IFN-Stimulated Gene LY6E in Monocytes Regulates the CD14/TLR4 Pathway but Inadequately Restrains the Hyperactivation of Monocytes during Chronic HIV-1 Infection

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IFN-Stimulated Gene LY6E in Monocytes Regulates the CD14/TLR4 Pathway but Inadequately Restrains the Hyperactivation of Monocytes during Chronic HIV-1 Infection

Xuan Xu,*† Chao Qiu,* Lingyan Zhu,* Jun Huang,* Lishuang Li,‡ Weihui Fu,* Linxia Zhang,* Jun Wei,§ Ying Wang,§ Yunqi Geng,† Xiaoyan Zhang,*‡ Wentao Qiao,‡ and Jianqing Xu*‡

Owing to ongoing recognition of pathogen-associated molecular patterns, immune activation and upregulation of IFN-stimulated genes (ISGs) are sustained in the chronically infected host. Albeit most ISGs are important effectors for containing viral replication, some might exert compensatory immune suppression to limit pathological dysfunctions, although the mechanisms are not fully understood. In this study, we report that the ISG lymphocyte Ag 6 complex, locus E (LY6E) is a negative immune regulator of monocytes. LY6E in monocytes negatively modulated CD14 expression and subsequently dampened the responsiveness to LPS stimulation in vitro. In the setting of chronic HIV infection, the upregulation of LY6E was correlated with reduced CD14 level on monocytes; however, the immunosuppressive effect of LY6E was not adequate to remedy the hyperresponsiveness of activated monocytes. Taken together, the regulatory LY6E pathway in monocytes represents one of negative feedback mechanisms that counterbalance monocyte activation, which might be caused by LPS translocation through the compromised gastrointestinal tract during persistent HIV-1 infection and may serve as a potential target for immune intervention. The Journal of Immunology, 2014, 193: 000–000.

Infection with HIV-1 not only results in the progressive loss of CD4+ T cells (1, 2) but also causes impairment of immune cell subsets, such as CD8+ T cells, NK cells, B cells, and dendritic cells (DCs) (3–5). The global upregulation of type I IFN (IFN-I)–stimulated genes (ISGs) has been concomitantly observed with the impairment of immune cells (6, 7). IFN-I induces expression of ISGs via both autocrine and paracrine mechanisms to promote effector functions of both the innate and adaptive immune responses in the face of infection to restrain viral replication (8, 9). A series of ISGs that function to restrict viral replication have been identified with in vitro cell line–based HIV infection models, and these genes include APOBEC3G, Tetherin/BST2, MX2, and ISG15 (8, 10, 11). Recent reports also suggest a causal role of IFN-I signaling in the control of immune activation and reduction of viral titers in a mouse model of lymphocytic choriomeningitis virus infection (12, 13). One possible explanation for these effects is that the host bolsters its immunoregulatory effects to prevent the immune system from systemic hyperimmune activation during the chronic stages of infection; however, these immunoregulatory attempts in turn effectively dampen the immune responses, resulting in inefficient control of virus replication (9, 14, 15).

Several pathways contribute to sustained activation of IFN-I signaling in SIV/HIV infection. First, plasmacytoid DCs directly recognize viral RNA through TLR7 or TLR9, resulting in considerable IFN-I production during HIV/SIV infection (16–18). Second, translocation of microbial products from the compromised gastrointestinal tract stimulates the innate immune cells (19), and phagocytosis of virus-infected cells also stimulates these cells to produce IFN-I (20). Third, monocyte-derived DCs have the potential to produce IFN-I upon recognition of new synthesis of the HIV capsid or viral cDNA (21–23).

Because plasmacytoid DCs are gradually lost after the establishment of HIV infection, and the engagement of myeloid DCs by HIV replication products requires the removal of several barriers in the presence of viral proteins Vpx or Vpr of SIV/HIV-2, which are not physiological conditions for HIV-1 infection (24), monocytes may function as the primary IFN-I–producing cells during chronic...
HIV/SIV infection. Furthermore, because monocytes are the major cell population that responds to translocated LPS from the damaged gastrointestinal tract, and LPS is considered to be one of the major triggers of innate immune activation during the chronic stages of HIV infection (19), understanding the regulatory mechanism of monocytes is vital to delineate the mechanisms of pathogenesis and develop new strategies for immune intervention during chronic HIV infection.

In this study, we filtered a subset of ISGs that were closely related to disease progression to determine those that are likely to exert immunomodulatory effects during HIV pathogenesis. Although most of these identified disease-associated ISGs are antiviral mediators, lymphocyte Ag 6 complex, locus E (LY6E) is known as an immune regulator. Thus, we further characterized the immunoregulatory role of LY6E in HIV disease pathogenesis. Gain-of-function and loss-of-function assays demonstrated that LY6E downregulates monocyte responsiveness by modulating CD14 expression; additionally, the upregulation of LY6E in monocytes during chronic HIV infection suggests that this pathway may provide negative feedback to the hyperimmune activation that results from increased LPS translocation from the compromised gut tract into the systemic circulation.

Materials and Methods

Subjects
Fifty-four HIV-1–infected individuals were recruited in this study. After enrollment, participants attended quarterly visits unless they were subjected to antiretroviral medications due to CD4 counts of <200 cells/μl or experienced AIDS-related illnesses. The CD4+ T cell count was measured at every visit, and the viral loads were quantified every 9 mo. The basic characteristics of the studied subjects are summarized in Table 1 and detailed information is listed in Supplemental Table I. Progressors were defined as HIV-infected individuals with a decline in CD4+ T cell counts of >50 cells/mm³/y. Nonprogressors were defined as HIV-infected individuals with no evidence of a CD4+ T cell count decline and a viral load of <5000 copies/ml.

Ethics statement
The study was reviewed and approved by the Ethics Committee of Shanghai Public Health Clinical Center. Written informed consents were provided by all participants. All participants were adults; no minor was involved in this study.

Analysis of microarray data
To study the expression of IFN-inducible genes in HIV progressors, 26 participants, including 15 nonprogressors and 11 progressors, were included. These individuals were all naive to antiretroviral therapy (ART). RNA from whole blood from these donors was extracted with a PaxGene Blood miRNA kit (Qiagen, Hilden, Germany). For the study of the mRNA profile of LY6E-silent THP-1 cells, RNA from the cell lines was prepared with a TRizol RNA extraction protocol. Microarray analysis was performed by United Gene according to a standard protocol on the HumanHT-12 version 4.0 expression beadchip (Illumina). Statistical analysis and hierarchical cluster analysis were performed using Biometric Research Branch–ArrayTools version 4.1.0 beta 2. Log-transformed data were normalized by the results of median normalization. Genes showing minimal variation or cases where the percentage of missing values was >20% across the set of arrays were excluded from further analysis. Class comparison analysis was used to identify mRNAs that were differentially expressed between two predefined classes of samples, and a specified threshold p value (<0.01) and a random variance model were used for these univariate tests. Specific criteria were applied for filtering the differentially expressed genes: a minimum of 1.2-fold change was used for arrays of HIV-1 disease-associated genes in whole blood samples from patients, and a 1.5-fold change was required for altered genes in THP-1 cells using short hairpin RNA (shRNA) against LY6E (THP-1-shLY6E cells) compared with THP-1 cells using shRNA control (THP-1-shCtrl cells).

Monocytes isolation
Enriched monocytes were isolated from PBMCs. CD14+ monocytes were separated using magnetic negative selection with the EasySep human monocyte enrichment kit (StemCell Technologies). Monocyte purity was >97% as determined by flow cytometry.

RNA isolation and RT-PCR
Total RNA was isolated from whole blood or monocytes using an RNeasy Mini kit (Qiagen). RNA (1 μg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Then, cDNA was used for target gene amplification using Platinum SYBR Green quantitative PCR (qPCR) SuperMix (Invitrogen). The primer sequences are listed in Table II. PCR was performed in a Mastercycler ep realplex (Eppendorf), with annealing temperatures of 60°C. Data are expressed as levels of target mRNA expression relative to GAPDH mRNA expression.

Cell lines, cell culture, and reagents
Promonocytic leukemia THP-1 cells were obtained from the Cell Bank of the Chinese Academy of Sciences and then cultured in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/ml), streptomycin sulfate (100 mg/ml), 2-ME (0.05 mM), and l-glutamine (2 mM). shRNA-silenced cell lines were generated through electroporation of THP-1 cells with a TRC2-LKO-puro construct containing shRNA directed against human LY6E (hairpin sequence, 5'-CATTTGGGAATCTCGTGACATT-3'), and then cells were selected for resistance to puromycin and cultured in the appropriate media supplemented with 400 ng/ml puromycin. LPS was derived from Escherichia coli O55: B5 (Sigma-Aldrich). Recombinant human IFN-α2b was obtained from Life Technologies.

Plasmids
The LY6E pGL2 basic promoterless luciferase reporter plasmid was provided by Dr. Sakamura V. Reddy (Charles P. Darby Children’s Research Institute, Medical University of South Carolina, Charleston, SC). The shRNA constructs were purchased from Sigma-Aldrich. shRNA-resistant LY6E (LY6E-R) and CD14 were subcloned in the pWP1 lentivirus expression vector (HIV-IRES-GFP) using the Spel and Ndel restriction endonucleases (Fermentas). All plasmids were prepared with the EndoFree Plasmid kit (Qiagen). The LY6E-R mutant was generated via overlap PCR methods using KOD Plus (Toyobo).

Cell stimulation
Monocytes and THP-1 cells (1 × 10⁶ cells/well) were treated with 1000 U/ml rIFN-α2b. Samples were collected 12–24 h after stimulation. RNA was isolated for quantitative RT-PCR (qRT-PCR) analysis, and whole-cell lysate was examined by Western blotting for target gene expression.

Cytokine measurement
THP-1-shLY6E and THP-1-shCtrl cells were plated in 96-well plates at 1 × 10⁵ cells in 100 μl culture medium per well and then treated with culture medium or LPS at 10 ng/ml or 100 ng/ml for 3–12 h. Cell-free supernatants were collected and stored at −80°C until analysis. Concentrations of inflammatory cytokines in the supernatants were assessed by the cytomagnetic bead array (CBA) (BD Biosciences) or ELISA (eBioscience) according to the manufacturers’ instructions. The cells were also collected for mRNA expression detection by qRT-PCR.

Intracellular cytokine staining
Brefeldin A (1 μg/ml) and monensin (1 μM) were added to monocytes cultures 1 h after the beginning of stimulation. After 6 h of incubation, cells were washed in PBS containing 2% newborn calf serum and stained with surface-specific Abs for 15 min at room temperature (25°C) before fixation with 100 μl fixation and permeabilization solution for 20 min at 25°C (BD Biosciences). Cells were then stained with anti–TNF-α-PE-Cy7 (BD Biosciences) for 30 min at 4°C. Data were collected on a FACScalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, San Carlos, CA). In all experiments, the Live/Dead blue dye (Invitrogen) was used to exclude dead cells.

Small interfering RNA/plasmid transfection of THP-1 and monocytes
Scrambled small interfering RNA (siRNA) and LY6E transiently silenced using an LY6E-specific siRNA (si-LY6E; seed sequence, 5′-CGTCTCCGACCCAGGACACTT-3′) were synthesized by Qiagen. Nucleofection was used to deliver siRNA and endotoxin-free plasmids. The Amaxa cell line Nucleofector kit or human monocyte Nucleofector kit (Lonza) was used for transfection in accordance with the manufacturer’s recommendations. In brief, 1 × 10⁶ THP-1 cells or 5 × 10⁶ monocytes were transfected with 30 nM siRNA or 500 ng plasmid in 1.5 ml RPMI 1640 medium supplemented with 10% FBS in 12-well plates. The knockdown efficiency was assessed by qRT-PCR or Western blotting.
**Phenotypic analysis**

Cells were stained with the following fluorochrome-conjugated Abs: CD14-PE (BioLegend), CD16-allophycocyanin-Cy7 (BD Pharmingen), CD86-Pacific Blue (BioLegend), PD-1-allophycocyanin (BioLegend), PD-L1-allophycocyanin (eBioscience), PD-L2-allophycocyanin (BioLegend), CD95–Pacific Blue (BioLegend), Fas ligand–PE (BioLegend), HLA-DR–PerCP (BioLegend), TLR4–allophycocyanin (eBioscience), CXC4R–PE-Cy7 (BioLegend), and CCR5–FITC (BD Biosciences). Data were collected on a FACSDiva flow cytometer and analyzed using FlowJo software.

**Lentiviral transduction**

To produce lentivirus, 293T cells were transfected with 2 μg pWPI-LY6E-R or pWPI-CD14 (lentiviral vector), 1 μg pSPAX (packing plasmid), and 0.3 μg VSV-G (envelope-encoding plasmid) using Lipofectamine 2000 (Invitrogen). Medium was replaced 8 h after transfection, and virus was harvested 48 h later and filtered through a 0.45-μm filter. THP-1-shLY6E and THP-1-shCtrl cells were transduced with VSV-G pseudotyped lentivirus to generate LY6E-overexpressing THP-1-shLY6E cells and CD14 overexpressing THP-1-shCtrl cells.

**Western blot analysis**

Cells were lysed in RIPA buffer containing 1% NP-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, protease inhibitor mixture (Thermo Fisher Scientific), and PhosSTOP phosphatase inhibitor mixture (Roche). Whole-cell lysates were resolved on SDS-PAGE, transferred to 0.22-μm polyvinylidene fluoride membranes, and probed with primary Abs and the corresponding HRP-conjugated secondary Abs. The following Abs were used: rabbit anti-LY6E polyclonal Ab (LifeSpan BioSciences), mouse anti-β-actin mAb (Santa Cruz Biotechnology), HRP-conjugated goat anti-mouse secondary Abs, and HRP-conjugated donkey anti-rabbit secondary Abs (Santa Cruz Biotechnology).

**Statistical analysis**

All data are expressed as means ± SD. The Mann–Whitney U test was used to compare the difference between two groups. Correlations were evaluated with the Spearman test. Comparison of different treatments in the same sample was achieved with a paired t test. These analyses were performed using the GraphPad Prism software. Differences were deemed statistically significant when p was <0.05.

**Accession codes**

Accession codes are as follows: Gene Expression Omnibus, microarray data, GSE56837 and GSE44794 (http://www.ncbi.nlm.nih.gov/geo/).

**Results**

**Identification of HIV-1 disease–associated regulatory ISGs**

We conducted transcriptomic analysis of whole-blood samples from patients with chronic HIV-1 infection. We applied several logic constraints to identify candidate ISGs that may exert im-

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**FIGURE 1.** LY6E is an IFN-inducible gene and is upregulated in HIV-1 progressors. (A) The preferentially expressed ISGs in HIV progressors. The ratio of ISG levels in whole blood from HIV-1 progressors to those in nonprogressors (y-axis) was plotted against their fold-changes in IFN-α–treated monocytes divided by those in untreated cells (x-axis). (B) Clustering of 29 upregulated ISGs in HIV-1 progressors. Average linkage hierarchical clustering was performed using centered correlation metrics on 29 ISGs in progressors (pink bars, n = 11) and nonprogressors (green bars, n = 15). (C) Levels of LY6E mRNA relative to levels of GAPDH mRNA (±SD, n = 3) as quantified by qRT-PCR in monocytes and THP-1cells. (D) Western blot analysis of LY6E expression. R.S.I., relative signal intensity. (E) Activation of the LY6E promoter reporter construct upon IFN-α stimulation. HeLa cells were transiently transfected with pGL2 basic luciferase reporter plasmid containing the LY6E promoter sequence for 36 h, followed by IFN-α stimulation for an additional 12 h. The cell lysates were assayed for luciferase activity. The pGL2 basic empty vector was used as a control. Transfection efficiency was normalized to β-galactosidase activity that was constitutively expressed in these cells (±SD, n = 3).
munoregulatory functions in HIV pathogenesis. First, because ISGs are genes that should be inducible by IFN-I, such as IFN-α treatment, we filtered the ISGs, which exhibited a 4-fold increase in expression in response to IFN-α treatment, based on publically deposited microarray data (Gene Expression Omnibus ID E-GEOD-1740, Fig. 1A, x-axis) (25). Second, the disease-related regulatory ISGs should be more significantly upregulated in progressors than in nonprogressors. According to retrospective longitudinal medical records, we defined progressors as HIV-infected patients with a decline in CD4+ T cell count of $50$ cells/mm$^3$/y, and defined nonprogressors as HIV-infected individuals with no evidence of CD4$^+$ T cell count drop and a viral load of $<5000$ copies/ml during the period of observation (26). Among 190 ISGs induced by IFN-I treatment, our analysis revealed that 29 genes were differentially expressed between progressors and non-progressors, as expected for being predominantly upregulated in progressors (Fig. 1A, y-axis, 1B). Third, to identify immunoregulatory genes, we excluded the candidate genes that are primarily

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<th>Table I. Characteristics of subjects in the study</th>
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<td>Duration after HIV confirmation (y)</td>
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<td>CD4$^+$ T cell count (cells/µl)</td>
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<td>Viral loads (copies/ml)</td>
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Data are presented as median value (range).
known as antiviral mediators according to their functional annotation, for example, OAS1, MX1, IFITM1, IFI35, and IFI44 (8, 11, 27). These analyses led to the identification of the inhibitory regulator of T cells, LY6E, which was one of the most significantly upregulated ISGs in progressors (p = 0.0000132, 2.4-fold; Supplemental Table II). qRT-PCR analysis of LY6E mRNA levels confirmed the microarray data (data not shown).

To verify that LY6E expression is indeed inducible by IFN-I, we assessed LY6E expression in various cell types in the absence and the presence of IFN-α treatment. Elevated LY6E expression was confirmed in IFN-α-treated purified CD14+ primary monocytes and THP-1 cells at the mRNA level using qPCR and at the protein level using Western blotting (Fig. 1C, 1D). Additionally, luciferase activity was only increased in IFN-α–treated HeLa cells that had been transfected with the pGL2 reporter construct, which contains the LY6E promoter, whereas HeLa cells transfected with the pGL2 reporter construct lacking the LY6E promoter failed to respond to IFN-α stimulation, indicating that LY6E expression is

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** CD14 is increased in LY6E-silenced THP-1 cells. (A) THP-1 cells were engineered to stably express shRNA targeting LY6E (THP-1-shLY6E) or scrambled shRNA (THP-1-shCtrl). LY6E expression in THP-1-shLY6E and THP-1-shCtrl cells was analyzed by qRT-PCR and Western blotting. (B) Microarray analysis revealed the genes that were differentially expressed between THP-1-shLY6E and THP-1-shCtrl cells. (C) Flow cytometric analysis of CD14 expression in THP-1-shLY6E and THP-1-shCtrl cells. CD14 expression was assessed after incubation with LPS for 0, 3, or 6 h. *p < 0.05, **p < 0.01.
induced in response to IFN-I (Fig. 1E). Taken together, these data revealed that LY6E is an IFN-I–inducible gene that is preferentially overexpressed in HIV progressors.

Ly6e, a human homolog with the murine LY6 family, also termed retinoic acid–induced gene E, or thymic shared Ag-1 is induced by either IFN-I or retinoic acid (28, 29). It encodes a GPI-anchored protein that is predominantly expressed in a proportion of myeloid cells and thymocytes (30, 31). However, the biological significance of LY6E remains incompletely defined.

**Correlation of elevated LY6E expression in monocytes to HIV disease progression and immune activation**

We examined LY6E expression in monocytes in relationship to the clinical parameters of HIV disease progression. In a set of

**FIGURE 4.** LY6E reduces the responsiveness of monocytes to LPS stimulation. (A) Intracellular TNF-α staining in THP-1-shLY6E and THP-1-shCtrl cells. Cells were stimulated with 10 ng/ml LPS for 6 h, and then 1 mM monensin and 1 µg/ml brefeldin A were added 1 h after LPS stimulation. TNF-α production was measured by intracellular staining. The frequency of TNF-α* THP-1 cells from five independent experiments is shown. (B) Proinflammatory cytokine production in THP-1-shLY6E cells was compared with that in THP-1-shCtrl cells. Production of TNF-α, IL-1β, IL-6, and IL-8 in culture supernatants was quantified by CBA assay after incubation with LPS for 6 h. (C) Levels of IFN-α and IFN-β mRNA relative to GAPDH in THP-1-shLY6E and THP-1-shCtrl cells were quantified by qRT-PCR. (D) and (E) Production of proinflammatory cytokines in LY6E-silenced and control primary monocytes. Primary monocytes were nucleofected with siRNA targeting LY6E (si-LY6E) or scrambled siRNA (si-Ctrl) for 72 h. LY6E expression was analyzed by qRT-PCR and Western blotting. After stimulation with LPS for 6 h, production of proinflammatory cytokines was determined by CBA. *p < 0.05, **p < 0.01.
cross-sectional samples, the LY6E levels in purified CD14⁺ monocytes were inversely correlated with absolute CD4⁺ T cell count \( (p = 0.031) \) but failed to be associated with viral load \( (p = 0.37, r = 0.18; \) Fig. 2A, 2B). Successful ART (viral loads of <50 copies/ml) significantly decreased LY6E expression in monocytes \( (p = 0.005; \) Fig. 2C). We categorized patients into the following categories (Table I): ART-naive subjects with high CD4 count (CD4⁺ T cell count ≥350 cells/µm³), ART-naive subjects with low CD4 count (CD4⁺ T cell count <350 cells/µm³), and ART-treated patients. Monocytes from patients of different categories were subjected to LY6E expression detection by immunoblotting. Consistent with previous reports, these data demonstrate that LY6E expression in monocytes is associated with HIV disease progression and immune activation.

We classified purified monocytes according to CD14 and CD16 expression and measured the LY6E levels in these subsets to exclude the possibility that the observed elevation in LY6E levels is due to perturbation in monocyte subsets in HIV infection (32, 33). LY6E expression was higher in THP-1-shLY6E cells than in THP-1-shCtrl cells (Fig. 2D). Furthermore, the LY6E levels in monocytes were also correlated with the frequencies of CD38⁺CD8⁺ T cells, a marker of T cell immune activation \( (p = 0.002; \) Fig. 2E). For longitudinal observation, Kaplan–Meier survival analysis demonstrated that patients with higher levels of LY6E expression were significantly more likely to experience a rapid CD4 decline (defined as a decline of >50 cells/µm³/y) in comparison with subjects with lower levels of LY6E \( (p = 0.0082; \) Fig. 2F). Altogether, these data demonstrate that LY6E expression in monocytes is associated with HIV disease progression and immune activation.

We observed that genes encoding cytokines \( (\text{IL}-1, \text{IL}-6, \text{IL}-10, \text{IFN}-\gamma) \) and transcription factors \( (\text{Nfkbia, Relb, and Ira}k2) \) were upregulated in THP-1-shLY6E cells (Fig. 3B), suggesting that LY6E exerts an inhibitory role on those genes and that knockdown of LY6E by shLY6E results in upregulation of these factors.

Because CD14 is a receptor for TLR4-mediated recognition of LPS, these data suggest that LY6E may exert its regulatory role via the CD14/TLR4 pathway. We further confirmed the expression of CD14 protein by flow cytometric analysis. Up-regulation of CD14 expression in THP-1-shLY6E cells was observed in comparison with THP-1-shCtrl cells both before and after LPS stimulation (Fig. 3C). We also characterized other important molecules involved in biological functions of monocytes, including the sensor for LPS (TLR4), the ligands for PD-1 (PD-L1 and PD-L2), molecules involved in cell death and apoptosis (CD95 and Fas ligand), Apo2Ag presentation factors (HLA-DR, CD86), and cell surface marker (CD16), and coreceptors for HIV entry (CCR5 and CXCR4). No obvious changes were observed for any of these molecules, except HLA-DR, and these findings are in accord with the transcriptomic data (Supplemental Fig. 1).

Because CD14 is the vehicle that transports LPS to the TLR4/MD2 complex, we explored the role of LY6E on the monocyte-mediated innate immune responses to LPS. After stimulation with LPS, THP-1-shLY6E cells produced significantly more TNF-α than did THP-1-shCtrl cells as quantified by intracellular cytokine staining \( (p = 0.006; \) Fig. 4A). The levels of proinflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-8, were also higher in the supernatants of LPS-stimulated THP-1-shLY6E cells than in those of LPS-treated THP-1-shCtrl cells (Fig. 4B). The levels of proinflammatory cytokine mRNAs in cells were consistent with the protein concentrations in culture supernatants (Table II, Supplemental Fig. 2A). The differences of proinflammatory cytokine response between THP-1-shLY6E and THP-1-shCtrl cells were consistent from 3 to 12 h of stimulation in the presence of various doses of LPS, which became diminished during prolonged incubation (Supplemental Fig. 2B). We also quantified the IFN-β production in response to LPS by qRT-PCR. IFN-β expression was markedly higher in THP-1-shLY6E cells than in THP-1-shCtrl cells \( (p < 0.01) \), whereas IFN-β expression did not differ between the two cell types (Fig. 4C). Similar results were observed with si-LY6E in THP-1 cells (Supplemental Fig. 2C) and in primary CD14⁺ monocytes from healthy donors (Fig. 4D, 4E). In contrast, TLR3, TLR7, and TLR8 agonists induced comparable inflammatory responses in THP-1-shLY6E and THP-1-shCtrl cells (data not shown).

To confirm the influence of LY6E on CD14 expression, we expressed the LY6E-R mutant in THP-1-shLY6E cells by transfection of a lentiviral vector expressing LY6E-R and GFP in cis. In GFP⁺ cells, LY6E-R profoundly reduced CD14 expression and dramatically reduced TNF-α production upon LPS stimulation as compared with the vector control (Fig. 5A, 5B).

As observed in transcriptional microarray analysis, we also determined CD14 mRNA levels in THP-1-shLY6E and THP-1-shCtrl cells. Knocking down LY6E resulted in up to a 12.7-fold modulation of CD14 expression by LY6E regulates monocyte responsiveness to LPS

To explore the role of LY6E in the monocyte-mediated innate immune responses, we established an in vitro model via stable knockdown of LY6E expression in THP-1-shLY6E cells. Treatment with shLY6E significantly reduced LY6E expression at both the RNA level (Fig. 3A, upper panel) and the protein level (Fig. 3A, bottom panel) in comparison with treatment with control shRNA (i.e., THP-1-shCtrl cells). Comparative transcriptome analyses were performed to define the genes that were differentially expressed between THP-1-shLY6E and THP-1-shCtrl cells. We observed that genes encoding cytokines \( (\text{Il}1b, \text{Il}8, \text{and Tnf}) \), cell surface molecules \( (\text{Cd}14, \text{Cd}83, \text{and Hla-dra}) \), and transcription factors \( (\text{Nfkbia, Relb, and Ira}k2) \) were upregulated in THP-1-shLY6E cells (Fig. 3B), suggesting that LY6E exerts an inhibitory role on those genes and that knockdown of LY6E by shLY6E results in upregulation of these factors.

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Table II. Primers

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increase of the abundance of CD14 mRNA (Fig. 5C), suggesting that CD14 expression is modulated at the transcriptional step.

To test whether the reduction in CD14 expression is sufficient for attenuated responsiveness to LPS stimulation mediated by LY6E, we exogenously overexpressed CD14 in THP-1-shCtrl cells using the same approach (Fig. 5D). LPS treatment invoked significantly higher TNF-α production in CD14 overexpressing THP-1-shCtrl cells than in cells transduced with vector control (Fig. 5E). These data demonstrate that LY6E attenuates the responsiveness of monocytes to LPS stimulation by downregulating CD14 expression.

LY6E represses the responsiveness of primary monocytes to LPS through downregulating CD14 expression

To evaluate the regulatory effects exerted by LY6E in monocytes in HIV chronically infected patients, we examined the relationship between LY6E levels and CD14 expression. The mean fluorescence intensity (MFI) of CD14 on monocytes was inversely correlated with the expression of LY6E during HIV infection. (A) The association of the expression of CD14, which is shown as MFI, with LY6E expression in monocytes of ART-naive HIV+ individuals was displayed (p = 0.048, n = 16). (B) CD14 levels in monocytes were compared among ART-naive subjects with low CD4 count (n = 9), ART-naive subjects with high CD4 count (n = 8), and ART-treated individuals (n = 8). (C) CD14 expression was compared in ART-naive subjects with low CD4 count (n = 11), ART-naive subjects with high CD4 count (n = 9), and ART-treated patients (n = 19) in CD14++CD16− (classical), CD14−CD16++ (nonclassical), and CD14++CD16− (intermediate) monocyte subsets.
with the abundance of \textit{LY6E} mRNA in ART-naive HIV\textsuperscript{+} individuals \cite{p=0.048; Fig. 6A}. CD14 levels on monocytes were lower in ART-naive subjects with low CD4 count than in those with high CD4 count \cite{p=0.036}, and these CD14 levels were partially restored in ART-treated individuals \cite{p=0.004; Fig. 6B}. Similar results were observed in CD14\textsuperscript{++}CD16\textsuperscript{--}, CD14\textsuperscript{+}CD16\textsuperscript{++}, and CD14\textsuperscript{++}CD16\textsuperscript{+} subsets (Fig. 6C). Collectively, these data indicated that CD14 expression was inversely correlated with \textit{LY6E} in primary monocytes from subjects chronically infected with HIV.

Similar to previous reports, the percentage of TNF-\alpha\textsuperscript{+} monocytes in response to LPS stimulation was profoundly increased in ART-naive patients when compared with healthy donors \cite{p=0.003}, and this percentage was moderately decreased in virologically suppressed patients \cite{p=0.11; Fig. 7A, 7B}, indicating that monocytes from HIV patients are in a heightened activation state perhaps owing to persistent viral replication, exposure to homeostatic cytokines, and to microbial products \cite{33–35}. Furthermore, to directly examine the immunosuppressive role of \textit{LY6E} in HIV infection, CD14\textsuperscript{+} monocytes from three viremic subjects were pooled and transfected with si-LY6E or si-Ctrl for 72 h followed by 6 h LPS stimulation. The results are from two independent experiments. Knockdown of \textit{LY6E} levels was examined by qPCR. Flow cytometric analysis of CD14 expression and intracellular TNF-\alpha staining. In the \textit{upper panel}, the MFI of CD14 is indicated. In the \textit{lower panel}, the numbers indicate the percentage and MFI of TNF-\alpha–producing cells. The percentage or the MFI of TNF-\alpha\textsuperscript{+} monocytes after LPS stimulation. The MFI of CD14 expression.
upon LY6E knockdown (Fig. 7F). These data collectively indicate that upregulation of the immunosuppressive LY6E pathway could downregulate CD14 expression in vivo and thereby repress the responses of monocytes to LPS stimulation, which may partially counterbalance persistent immune activation during chronic HIV infection.

Discussion

The role of the ISG LY6E in viral infection has not been fully defined. Reports indicate that LY6E enhances the replication of yellow fever virus in *STAT1−/−* fibroblasts and Huh-7 cells but does not significantly affect replication of West Nile virus (8, 36). Besides, LY6E could restrict vesicular stomatitis virus replication (37). In vitro whole-genome analysis identified a susceptibility locus on human HSA8q24 that exerts influence on the cellular susceptibility to HIV-1. This locus is flanked on both sides by genes of the *LY6/uPAR* family, suggesting a potential role of the LY6 family of GPI-anchored proteins in HIV-1 infection (38). In mice, LY6E, also called thymic shared Ag-1, is a marker for T cell activation, and a signal through this molecule has a negative feedback role to limit IL-2 production from activated T cells stimulated through the TCR. Thus, LY6E functions as a modulator of the TCR signaling pathway (30, 39–41). The function of LY6E in human monocytes may be likewise a regulator that had a negative feedback role in innate immune activation. In our study, we demonstrated that HIV infection results in upregulation of LY6E and that LY6E levels are correlated with HIV disease progression and immune activation. The upregulation of LY6E has also been reported in multiple sclerosis (42, 43), wherein persistent immune activation is one of the complications of this autoimmune disease, suggesting that LY6E is an immunomodulator in a broad spectrum of chronic immune dysfunction diseases.

To dissect the role of LY6E in monocytes, we used THP-1, a monocytic cell line, as our model in vitro, which allows us to determine the function of LY6E in monocytes in the absence of immune activation that resulted from HIV-1 persistent replication. Our data showed that LY6E negatively regulates the LPS-stimulated innate immune responses in THP-1 cells through downregulating CD14 expression. The silence of LY6E significantly increased CD14 expression and thereby enhanced the immune responses to LPS stimulation, whereas the overexpression of LY6E brought down CD14 expression and decreased the LPS-stimulated immune responses. The regulatory role of LY6E in THP-1 cells was also confirmed in primary monocytes derived from HIV-1-infected subjects. Overall, our data in vitro established a regulatory role of LY6E in monocytes in responding to LPS stimulation.

HIV-1 infection can result in the impairment of mucosal integrity in the gastrointestinal tract and subsequently lead to the translocation of microbial products, LPS, which is a major structural component of the outer wall of Gram-negative bacteria and an effective CD14/TLR4 trigger, has been shown to be gradually increased in peripheral blood with the disease progression in HIV-1-infected subjects (19, 44–48). Because LY6E is upregulated during HIV-1 infection and plays a regulatory role in monocytes through modulating the expression of CD14, the innate immune responses to LPS stimulation in monocytes are likely to be attenuated via downregulating CD14/TLR4 pathway, as shown in our data ex vivo (Fig. 7). The functional damage to monocytes during HIV-1 infection may facilitate the development of exacerbated clinical complications and accelerate the disease progression when a coinfection with Gram-negative bacteria occurs. For instance, HIV-infected individuals are vulnerable to *Mycobacterium tuberculosis* infection, and reactivation of latent *M. tuberculosis* occurs more often in HIV-infected individuals than in those who are not infected with HIV (49, 50). Coinfection with HIV and *M. tuberculosis* is likely to result in dissemination of *M. tuberculosis* (51). Lung granulomas from *M. tuberculosis* HIV-1–coinfected patients display decreased in situ TNF production (52).

Our data revealed that LY6E exerts its role through downregulating CD14 expression at the transcriptional step but not the stage of protein stability or degradation. The possibility remains that the transcriptional machinery involved in CD14 expression, such as the activity of transcription factors and the transcriptional status of the promoter region, may be influenced by the LY6E pathway. Another avenue for further investigation is how the LY6E pathway is triggered. To date, no LY6E ligand has been identified. It would also be interesting to uncover how LY6E, as a GPI-anchored protein, introduces an intracellular signaling cascade. Further investigations will be pursued to address these questions.

Because IFN-α is important for initiating innate and adaptive immune responses against infection (53–55), the activation of ISGs is likely to arm the immune system to fight against pathogen infection during the early stages. Meanwhile, some ISGs play a regulatory role in stopping the immune response and protecting the host from deterioration due to excessive immune activation. The failure to eradicate pathogens at this early stage, however, results in persistent infection, which in turn keeps the immune system alerted to produce IFN-α and upregulate immunosuppressive molecules in IFN-I signaling, including some ISGs that practically restrain the immune system from launching sufficient immune responses to clear invaded pathogens. Overall, IFN-α and ISGs together may indeed play an important role in the elimination of pathogens during early infection, but these players may ultimately aid the pathogens during chronic infection as suggested by the association of IFN-1 and CD4+ T cell depletion in a humanized mice model (12, 13, 56).

Although LY6E is upregulated in monocytes from chronic HIV-infected patients, its negative regulatory effect is insufficient to ameliorate the ready-heightened responsiveness of monocytes caused primarily by persistent viral replication, exposure to homeostatic cytokines, and exposure to microbial products and thus still remain prone to produce inflammatory cytokines upon recognizing LPS (19, 21, 55). Owing to the persistent elevation of immunoegulatory ISGs, such as LY6E, during chronic HIV infection, therapeutic immune intervention will be unlikely to be effective. Effective ART may reduce LY6E to a similar level to that in HIV-seronegative individuals and thereby remove the immunosuppression, which would be likely to promote the effectiveness of immune therapeutics and to reinvigorate the functionally impaired immune cells.

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Data deposition

The genome-wide mRNA expression data have been deposited in the GEO database (Accession number GSE81544).

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Disclosures
The authors have no financial conflicts of interest.

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