HIV-1 infection–induced apoptotic microparticles inhibit human DCs via CD44

Davor Frleta,[1] Carolyn E. Ochoa,[1] Holger B. Kramer,[2] Shaukat Ali Khan,[1] Andrea R. Stacey,[3] Persephone Borrow,[3] Benedikt M. Kessler,[2] Barton F. Haynes,[4] and Nina Bhardwaj[1]

[1]New York University Langone Medical Center, New York, New York, USA. [2]Henry Wellcome Building for Molecular Physiology, Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom. [3]Nuffield Department of Medicine, University of Oxford, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford, United Kingdom. [4]Duke University Human Vaccine Institute, Durham, North Carolina, USA.

Acute HIV-1 infection results in dysregulated immunity, which contributes to poor control of viral infection. DCs are key regulators of both adaptive and innate immune responses needed for controlling HIV-1, and we surmised that factors elicited during acute HIV-1 infection might impede DC function. We derived immature DCs from healthy donor peripheral blood monocytes and treated them with plasma from uninfected control donors and donors with acute HIV-1 infections. We found that the plasma from patients with HIV specifically inhibited DC function. This suppression was mediated by elevated apoptotic microparticles derived from dying cells during acute HIV-1 infection. Apoptotic microparticles bound to and inhibited DCs through the hyaluronate receptor CD44. These data suggest that targeting this CD44-mediated inhibition by apoptotic microparticles could be a novel strategy to potentiate DC activation of HIV-specific immunity.

Introduction

HIV-1 infection is a worldwide health problem that is yet to be controlled due to lack of an effective vaccine (1). AIDS results from HIV-1 infection and is characterized as a chronic infection caused by a compromised immune system that renders individuals susceptible to opportunistic infections (1). Recent studies have indicated that immune system dysregulation occurs very early after HIV-1 infection (1–5). Acute HIV-1 infection (AHIV) is classified into specific stages (Fiebig stages 1–6) indicated by increasing viral load, elevation of soluble viral proteins, and appearance of HIV-specific antibodies (6). After viral transmission and prior to detectable virus in blood, there is a 7- to 10-day "eclipse phase" (6). The eclipse phase is followed by viral ramp-up (VR) during Fiebig stage 1, in which viral copies in the blood increase, after which peak viremia is reached (Fiebig stages 2 and 3). Viral titers subsequently decrease and plateau at a viral set point (Fiebig stages 4–6) (6). The early stages of AHIV (Fiebig stages 1 and 2) are also defined by an explosive production of proinflammatory and antiviral cytokines (7), yet adaptive immune responses are either compromised or substantially delayed (3, 5, 8). Studies of the events that transpire from initial infection to onset of plasma viremia are essential to understanding why effective immune responses are not induced soon after virus transmission and to identifying the barriers a vaccine must surmount.

DCs are professional antigen-presenting cells that are critical for initiating innate and adaptive immune responses (9, 10). Recognition of microbial stimuli by DCs via different pathogen-associated pattern recognition receptors induces DC activation and cytokine production that conditions subsequent T cell responses (9, 10). For example, detection of viral nucleic acid by DCs through TLR3 and TLR8 induces IL-12p70 production, which promotes a Th1 CD4+

T cell response that mediates cellular immunity and qualitatively influences antiviral antibody responses (9, 10). Furthermore, DCs can regulate innate immune responses through production of inflammatory cytokines, such as IL-6 and TNF-α, as well as stimulating NK cells (11, 12). Because of their critical role in initiating antiviral immunity, we examined the effect of AHIV on human DCs.

Previous studies have indicated that DCs are reduced in the blood of patients with HIV, the drop occurring acutely and remaining persistent in the absence of antiretroviral therapy (4). Reports on the functional capacity of myeloid-derived DCs (mDCs) in patients with HIV-1 have varied, with some indicating that isolated DCs are either hyperresponsive to stimuli or show impairment in their ability to produce proinflammatory cytokines or promote T cell activation (4, 13). One reason for these differences may be the different stages of HIV-1 infection at which patient samples were taken. These studies, which used isolated DCs, focus on the intrinsic capacity of DCs, without taking into account what is present during HIV infection that may impact DC function. During AHIV, substantial CD4+ T cell loss occurs in the gut and in the peripheral blood (3, 14). While there is significant infection of tissue CD4+ T cells, in the blood many dying CD4+ T cells, as well as other dying peripheral blood monocytes (PBMCs), are uninfected, and so death results from indirect mechanisms, such as proinflammatory factors, dysregulated cellular activity, and viral products generated during abortive viral replication (15–18). Concurrently, there is a substantial production of apoptotic microparticles (MPs), small membranous fragments (0.1–1 μm) that are released from apoptotic cells into the plasma, a subset of which express phosphatidylserine on the MP surface and have been implicated in suppression of a variety of immune functions (15). We theorized that such factors present during AHIV in patient plasma may affect DCs such that innate and adaptive immune responses are dysregulated.

Results

Monocyte-derived immature DCs generated from normal donor PBMCs were treated with plasma from uninfected control donors

or plasma from subjects during AHIV (AHIV plasma). DCs were initially pretreated with AHIV plasma obtained during VR to peak viremia (Fiebig stages 1 and 2). We then measured the responsiveness of plasma-treated DCs to selected TLR stimuli. There were no differences between DCs treated with uninfected donor plasma or AHIV plasma, regarding their phenotype (expression of MHC molecules, costimulatory molecules, or markers of maturation, e.g., CD83, CD80, and CD86; data not shown). However, DCs treated with 10% AHIV plasma were substantially compromised in cytokine production (IL-12p70, TNF-α, IL-6) upon TLR stimulation, as shown in one representative experiment depicted in Figure 1A. Specifically, AHIV plasma–treated DCs produced reduced levels of IL-6, TNF-α, and IL-12p70 relative to uninfected donor plasma–treated DCs (Figure 1A). Inhibition with 10% AHIV plasma was consistent over multiple experiments; DCs treated with 23 different AHIV plasma samples showed substantially less cytokine production when normalized to average cytokine levels produced by DCs treated with multiple different uninfected control donor plasma samples (Figure 1B). These 23 different AHIV plasma samples were tested on different donor DCs in multiple experiments normalized against 2 to 5 uninfected control plasma per experiment (the number of experiments ranges from $n = 6$ to $n = 50$ per individual AHIV plasma samples). This inhibition was also observed irrespective of the source of samples from patients with AHIV, in that AHIV plasma samples purchased from Zepto-Metrix Corp. and those collected through the Center for HIV/AIDS Vaccine Immunology (CHAVI) in North Carolina, USA, and Malawi, Africa elicited comparable DC inhibition (Figure 1B). For most experiments, DCs were stimulated with the TLR3 agonist poly I:C, because we found that poly I:C is most potent at inducing IL-12p70 production from DCs. Inhibition by AHIV plasma of DC cytokine production was also observed when using other TLR agonists, such as LPS (TLR4 agonist), peptidoglycan (TLR2 agonist), and R848 (TLR7/8 agonist) (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI64439DS1). Culturing DCs in 10% AHIV plasma overnight inhibited DC cytokine secretion, but treating DCs for as little as 3 hours also induced inhibition (data not shown). Titering plasma indicated that as little as 1% AHIV plasma could be inhibitory (data not shown), although most consistent results were obtained using 10% plasma levels. Furthermore, DCs treated with AHIV plasma had reduced cytokine production relative to that of both DCs treated with normal donor plasma or DCs under plasma-free conditions (Supplemental Figure 2), indicating that AHIV plasma specifically inhibits DCs, rather than being qualitatively poorer for DC stimulation than control donor plasma. Furthermore, DC inhibition by AHIV plasma was not a result of decreased DC viability because AHIV plasma–treated DCs were as viable after treatment as DCs exposed to uninfected donor plasma (data not shown).

DC-mediated antigen presentation is critical for activation of effector CD4+ and CD8+ T cells, and the cytokine milieu produced by DCs influences the subsequent adaptive immune response elicited (10). The negative effect AHIV plasma has on TLR-stimulated DC cytokine production would be expected to subsequently impact downstream T cell responses. IL-12p70 is critical for polarizing CD4+ T cells toward a Th1 phenotype (9). Because of the decreased levels of IL-12p70, we hypothesized that AHIV plasma–treated DCs would have a reduced capacity to prime Th1 CD4+ T cells. Accordingly, poly I:C–stimulated DCs treated with plasma

from 3 separate donors with AHIV failed to induce Th1 CD4+ T cells (as measured by IFN-γ production from the T cells), whereas poly I:C–stimulated DCs treated with uninfected donor plasma primed potent Th1 responses from naive allogeneic CD4+ T cells (Figure 1C). NK cells expressing certain receptors have recently been shown to be associated with control of HIV infection (19), and NK cells are one of the earliest sources of IFN-γ for promoting Th1 responses (20). DCs also activate and induce IFN-γ secretion by NK cells through DC production of IL-12p70 (12). Accordingly, NK cells cocultured with AHIV plasma–treated DCs exhibited reduced IFN-γ secretion relative to that of NK cells cocultured with uninfected plasma–treated DCs (Figure 1D), indicating that the deficiency in DC activity induced by AHIV plasma manifests on both CD4+ T cell and NK cell activation. Inhibited NK cell activation by AHIV plasma–treated DCs results from the defective IL-12p70 and IL-15 production by DCs, as both cytokines can reverse the observed inhibition of NK cells (data not shown). Consistent results were obtained when plasma from multiple additional donors with AHIV was tested on DCs from different normal donors; altogether 12 AHIV plasma samples were tested in T cell priming experiments, and 9 AHIV plasma samples were tested in NK cell activation experiments.

Since TLR-stimulated DC cytokine production was inhibited by AHIV plasma, we also tested whether plasma from subjects with HIV-1 at other stages of infection inhibits DCs. Plasma from patients with chronic HIV who were not on antiretroviral therapy or long-term nonprogressors (LTNPs; patients with HIV who have maintained stable CD4+ T cell counts) also inhibited DC cytokine production, although the inhibition was not as potent as with AHIV plasma (Supplemental Figure 3A). We also tested the capacity of DCs treated with different plasma to prime Th1 responses, with AHIV plasma–treated DCs promoting very poor Th1 responses, whereas untreated chronic HIV- and LTNP plasma-treated DCs elicited Th1 responses more comparable to those of DCs treated with uninfected donor plasma (Supplemental Figure 3B). Because of the higher potency of inhibition by AHIV plasma, we focused our studies on elements of AHIV plasma that inhibit DCs.

In order to determine at which time point plasma from patients with AHIV becomes inhibitory to DCs, we tested plasma isolated from patients with AHIV over sequential time points during the acute phase of infection, including the eclipse phase and VR, spanning approximately 45 days before and after viremia (8 panels of AHIV plasma). As shown in Figure 2, the time point when viremia was first detected is indicated as day 0 and the following phase is noted as VR. Plasma collected from uninfected donors over sequential time points did not mediate any significant inhibitory effects on DCs, therefore cytokine levels from DCs treated with plasma from infected donors were normalized to levels produced by uninfected donor plasma–treated DCs. As is shown in Figure 2, the inhibitory effect of AHIV plasma on TLR-stimulated DC production of IL-12p70 occurs concurrently with VR, and, for some AHIV samples, the inhibitory effect is transient, whereas in others it is maintained during high viral load. Plasma collected from donors with acute HCV or acute HBV did not mediate inhibition of DC IL-12 production, even at time of VR, indicating that the inhibition mediated by AHIV plasma is not a generalized feature of plasma from the acute phase of all virus infections (Figure 2). The same kinetics of inhibition was observed on poly I:C–stimulated DC production of IL-6 and TNF-α upon AHIV but not acute HCV plasma treatment of DCs (data not shown).
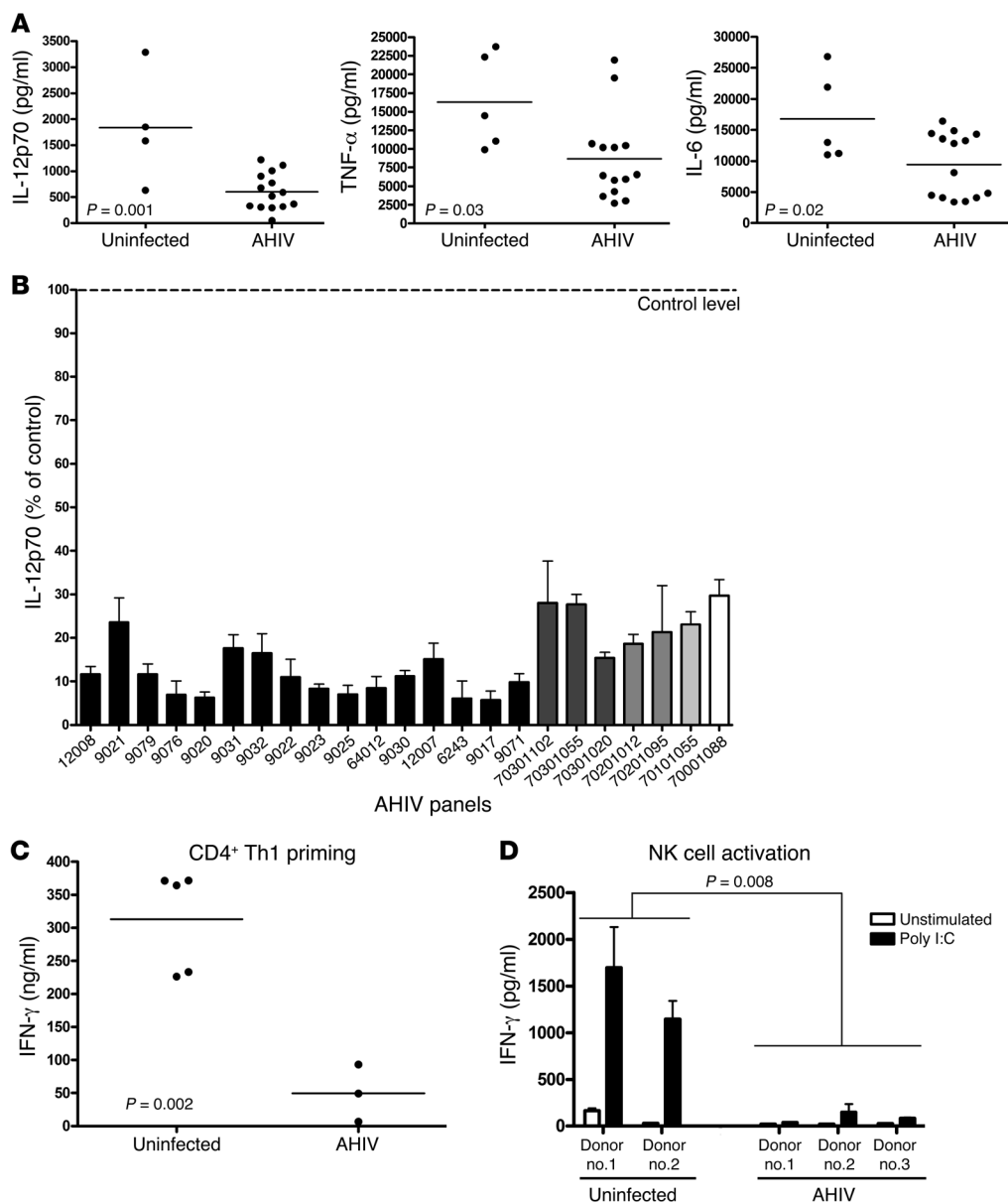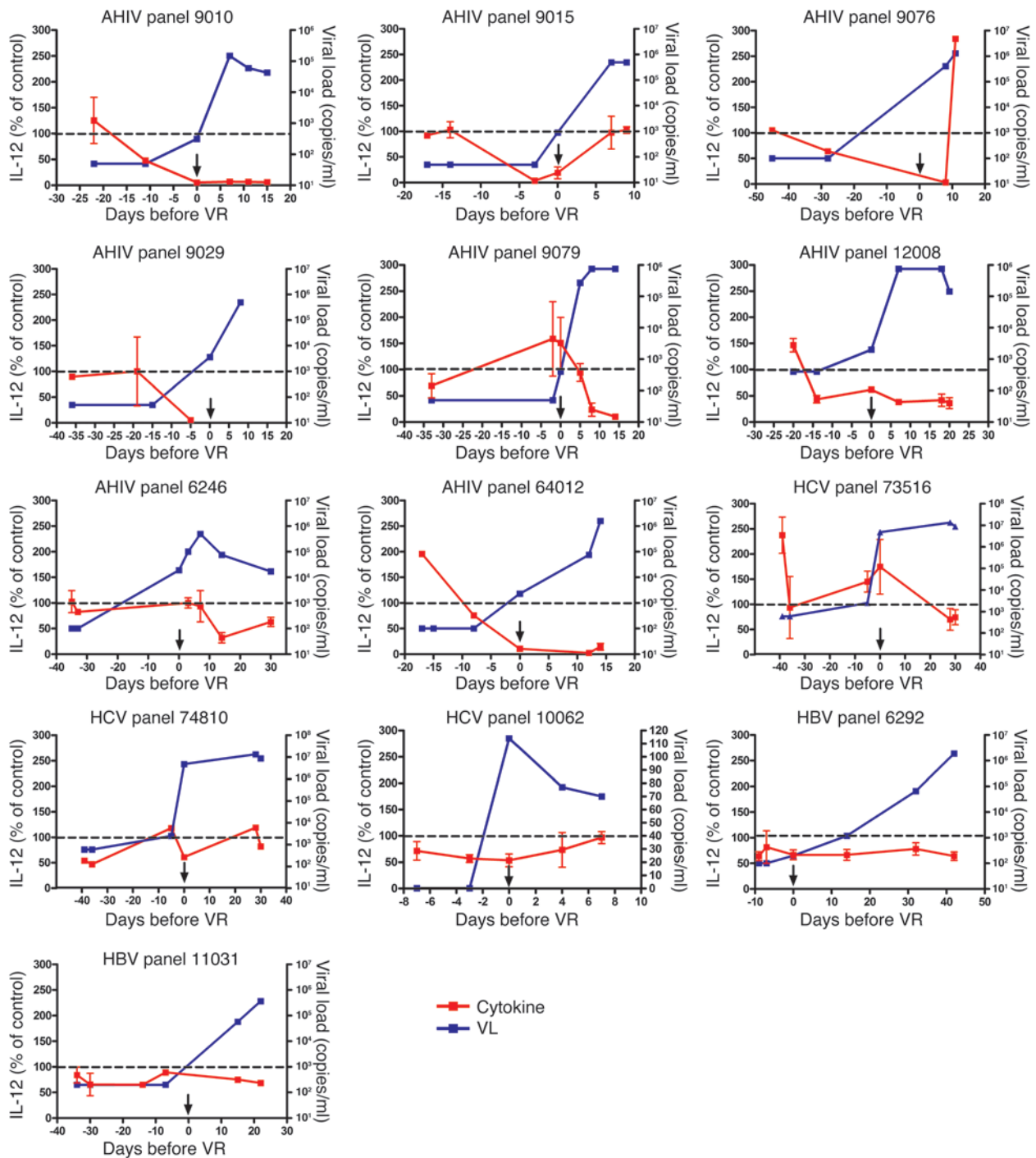
Figure 1
AHIV plasma inhibits TLR-stimulated DC function. (**A**) DCs were treated overnight with 10% uninfected donor plasma or plasma taken from patients with AHIV during Fiebig stages 1 and 2. The next day, DCs were stimulated with the TLR3 agonist poly I:C, and cytokine production was analyzed the following day. Mean is shown by horizontal bars, with each dot representing individual plasma samples. (**B**) DCs were tested as described in **A** with AHIV plasma (Fiebig stages 1 and 2), including seroconversion panels obtained from ZeptoMetrix Corp. (black bars), University of North Carolina Center for Infectious Diseases (white bar), Duke University Medical Center (light gray bar), Queen Elizabeth Central Hospital (medium gray bars), and Lilongwe Central Hospital (dark gray bars). The level of IL-12p70 production of AHIV plasma–treated DCs is shown, expressed as a percentage of the IL-12p70 levels produced by uninfected donor plasma–treated DCs (100% represents no inhibition). This figure combines multiple experiments compared against multiple uninfected control donor plasma samples (ranging from $n = 12$ to $n = 50$). (**C**) DCs were treated with plasma as described in **A**. DCs were then stimulated with poly I:C, washed, and cocultured with allogeneic naive CD4+ T cells. T cell cytokine production was analyzed at day 6. Mean is shown by horizontal bars, with each dot representing individual plasma samples. (**D**) DCs were treated as described in **C** and then cocultured with NK cells. NK cell cytokine production was analyzed the next day. Representative experiments are shown.

Because AHIV plasma inhibits TLR-stimulated DC cytokine production at time points in infection corresponding to VR, it is possible that the elevated levels of HIV virions in the plasma may be responsible for these effects. However, treating DCs with live HIV-1, a laboratory strain (ADA) or a founder strain, added together with uninfected donor plasma did not inhibit cytokine production (Figure 3A). This indicates that the inhibitory effects of AHIV plasma are mediated by factors resulting from acute infection and not by
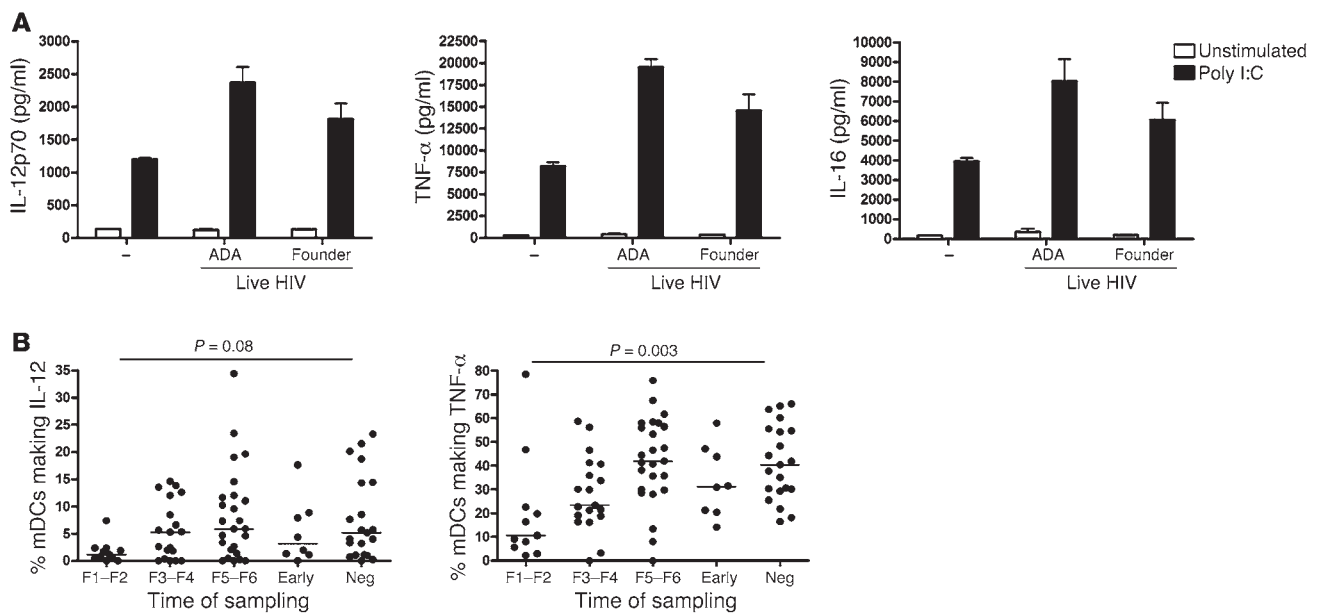
Figure 2
AHIV plasma–mediated DC inhibition occurs concurrently with VR. DCs were treated overnight with 10% AHIV, acute HBV, or acute HCV plasma collected at indicated time points before and after VR. DCs were also treated with 10% normal, uninfected donor plasma taken at various time points, generally 2–7 days apart. DCs were then poly I:C stimulated. IL-12p70 levels (as percentages of the levels from uninfected plasma–treated DCs) secreted by DCs treated with plasma from before or after viremia (red line) are graphed versus the plasma viral load (VL) (blue line). The dotted line denotes 100% of control IL-12p70 levels, indicating no inhibition. Arrows indicate start of VR.

the virus itself. It should be noted that HIV-1 by itself, when added to uninfected donor plasma, was not inhibitory to DCs across a wide range of doses, including supraphysiological doses corresponding to 300 ng/ml of p24 protein (data not shown).

Because of the effects observed with acute HIV plasma, we evaluated the function of primary mDCs from patients with acute HIV at different Fiebig stages for their capacity to produce inflammatory cytokines upon TLR stimulation. Whole PBMCs were stimu-
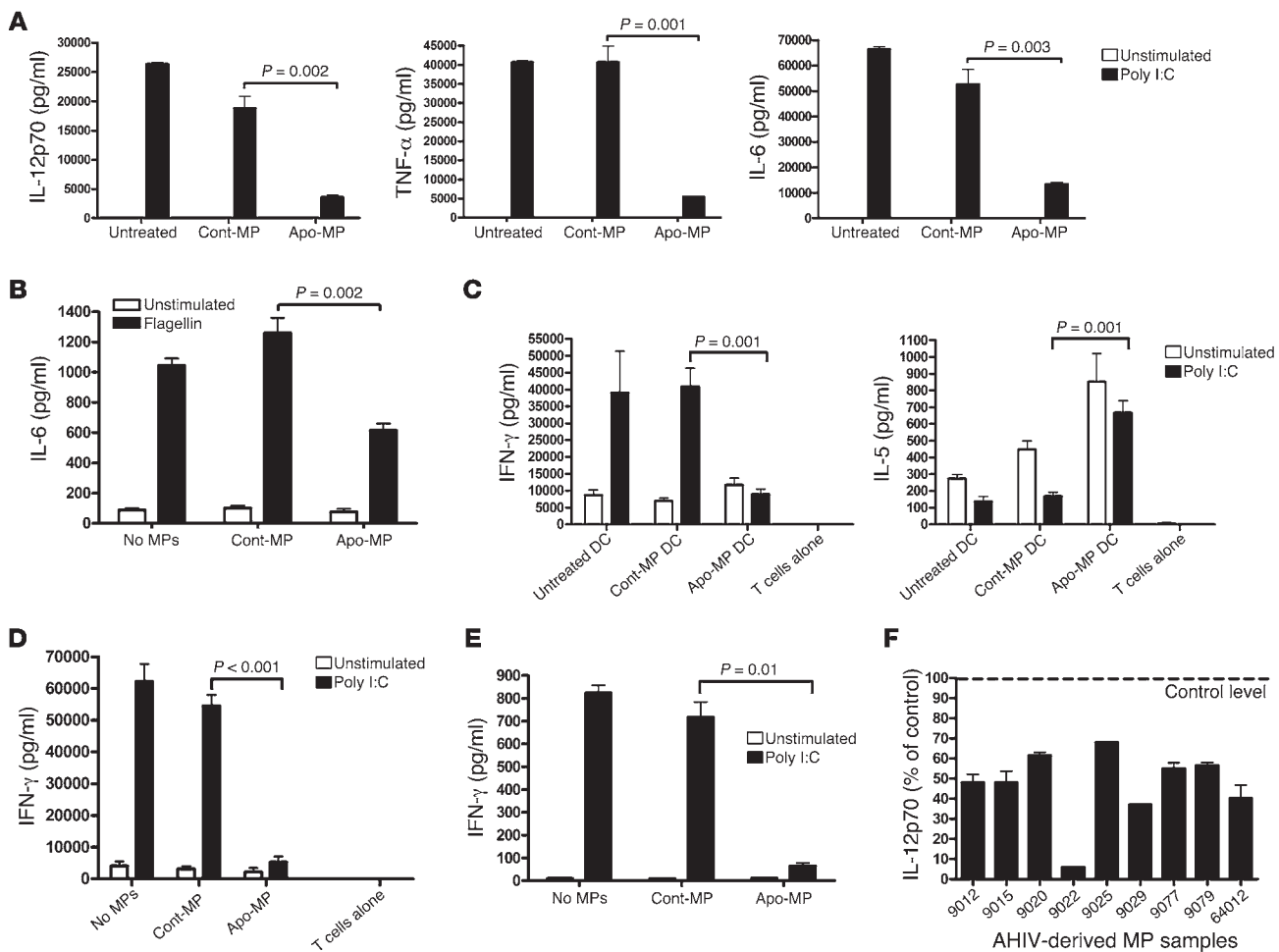
Figure 3
AHIV plasma–mediated DC inhibition is independent of virus and occurs in primary myeloid DCs. (**A**) DCs were treated overnight with 10% uninfected plasma containing live HIV-1 (ADA laboratory strain or founder strain; dose of 300 ng/ml of p24 protein) or no virus (–). DCs were subsequently stimulated and cytokine production assessed. (**B**) PBMCs from subjects at different stages of HIV infection (Fiebig stages [F] 1–6 or a time point in early infection, approximately 6 months after seroconversion) or uninfected subjects were stimulated with CL097 (Neg) (TLR7/8 agonist). Percentages of IL-12+ or TNF-α+ myeloid DCs assessed by intracellular cytokine staining are indicated. Mean is shown by horizontal bars, with each dot representing individual patient samples. The P values (Kruskal-Wallis test followed by Dunn's multiple comparison post-test) for the indicated comparisons are shown.

lated with CLO97, a TLR7/8 agonist, and cytokine production by mDCs was assessed. In agreement with our in vitro data, mDCs from patients with AHIV at Fiebig stages 1 and 2 exhibited an impaired capacity to produce cytokines, with significantly fewer cells producing TNF-α ($P = 0.003$) and results for IL-12 production that approached significance ($P = 0.08$) (Figure 3B). As with the inhibitory effects of AHIV plasma on monocyte-derived DCs, this phenomenon seemed transient in that the inhibition manifested at the earliest Fiebig stages (Fiebig stages 1 and 2) of AHIV when VR occurs (Figure 3B). Therefore, mDCs are inhibited by factors elicited early on in AHIV.

During AHIV, the apoptosis of CD4+ T cells results in elevated levels of apoptotic MPs, which are released from the apoptotic cells into the plasma (15). Elevated MPs occur concurrently with VR (15). Accordingly, we detected elevated apoptotic MPs in AHIV plasma relative to those in uninfected control plasma (Supplemental Figure 4A). Based on our data indicating that AHIV plasma becomes inhibitory at the time of VR (Figures 2 and 3), we surmised that apoptotic MPs elevated during VR impede DC function. Apoptotic MPs can also be experimentally generated from apoptotic PBMCs and isolated by ultracentrifugation. Isolated MPs were clearly apoptotic, as determined by interaction with annexin V, which binds phosphatidylserine (ref. 21 and data not shown). Electron microscopy of isolated MPs indicated small membrane-bound fragments, ranging in size from 0.1 to 1 μm (Supplemental Figure 5), as would be expected for plasma MPs (20). Mass spectrometric analysis of apoptotic MPs indicated that they were enriched in ER-derived proteins, such as Erp44, as well as stress-related proteins, such as hypoxia upregulated protein 1α

(HIP-1α) and various heat shock proteins (HSPs) (Supplemental Figure 6 and Supplemental Table 1, A and B). Using apoptotic MPs isolated from the supernatant of UV-irradiated PBMCs (to induce apoptosis), we tested the effects of experimentally derived apoptotic MPs on DC function. As controls, DCs were treated with MPs from the supernatant of non-UV-irradiated PBMCs or no MPs. Apoptotic MPs inhibited cytokine production from poly I:C–stimulated DCs (Figure 4A), although upregulation of DC activation markers and viability was unaffected (data not shown). Apoptotic MPs also inhibited DC cytokine production stimulated by TLR agonists, such as flagellin (Figure 4B), LPS, and R848 (data not shown). The dose of experimental MPs used was comparable to the number of MPs observed in AHIV plasma preparations by FACS analysis (Supplemental Figure 4A). As with AHIV plasma, apoptotic MP-treated DCs also exhibited reduced priming of Th1 CD4+ T cells, as indicated by reduced levels of IFN-γ and elevated IL-5 production (indicative of Th2 skewing) from cocultured CD4+ T cells (Figure 4C). In accordance with reduced Th1 priming, apoptotic MP-treated DCs did not prime naive CD8+ T cells, as indicated by inhibited levels of IFN-γ production from CD8+ T cells cocultured with apoptotic MP-treated DCs (Figure 4D). Moreover, apoptotic MP-treated DCs did not activate NK cells (Figure 4E). We next isolated MPs from 9 different AHIV plasma samples (at time points when plasma is inhibitory to DCs) and confirmed that MPs derived from AHIV plasma inhibit TLR-stimulated DC cytokine production (Figure 4F). DCs were treated with an equal number of AHIV- and control plasma-derived MPs, and yet only the MPs derived from AHIV plasma were inhibitory (Figure 4F). Therefore, in addition to exhibiting elevated levels,

Figure 4
Apoptotic MPs inhibit TLR-stimulated DC function. (**A**) DCs were treated overnight with apoptotic MPs (Apo-MP), control MPs (Cont-MP), or no MPs. DCs were subsequently poly I:C stimulated, and cytokine production was analyzed. (**B**) DCs were treated with MPs as described in **A**. DCs were then stimulated with 100 ng/ml flagellin, and IL-6 production was measured (flagellin-stimulated DCs do not produce substantial IL-12p70). (**C**) DCs were treated with MPs as described in **A**. After being poly I:C stimulated for 4 to 6 hours, DCs were washed, cocultured with isolated allogeneic naive CD4$^+$ T cells or (**D**) naive CD8$^+$ T cells for 6 days, and T cell cytokine production was assessed. (**E**) DCs were treated with MPs and stimulated as described in **C**. DCs were then cocultured overnight with isolated NK cells, and NK cell cytokine production was assessed. Data are representative of at least 3 independent experiments. The P values (unpaired Student's t test) for indicated comparisons are shown. (**F**) DCs were treated with MPs derived from AHIV plasma from indicated donors. DCs were then poly I:C stimulated and IL-12p70 levels were analyzed. IL-12p70 levels were normalized to levels from DCs treated with MPs derived from uninfected plasma (at least 2 different control donors per experiment). The dotted line denotes 100% of control levels, indicating no inhibition.

MPs from AHIV plasma are also qualitatively different than MPs derived from control plasma. Relative to uninfected MPs derived from control plasma, MPs derived from AHIV plasma are CD41$^-$, indicating that they are predominantly nonplatelet derived (Supplemental Figure 4, B and C). MPs derived from AHIV plasma and uninfected plasma were analyzed for specific lineage markers (CD3, CD19, CD14, and CD16), and overall the expression for any of these markers was low. CD41 is highly expressed on MPs derived from uninfected plasma, indicating that control MPs are primarily platelet derived, whereas MPs in AHIV plasma are probably derived from many different PBMC subsets that undergo apoptosis during AHIV (Supplemental Figure 4, B and C).

Because MPs are elevated in AHIV plasma during VR, we tested whether there is a synergy between virus and MPs that elicits DC

inhibition. Plasma from patients with acute HCV or HBV was not inhibitory (Figure 2), and accordingly, MPs derived from acute HCV plasma did not inhibit DC cytokine production even when DCs were treated with MPs derived from acute HCV plasma combined with live HIV-1 (Supplemental Figure 7A). Additionally, to further rule out the role of virus in MP-mediated DC dysregulation, apoptotic MPs were generated by UV irradiation of HIV-infected PBMCs. Apoptotic MPs from HIV-infected PBMCs did not elicit greater DC inhibition than apoptotic MPs from uninfected PBMCs (Supplemental Figure 7B). Finally, filtering out most MPs from supernatant of HIV-infected, irradiated PBMCs by passage through a 0.2-μM filter (which allows HIV-1 virus to pass through) showed that MPs and not virus are responsible for DC inhibition because removal of most MPs ameliorates DC inhibition (Supplemental Figure 7B).

Table 1
MP-derived proteins bound to DCs identified by MS/MS

Acc. No.[A]	Protein name	Score[B]	No. of MS/MS queries	Seq. cov. (%)	Subgroup
P16070	CD44 antigen	112	2	3.1	DC+ apoptotic MPs
P17927	CD35 antigen	52	2	1.2	DC+ apoptotic MPs
P61224	Ras-related protein Rap-1B	60	2	13.6	DC+ apoptotic MPs
Q9Y286	CDw328, sialic acid–binding Ig-like lectin 7	45	1	2.6	DC+ apoptotic MPs
P05556	CD29 antigen	110	3	4.8	DC+ apoptotic/control MPs
P02786	CD71, TfR1	57	2	3.3	DC+ apoptotic/control MPs
P16050	Arachidonate 15-lipoxygenase	72	2	1.8	DC+ apoptotic/control MPs
P16284	CD31, PECAM-1	51	2	1.5	DC+ apoptotic/control MPs
P31994	CD32, IgG Fc receptor II-b	61	1	6.1	DC+ apoptotic/control MPs
Q07954	CD91, LRP	81	3	0.9	DC control MPs
P61026	Ras-related protein Rab-10	69	1	5.5	DC control MPs

DCs were surface biotinylated and then treated with no MPs, control MPs, or apoptotic MPs. MPs were cleaved off by trypsin treatment, and DCs were removed by centrifugation. Collected supernatant was then ultracentrifuged to collect MPs (with bound DC-derived proteins). MPs were lysed and DC-derived proteins were isolated by streptavidin column to collect biotinylated proteins. Apoptotic MP and control preparations were analyzed by MS/MS. [A]Accession number (Acc. No.) given for the SwissProt database (UniprotKB). [B]Mascot protein score indicates the log value of the probability that this protein hit is a random event. Seq. cov., sequence coverage.

Based on our data, we surmised that elevated apoptotic MPs in AHIV plasma contribute to the inhibition of DCs that accounts for dysfunctional Th1 priming as well as NK cell activation. We strove to elucidate the mechanism by which apoptotic MPs inhibit human DCs. In order to determine which receptors on DCs could be mediating the inhibitory effects of apoptotic MPs, we isolated MP-binding proteins from surface-biotinylated DCs and performed mass spectrometry (MS). Cell surface proteins that specifically bind apoptotic MPs were identified by comparing mass spectrometric analysis of apoptotic MP pull-down preparations and excluding proteins that appeared in control MP preparations. A list of DC surface proteins (or associative signaling molecules) that specifically bound apoptotic MPs is shown in Table 1. Several candidate proteins were identified, including CD35 (complement receptor 1), CD44 (phagocytic glycoprotein 1), and FcγRIIb (CD32b; inhibitory Fc receptor). All of these were sequentially tested, but only CD44 was determined to play a role in apoptotic MP-mediated DC inhibition.

CD44 is upregulated on circulating mDCs from patients with AHIV and early HIV (Supplemental Figure 8). It is possible that the upregulation results from inflammation associated with early HIV infection (7). Binding of CD44 selectively to apoptotic MPs was confirmed by staining apoptotic MPs with CD44-Fc chimeric protein (Figure 5A). Blockade of CD44 on DCs with anti-CD44 monoclonal antibody (data not shown) or competing for MP interaction with CD44 on DCs using soluble CD44-Fc chimeric protein (Figure 5B) relieved apoptotic MP-mediated inhibition of poly I:C–stimulated DC cytokine production. Blocking CD35 or FcγRIIb did not have any effect on apoptotic MP-mediated DC inhibition of cytokine production (data not shown). Blocking CD44 with anti-CD44 monoclonal antibody, or pretreating MPs derived from AHIV plasma with CD44-Fc, ameliorated the inhibitory effects of MPs derived from AHIV plasma on DCs, as indicated by restoration of IL-12p70 production from DCs stimulated with poly I:C (Figure 5C). In order to test whether CD44 ligation of DCs is sufficient to inhibit DCs, we used receptor-targeted antibodies crosslinked on beads (22). As shown in Figure 5D, crosslinking CD44 on DCs inhibits IL-12p70 production in response to poly I:C stimulation and IL-6 produc-

tion in response to LPS. Thus, signaling through CD44 on DCs is sufficient to inhibit TLR-stimulated DC cytokine secretion. Despite binding to annexin V, treatment of apoptotic MPs with annexin V failed to alleviate their inhibition of DCs (Supplemental Figure 9A); moreover, neither did treatment of apoptotic MPs with another phosphatidylserine binding protein, MFG-E8 (Supplemental Figure 9B) alleviate inhibition of DCs, indicating that apoptotic MP-mediated DC inhibition is not due to any classical phosphatidylserine receptor, such as Tim-4, BAI, or Stabilin-2 (23).

Interestingly, CD44 also mediates endocytic uptake by DCs (24–28), which led us to examine whether uptake of apoptotic MPs is required for their MP-mediated DC inhibition. Receptor-mediated uptake by DCs is commonly regulated by the GTPase Rac1, which controls actin filament nucleation (29, 30). Blocking Rac1 signaling abrogates DC uptake capacity (29, 30). Rac1 and associated molecules such as Rap-1b also appeared in our mass spectrometric analysis (though for both control and apoptotic MPs; Table 1). We hypothesized that the inhibitory effect of apoptotic MPs is linked to their uptake by DCs. Supporting this, inhibition of Rac1 signaling via a specific chemical inhibitor almost completely reversed the inhibitory effects of apoptotic MPs on DC cytokine production (Figure 6A). Using CFSE-labeled MPs and confocal microscopy, we determined that Rac1 inhibition abrogates engulfment of MPs by DCs but still allows DCs to bind MPs (Figure 6B). This is in agreement with the role of Rac1 in regulating actin polymerization during phagocytosis/endocytosis. Thus, blocking MP uptake with cytochalasin D treatment (which disrupts actin filament formation) of DCs completely abrogated the inhibitory effects of apoptotic MPs on DC cytokine production (Figure 6C). Although these data suggest that engulfment of MPs is required for DC inhibition, it should be kept in mind that downstream signaling events could also be affected by Rac1 inhibition and actin filament disruption, irrespective of MP uptake by DCs.

Discussion

With emerging evidence of early immune dysregulation during AHIV, it is imperative to understand the microenvironment
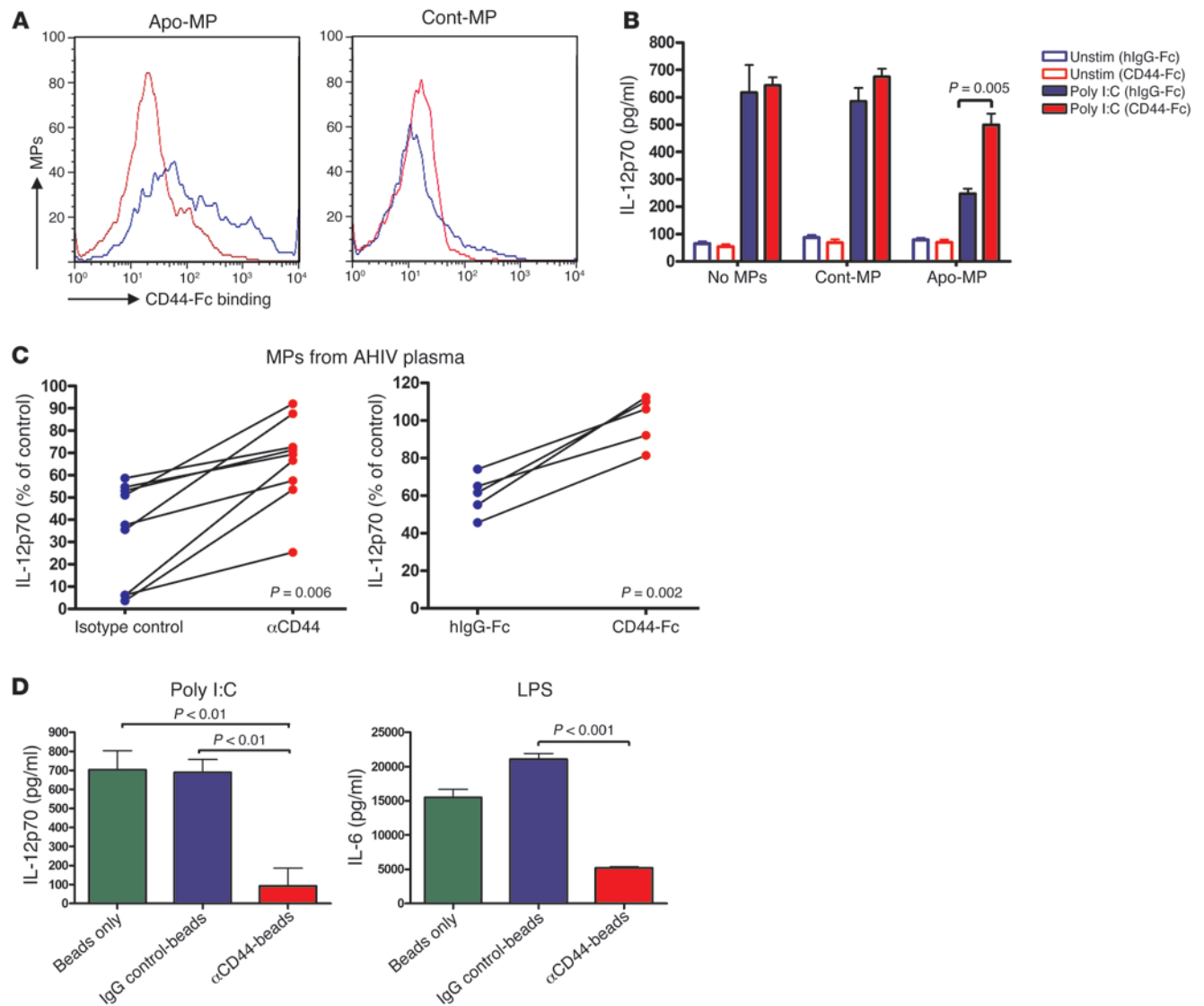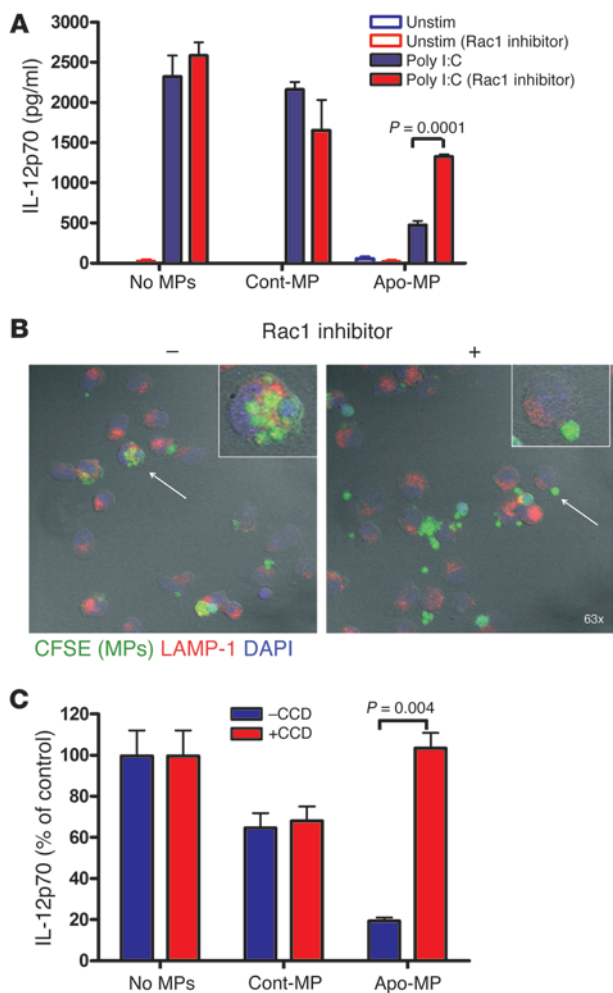
Figure 5
Inhibition of DCs by AHIV plasma is dependent on CD44. (**A**) Control or apoptotic MPs were stained with FITC-labeled CD44-Fc (30 μg/ml; blue line) and analyzed by FACS. Control staining was with FITC-labeled human IgG-Fc (red line). (**B**) MP preparations were pretreated with 50 μg/ml CD44-Fc chimeric protein or human IgG-Fc protein (hIgG-Fc). DCs were treated overnight with control or apoptotic MPs and subsequently poly I:C stimulated, and cytokine production was analyzed the following day. Data are representative of at least 3 independent experiments. The *P* value (unpaired Student's *t* test) for indicated comparisons is shown. (**C**) DCs were pretreated with 50 μg/ml anti-CD44 blocking monoclonal antibody (BD Biosciences; clone 515) or mouse IgG isotype control. An alternative method involved pretreating plasma-derived MPs with CD44-Fc or hIgG-Fc control. DCs were then treated with MPs derived from uninfected or AHIV donors and subsequently stimulated with poly I:C. IL-12p70 secretion by DCs was analyzed the next day, and levels of IL-12p70 from AHIV MP-treated DCs were normalized to levels from DCs treated with MPs derived from uninfected donor plasma. Results are representative of at least 5 separate experiments with different control and AHIV donors. The *P* values (paired Student's *t* test) for indicated comparisons are shown. (**D**) DCs were treated with microbead-bound anti-CD44, mouse IgG1 control, or microbeads only. DCs were subsequently poly I:C or LPS stimulated, and cytokine levels were assessed (IL-12p70 for poly I:C; IL-6 for LPS; LPS-stimulated DCs do not produce substantial IL-12p70). The *P* values (unpaired Student's *t* test) for indicated comparisons are shown.

induced during AHIV that results in such dysregulation (1, 3). Despite the generation of proinflammatory cytokines in plasma from patients with AHIV (7), we have shown that the plasma milieu inhibits DC function, resulting in subsequently perturbed Th1 and NK cell responses. Because DCs are critical for initiation of adaptive immunity, we surmise that such inhibition can seriously impact establishment of controlling HIV-specific immunity.

Previous studies of the functional quality of mDCs in patients with HIV-1 have given variable results (1, 4, 13), possibly as a consequence of the stage of infection at which DCs were analyzed as well as the use of isolated DCs, effectively removing them from the microenvironment that modulates their function. Accordingly, the inhibitory effects of AHIV plasma on DCs were observed with striking kinetics, i.e., concurrent with VR, and primary mDCs iso-

Figure 6
Mechanisms of apoptotic MP-mediated DC inhibition. (**A**) DCs were pretreated with 50 μM Rac1 inhibitor (EMD Chemicals) for 30 minutes and then treated with control MPs, apoptotic MPs, or no MPs. DCs were subsequently washed and poly I:C stimulated, and cytokine production was analyzed the following day. (**B**) DCs were pretreated with 50 μM Rac1 inhibitor for 30 minutes and then treated with of CFSE-labeled control or apoptotic MPs (green). After 3 hours, DCs were mounted, stained with LAMP-1 (red) and DAPI nuclear stain (blue), and analyzed by confocal microscopy (original magnification, ×63; inset, ×252). Arrows indicate individual cells shown in insets. (**C**) DCs were pretreated with 10 μM cytochalasin D (CCD; Sigma-Aldrich) for 30 minutes and then treated with control MPs, apoptotic MPs, or no MPs. DCs were subsequently washed and poly I:C stimulated, and cytokine production was analyzed the following day.

that HIV-1 directly inhibits DCs during AHIV. Furthermore, addition of supraphysiological doses of live HIV-1 to control plasma did not inhibit DCs, indicating once again that plasma factors generated during specific stages of AHIV modulate DC function.

During AHIV, there is significant cell death occurring in all PBMC subsets, mostly in uninfected cells because of inflammation-derived bystander mechanisms (15, 16). Subsequently, during VR, there is a substantial increase in apoptotic MPs, and we show that these apoptotic MPs can inhibit DC function. In addition to being elevated during VR in AHIV, apoptotic MPs are qualitatively different from circulating MPs found in control plasma, as shown by the fact that when equal numbers of MPs are used to treat DCs, MPs derived from AHIV plasma inhibit DC function whereas circulating MPs from control plasma do not. In uninfected donor plasma, the majority of circulating MPs (60%–90%) are platelet-derived, as denoted by CD41 expression (40, 41). MPs derived from CD41⁺ platelets transport bioactive molecules; participate in hemostasis, thrombosis, and inflammation; and are associated with atherosclerosis and certain malignancies (40, 41). Inhibitory apoptotic MPs from AHIV plasma differ from uninfected MPs derived from control plasma and lack CD41 expression. This would indicate that the inhibitory MPs arising during AHIV are from a different cellular source, not platelet derived. Although it is clear that MPs from AHIV plasma are of leukocyte origin (data not shown), the percentage that expressed any specific lineage marker (CD3, CD19, CD14, and CD16) is overall low, possibly because there is no selective enrichment of any particular PBMC subset as a source of MPs, as many different subsets undergo apoptosis during AHIV (Supplemental Figure 4C). Alternatively, during AHIV, leukocytes may downregulate expression of lineage markers, which would account for low level of expression on subsequently generated MPs.

Despite elevated MP levels during VR, MP count does not correlate with viral load (data not shown), which is expected since most dying cells are uninfected (15). Furthermore, AHIV plasma samples are comparably inhibitory, with samples that have higher MP levels not having significantly greater inhibitory capacity than AHIV plasma samples with lesser MPs. This is probably due to the qualitative versus quantitative characteristics of the MPs present during AHIV that mediate such potent DC inhibition. This suggests that the proapoptotic environment elicited during AHIV may influence how MPs are generated from PBMCs. Stacey et al. reported a transient increase in IL-15 and IFN-α at the time of VR in these same AHIV plasma samples as well as a more prolonged increase in TNF-α and MCP-1 and later increases in

lated from patients with AHIV indicated inhibited function only at Fiebig stages 1 and 2, corresponding to the earliest stages of AHIV. Our group previously showed that mDCs from patients with AHIV exhibited hyperresponsiveness to TLR stimulation upon isolation (4); however, at the time, very few Fiebig stage 1 and 2 samples were available for analysis, and most studies were performed with mDCs isolated later in AHIV. Our current data indicate that the inhibitory effects of AHIV plasma are observed only at the earliest stages of AHIV.

Interestingly, despite setting in during VR, AHIV plasma–mediated DC inhibition was not a result of virus itself, but rather resulted indirectly from factors elicited during AHIV. mDCs are not responsive to HIV-1 due to an inability of sensing HIV-1 or HIV-1–infected cells, whereas plasmacytoid DCs are triggered upon HIV-1 infection via TLR7 (31–33). The virus has been shown to bind to mDCs via C-type lectins, which promotes transmission of HIV from migratory tissue-derived mDCs to CD4⁺ T cells localized in secondary lymphoid organs (34, 35). However, HIV-1 cannot establish a productive infection in mDCs due to inhibition of viral replication by SAMHD1 (36, 37). It has been shown that virus expressing Vpx can overcome this replication blockade and trigger IFN production from mDCs via a cryptic cytoplasmic sensor (38, 39). Nevertheless, active HIV-1 infection of mDCs has not been conclusively shown during AHIV (1, 4). Therefore, it is unlikely

IL-6, IL-8, IL-10, IL-18, and IFN-γ (7). The inhibitory effects of the AHIV plasma occur at time points before the observed cytokine increase, so it is unlikely that the qualitative differences in our MPs are due to plasma cytokines.

It would be interesting to evaluate whether the DC inhibition observed with these AHIV plasma samples correlates to physiological parameters, such as CD4^{+} count and disease progression. However, these studies are impossible because (a) most of our AHIV plasma samples are derived from anonymous donors for whom demographic and etiologic data are not available and (b) such studies would require donors to abstain from antiretroviral therapy, which, due to current treatment practices, is untenable (42, 43).

We have made the observation which we believe to be novel that CD44 is a receptor for apoptotic MPs on DCs, and that it promotes MP-mediated DC inhibition. CD44 is a cell surface proteoglycan that mediates binding of immune cells to the extracellular matrix (44, 45). CD44 is also a phagocytic receptor and is described as the receptor for hyaluronic acid (44, 46). Additionally, binding to CD44 by different ligands elicits both proinflammatory and antiinflammatory responses. For example, CD44 interaction with osteopontin has been implicated in DC-mediated contact hypersensitivity reactions (47), whereas binding of hyaluronic acid disrupts LPS signaling (48). CD44 binding to MPs is likely not through hyaluronic acid, as we failed to detect any hyaluronate on the MPs (data not shown); therefore the CD44 ligand on apoptotic MPs remains to be characterized and is the subject of a separate set of studies. We surmise that heterogeneous expression of CD44 ligand(s) on MPs accounts for the heterogeneity in CD44 binding. Apoptotic MPs are enriched in stress-related proteins, such as HSPs and HIP-1α, which would suggest that the ligands could also be dependent on the cellular death process that generated the MPs.

Previous reports have indicated that CD44 activation enhances phagocytosis of apoptotic neutrophils by macrophages (28, 49). CD44-mediated phagocytosis of beads also induces inside-out activation of complement receptor 3 (CR3; CD11b/CD18 complex) (50), and we and others have reported that signaling through CR3 can inhibit DC function (22, 27, 51). CD44 also has been shown to negatively regulate TLR signaling and subsequent inflammation due to the fact that *Cd44*^{−/−} mice exhibit increased NF-κB signaling, proinflammatory cytokine production by TLR-stimulated macrophages, and consequentially more severe TLR-induced arthritis (52). However, this is the first report to our knowledge of CD44 on DCs binding apoptotic debris and playing a direct role in the inhibitory effects of apoptotic MPs. It is interesting to note that CD44 is widely expressed on immune cells, such as DCs, T cells, and NK cells (44). Yet, apoptotic MPs do not directly inhibit activation of NK cells or T cell differentiation (data not shown), rather they act indirectly through blockade of DC cytokine production. One possible reason for this may be differential CD44 isoform expression on DCs. Due to differential mRNA splicing, at least 20 different isoforms of CD44 exist (53), and it remains to be characterized whether there is selective interaction of apoptotic MPs with specific CD44 isoforms. Another possible reason may be that CD44 mediates MP binding, but internalization of MPs is required for their inhibitory effect as is suggested by our data with Rac1 and actin polymerization blockade. NK cells and T cells do not engulf MPs comparably to DCs. Thus, the clearance of apoptotic MPs by DCs via CD44 binding may be linked to the MP inhibitory effects as indicated by our data with Rac1 blockade and actin filament disruption. However, our data also indicate that CD44 ligation is sufficient to inhibit TLR-stimulated DC cytokine production, and so it seems unlikely that engulfment of MPs is necessary if there is adequate CD44 triggering. We therefore surmise that Rac1 signaling is an important downstream pathway of CD44 that elicits DC inhibition and may also represent a target that can be exploited to enhance DC-based immunity.

DCs are critical in vivo for initiating HIV-specific immunity, and in vitro–derived DCs are being developed in HIV vaccine strategies. Furthermore, in vivo DC strategies for generation of HIV-specific immunity are also being tested. Various groups have targeted HIV-specific antigens to DCs in vivo through DC-specific targeting antibodies, HIV-infected apoptotic cells, or even direct immunization with viral vectors (54–56). These in vivo strategies require an understanding of how factors, such as apoptotic MPs, may impede DC function. Determining factors that block TLR-stimulated DC function is paramount to developing effective vaccines for HIV.

Methods

DC preparation. PBMCs were prepared by centrifugation over Ficoll-Hypaque gradients (BioWhittaker) from healthy donor buffy coats (New York Blood Center). Monocytes were isolated from PBMCs by adherence and then treated with 100 U/ml GM-CSF and 300 U/ml IL-4 in RPMI plus 1% human plasma or 5% human serum. Cells were fed with additional GM-CSF/IL-4 on day 2 and later on day 4 of coculture and harvested for use on days 5–7. For all experiments, harvested DCs were washed and equilibrated in serum-free X-Vivo 15 media (Lonza).

Reagents. TLR agonists for TLR3 (poly I:C) and TLR5 (flagellin) were purchased from Invivogen. Salmonella-derived LPS (TLR4 agonist) was purchased from Sigma-Aldrich. The founder strain of live HIV-1 was provided by Beatrice Hahn (University of Pennsylvania, Philadelphia, Pennsylvania, USA).

Plasma samples. AHIV plasma samples (*n* = 16) were seroconversion panels obtained from ZeptoMetrix Corp. and provided by CHAVI. Each panel consisted of sequential aliquots of plasma (range, 4–30 aliquots) collected approximately every 3 days prior to and during the time of acute infection with HIV-1. Seven additional AHIV samples (Fiebig stage 1 and 2) were obtained from CHAVI 001 longitudinal clinical studies, including those collected at University of North Carolina Center for Infectious Diseases (Chapel Hill, North Carolina, USA), Duke University Medical Center (Durham, North Carolina, USA), Queen Elizabeth Central Hospital (Blantyre, Malawi), and Lilongwe Central Hospital (Lilongwe, Malawi). Acute HCV panels were also obtained via CHAVI from ZeptoMetrix Corp. Uninfected control donor plasma were panels obtained from ZeptoMetrix Corp. or from healthy volunteers at New York University Langone Medical Center. For some experiments to isolate MPs, plasma was diluted approximately 20 fold in X-Vivo 15 and ultracentrifuged at 100,000 *g* for 30 minutes.

Analysis of PBMCs from patients with HIV. PBMCs were cryopreserved at sequential time points during acute and early HIV infection and were from subjects with AHIV from the United States, United Kingdom, and Africa and population-matched HIV-seronegative controls. The majority of subjects with AHIV were enrolled in the CHAVI 001 acute infection cohort, but some samples were obtained from subjects with AHIV recruited from the Mortimer Market Centre, London, United Kingdom, and from the University of Alabama, Birmingham, Alabama, USA. Ethical approval for these studies was obtained from local ethics committees, and all blood samples were withdrawn with written informed consent. Subjects with AHIV were Fiebig-staged to enable temporal alignment of sample series from different individuals. Only data from time points at which subjects were not receiving antiretroviral therapy was included in this study.

For ex vivo analysis of CD44 expression on mDCs, PBMCs were stained with monoclonal antibodies to CD3-PB, CD14-PB, CD16-PB, and HLA-

DR PerCP (all from BD Pharmingen); CD19-PB and CD20-PB (both from AbD Serotec); CD56-PB (custom conjugate, ReaMetrix); and CD123 PE-Cy7 (eBioscience) to allow identification of mDCs (DR$^+$, CD123$^-$) as well as with CD44-FITC (BD Pharmingen). For analysis of TLR-stimulated mDC cytokine production, PBMCs (5×10^5 cells per well) were stimulated with 10 µg/ml CL097 (TLR7/8 agonist; Autogen Bioclear) for 20 hours with the addition of 1 µl/ml Golgiplug (BD Pharmingen) 1 hour after the start of the incubation. Cells were then surface stained for identification of mDCs as above. After washing, cells were incubated with CytoFix/CytoPerm (BD Pharmingen), followed by IL-12–PE and TNF-α–APC (Miltenyi Biotech). 200,000 events were acquired for each sample using a CyAn ADP Analyzer (Beckman Coulter), and data obtained were analyzed using FlowJo (Treestar).

Apoptotic MP preparation. PBMCs isolated from healthy donor whole blood buffies (New York Blood Center) were UV irradiated with 4,000 joules. PBMCs were then cultured overnight in RPMI and 1% normal human plasma. Controls were nonirradiated PBMCs. The next day, supernatant was collected from irradiated or nonirradiated preparations and spun at 400 g for 5 minutes twice to remove any cells. Supernatant was then ultracentrifuged at 100,000 g for 30 minutes, and pelleted MPs were resuspended in 1 ml of media for every 100 million starting PBMCs.

DC treatment. DCs were cultured in 96-well U-bottom plates (2×10^5 cells per well) in X-Vivo 15 media supplemented with 100 U/ml GM-CSF and 300 U/ml IL-4. Cells were treated with AHIV, acute HCV, or normal donor plasma at a concentration of 10%. Dose titrations of 33% to 0.1% plasma were performed in some experiments. Experiments with MPs involved DCs being treated with 10 to 50 µl of MP preparations. After overnight treatment with plasma or MPs, DCs were either unstimulated or stimulated with various TLR agonists: poly I:C (TLR3 agonist) used at 2 µg/ml, LPS (TLR4 agonist) used at 100 ng/ml alone or in combination with 500 U/ml IFN-γ, peptidoglycan (PGN; TLR2 agonist) used at 5 µg/ml, R848 (TLR7/8 agonist) used at 10 µM, and flagellin (TLR5 agonist) used at 100 ng/ml. Final volume after treatment and stimulation was 200 µl per well (cell concentration, 2×10^6/ml). DC phenotype and cytokine secretion were assessed approximately 24 hours after stimulation with agonists.

CD4$^+$ and CD8$^+$ T cell priming. Naive CD4$^+$ or CD8$^+$ T cells were isolated (>95% purity) from allogeneic PBMCs using EasySep Naive Human CD4$^+$ or Naive Human CD8$^+$ T Cell Enrichment Kits (StemCell Technologies) according to manufacturer's instructions. For some experiments to measure T cell proliferation, T cells were labeled with 1 µM CFSE. Naive allogeneic CD4$^+$ or CD8$^+$ T cells were cocultured with DCs treated as described above at a ratio of 1:10 in 96-well U-bottom plates (3.5×10^5 to 4.5×10^5 T cells per well) in 200 µl of RPMI media with 5% human serum. After 5 to 7 days of DC/T cell coculture, T cells were harvested for FACS analysis and supernatant was harvested for cytokine analysis.

NK cell activation. NK cells (>95% purity) were isolated from allogeneic PBMCs using the EasySep NK Cell Enrichment Kit (StemCell Technologies) according to manufacturer's instructions. NK cells were cocultured with DCs treated as described above at a ratio of 1:5 in 96-well U-bottom plates (3.5×10^5 to 4.5×10^5 NK cells per well) in 200 µl of RPMI media with 5% human serum. After overnight coculture, NK cells were harvested for FACS analysis and supernatant was harvested for cytokine analysis.

Cytokine analysis. Supernatant DC cultures or DC/T cell cocultures were analyzed by cytokine bead array (CBA; BD Biosciences) according to manufacturer's instructions. For DC cultures, supernatant was analyzed by the Human Inflammation Kit (BD Biosciences, which measures IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α levels. For DC/T cell cocultures or DC/NK cell cocultures, supernatant was analyzed by the Th1/Th2 CBA Kit, which measures IL-2, IL-4, IL-5, IL-10, IFN-γ, and TNF-α levels.

FACS analysis. DCs (distinguished by CD11c expression) treated as described above were analyzed for CD80, CD86, CD40, HLA-ABC, and

HLA-DR levels by FACS. DC viability was assessed by forward/side scatter gating. In cocultures of T cells and DCs, CFSE-labeled T cells were analyzed for proliferation after 5 to 7 days of coculture by decreased CFSE intensity. NK cells were analyzed for activation by staining for CD56 (NK marker) and CD69 (activation marker).

Mass spectrometric analysis of MP-binding proteins. DCs were surface biotinylated with EZ-link NHS sulfo-LC biotin (Pierce) and washed with RPMI and 5% human serum. DCs were treated with no MPs, control MPs, or apoptotic MPs for 30 minutes to 1 hour and then MPs were cleaved off by trypsin treatment. DCs were spun at 400 g for 5 minutes, and supernatant was collected and respun at 400 g for 5 minutes to remove any cells. Collected supernatant was then ultracentrifuged at 100,000 g for 30 minutes, and pelleted fractions were washed twice with PBS. Pellets were then lysed with cold PBS plus 1% Triton X-100 plus protease inhibitor cocktail (Calbiochem). Lysate was then added to a streptavidin column (to isolate biotinylated DC-derived proteins) and washed 3 times, and DC-derived proteins were eluted by cleaving linker arm of the EZ-link NHS sulfo-LC biotin with DTT. Eluants from apoptotic MP and DC preparations and control preparations were precipitated with methanol-chloroform and in-solution digested with trypsin. The digested material was subjected to nano–ultra performance liquid chromatography tandem mass spectrometry (nano-UPLC-MS/MS) analysis using a 75-µm-inner diameter × 25-cm C$_{18}$ nanoAcquity UPLC column (1.7-µm particle size; Waters) and a 90-minute gradient of 2% to 45% solvent B (solvent A, 99.9% H$_2$O, 0.1% HCOOH acid; solvent B, 99.9% MeCN, 0.1% HCOOH acid) on a Waters nanoAcquity UPLC system (final flow rate, 250 nl/min; 7,000 psi) coupled to a Q-TOF Premier Tandem Mass Spectrometer (Waters) run in positive ion mode. Data were acquired in data-directed analysis mode (1-second MS survey scans, MS to tandem mass spectrometry [MS/MS] switching at precursor ion counts greater than 10 and 1-second MS/MS scans with collision energy dependent on precursor ion mass and charge state). All raw MS data were processed using the PLGS software (version 2.2.5), including deisotoping and deconvolution (converting masses with multiple charge states to $m/z = 1$). The mass accuracy of the raw data was corrected using Glu-fibrinopeptide (200 fmol/µl; 700 nl/min flow rate; 785.8426 Da [M + 2H]$^{2+}$) that was infused into the mass spectrometer as a lock mass during analysis. MS and MS/MS data were calibrated at intervals of 30 seconds. MS/MS spectra were searched against the UniProtKB/SwissProt database (release 14.8; 408,099 entries) with the following parameters: peptide tolerance, 0.2 Da; fragment tolerance, 0.1 Da; trypsin missed cleavages, 2; fixed modification, Cys carbamidomethylation; variable modifications, Met oxidation and N/Q deamidation; and instrument type, ESI-Q-TOF. All database searches were restricted to human species because of the complexity of the searches when combined with multiple modifications. The interpretation and presentation of MS/MS data were performed according to published guidelines. DC proteins that bind apoptotic MPs were determined by candidate proteins that appear only in apoptotic MP and DC preparations and not in control preparations. MP-derived proteins were also analyzed from 3 separate preparations of MPs.

CD44 blockade or ligation. DCs were pretreated with 20 to 50 µg/ml of anti-CD44 monoclonal antibody (515 clone; BD Biosciences) or mouse IgG1 isotype control (BD Biosciences) before being treated with MPs as described above. Alternatively, experimentally or plasma-derived MPs were pretreated with 50 µg/ml CD44-Fc chimeric protein (R&D Systems) or human IgG-Fc (Jackson ImmunoResearch Inc.) control before being added to DCs. In experiments involving crosslinking CD44 on DCs, Dynabeads Pan Mouse IgG (Invitrogen) were ligated with anti-human CD44 (Biolegend) or purified mouse IgG1 (Biolegend) according to manufacturer's instructions. Antibody-coated or uncoated beads were incubated with DCs at a ratio of 4:1 at 4°C for 30 minutes, followed by incubation at 37°C for 2 hours prior to stimulation with poly I:C or LPS. After 24 hours, supernatant was collected and cytokine levels were determined by CBA.

Statistics. All data are shown as mean + SEM. Unpaired 2-tailed Student's *t* tests were performed on indicated comparisons (see figure legends) of DC, T cell, and NK cell cytokine production using GraphPad Prism 4 (GraphPad Software Inc.). Where indicated, paired 2-tailed Student's *t* test was performed on experiments involving CD44 blockade of AHIV plasma–mediated DC inhibition or AHIV plasma–derived MP-mediated DC inhibition. One-way ANOVA tests with Tukey post-test or Kruskal-Wallis test followed by Dunn's multiple comparison post-test were performed on indicated comparisons of disease plasma on DCs. *P* values of less than 0.05 were considered statistically significant.

Study approval. All nonexempt human samples were collected and processed in accordance with New York University Langone Medical Center IRB approval (IRB no. 06-480, 06-541, and 07-369). HIV seroconversion panels were obtained from ZeptoMetrix Corp. and are considered commercial products.

Address correspondence to: Nina Bhardwaj, 1307 Smilow Research Bldg., New York University Langone Medical Center, 522 1st Avenue, New York, New York 10016, USA. Phone: 212.263.5814; Fax: 212.263.6729; E-mail: Nina.Bhardwaj@nyumc.org.

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