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Human immunodeficiency virus type 1 (HIV-1) infection is characterized by persistent viral replication in the context of CD4+ T cell depletion and elevated immune activation associated with disease progression. In contrast, simian immunodeficiency virus (SIV) infection of African-origin sooty mangabeys (SM) generally does not result in simian AIDS despite high viral loads and therefore affords a unique model in which to study the immunologic contributions to a nonpathogenic lentiviral disease outcome. A key feature of these natural SIV infections is the maintenance of low levels of immune activation during chronic infection. Our goal was to delineate the contribution of monocytes to maintaining low levels of immune activation in SIV-infected SM. Utilizing an ex vivo whole-blood assay, proinflammatory cytokine production was quantified in monocytes in response to multiple Toll-like receptor (TLR) ligands and a specific, significant reduction in the tumor necrosis factor alpha (TNF-α) response to lipopolysaccharide (LPS) was observed in SIV-infected SM. In contrast, monocytes from hosts of pathogenic infections (HIV-infected humans and SIV-infected Asian macaques) maintained a robust TNF-α response. In SIV-infected SM, monocyte TNF-α responses to low levels of LPS could be augmented by the presence of plasma from uninfected control animals. The impact of LPS-induced TNF-α production on immune activation was demonstrated in vitro, as TNF-α blocking antibodies inhibited downstream CD8+ T cell activation in a dose-dependent manner. These data demonstrate an association between nonpathogenic SIV infection of SM and a reduced monocyte TNF-α response to LPS, and they identify a role for monocytes in contributing to the suppressed chronic immune activation observed in these natural hosts.

While the role of immune activation in lentiviral pathogenesis is well characterized, the precise causes of elevated immune cell activation are unclear. Numerous groups have reported a higher incidence of immune cell proliferation, activation, and apoptosis in human immunodeficiency virus (HIV)-infected subjects (48), and the levels of activation provide a stronger correlate of HIV disease progression than viral load or CD4+ T cell count (16, 17). The causes of these changes are likely multifactorial and include the direct effects of stimulatory viral nucleic acids and proteins, the consequences of antiviral immune responses, and immune cell stimulation by bacterial products translocating through a compromised gut mucosa (9, 43, 48). One mechanistic approach to assessing the causes and consequences of lentivirus-associated immune activation is through the use of the simian immunodeficiency virus (SIV)-infected monkey models. These monkey models include Asian-origin macaques, which undergo a pathogenic disease course similar to HIV infection, as well as the nonpathogenic SIV infections of African-origin monkey species such as sooty mangabeys (SM). Natural SIV infections represent virus-host relationships that are evolutionarily older than the HIV pandemic in humans and generally do not result in clinical signs of simian AIDS (52, 54). The natural SIV infection of SM recapitulates many of the key observations of pathogenic HIV and SIV infections, including similar viral loads, comparable antiviral immune responses, and, in some instances, CD4+ T cell depletion (31, 38, 45, 47, 48). However, a key difference between pathogenic and nonpathogenic infections is the ability to resolve early increases in immune activation. In SIV-infected natural hosts, the early induction of immune activation during the acute phase of infection is transient, and the chronic stage of infection (after 4 weeks) is characterized by low levels of systemic immune activation as determined by cytokine and chemokine levels in peripheral blood and the activation state of multiple immune cell subsets (5, 22, 33). It is clear that the ability to restrict chronic immune activation is a critical component of a nonpathogenic disease outcome in SM (37, 46, 48), and elucidating the mechanisms underlying this phenotype will have important implications for understanding and treating the pathogenesis of HIV disease.

Monocytes are macrophage precursors present in the blood and are critical effectors of the innate immune system. Among their varied functions as phagocytes and antigen-presenting cells, monocytes are potent producers of proinflammatory cytokines and chemokines which are beneficial in the programming of adaptive immune cells but are potentially harmful, as an overabundance of these molecules leads to aberrant immune cell activation, apoptosis, and tissue destruction (25, 42). Interestingly, many of...
the immunologic events thought to propagate lentivirus-associated immune activation originate with proinflammatory cytokine production from cells such as monocytes (9). For example, the HIV type 1 (HIV-1) genome encodes Toll-like receptor 8 (TLR8) ligands capable of eliciting tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) production from monocytes (28). In addition, bacterial products that have translocated from the intestinal lumen into the plasma are recognized by multiple TLRs on monocytes, leading to the production of cytokines and chemokines (8, 9). Moreover, a primary indicator of immune activation is increased levels of plasma proinflammatory cytokines such as TNF-α (21, 27, 41). Because SIV-infected SM are able to maintain low levels of chronic immune activation, we hypothesized that the production of proinflammatory cytokines by monocytes would be tightly regulated during this nonpathogenic infection. To investigate this, an *ex vivo* comparative assessment of monocyte effector functions was performed in SIV-infected SM, SIV-infected Asian macaques, and HIV-infected individuals. A dramatic decline in monocyte TNF-α production that was specific to lipopolysaccharide (LPS) stimulation was observed only in SIV-infected SM and was not observed with other TLR ligands (lipoteichoic acid [LTA] and single-stranded RNA [ssRNA]). In contrast, pathogenic HIV was not observed with other TLR ligands (lipoteichoic acid [LTA] and single-stranded RNA [ssRNA]). In contrast, pathogenic HIV was not observed with other TLR ligands (lipoteichoic acid [LTA] and single-stranded RNA [ssRNA]).

**MATERIALS AND METHODS**

**Human subjects.** All protocols involving human subjects were approved by the institutional review board of the University of Texas Southwestern Medical Center at Dallas and Seattle Biomedical Research Institute. Written informed consent was obtained from all study participants. HIV-infected donors were chronically infected and treatment naïve for at least 1 year. Viral loads ranged from 50 to 64,000 copies/ml of plasma, and CD4+ T cell counts ranged from 4 to 833 cells/μl of blood.

**Animals and viral infection.** All animal experimentation was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals (34a)* of the National Institutes of Health and under the approval of the Institutional Animal Care and Use Committees (IACUC) of the Yerkes National Primate Research Center (YNPRC), the Washington National Primate Research Center (WNPRC), and the Oregon National Primate Research Center (ONPRC). SM were either uninfected controls or naturally infected at the YNPRC (Atlanta, GA), except for sooty mangabey 7 (SM7), SM8, and SM9, which were infected intravenously (i.v.) with 1 ml of plasma from an SIV-infected mangabey as previously described (30), and the SM represented in Fig. 3b, which were infected with an uncloned SIVsmm obtained from a naturally infected mangabey in the Yerkes colony as described previously (5). Rhesus macaques (RM) represented in Fig. 2 were housed at the ONPRC (Beaverton, OR) and were either uninfected controls or infected with SIVmac239 i.v. using 5-ng equivalents of SIV p27 (1.0 × 10^7 infectious centers); all samples were obtained from chronic-phase time points (≥50 days postinfection). RM represented in Fig. 3d were housed at the YNPRC and infected with an uncloned SIVsmm as described previously (5). Pig-tailed macaques were housed at the WaNPRC (Seattle, WA) and were infected intraarterially with 1 ml of undiluted stock of SHIV-117p3d3N4 (SHIV is simian-human immunodeficiency virus) with a p27 concentration of 95 ng/ml and an *in vitro* infectivity as determined by 50% tissue culture infectious doses (TCID₅₀) of 10^7 per ml as titrated in TZM-bl cells (20).

**Blood collection.** For *ex vivo* TLR stimulation experiments, all blood samples were collected in Vacutainers containing the anticoagulant EDTA, as previous studies have demonstrated that the presence of heparin contributes to augmented monocyte responses to LPS via the activity of heparin-binding protein (10, 19). For phagocytosis and oxidative-burst assays, blood samples were collected in Vacutainers containing the anticoagulant heparin to prevent the inhibitory effects of EDTA on these functions (4). For enzyme-linked immunosorbent assays (ELISAs), real-time PCR, and TNF blocking experiments, blood was collected in Vacutainers containing EDTA. Nonhuman primate blood samples were shipped overnight from national primate centers, and blood collected from human donors was held overnight (mock shipped) to control for changes due to shipping and time postvenipuncture.

**Quantification of cytokine secretion from whole-blood cells.** Whole blood was aliquoted in 96-well U-bottom plates or 5-ml polystyrene culture tubes in 100-μl volumes. Samples in Fig. 1 were stimulated with 10 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO) for 6 h; samples in Fig. 3c and d were stimulated with 0.1 μg/ml LPS for 4 h. Cytokine secretion was measured in supernatants by cytometric bead array (BD Biosciences, San Diego, CA) according to the manufacturer’s recommendations. Briefly, cell supernatants were diluted 1:2 with phosphate-buffered saline (PBS) and incubated for 3 h at room temperature with capture beads and phycoerythrin (PE) detection reagent. Following a wash step, samples were collected on a BD LSRII (BD Biosciences) and analyzed with CBA analysis software (BD Biosciences). All reported results were within the limit of detection of the array. The cross-reactivity between the kit antibodies and SM is inferred, as the majority of cytokines have similar levels between the two species, with the exception of IL-6, which was generally lower in SM than in humans.

**Whole-blood stimulation and staining for flow cytometry.** Whole blood was aliquoted in 96-well U-bottom plates or 5-ml polystyrene culture tubes in 100-μl volumes. Samples were stimulated with 10 μg/ml LPS (Sigma-Aldrich, St. Louis, MO), 200 μg/ml lipoteichoic acid (LTA; Sigma-Aldrich), or 5 μg/ml single-stranded RNA (ssRNA; Invitrogen, Carlsbad, CA) for 1 h at 37°C and 5% CO₂. Following the addition of the Golgi blocker brefeldin A (BFA; Sigma-Aldrich), stimulations were continued for 5 h at 37°C and 5% CO₂. Cells were stained for 30 min on ice with anti-CD3-fluorescein isothiocyanate (FITC) (SP34), anti-CD14-phycocerythrin-cyanine-7 (PE-Cy7) (M5E2), anti-CD16-Alexafluor 647 (3G8), and Live/Dead Aqua (Invitrogen, Carlsbad, CA). Following a wash in PBS plus 2% fetal bovine serum (FBS), cells were fixed and permeabilized in FACS juice (BD FACS lysing solution plus Tween 20) and stained with anti-TNF-phycocerythrin (PE) (MABII). Cells were collected on a BD LSRII and analyzed with FlowJo software (Tree Star, Inc., Ashland, OR).

**Enrichment of monocytes and CD8⁺ T cells by magnetic bead sorting.** Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Hypaque gradient centrifugation. A total of 30,000 PBMCs were incubated with anti-CD14 or anti-CD8 microbeads (Miltenyi, Auburn, CA) for 15 min at 4°C, washed with bead buffer (PBS plus 2 mM EDTA and 0.5% bovine serum albumin [BSA], pH 7.2), and collected by magnet-associated cell sorting (MACS) on a large-selection (LS) column (Miltenyi). Purity was assessed by flow cytometry and was on average 55 to 60% (monocytes) or 80 to 85% (CD8⁺ T cells). For analysis of cytokine mRNA expression, 100,000 enriched monocytes were plated on 24-well flat-bottom polystyrene plates in 0.5 ml complete or serum-free RPMI medium and stimulated with 1 μg/ml LPS for 18 h.

**Evaluation of plasma effects on monocyte LPS responses.** PBMCs were isolated from SIV-infected SM whole blood by Ficoll-Hypaque gradient centrifugation. Two million PBMCs in 100 μl complete RPMI medium were plated in 96-well U-bottom plates with 10 μg autologous plasma or plasma obtained from one of six SIV-uninfected SM. Plasma was freshly isolated from whole blood or was previously frozen; no difference was detected between experiments using fresh or frozen plasma. Cells were stimulated with 0.1 μg/ml or 10 μg/ml LPS for 4 h at 37°C and 5% CO₂, and then BFA was added and cells were incubated for an addi-
tional 5 h. Monocyte cytokine production was assessed by flow cytometry as described above.

ELISA quantification of sCD14 and EndoCAB. ELISA kits were utilized to detect soluble CD14 (sCD14) (R&D Systems) and endotoxin core antibody (EndoCAB) (Cell Sciences) in plasma samples from SM7, SM8, and SM9 as previously described (9).

Total RNA isolation, cDNA synthesis, and quantitative real-time PCR for immune modulators. Cells were stimulated with 20 μg/ml LPS (Escherichia coli O55:B5, Invitrogen, Carlsbad, CA) as previously described (9). To detect soluble CD14 (sCD14) (R&D Systems) and endotoxin core antibody, quantitative real-time PCR, a Mann-Whitney U test (nonparametric, two-tailed) was performed. The TNF-α blocking assay was analyzed using a paired t test (one tailed). For all assays, statistical significance is identified as P values less than 0.05 (95% confidence interval).

RESULTS

Down-modulation of TNF-α production following SIV infection of sooty mangabeys but not HIV or SIV infection of nonnatural hosts. To assess the impact of SIV infection on immune cell inflammatory responses in SM, whole blood obtained from control (SIV-uninfected) or SIV-infected SM was stimulated with the Gram-negative bacterial cell wall component LPS. The resulting secretion of the proinflammatory cytokines TNF-α, IL-6, and IL-8 was measured by cytometric bead array (Fig. 1). Peripheral blood cells from SIV-infected SM were found to secrete significantly less TNF-α than those from control SM (300 ± 188 pg/ml, compared to 700 ± 517 pg/ml [Fig. 1a]). In contrast, the ex vivo peripheral blood cell LPS response in HIV-infected patients was similar to that in healthy control volunteers (1,000 ± 565 pg/ml, compared to 770 pg/ml ± 823 pg/ml [Fig. 1b]). The production of IL-6 and IL-8 was maintained regardless of infection status in both SM and humans (though SM secreted overall lower levels of IL-6 than did humans), indicating that LPS-induced TNF-α production was specifically down-modulated in SIV-infected SM (Fig. 1c to f). For all analytes tested, unstimulated controls were consistently below the levels of detection in both SM and humans (data not shown).

Monocytes specialize in detecting microbial antigens through the expression of numerous pattern recognition receptors, including the LPS receptors CD14 and TLR4. Utilizing intracellular cytokine staining (ICS), monocyte TNF-α production was assessed following LPS stimulation in whole-blood samples obtained from SM, rhesus macaques (RM), and human volunteers (Fig. 2). In control SM, TNF-α was detected in an average of 20% (±13.77%) of monocytes (identified as CD14+517) following ex vivo LPS stimulation (Fig. 2b). In contrast, SIV-infected SM mounted a significantly reduced LPS response, with an average of 7% (±5.19%) of monocytes producing TNF-α (Fig. 2b). This reduced monocyte LPS response was not observed in hosts of a pathogenic infection, as both HIV-infected humans and SIV-infected RM generated a monocyte LPS response that was comparable to that of uninfected controls (Fig. 2c and d). Taken together, these results indicate that SIV-infected SM but not SIV-infected RM or HIV-infected humans experience a reduction in the monocyte LPS response.

Rapid reduction in monocyte LPS response following multistropic SIV infection in sooty mangabeys. We previously described a cohort of SIV-infected SM that developed a multistropic (including R5/X4/R8) SIV infection resulting in a dramatic decline of CD4+ T cells, to fewer than 50 cells/μl blood, yet remained free of AIDS and opportunistic infections (31). Passage of plasma from one SIV-infected CD4-low mangabey to three additional SM, SM7, SM8, and SM9, initiated a rapid, systemic depletion of CD4+ T cells within 21 days postinfection (dpi) (30). To assess the impact of virally mediated CD4+ T cell depletion on monocyte function in SM, an ex vivo longitudinal analysis of monocyte responses to LPS was undertaken in whole blood obtained from SM7, SM8, and SM9. Prior to infection, 15 to 30% of whole-blood monocytes produced TNF-α upon ex vivo LPS stimulation, comparable to what was observed in other control SM (Fig. 3a). In contrast, by 7 dpi, the fraction of monocytes producing TNF-α...
after LPS stimulation declined to 2 to 7%, and it remained in this lower range throughout the study period (250 dpi [Fig. 3a]). The decline in monocyte LPS responses at 7 dpi preceded the decrease in peripheral CD4+ T cells, which occurred between 15 and 21 dpi (30), indicating that the restriction of monocyte TNF-α production was unlikely to result from low CD4+ T cell levels. Despite persistent viral replication and the rapid decline in both monocyte LPS responses and CD4+ T cells, SM7, SM8, and SM9 maintained low levels of immune activation during chronic infection and remained free of opportunistic infections (30). We found similar kinetics for the LPS response in a group of four SM infected with an uncloned SIVsmm that maintained healthy peripheral CD4+ T cell counts. Analysis of this group revealed a dramatic decrease in TNF-α secretion from whole-blood cells, from 74 (±12) pg/ml to 18 (±9) pg/ml by 10 dpi (Fig. 3b). In contrast, Asian-origin macaques undergoing pathogenic SHIV or SIV infection generally maintained the TNF-α response to LPS (Fig. 3c and d). A group of pig-tailed macaques infected with SHIV-1157 maintained the pre-infection ex vivo LPS response of 10 to 55% of monocytes producing TNF-α throughout 300 days of observation (Fig. 3c). This robust monocyte response was preserved despite an early and rapid depletion of CD4+ T cells which was comparable to that observed in SM7, SM8, and SM9 (20). Moreover, in a separate group of four RM infected with SIVsmm there was generally a slight decline but no significant change in TNF-α secretion from whole blood stimulated with LPS (values ranged between 867 [±176] at day 0 and 526 [±195] pg/ml at day 48) (Fig. 3d). Taken together, these data indicate that the monocyte TNF-α response to LPS is reduced in SIV-infected SM regardless of CD4+ T cell status, while monocyte TNF-α production remains robust during pathogenic infections of Asian macaques.

The monocyte response to lipoteichoic acid and single-stranded RNA is maintained in SIV-infected sooty mangabeyes. To assess whether the down-modulation of monocyte TNF-α production in nonpathogenic SIV infection was specific to LPS stimulation, whole blood obtained from control and SIV-infected SM was stimulated ex vivo with the TLR2 agonist LTA or the TLR8 agonist ssRNA, and the resulting monocyte TNF-α response was quantified by ICS. In contrast to the decline in the monocyte response to LPS, a preserved response to LTA and ssRNA was ob-
served in SIV-infected SM (average of 4 to 5% of monocytes producing TNF-α, similar to the value for control SM) (Fig. 4). While the overall frequency of monocytes responding to LTA and ssRNA was lower than that observed for LPS in these animals (Fig. 2a and 4), the decline in monocyte TNF-α production observed in response to LPS in SIV-infected SM was not recapitulated with these TLR ligands. Moreover, we also found that human monocytes responded to LTA and ssRNA at lower levels than to LPS (data not shown), demonstrating that at the concentrations used in this study, LTA and ssRNA elicit a lower TNF-α response than LPS in multiple species.

Phagocytosis and oxidative burst are not altered during non-pathogenic SIV infection of sooty mangabeys. HIV infection is associated with a deficiency in monocyte/macrophage phagocytosis and oxidative burst (3, 6, 40). To determine if nonpathogenic SIV infection impacts monocyte effector functions beyond TNF-α production, the ability of monocytes to take up Gram-negative bacteria by phagocytosis and kill ingested bacteria through the production of reactive oxygen species (ROS) was also assessed. Whole blood from control and SIV-infected SM was incubated with FITC-labeled E. coli particles at 37°C, and flow cytometry was utilized to quantify the geometric mean fluorescence intensity (MFI) of the FITC signal in monocytes (Fig. 5a). The FITC MFI was normalized to that for sample-matched controls, which were incubated at 4°C, thus preventing efficient uptake of the bacteria, and a MFI was generated for each sample, where ΔMFI = MFI37°C − MFI4°C (Fig. 5). Monocytes from control and SIV-infected SM had comparable capacities to phagocytose E. coli, with a mean ΔMFI for each group of approximately 2,000 units (Fig. 5b). In addition, the percentages of monocytes ingesting FITC-labeled E. coli were generally similar among SIV-infected and uninfected SM (average, 60%) (data not shown). The oxidative-burst capacity of monocytes from control and SIV-infected SM was assessed utilizing the fluorescent probe dihydroethidium (DHE), which emits a red fluorescent signal (maximum wavelength [λmax] = 600 nm) when reduced by superoxide, one of the earliest reactive oxygen intermediates produced by phagocytes. Whole blood was incubated with E. coli in the presence of DHE, and the MFI of reduced DHE was measured within a monocyte gate for each sample. Similar to the phagocytosis assay, a MFI was calculated utilizing a sample-matched negative control. The mean ΔMFI of DHE for both SIV-infected and control SM was approximately 1,000 units, indicating that nonpathogenic SIV infection does not impact the ability of monocytes to produce reactive oxygen species in response to E. coli (Fig. 5c). Therefore, the reduced TNF-α response by SIV-infected mangabey monocytes exposed to LPS was not associated with a reduced ability of these cells to phagocytose and kill E. coli.

**FIG 2** Monocyte TNF-α production in response to LPS stimulation in sooty mangabeys, human donors, and macaques. (a) Representative flow cytometry data and gating strategies to identify the percentage of monocytes producing TNF-α in response to LPS stimulation or a medium control. Whole blood from SM (b), human donors (c), and RM (d) was stimulated ex vivo with LPS, and the resulting TNF-α production by monocytes was quantified by intracellular cytokine flow cytometry. Uninfected control subjects (SIV negative [SIVneg] and HIVneg) were compared to infected subjects (SIV positive [SIV+] and HIV+). Values obtained for LPS-stimulated samples were normalized to the background staining on sample-matched medium controls, which were consistently less than 1%.

Assessment of mRNA levels of TLR4, IL-10, and TNF-α following LPS stimulation of SIV-infected mangabey monocytes. To examine the potential mediators of the decline in TNF-α production by monocytes exposed to LPS in SIV-infected SM, we measured the expression of TLR4 and IL-10 in a cross-sectional analysis of SIV-infected and uninfected SM. One possibility is that reduced TLR4 expression resulting in decreased sensing of LPS could mediate a decline in monocyte LPS responses. Because TLR4 protein expression on SM monocytes could not be measured directly (due to a lack of antibodies cross-reactive with non-human primate TLR4), real-time PCR was performed to quantify the transcript levels of TLR4 in enriched monocyte populations obtained from control and SIV-infected SM. No discernible difference was observed in the relative expression of TLR4 mRNA in
monocytes from control and SIV-infected SM (Fig. 6a). IL-10 is an anti-inflammatory cytokine capable of acting in an autocrine and paracrine manner to prevent the production of TNF-α and other proinflammatory cytokines. Real-time PCR assessment of enriched monocytes stimulated with LPS identified comparable transcript levels of IL-10 mRNA in mangabey monocytes regardless of SIV status (Fig. 6b). Taken together, these results suggest that the reduced monocyte LPS response in SIV-infected SM is not associated with alterations in the expression of TLR4 or IL-10.

To assess the level of TNF-α transcripts in SIV-infected SM monocytes, real-time PCR was performed on enriched monocytes following LPS stimulation in serum-free medium. In contrast to the reduction in TNF-α protein detected in whole-blood monocytes (Fig. 1 to 3), TNF-α mRNA production was maintained in enriched cultures of monocytes obtained from SIV-infected SM (Fig. 6c). These data indicate that the reduction in monocyte TNF-α production in SIV-infected SM is not due to the down-modulation of TNF-α at the level of mRNA transcription, and they suggest that the regulation of this response may occur post-transcriptionally.

Plasma contributes to the monocyte LPS response in sooty mangabeys. Plasma contains multiple soluble components capable of binding LPS and/or modulating immune cell responses to LPS. To assess the role of plasma in mediating monocyte LPS...
responses in SM, we stimulated PBMCs from SIV-infected SM in the presence of plasma obtained from SIV-uninfected SM or autologous plasma. When autologous plasma was present, a low dose of LPS (0.1 μg/ml) elicited TNF-α production in approximately 15 to 55% of monocytes (Fig. 7a). In the presence of SIV-uninfected plasma, the monocyte TNF-α response was augmented to 35 to 80%, and this increase was statistically significant (P = 0.0321) (Fig. 7a). However, when cells were stimulated with a higher concentration of LPS (10 μg/ml), there was no significant difference in TNF-α responses between monocytes mixed with autologous or SIV-uninfected plasma. These data indicate that the plasma plays a role in modulating LPS responses in SM and suggest that a factor (or multiple factors) present in the plasma contribute the downregulation of TNF-α responses observed in SIV-infected SM. However, the influence of this putative plasma factor(s) to suppress LPS responsiveness in SIV-infected SM can be overcome by a higher dose of LPS.

We next assessed the levels of two plasma molecules capable of sequestering LPS and preventing its recognition by TLR4, soluble CD14 (sCD14) and endotoxin core antibody (EndoCAb) (12, 34), during the course of nonpathogenic SIV infection of SM7, SM8, and SM9. Despite the rapid and dramatic decline in monocyte TNF-α responses observed in these SM (Fig. 3a), the levels of sCD14 and EndoCAb remained relatively stable throughout infection (Fig. 7c and d). Thus, it is unlikely that fluctuations in these plasma mediators were responsible for the decline in monocyte responses.

**LPS-induced TNF-α contributes to activation of sooty mangabey T cells.** To assess the impact of reducing TNF-α production on the activation state of peripheral blood T cells in SM, PBMCs were stimulated with LPS in the presence of a TNF-α specific antibody, and the activation state of CD8+ T cells was assessed via the expression of HLA-DR and CD38 (Fig. 8). In the absence of TNF-α blocking antibody, LPS stimulation resulted in a 5% increase in the frequency of activated (CD38+ HLA-DR+) CD8+ T cells compared to unstimulated controls (Fig. 8a). However, addition of a TNF-α blocking antibody (0, 2.5, or 10 μg/ml) led to a dose-dependent decrease in mangabey CD8+ T cell activation (Fig. 8a). In contrast, when enriched CD8+ T cells were stimulated with LPS, only a 1% increase in activation was observed, suggesting that mangabey CD8+ T cells are not activated directly by stimulation by LPS (Fig. 8b). Stimulation of enriched CD8+ T cells with phytohemagglutinin (PHA), on the other hand, led to a 5.5% increase in CD38 and HLA-DR coexpression (Fig. 8b). These findings provide evidence that the reduced monocyte TNF-α response to LPS in SIV-infected SM contributes to the lower levels of CD8+ T cell activation observed during the chronic phase of infection in SIV-infected SM.

**DISCUSSION**

HIV-associated immune activation is a correlate of disease progression and is characterized by increases in proinflammatory cytokines and immune cell activation (16, 17, 36, 48). In contrast, SIV-infected natural hosts maintain low levels of chronic immune activation and do not progress to AIDS (38, 46, 48). Here we demonstrate a role for monocytes in the ability of SM to resist increases in systemic immune activation following SIV infection. These data demonstrate that in SM, SIV infection induces a rapid and dramatic decline in the monocyte TNF-α response to LPS. This response is specific to LPS, as the TNF-α response to addi-

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*FIG 5* Monocyte phagocytosis and oxidative burst in uninfected controls and SIV-infected sooty mangabeys. (a) Representative flow cytometry data and gating strategy for phagocytosis assay. Oxidative-burst assays were gated in a similar strategy, measuring the fluorescence of the oxidized DHE probe rather than the *E. coli*-FITC. (b and c) Phagocytosis capacity (b) and oxidative-burst capacity (c) in response to *E. coli* in monocytes from uninfected controls (SIVneg) and SIV-infected SM. ΔMFI represents the average of triplicate assays in which the MFI of experimental samples (37°C) was normalized to the MFI of control samples (4°C). Box plots represent 12 samples.
Our studies indicate that the down-modulation of monocyte TNF-α responses to LPS in SM occurs rapidly, within 10 days following SIV infection. It is now evident that the majority of the initial acute-phase immune activation events in SIV-infected natural host species resolve during the transition from acute to chronic phase, which generally occurs later, around 21 to 28 days postinfection (5, 14, 18, 22, 26, 33). Therefore, down-modulating the LPS-specific monocyte TNF-α response in SIV-infected SM may be an early step in establishing the low levels of immune activation observed during the chronic phase of the infection. Maintaining low levels of aberrant immune activation during chronic infection is an important aspect of resisting disease progression in SIV natural host monkey species (even when CD4+ T cell levels are low) (30, 31, 46). The rapid regulation of monocyte LPS responses in SIV-infected SM suggests that monocytes, which are powerful contributors to inflammation, must be tightly regulated to avoid tipping the balance toward immune activation and disease progression. Additionally, because the monocyte TNF-α response to LPS is robust in healthy SM, it may warrant specific regulation, whereas responses to other microbial antigens (LTA and ssRNA) involve an overall lower frequency of TNF-α production by monocytes. Interestingly, this decreased TNF-α response does not coincide with any specific impact on phagocytosis or oxidative-burst capabilities in these monocytes. Hence, innate effector functions that contribute to clearing opportunistic infections (phagocytosis and oxidative burst) are maintained, while those with the capacity to promote immune activation (robust TNF-α production) are suppressed in the SIV-infected SM.

One way to assess the inherent differences in the LPS responses between humans, macaques, and SM is to compare the baseline (preinfection) levels of the three hosts. Utilizing flow cytometry, we found similar frequencies of TNF-α+ monocytes in uninfected SM and RM following a high-dose LPS stimulation (Fig. 2). Likewise, levels of TNF-α secretion were comparable between humans and SM at a high dose of LPS (Fig. 1). In contrast, when a lower dose of LPS was utilized, the secretion of TNF-α was lower in SM than RM prior to SIVsmm infection (Fig. 3c and d). This difference highlights an inherent caveat of comparative studies involving multiple species, namely, the difficulty of undertaking direct species-to-species comparisons. For this reason, our study focused on the changes that occur to the monocyte LPS response within each host in response to HIV/SIV infection and compared these changes between the different species to identify important differences.

The specific decline in the LPS response in SM (but not RM) is triggered by the presence of SIV, and the mechanism of this suppressed response was assessed. Utilizing plasma from uninfected and SIV-infected SM, we determined that the LPS response is regulated in part by a factor (or factors) found in the plasma of SIV-uninfected SM and can be observed when relatively low levels of LPS are present. However, the ability of plasma to suppress the TNF-α response was overcome by a higher dose of LPS. In addition, our findings point to a cellular influence on the regulation of TNF-α-specific monocyte LPS responses in SIV-infected SM, as IL-6 and IL-8 expression is maintained while TNF-α expression is suppressed. Thus, LPS can be detected by the cells, resulting in the production of IL-6 and IL-8 and indicating that a plasma factor does not completely block the bioavailability of LPS. Moreover, the conserved upregulation of TNF-α mRNA levels in response to LPS in SIV-infected SM monocytes indicates that the cells recognize and bind the LPS to some extent and provides evidence for posttranscriptional regulation. Taken together, these data suggest a multifactorial regulation of monocyte TNF-α responses during nonpathogenic SIV infection of SM, likely involving both plasma and cellular factors. Our findings suggest that the regulation does

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**FIG 6** Assessment of potential mediators of the LPS response in monocytes obtained from uninfected controls and SIV-infected sooty mangabeys. (a) The relative expression of TLR4 was quantified by real-time PCR in cultures of enriched monocytes obtained from uninfected controls (SIVneg) and SIV-infected SM. Values shown are relative to the housekeeping GAPDH gene in the same sample. (b and c) Enriched monocyte cultures were stimulated with LPS, and the transcript levels of IL-10 and TNF-α were quantified by real-time PCR. The fold change was calculated by comparing cytokine expression between LPS-stimulated and unstimulated matched samples. Columns represent the means and standard errors of 8 to 10 samples.
not result from alterations in the expression of TLR4 or IL-10 or from changes in the levels of sCD14 or EndoCAb in plasma. At the cellular level, the TNF-α response to LPS may be regulated post-transcriptionally. Posttranscriptional regulation of TNF-α has been reported to occur in both human and murine systems, including mechanisms mediated by transforming growth factor β (TGF-β) (11) and IL-1-associated receptor kinase 2 (IRAK-2) (51). Specific regulation of TNF-α production by SIV-infected SM may be beneficial, as TNF-α is one of the earliest and most abundant proinflammatory cytokines produced in response to LPS (34). Moreover, TNF-α is a major contributor to apoptosis and the physiological outcomes of LPS exposure, as evidenced by studies demonstrating that TNF-α blocking antibodies can alleviate the symptoms of septic shock, while administration of TNF-α can recapitulate septic shock in both animal models and human subjects (39, 49). Moreover, the decline in monocyte LPS responses is unlikely to result from direct infection of monocytes by SIV, as monocytes are generally infected at very low levels during HIV or SIV infection (2, 53), and we did not detect SIV DNA in sorted monocytes from a representative mangabey, SM8.

The observation that LPS-induced TNF-α can contribute to increased CD8+ T cell activation in sooty mangabey peripheral blood cells illustrates the importance of regulating monocyte LPS responses during nonpathogenic SIV infection. It may be that an early reduction in monocyte TNF-α production in SIV-infected SM prevents tipping the balance of the immune environment toward a proinflammatory and proapoptotic landscape during acute infection. Indeed, it has been demonstrated that natural hosts begin to establish an anti-inflammatory environment during acute infection by increasing IL-10 and TGF-β production and have lower levels of peripheral T cell activation and apoptosis during both acute and chronic infections (5, 26, 29, 31, 50). Moreover, the LPS-specific nature of the observed decline in the monocyte TNF-α response may indicate that Gram-negative bacteria were a strong driving force in the evolution of SM with SIV. The contribution of LPS to lentiviral pathogenesis is demonstrated by the correlation between elevated plasma LPS concentrations, increased immune cell activation, and advanced disease progression in HIV-infected subjects and SIV-infected macaques (9, 24). SM seem to have evolved multiple mechanisms to avoid the detrimental effects of LPS during SIV infection, including suppressing monocyte LPS responses and inhibiting microbial translocation during chronic infection via the selective preservation of mucosal Th17 cells and maintained expression of intestinal tight junction proteins such as occludin (7, 30). Importantly, this study implies that unrestrained monocyte TNF-α production could be detrimental during pathogenic HIV and SIV infections. Indeed, monocytes were found to maintain a robust response to LPS in HIV-infected patients and SIV-infected Asian macaques, suggesting that monocyte-derived proinflammatory cytokines contribute to an environment of immune activation. These findings suggest that therapeutic intervention aimed at reducing TNF-α may be beneficial for reducing immune activation during HIV infection. Indeed, TNF-α inhibitors have been utilized for the treatment of inflammatory conditions in HIV-infected patients (15, 23, 35, 44). However, none of the studies performed to date has measured indicators of immune activation or monocyte function following treatment. Moreover, targeting TNF-α may be most beneficial during the early stages of infection. In another study of HIV- positive (HIV+) patients, plasma LPS levels were found to be in-

FIG 7 Plasma contributes to monocyte LPS responses in sooty mangabeys. (a and b) PBMCs from SIV-infected SM were stimulated with LPS in the presence of autologous plasma or plasma obtained from SIV-uninfected SM. TNF-α production by monocytes was quantified by flow cytometry. Data shown represent 9 SIV-infected SM; SIV-uninfected plasma was obtained from 6 SM and added separately to PBMCs. (c and d) Soluble CD14 and EndoCAb were quantified in plasma samples obtained pre- and post-SIV infection from SM7, SM8, and SM9.
and standard error for 6 samples. HLA-DR was measured by flow cytometry. Each column represents the mean or PHA, and the resulting expression of the activation markers CD38 and 10.

versely correlated with monocyte TNF-α and IL-1 production following LPS stimulation of PBMCs isolated via Ficoll-Hypaque centrifugation (9). This HIV-associated reduction in monocyte cytokine production is likely facilitated by a tolerance to elevated levels of plasma LPS during advanced disease, which is distinctive from the rapid suppression of the LPS response we have observed following SIV infection of SM. In the present study, we did not detect any difference in monocyte TNF-α production in HIV+ patients compared to healthy controls, which is distinct from the previous study and could be due to differences in the assays, including the use of a whole-blood assay in this study rather than isolated PBMCs.

Overall, the current study further emphasizes the importance of controlling immune activation for a nonpathogenic disease outcome and demonstrates that monocytes contribute to inhibiting immune activation during the transition from acute to chronic phase of nonpathogenic SIV infection. Therapeutic compounds capable of down-modulating monocyte inflammatory responses administered alone or in combination with antiretroviral therapy may benefit HIV-infected patients by inducing a similar resolution of immune activation regardless of CD4+ T cell status.

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FIG 8 Impact of TNF-α on CD8+ T cell activation in sooty mangabey PBMCs. (a) PBMCs obtained from uninfected control SM were stimulated with LPS in the presence of 0, 2.5, or 10 µg/ml TNF-α blocking antibodies, and the resulting activation of CD8+ T cells, as determined by coexpression of CD38 and HLA-DR, was quantified by flow cytometry. (b) Enriched cultures of CD8+ T cells obtained from SIV-infected SM were stimulated with LPS or PHA, and the resulting expression of the activation markers CD38 and HLA-DR was measured by flow cytometry. Each column represents the mean and standard error for 6 samples.


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