slides with ProLong anti-Fade reagent (Molecular Probes). Stained cells were analyzed by confocal microscopy using a Zeiss laser confocal microscope (Confocal shared facility, Department of Pathology, Emory University, Atlanta, GA).

Limiting dilution analysis

A dilution series consisting of a constant number of 100,000 total cells, with the proportion of latently infected MCs in a background of (time-in culture)-matched mature uninfected (and HIV infection-resistant) MCs decreasing by 0.5 log with each dilution, was set up in six-well replicates in a Transwell culture system. Most cells were cultured in the apical chambers in growth medium in the presence or absence of a mixture of TLR-4 agonists, Escherichia coli LPS and Chlamydia heat shock protein 60 (HSP60), TLR-2 agonist Staphylococcus aureus peptidoglycan (PGD), and TLR-9 agonist unmethylated CpG-DNA (InVivoGen, San Diego, CA), at experimentally derived optimal doses. Simultaneously, a total of 100,000 uninfected (HIV-susceptible) A301 sentinel cells were set up in culture in the corresponding basal chambers. After 18 days in culture, levels of HIV-1 p24 were determined for each replicate culture by ELISA. Significantly high levels of HIV-1 p24 ranging from 456 to 512 pg/ml were detected in culture supernatant fluids of A301 cells cultured in the presence of the highest numbers of latently infected MCs, thus confirming their ability to become productively infected in this culture system. Using a cutoff value of 30 pg/ml, the total number of positive and negative HIV-1-infected replicate cultures at each dilution was used to determine the frequency of productively infected MCs by the maximum-likelihood method, using L-Calc limiting dilution analysis software version 1.1 for Microsoft Windows (StemSoft Software, Vancouver, British Columbia, Canada).

PCR procedures

The mean number of copies of integrated proviral DNA per cells was determined using a modification of the previously described linker-primer PCR (LP-PCR) method (16) adapted for real-time PCR. Chromosomal (pellet), and extrachromosomal (supernatant) DNA fractions were prepared from mock-infected or HIV-1-infected MCs and from control standards using the method described by Hirt (17). HIV-1-integrated proviral DNA copy dilution standards were prepared from chromosomal DNA fractions isolated from a series of 10 samples of cell suspensions with constant predetermined numbers containing log dilutions of the latently HIV-1-in- fected OM-10.1 cells (which harbor a single copy of integrated HIV-1 proviral DNA per cell, Ref. 18) in uninfected Jurkat cells. To prevent amplification of HIV-1-specific products from possible contaminating unintegrated circular HIV-1 DNA, chromosomal DNA preparations were subjected to additional digestion with BglI followed by treatment with Klenow polymerase as previously described (16). The number of DNA cell equivalents was determined by calculating the average real-time PCR threshold cycle number for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene amplification products corresponding to the total number of DNA cell equivalents isolated from each proviral DNA copy dilution standard. A dilution series with predetermined DNA cell equivalents along with the full set of proviral DNA copy dilution standards were then run with each nested PCR. First-round reactions were run in a total volume of 100 µl consisting of 1 X reaction buffer with 2 mM MgCl2, (Promega, Madison, WI), 0.2 mM concentrations of each dNTP, 150 pmol of LPNV primer (5’-CTATGATCAATGGACGATACATAG-3’), and 100 pmol of U3NV primer (5’-GGTCCTTCTATCTTCTGGCT-3’), 1 µl of sample, and 2 U of Taq DNA polymerase (Promega, Madison, WI, U3 primed reaction) performed at 95°C for 3 min, then 29 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, and 72°C for 10 min. Second-round (nested) PCR was performed on 1/100th of the first-round PCR product in triplicate in a final volume of 50 µl of 1 X SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA) 50 pmol of U3.1 (5’-GGAAGGCTAATTCACTC-3’) primer, and 50 pmol U3-106 (5’-CCTGCCCTTGTTGATGTC-3’) primer. Separate reactions were set up identically in the same PCR plate using GAPDH-specific primer pairs (GenBank accession number J04038). The nested PCR cycles were performed at 95°C for 3 min, then 29 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s and then held at 4°C. Reverse transcriptase PCR for expression of human TLR mRNA was performed as previously described using primer pairs specific for TLR2, TLR4, and TLR9 (19).
FIGURE 1. HIV-1-susceptible human progenitor CBMCs differentiate into mature (tryptase/chymase) MCs with limited population doublings. After 4 wk in culture in vitro in MC medium, human cord blood-derived CD34+ stem cells differentiate into phenotypically characteristic progenitor MCs that are susceptible to infection with monocytotropic HIV-1Bal. After 8 more weeks in culture (wk 12), progenitor MCs mature into HIV-1-resistant tryptase/chymase mature forms without significant proliferation. Four-week-old progenitor CBMCs are phenotypically CD117+, CD13+ and CD14+ by FMF (A), stain positive (with anti-Hu-tryptase-FITC Ab conjugate) for MC tryptase (B), and are susceptible to infection with HIV-1Bal. C, At day 14 PI, FcεRIα+ (blue (Cy5)), CD117+ (red (PE)), HIV-1 core Ag-positive (green (FITC)) progenitor MCs can be imaged by confocal microscopy. D, From wk 4 to wk 12, progenitor MCs differentiate into mature forms without significant proliferation. Cell proliferation is expressed as mean total cell counts from three separate cultures of HIV-1-infected or mock infected (UI) CBMCs determined at weekly intervals (viability >95% for each data point shown).
Characterization of induced apoptotic pathways in latently infected MCs

Qualitative and semiquantitative comparative analyses of the expression of proteins involved in intrinsic and extrinsic apoptosis-signaling pathways were performed on cell lysates prepared from age-matched populations of TLR-stimulated latently infected MCs and for purposes of comparison on uninfected MCs. A total of 2–4 × 10^6 latently infected MCs were cultured in growth medium supplemented with a mixture of TLR agonists—E. coli LPS, Chlamydia HSP60, S. aureus PDG, and bacterial CpG-oligodeoxynucleotide (ODN) (InVivogen, San Diego, CA) at experimentally derived optimal doses. An equal number of age-matched resting uninfected MCs were set up in growth medium alone as controls. After 7 days in culture, MCs were harvested and pelleted, and total cell lysates were suspended in ice-cold lysis buffer consisting of Ca^2+/-Mg^2+ free PBS containing 2 mM EGTA, 5 mM EDTA, 0.5% Nonidet P-40, and 1 μM protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO) were sonicated twice for 15 s and clarified by ultracentrifugation for 30 min at 100,000 × g. After determination of the final protein concentration by Bradford assay (Bio-Rad Laboratories), samples were adjusted to 1 mg/ml in SDS-PAGE sample buffer consisting of 31.25 mM Tris-HCl (pH 6.8), 1% SDS (w/v), 12.5% glycerol (v/v), 0.02% bromphenol blue (w/v), and 1.25% 2-ME and then boiled for 4 min at 100°C. The prepared samples were used in a Kinetworks apoptosis protein screen (Kinexus Bioinformatics, Vancouver, British Columbia, Canada).

ELISAs

Standard commercial colorimetric quantitative Ag capture (sandwich) ELISAs were used according to the manufacturer’s instructions to determine culture supernatant levels of HIV-1 p24 Ag and human cytokines TGF-β, TNF-α, IL-6, and IL-10. HIV-1 p24 levels were determined by Coulter HIV-1 p24 Ag assay (Beckman Coulter, Miami, FL), and human cytokine levels were determined by Cytoscreen ELISA kits (BioSource International, Camarillo, CA) specific for each cytokine measured.

Results

Growth characteristics of HIV-1-infected and HIV-1-uninfected MCs

As has been previously reported, enriched cultures of progenitor MCs can be derived from purified populations of cord blood-derived CD34^+ cells cultured in vitro in the presence of human IL-6, IL-10, and SCF (14). After immunomagnetic positive selection and depletion of CD14^+ cells, 4-wk-old cultures of CD117^+ CD13^+ CD14^+ tryptase^+ progenitor MCs (Fig. 1, A and B) were experimentally infected or mock infected with HIV-1Bal as described. By 10–14 days postinfection (PI), HIV-1 p24 Ag was

![FIGURE 2. Infected progenitor CBMCs mature into HIV-1 infection-resistant, latently infected mature CBMCs. A, Independent groups of CBMCs were infected after increasing time intervals in culture (x-axis). Analysis of HIV-1 p24 levels at different time points PI (different bar graphs) indicates that maturing CBMCs lose their susceptibility to infection with HIV-1 by day 56 in culture. B, HIV-1 p24 levels measured at weekly intervals PI in five independent groups of infected CBMCs (∆, ×, ♦, ○, □) revealed that maturing CBMCs became latently infected at 4–5 wk PI. C, Culture supernatant fluids and DNA isolated from independent cultures of CBMCs at increasing intervals PI were assayed by HIV-1 p24 ELISA and by real-time LP-PCR. HIV-1 p24 levels and the number of copies of integrated HIV-1 proviral increase as CBMCs mature. D, Extrachromosomal HIV-1 DNA increases PI and then decreases as maturing CBMCs become infection resistant. HIV-1 p24 levels also begin to recede as infected maturing CBMCs approach latency.](http://www.jimmunol.org/)
readily detected in culture supernatants by ELISA and productively (HIV-1) infected FcεRIα⁺CD117⁺ dual positive cells could be imaged by confocal microscopy (Fig. 1C). After prolonged culture in vitro, HIV-1-infected progenitor MCs remained productively infected as they evolved into tryptase/chymase mature forms. However, the in vivo process of maturation in both infected and uninfected MCs was remarkable for its low levels of observed cellular proliferation. Three separate cultures of CBMCs were HIV-1 infected or mock infected at day 28 in culture, then total cell number and viability counts were subsequently performed at weekly intervals for 8 wk (after a total of 12 wk in culture). Both infected and uninfected MC cultures maintained high viability during this interval (>95%; data not shown) and significant differences in the total number of cells were not observed between these two populations during this period (Fig. 1D). The total number of cells in both populations doubled by 4 wk postinfection and then declined slightly during the subsequent 4-wk period (by wk 12 in culture), whereas cell viability and phenotype remained stable among both infected and uninfected groups. Therefore, both virally infected and uninfected groups showed similar patterns of maturation in vitro, characterized by low levels of proliferation with high viability and longevity.

**Latent HIV-1 infection corresponds with increased copies of integrated proviral DNA in mature MCs**

MC maturation in vitro is also associated with the loss of expression of viral coreceptors CCR5, CD4, and CXCR4 as well as susceptibility to infection with HIV-1 (14). To carefully define the kinetics of these HIV-1 infection-related phenotypic maturational changes for this study, independent groups of triplicate cultures of CBMCs were infected at weekly intervals from days 14 to 63 in culture. Culture levels of HIV-1 p24 determined at weekly intervals from days 7 to 28 after each infection time point indicated that the susceptibility of CBMCs to infection with HIV-1 peaked at day 14 PI in CBMCs infected at day 28 in culture. However, the maximal levels of p24 steadily declined with each subsequent infection time point as CBMCs matured, and by day 56 levels had receded beyond detectable (10 pg/ml ≤) (Fig. 2A). HIV-1 replication was also shown to be closely associated with MC maturation in vitro. Mean levels of HIV-1 p24 were measured at weekly intervals PI in five independent groups of triplicate cultures of CBMC infected with HIV-1Bal on day 28 in culture. As shown in Fig. 2B, viral replication steadily increased to peak levels (>20 ng/ml) at day 28 PI and then declined to below detectable levels (10 pg/ml ≤) as mature CBMCs became latently infected by day 63–70 PI.

The diminishing levels of HIV-1 replication observed in maturing CBMCs could have been caused by the loss of integrated HIV-1 proviral DNA in infected cells or by mechanisms of postintegration latency. To distinguish between these two possibilities, chromosomal and extrachromosomal DNA fractions were collected at various times during a 6-wk period PI from independent groups of day 28 HIV-1-infected CBMCs, and the number of copies of integrated proviral DNA per 1000 cell equivalents was

![Figure 3](http://www.jimmunol.org/Downloadedfrom)
determined by the modified LP-PCR method adapted for real-time PCR as described in Materials and Methods. The specificity of the real-time LP-PCR method was confirmed by 1) absence of detectable HIV-1 long terminal repeat (LTR)-specific nested PCR products by either real-time or conventional PCR in uninfected Jurkat (Fig. 3, A and B) and by sequence homology of the 104-bp nested PCR product and HIV-1 U3-LTR (data not shown). The sensitivity of the real-time LP-PCR method was determined to be 1 copy of proviral DNA per 10,000 cell equivalents (Fig. 3C). The average number of copies of integrated proviral DNA per 1000 cell equivalents steadily increased to ≥340 copies/1000 cells by 4 wk PI (Fig. 2C). Analysis of extrachromosomal DNA fractions showed that unintegrated HIV-1 DNA peaked at ~7000 copies/1000 cell equivalents by 3 wk PI and then rapidly declined to <200 copies/1000 cells during a period corresponding to loss of susceptibility to HIV-1 infection (Fig. 2A). Elevated p24 levels during this same period also indicated that the maturing CBMCs remained productively infected (Fig. 2, C and D). These data indicate that as infected progenitor MCs matured, they remained productively infected even as they lost their susceptibility to infection. Eventually a stable population of latently infected MCs was established, containing an average of ~340 copies of integrated proviral DNA per 1000 infected mature CBMCs (Fig. 2C).

**TLR agonists increase HIV-1 replication in progenitor MCs**

Recent studies with HIV-1 transgenic mice and human microvascular endothelial cells transfected with TLR2 cDNA have shown that signaling through TLR2, TLR4, or TLR9 triggers NFkB-dependent HIV-1 LTR trans activation (20, 21). Therefore, TLR2, TLR4, and TLR9 signaling pathways were selected in this investigation to examine their role in reactivation of viral replication in latently infected MCs.

However, the expression of these TLRs on human MCs, which has remained controversial (22, 23), first had to be confirmed. Gene expression of TLR2, TLR4, and TLR9 in 28- to 40-day-old HIV-1-infected and uninfected CBMCs was confirmed by RT-PCR (Fig. 4B). Furthermore, colocalization of TLR4 and FcεRIα expression on uninfected CBMCs was shown by FMF (Fig. 4A).

Next, the effects of biologically active agonists, specific for TLR2, TLR4, and TLR9, were tested for their effects on viral

**FIGURE 4.** HIV-1-infected and uninfected human CBMCs express TLR2, TLR4, and TLR9. A, FMF analysis revealed colocalization of FcεRIα and TLR4 on populations of 28-day-old CBMCs. Contour plots represent isotype control, expression profiles for each ligand, and a coexpression profile for TLR4 and FcεRIα. B, Gene expression of TLR2, TLR4, TLR9, and GAPDH in HIV-1-infected and mock infected CBMCs was revealed by reverse transcriptase-PCR. MW STD, molecular weight standard; FL, fluorescence."
replication in day 28 HIV-1-infected progenitor MCs. After over-night infection with HIV-1 Bal, replicate cultures of CD14-de-pleted progenitor MCs were washed and resuspended in culture with varying concentrations of E. coli LPS (TLR4) (A), Chlamydia HSP60 (TLR4) (B), S. aureus PDG (TLR2) (C), bacterial CpG ODN (TLR9) (D). Culture supernatant fluids were sampled at days 9, 14, and 18 PI, corresponding to days 37, 42, and 46 in culture (indicated as 9/37, 14/42, and 18/46), and HIV-1 p24 levels were determined by ELISA. Levels of HIV-1 p24 for each dose of TLR agonists plotted vs time points PI revealed significant (10- to 20-fold) dose- and time-dependent increases in HIV-1 replication in infected progenitor CBMCs in response to each of the TLR agonists.

TLR agonists reactivate viral replication in latently infected MCs

An essential requirement for a viral reservoir is that the virus be maintained in a population of latently infected cells in some transcriptionally silent yet replication-competent form capable of infecting a new generation of target cells on reactivation. To test whether HIV-1-infected MCs could meet this requirement, latently infected CBMC (10 wk PI when P24 levels were below detectable limits) were set up in triplicate cultures with dilutions of specific TLR agonists as described above, and HIV-1 p24 levels were determined for an additional 18-day period. A time- and dose-de-pendent elevation of HIV-1 p24 levels was observed for each TLR agonist (Fig. 6). Activation through TLR2 and TLR4 resulted in peak p24 response levels by day 7 poststimulation (Fig. 3A–C), whereas activation through TLR9 resulted in peak p24 responses at day 14 poststimulation (Fig. 3D).

Although activation with TLR agonists resulted in a dose- and time-dependent reactivation of HIV-1 replication in latently infected MCs, the overall maximum levels of induced HIV-1 p24 were significantly lower (1000-fold ≤) than those observed under similar experimental conditions with TLR-stimulated HIV-1-in-fected progenitor MCs (Fig. 5). Thus, to evaluate the functional significance of these results, a limiting dilution analysis was performed to determine the frequency of latently infected MCs capable of initiating productive infections after activation by TLR agonists. To conduct this analysis, a 0.5-log dilution series ranging from 100,000 to 1 latently infected MCs in a background of age-matched uninfected and HIV-1 infection-resistant MCs was cul-tured in six-well replicates in apical chambers of Transwell culture inserts in the presence or absence of experimentally derived
optimal doses of TLR agonists, whereas a constant number of un-infected, HIV-1-susceptible A301 cells were cultured in the basal chamber as described in Materials and Methods. Samples of culture supernatant fluids were assayed for p24 levels by ELISA at days 14 and 18 poststimulation, and the number of positive wells was used in a maximum likelihood-limiting dilution analysis to determine the frequency of productively infected MCs. By day 18 poststimulation with TLR agonists, one in four MCs were shown to be productively infected, a precursor frequency that generally corresponded to the frequency of integrated HIV-1 proviral DNA in populations of latently infected CBMCs. These results confirmed that stimulation through TLR-mediated pathways could induce reactivation of viral replication and productive infection in latently HIV-1-infected MCs, thus providing further support for the role of MCs as a potential viral reservoir for HIV-1 infection.

TLR agonists or viral replication do not induce apoptosis

Another essential requirement for a viral reservoir is that the cellular population that harbors the virus must be not only able to survive the viral infection but also long-lived. Acute HIV-1 infection of CD4+ T cells leads to active viral replication, which causes death of both infected and uninfected bystander cells by multiple apoptotic and necrotic mechanisms (24, 25). In addition, apoptotic signaling pathways have also been shown to be triggered by activation through TLR-mediated pathways (26). Thus, the potential for latently infected MCs to undergo apoptosis after TLR-mediated reactivation of viral replication was assessed by performing a quantitative analysis of proteins involved in multiple apoptotic pathways. Cell lysates were prepared from equal numbers of age-matched uninfected and TLR-stimulated latently infected MCs and then subjected to a Kinexus apoptosis protein screen (Fig. 7A) as described in Materials and Methods. Procaspases 1 and 1β were significantly up-regulated (Fig. 7B), and procaspase 3 and Fas ligand (Fas-L) were significantly down-regulated relative to levels detected in uninfected controls. Although this analysis could not discriminate between active and inactive forms of the caspases measured, the absence of detectable levels of apoptosis-associated substrates of the enzymes (particularly ADP ribosyltransferase (PARP) and DNA fragmentation factor α (DFF45)) or of detectable levels of other apoptosis-associated activated caspases in cell lysate preparations from infected or uninfected MCs indicated that active apoptotic processes were not involved. Furthermore, the relative down-regulation in the expression of FAS-L in TLR-stimulated latently infected MCs suggested that apoptosis of uninfected cell would also be potentially limited.

FIGURE 6. Stimulation through TLR2, TLR4, or TLR9 causes a reactivation of virus replication in latently HIV-1-infected CBMCs. At 10 wk PI when HIV-1 p24 levels had fallen below detectable levels, E. coli LPS (TLR4) (A), Chlamydia HSP60 (TLR4) (B), S. aureus PDG (S. aur pep.; TLR4) (C), or bacterial CpG ODN (TLR9) (D) were added in varying concentrations to cultures of latently infected mature MCs. Culture supernatant fluids were sampled at days 4, 7, 14, and 18 PI, corresponding to days 74, 77, 84, and 88 in culture (indicated as 4/74, 7/77, 14/84, and 18/88) and HIV-1 p24 levels were determined by ELISA. Levels of HIV-1 p24 for each dose of TLR agonists plotted vs time points PI revealed significant dose- and time-dependent increases in HIV-1 replication in latently CBMCs in response to each of the TLR agonists. However, maximum levels of HIV-1 replication achieved in TLR stimulated latently infected CBMCs were significantly lower than those observed in TLR stimulated progenitor CBMCs.
a delayed expression of cytokines and inflammatory mediators that can potentially positively or negatively interfere with viral infection of neighboring cells. Therefore, the activation of HIV-1-infected progenitor MCs after stimulation by the TLR-4 agonist LPS was measured to characterize early and delayed release of mediators that could potentially influence viral infection of susceptible bystander cells. Stimulation of infected MCs with LPS did not result in degranulation or immediate release of detectable levels of histamine or tryptase. However, elevated levels of TNF-α could be detected by 6 h, and elevated levels of IL-6, IL-10, and TGF-β were detected by 18 h after stimulation with LPS (Fig. 8).

Discussion
By definition, a viral reservoir must fulfill two important requirements: it must be able to maintain and preserve the virus and its ability to replicate within the host; and it must be able to restore the population of infected cells as they are eliminated by viral or host immune-mediated cytotoxic mechanisms. In this report, we have demonstrated that MCs are able to fulfill both of these requirements for a viral reservoir in HIV-1 infection. Evidence for the first of these requirements was met along several lines. First, we showed that latent HIV-1 infection could be established in mature, resting MCs in vitro by 8 wk PI, a time point when HIV-1 p24 levels had receded in culture supernatants to below detectable limits, where there was no evidence of virus production, and where integrated HIV-1 proviral DNA was maintained at a level of 340 copies/1000 MCs. Second, we showed that the integrated provirus preserved in these latently infected cells was replication competent and that replication could be reactivated by biologically active agonists that trigger signaling through major TLR pathways without inducing MC degranulation, apoptosis, or replication. These findings were significant because the persistence of virus infection in the host can be considered to be proportional to the life span of the infected cells comprising the viral reservoir.
Like memory T cells, MCs are long-lived (27). However, our findings show that unlike HIV-1-infected memory T cells, latently infected MCs can respond to environmental signals that reinitiate productive virus infection without triggering cell proliferation or virus-induced cell death. These contrasting characteristics reflect important fundamental differences in innate vs adaptive immune responses. Activation of latently infected memory T cells, which in vivo generally occurs only through cognate antigenic signals delivered through clonally rearranged TCRs, results in rounds of proliferation of emerging populations of effector T cells. Proliferation triggers viral replication in infected cells and leads to virally induced T cell death (24, 28). In contrast, activation of fully differentiated mature MCs can occur through both Ag-dependent and independent pathways, which can lead to separate or overlapping effector responses without inducing MC-proliferative responses (29). Ag-dependent signaling in MCs occurs via cross-linking of IgE bound to high affinity membrane-expressed FcεRIα receptors, resulting in MC degranulation and release of preformed mediators. Ag-independent signaling occurs through MC-expressed receptors specific for various bioactive compounds, including cannabinoids (30), adenosine (31), and neuroepitides (32), and through conserved germline-encoded pattern recognition receptors that recognize pathogen-associated molecular patterns that are common to many prokaryotic pathogens (23). The latter include the family of Toll-like receptors, type 1 transmembrane receptors that have a characteristic cytoplasmic Toll-IL-1 receptor domain and an extracellular leucine-rich repeat.

At least 10 different TLRs, characterized by their specificities for different pathogen-associated molecular patterns, are expressed on diverse groups of mammalian cells, including dendritic cells, B cells, macrophages, endothelial cells, microglial cells, and MCs (22, 30–35), but little or no TLR expression has been detected on T cells (19). TLR signaling uses a common MyD88-IRAK4-TRAF6 signal transduction pathway that results in activation of NFκB (36). NFκB has been shown to be involved with HIV-1 Tat and host cellular factors, such as positive transcription elongation factor B, in promoting elongation of transcription of integrated HIV-1 proviral DNA (37, 38). Triggering through Toll receptor pathways as well as viral replication have lethal cytotoxic effects on host cells (25, 26). Nevertheless, in the studies reported herein, a significantly high viability (≥95%) was observed during a 10-wk period of productive infection in CBMC (Fig. 1C). Furthermore, our inability to detect evidence of apoptosis-associated proteins or important caspase degradation products that are present in central apoptotic pathogens (23). The latter include the family of Toll-like receptors, type 1 transmembrane receptors that have a characteristic cytoplasmic Toll-IL-1 receptor domain and an extracellular leucine-rich repeat.

Although the antiapoptotic in vitro effect of SCF-supplemented culture medium cannot be disregarded (39), in vivo MC survival and longevity are maintained through interactions with neighboring tissues as well as with bound monomeric IgE (40). The fact that viral replication occurs in nonreplicating MCs emphasizes the fact that processes of HIV-1 infection in MCs are more similar to those seen in HIV-1-infected differentiated macrophages and dendritic cells than in T cells. The process of HIV-1 infection, proviral integration, and viral replication requires a threshold basal metabolic level that for T cells is met only in actively dividing cells (28). In this regard, MCs appear to have more in common with other differentiated HIV-1-susceptible cells (e.g., macrophages and dendritic cells) in that they can maintain productive infection in the absence of proliferation. However, the enhancement of virus production in MCs through multiple TLR-mediated pathways and their resistance to virally mediated cytopathology suggest that MC viral reservoirs are potentially more stable, perdurable, and virulent than macrophage, dendritic cell, or memory T cell viral reservoirs and thus may significantly contribute to persistent HIV-1 infections.

Our data also show that HIV-1-infected MCs also meet the second requirement for a viral reservoir, the ability to replenish the population of infected cells. Not only were HIV-1 p24 levels elevated in latently infected MCs after stimulation through TLRs, but also one in four latently MCs were capable of infecting susceptible A301 cells in coculture. The same population of infected MCs exhibited physiologically normal cytokine responses to TLR stimulation (Fig. 6). Mast cell expressed products (e.g., LTβ, TNF-α, IL-1, IL-4, IL-6, IL-8, IL-13, and IL-16) can work directly or indirectly to promote T cell recruitment to extravascular tissues, facilitating MC-T cell interactions (11, 41, 42) and thus creating the potential for disseminating viral infection. Furthermore, the widespread anatomical distribution of MCs positions them not only in tissue sites (e.g., the lung, skin, and mucosal surfaces) that are exposed to the external environment where they may be likely to encounter TLR ligands but also in lesser exposed tissues, including the nervous system, heart, and even immunologically privileged sites, such as the brain (43) and testes (44). The results of this study by no means suggest that TLR-mediated signaling pathways represent a unique mode of activation of HIV-1 replication in latently infected MCs. It would be interesting to investigate whether non-TLR and nonclassical agonists would be available and able to trigger reactivation of viral replication in latently infected MCs sequestered in remote anatomical compartments protected from immune effector mechanisms or antiretroviral therapeutic agents. Indeed, our laboratory has recently demonstrated that cross-linking IgE bound to high affinity FcεRIα receptors expressed on infected CBMCs also induces HIV-1 replication (our manuscript in preparation).

To date there have only been limited reports of circulating HIV-1-infected FcεRIα+ cells with a progenitor phenotype isolated from AIDS patients with allergic disorders (15). Therefore, more results from in vivo studies are needed to verify that progenitor MCs can become latently infected and then home to, function, and persist in extravascular tissues in HIV-1-infected individuals. Recent studies by our laboratory have documented the finding of SIV-infected MCs in the lungs and other extravascular tissues spaces in SIVmac239-infected rhesus macaques. Thus, these new data (our manuscript in preparation) will support the paradigm that (HIV-1) susceptible progenitor MCs can become infected in vivo and mature into latently infected MCs that home to different tissue environments, where they can potentially serve as reservoirs for persistent HIV-1 infection.

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References
