**Chlamydia trachomatis** Stimulation Enhances HIV-1 Susceptibility through the Modulation of a Member of the Macrophage Inflammatory Proteins

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Sexually transmitted infections such as *Chlamydia trachomatis* can enhance HIV-1 infection. However, the molecular mechanisms modulating the enhancement of HIV-1 infectivity and replication during HIV-1/sexually transmitted infections coinfection remain elusive. In this study, we performed an ex vivo infection of HIV-1 in PBMCs of *C. trachomatis*-infected patients and observed a significant increase in HIV-1 p24 levels compared with those in cells from healthy donors. Similarly, *C. trachomatis*-stimulated PBMCs from healthy donors showed enhanced susceptibility to HIV-1. *C. trachomatis*-stimulated CD4 T cells also harbored more HIV-1 copy numbers. RNA sequencing data revealed the upregulation of CCL3L1/CCL3L3, a paralog of CCL3 in *C. trachomatis*-stimulated CD4 T cells infected with HIV-1. Furthermore, an increase in CCL3L1/CCL3L3 expression levels correlated with HIV-1 replication in *C. trachomatis*-stimulated cells. However, the addition of exogenous CCL3L1 reduces HIV-1 infection of healthy cells, indicating a dual role of CCL3L1 in HIV-1 infection. Further investigation revealed that a knockout of CCL3L1/CCL3L3 in Jurkat T cells rescued the increased susceptibility of *C. trachomatis*-stimulated cells to HIV-1 infection. These results reveal a role for CCL3L1/CCL3L3 in enhancing HIV-1 replication and production and highlight a mechanism for the enhanced susceptibility to HIV-1 among *C. trachomatis*-infected patients.


**INTRODUCTION**

A combination of viral and host cell factors determines the infectivity of HIV-1 and the progression of the disease (Douek et al., 2003). Two crucial factors that play important roles in the rapid spread of HIV-1 infection are the efficiency of HIV-1 transmission and the duration of infectiousness (May and Anderson, 1987). Sexual intercourse remains the major means of HIV-1 transmission even though it has low efficiency (Galvin and Cohen, 2004). Many researchers have suggested an interplay of other sexually transmitted infections (STIs) and opportunistic infections in the widespread of HIV-1 because they are all transmitted through similar risk behaviors (Hu et al., 2013; Lawn et al., 2001; Liu et al., 2016; Sonnenberg et al., 2005).

The impact of different pathogens on the susceptibility of the CD4 T cells remains relatively unexplored. The activation or deactivation of the host immunity by STIs influences the surface expression of coreceptors of normal mononuclear cells and also compromises immune cells’ ability to fight HIV-1 infection (Grassey et al., 2005; Lawn et al., 2001). For example, changes in the gene expression profiles of CD4 T cells as a result of pathogen stimulation are associated with the response of the pathogen-specific CD4 T cells to HIV-1 infection (Auclair et al., 2018; Hu et al., 2013; Liu et al., 2016). Nevertheless, the treatment of STIs failed to show lower HIV-1 incidence in Africa (Hayes et al., 2010; Stillwagon and Sawers, 2015). This suggests that the elimination of STIs in patients was insufficient in reducing HIV-1 infection and that the antigen-specific memory stimulation of CD4 T cells may remain for a long time resulting in enhanced susceptibility even after treatment of the STIs (Hayes et al., 2010). Thus, the high prevalence of STIs may partially explain the high incidence of the heterosexual HIV-1 epidemic in Africa and could serve as an important indicator for intervention to combat HIV-1 infection (Plummer et al., 1991).
Kofoed et al., 2006; Masese et al., 2015). C. trachomatis infection has been suggested to facilitate the transmission of HIV-1 among African prostitutes (Plummer et al., 1991). Previous studies have suggested that STI treatment continues to be a cost-effective strategy for HIV-1 prevention, although it is insufficient by itself to result in a significant reduction in HIV-1 incidence (Johnson et al., 2012; Mlisana et al., 2012). A recent report has shown that there was a high recurrence of asymptomatic C. trachomatis urethritis among people living with HIV-1 infection (Silva et al., 2018). Coinfection with C. trachomatis and HIV-1 has been associated with an increase in HIV-1 susceptibility among men who have sex with men (Lewis et al., 2012; Silva et al., 2018; Smith et al., 2017). However, the detailed mechanism through which C. trachomatis infection facilitates HIV-1 susceptibility and transmission has not been previously elucidated.

In this study, we systematically examined the effect of C. trachomatis infection or stimulation on the HIV-1 infectivity of CT-stimulated CD4 T cells. (a) Representative flow cytometry dot plot analysis of the susceptibility of CD4 T cells from PBMCs isolated from CT-infected patients. (b) Representative flow cytometry dot plot analysis of the HIV-1 susceptibility of CD4 T cells from CT-infected patients (n = 10). (c) Susceptibility of CD4 T cells from healthy donors after 3 days ex vivo stimulation with CT. (d) Analysis of the HIV-1 susceptibility of CT-stimulated CD4 T cells from healthy donors (n = 10). HIV-1 p24 levels in unstimulated PBMCs from the same donors were used as controls. (e) HIV-1 copy number in CT-stimulated and -unstimulated CD4 T cells. Student’s t-test, *P < 0.05, ***P < 0.001. CT, Chlamydia trachomatis.
infectivity and susceptibility of CD4 T cells. We found that PBMCs from *C. trachomatis*–infected patients or healthy *C. trachomatis*–stimulated cells show a significant increase in HIV-1 infection and harbor more HIV-1 copies than unstimulated cells. Through RNA sequencing, CRISPR/Cas9, and small interfering RNA technologies, we document a mechanistic role for the chemokine CCL3L1/CCL3L3, a paralog of CCL3, as a major regulator of HIV-1 replication and viral release during *C. trachomatis* infection. This study attempted to decipher the molecular role of *C. trachomatis* on the susceptibility of CD4 T cells and the influence of intracellular CCL3L1/CCL3L3 on HIV-1 replication during coinfection with *C. trachomatis*.

**RESULTS**

*C. trachomatis*–stimulated CD4 T cells exhibit enhanced susceptibility to HIV-1

We tested whether PBMCs extracted from blood samples collected from clinically diagnosed patients with *C. trachomatis* exhibit any significant difference in their susceptibility to HIV-1 infection. We observed a significant increase in the HIV-1 infection of CD4 T cells from *C. trachomatis*–infected patients compared with those from healthy blood donors (Figure 1a and b and Supplementary Table S1). We then extracted PBMCs from healthy donors and divided each sample into two groups whereby one group was stimulated ex vivo with purified *C. trachomatis* elementary bodies for 3 days before HIV-1 infection. CD4 T cells of *C. trachomatis*–stimulated PBMCs from healthy donors also showed a significant increase in the susceptibility to HIV-1 infection compared with those of unstimulated cells using R5 (Figure 1c and d) or X4 HIV-1 (Supplementary Figure S1a).

Next, using quantitative RT-PCR, we examined the amount of HIV-1 DNAs in the cells as previously described (Douek et al., 2002; Wei et al., 2019). HIV-1 Gag and GAPDH plasmids were used to generate standard curves (Supplementary Figure S1). We observed that *C. trachomatis*–stimulated CD4 T cells showed increased amounts of HIV-1 copy number compared with the unstimulated cells (Figure 1e). Interestingly, there was no significant difference in the expression levels of the CD4 T-cell early activation marker CD69, late activation marker HLA-DR, and the CCR5 coreceptor among healthy subjects and *C. trachomatis*–infected patients (Supplementary Figure S2a–c). These results indicate that infection with *C. trachomatis* enhances the susceptibility of CD4 T cells to HIV-1 infection and significantly increases the amount of HIV-1 DNA in the cells.

**Gene expression dynamics during HIV-1 and *C. trachomatis* coinfection**

RNA sequencing analysis of the isolated CD4 T cells after *C. trachomatis* stimulation and HIV-1 infection (Figure 2a) showed that a large proportion of genes were downregulated in the *C. trachomatis*–stimulated and HIV-1–infected cells, with a small number of upregulated genes (Figure 2b and c). HIV-1–infected and *C. trachomatis*–stimulated/HIV-1–infected cells showed 1,625 and 780 significantly upregulated genes, respectively, and only 407 genes were shared (Figure 2d). HIV-1–infected and *C. trachomatis*–stimulated HIV-1–infected cells showed 1,890 and 2,379 downregulated genes, respectively (Figure 2e). Among the top 50 upregulated genes in the *C. trachomatis*–stimulated group, we identified the genes that were previously shown to play important roles in HIV-1 susceptibility (Figure 2f and g).

**Effect of gene knockdown on the HIV-1 susceptibility of *C. trachomatis*–stimulated CD4 T cells**

Gene ontology analysis of molecular pathways among the *C. trachomatis*–HIV-1–upregulated genes showed the upregulation of processes such as CCR chemokine receptor activity, cytokine activity, DNA-binding transcription activation, chemokine receptor binding, and signaling receptor binding (Figure 3a). RT-qPCR verification of the mRNA sequencing data showed a corresponding significant upregulation in the levels of CCL3L1, IL6, and SOX5 among *C. trachomatis*–HIV-1 CD4 T cells (Figure 3b).

We then tested the knockdown efficiency of small interfering RNAs against these selected candidate genes (Figure 3c–e). Small interfering RNA knockdown of CCL3L1 but not IL6 or SOX5 in *C. trachomatis*–stimulated Jurkat T cells resulted in reduced HIV-1 infectivity (Figure 3f and g and Supplementary Figure S3). The elimination of *C. trachomatis* also showed decreased susceptibility even on CCL3L1 knockdown (Figure 3a). These findings show that *C. trachomatis* modulates enhanced susceptibility of CD4 T cells to HIV-1 through the pathways involving CCL3L1.

CCL3L1 modulates the enhanced HIV-1 susceptibility in *C. trachomatis*–stimulated cells

As shown in Figure 3f and g, CCL3L1 knockdown rescues the enhanced susceptibility of *C. trachomatis*–stimulated Jurkat T cells. Next, we designed guide RNAs to knockout (KO) all copies of the CCL3L1 or CCL3L3 paralogs in the Jurkat cell line using CRISPR/Cas9 (Figure 4a). Three individual clones were selected after puromycin treatment and confirmed by PCR, DNA sequencing, and RT-qPCR analysis (Figure 4b and c). However, immunoblot analysis could not readily verify the KO because most available antibodies against CCL3L1 also target the first 10 amino acids of the CCL3 protein in the cell (Figure 4d).

Next, the wild-type Jurkat T-cell lines and our CCL3L1-KO–sequence variation cell lines (KO-1, KO-2, and KO-3) were stimulated with *C. trachomatis* for 3 days, as previously described, followed by 3 days of HIV-1 infection to identify the direct role of CCL3L1 in the HIV-1 susceptibility. Flow cytometry analysis showed no significant difference in the infectivity of HIV-1 among *C. trachomatis*–stimulated and unstimulated sequence variation cells. However, Jurkat cells expressing CCL3L1 showed increased levels of HIV-1 p24 after *C. trachomatis* stimulation (Figure 4e and f). These data show a role for CCL3L1 in the enhancement of HIV-1 infectivity or susceptibility of T cells to HIV-1 infection.

Expression of CCL3L1/CCL3L3 correlates with HIV-1 replication on *C. trachomatis* stimulation

To understand the relationship between CCL3L1 and HIV-1 replication on *C. trachomatis* stimulation, we measured the levels of CCL3L1 post–HIV-1 infection in Jurkat T cells using HIV-1 gp120 as a positive control of replication. RT-qPCR analysis showed that CCL3L1 mRNA levels increased 3 days after infection, reaching a peak on day 4 after infection.
without *C. trachomatis* stimulation. The levels of CCL3L1 remained relatively stable at about two-folds, whereas gp120 levels increased significantly after day 4 of infection before slightly decreasing by day 6 (Figure 5a). No correlation was observed between CCL3L1 and gp120 expression levels ($R^2 = 0.1907; P = 0.3866$). However, on *C. trachomatis* stimulation, CCL3L1 levels significantly upregulated within 2 days after HIV-1 infection. Surprisingly, CCL3L1 but not
Figure 3. Chemokine modulation of HIV-1 susceptibility in CT-stimulated CD4 T cells. (a) Molecular pathways of significantly upregulated genes in CT-stimulated CD4 T cells. (b) RNA-seq and RT-qPCR analysis of the top upregulated genes. (c) siRNA knockdown of CCL3L1/CCL3L3. (d) siRNA knockdown of IL6. (e) siRNA knockdown of SOX5. Data are the mean ± SEM (n = 3). (f) CCL3L1/CCL3L3 regulates HIV-1 susceptibility during CT infection. Jurkat cells were stimulated with CT for 3 days, followed by siRNA knockdown of CCL3L1/CCL3L3 and HIV-1 infection for 3 days. NC represents negative controls. (g) Analysis of the effect of CCL3L1/CCL3L3 knockdown on HIV-1 susceptibility of Jurkat T cells. Data are the mean ± SEM (n = 4). Student’s t-test; * P < 0.05, ** P < 0.01, and *** P < 0.001. CT, Chlamydia trachomatis; Ctrl, control; FC, fold change; FSC-A, forward scatter area; NC, negative control; n.s., not significant; RNA-seq, RNA sequencing; siCCL3L3, CCL3L3 small interfering RNA; si-Ctrl, control small interfering RNA; siRNA, small interfering RNA; tRNA, transfer RNA.
Figure 4. CRISPR/Cas9 KO of CCL3L3/CCL3L1 rescues the enhanced HIV-1 susceptibility in CT-stimulated Jurkat T cells. (a) Overview of the CCL3L1/CCL3L3 gene and the position of the sgRNAs. (b) PCR screening of CCL3L1/CCL3L3-KO cell lines. (c) Relative mRNA expression level of CCL3L1/CCL3L3 in wild-type and KO Jurkat T-cell cell lines. (d) Western blot analysis of selected sequence variation cell lines. (e, f) KO of CCL3L1/CCL3L3 reduces the percentage of infected cells after CT stimulation. Student’s t-test; *** P < 0.001. Data are the mean ± SEM (n = 3). bp, base pair; CT, Chlamydia trachomatis; KO, knockout; NC, negative control; n.s., not significant; sgRNA, single guide RNA; SSC-A, side scatter area; WT, wild type.
CXCL8 expression level was positively correlated with the level of gp120 in cells that have been stimulated with *C. trachomatis* for 3 days before infection ($R^2 = 0.9052; P = 0.0035$) (Figure 5b). We then inoculated *C. trachomatis*-stimulated and -unstimulated cells with heat-inactivated HIV-1 R5 virus and found that CCL3L1 levels increased slightly within 3 days of virus inoculation and then decreased drastically in *C. trachomatis*-treated cells. Unstimulated cells show no change in CCL3L1 levels within the 6 days of analysis (Figure 5c). Taken together, these data showed that *C. trachomatis* infection promotes CCL3L1 upregulation and correlates with HIV-1 replication during coinfection.

**Expression levels of CCL3L1 in *C. trachomatis*-infected patients**

Next, we analyzed the relative expression levels of CCL3L1 in primary CD4 T cells and two other T-cell lines on *C. trachomatis* stimulation for 3 days. *C. trachomatis* stimulation resulted in significant upregulation of CCL3L1 in both primary CD4$^+$ T cells and T-cell lines after 3 days of
stimulation (Figure 5d). CCL3L1 ELISA analysis of 24 clinical specimens showed that *C. trachomatis*-infected patients exhibited increased levels of CCL3L1 compared with healthy donors (Figure 5e).

CCL3L1/CCL3L3 and CCL3 were the most crucial ligand of the CC chemokine receptor CCR5, which is a major coreceptor of HIV-1 (Gonzalez et al., 2005). The addition of different concentrations of recombinant CCL3L1 to CD4⁺ T cells from healthy donors decreased HIV-1 infectivity of CD4⁺ T cells (Figure 5f and g).

*C. trachomatis* infection enhances HIV-1 replication and release

As described in Figure 5, CCL3L1 levels correlate with the replication of HIV-1 in *C. trachomatis*-treated cells. However, the addition of recombinant CCL3L1 reduces HIV-1 infection of Jurkat cells, similar to earlier reports (Figure 5f and g). We then hypothesized that CCL3L1 may play different roles intracellularly and extracellularly. To determine the influence on viral entry, we utilized the CD4⁺, CCR5⁺, CXCR4⁺ HeLa-derived indicator cell line TZM-bl containing a luciferase reporter under the control of the HIV-1 long terminal repeat promoter for single-cycle virus infectivity assay (Sarzotti-Kelsoe et al., 2014; Wei et al., 2002). Both *C. trachomatis*-stimulated and -unstimulated TZM-b1 cells were infected with high titers of viral stocks pseudotyped with VSV-G. No significant difference in HIV-1 infectivity was observed between *C. trachomatis*-stimulated and -unstimulated cells (Figure 6a). To investigate the intracellular role of CCL3L1 upregulation in response to *C. trachomatis* infection, we infected PBMCs from healthy donors and *C. trachomatis*-infected patients. ELISA analysis of the cell
supernatants showed a significant increase in HIV-1 p24 levels in C. trachomatis–infected patients after 3 days of culture compared with those in healthy persons (Figure 6b and c).

Next, we infected Jurkat and MT4 T-cell lines as well as three CCL3L1 KO cell lines (KO-1, KO-2, and KO-3) as described earlier. Interestingly, p24 levels in the cell lines treated with C. trachomatis significantly increased day 3 after infection and peaked by day 6 after infection compared with those in the untreated cells. However, all the three CCL3L1 KO cell lines showed no significant change in the levels of p24 with or without C. trachomatis stimulation (Figure 6d). Western blot examination of the p24 levels in the cell lysates and supernatants of both Jurkat and MT4 T cells showed increased p24 levels in the supernatants but not in the cell lysates of C. trachomatis–stimulated cell lines (Figure 6e). No significant difference was observed between C. trachomatis–treated and −untreated cells when comparing the p24 levels in the cell lysates (Figure 6f). Analysis of the viral release efficiency showed increased efficiency in C. trachomatis–treated Jurkat T cells but not in MT4 T cells. Overall, these results suggest a model by which C. trachomatis infection increases HIV-1 replication and viral release through the upregulation of CCL3L1 levels in the cells.

**DISCUSSION**

With the increasing rate of C. trachomatis incidence and morbidity, especially among people living with HIV-1 (Johnson et al., 2012; Silva et al., 2018, 2012), it has become extremely important to address the underlying role of C. trachomatis on HIV-1 infectivity and susceptibility. Several STIs such as C. trachomatis stimulate the immune system and cause changes in CD4+ T cells in the absence of HIV-1 infection (Buckner et al., 2016; Schust et al., 2012). Hence, exposure to C. trachomatis before HIV-1 infection increases HIV-1 infectivity and susceptibility of the host cell, which may in turn increase the transmission of HIV-1 among C. trachomatis–infected people (Schust et al., 2012). Given the fact that most C. trachomatis infections in humans are asymptomatic, it would therefore imply that C. trachomatis infection may be one of the underlying reasons for the increased rate of HIV-1 infection in Africa and other less developed countries with a high incidence of STIs (Dzakah et al., 2021; Grosskurth et al., 1995; Guthrie et al., 2009; Masha et al., 2019). However, the mechanism involved in the susceptibility of antigen-specific CD4+ T cells and HIV-1 transmission remains elusive. In this study, we seek to systematically elucidate the role of C. trachomatis in the enhancement of HIV-1 infectivity, susceptibility, and transmission during C. trachomatis–HIV-1 coinfected. Although dendritic cells or Langerhans cells play an important role as initial target cells for HIV-1 infection, our study focused on the gene regulation in C. trachomatis–stimulated CD4+ T cells and its role in modulating HIV-1 infection. This can serve as a model for the understanding of the increased HIV-1 susceptibility among patients with STI.

Interestingly, C. trachomatis–infected patients tend to be more susceptible to HIV-1 infection than healthy uninfected donors (Figure 1a and b). Similarly, comparative analysis of HIV-1 DNA copies in PBMCs obtained from healthy donors with or without C. trachomatis stimulation revealed that CD4+ T cells from C. trachomatis–stimulated PBMCs harbor more HIV-1 copies than unstimulated CD4+ T cells from the same donors (Figure 1c–e). However, CD4 T cells from C. trachomatis–infected patients and healthy donors showed no significant difference in the expression levels of CD4 activation markers CD69 and HLA-DR as well as CCR5 coceptor. These results suggested that the enhanced susceptibility or infectivity was probably due to factors other than increased expression levels of CD4 activation markers (Heigele et al., 2015; Rubbo et al., 2011).

The CCL3L1 or CCL3L3 protein is a member of the chemokine receptor ligands that have been designated as macrophage inflammatory proteins (MIP1α and MIP1β) and are well-known to influence HIV-1 replication (Menten et al., 2002). CCL3L1 or CCL3L3 has previously been shown to be the most crucial ligand of the CCR5, which is a major co-receptor of HIV-1 (Gonzalez et al., 2005). Hence, the blocking of CCR5 by CCL3L1/CCL3L3 may result in reduced HIV-1 susceptibility (Dolan et al., 2007). However, there are conflicting reports on the association between CCL3L1/CCL3L3 and HIV-1 susceptibility in humans. Previous reports have implicated the copy number of this gene in HIV-1 susceptibility (Gonzalez et al., 2005; Menten et al., 2002), whereas others found no association between HIV-1 and CCL3L1/CCL3L3 (Urban et al., 2009). In addition, a nonrandom distribution of CCL3L1 gene copy numbers among individuals of different geographical ancestry and the possession of a CCL3L1 gene copy number above or below the population-specific median did not influence the risk of HIV infection, viral load, or disease progression (Bhattacharya et al., 2009).

Our data showed that CCL3L1 mRNA levels increased significantly in C. trachomatis–stimulated cells, and this increase corresponds to HIV-1 replication (Figure 5a and b). These results indicated a role for CCL3L1 in HIV-1 replication during C. trachomatis infection. Analysis of mRNA levels in different cells after C. trachomatis treatment and comparison between serum levels in healthy donors and C. trachomatis–infected patients showed a significant increase in CCL3L1 levels as previously reported (Masson et al., 2014). Interestingly, the increased serum CCL3L1 levels in C. trachomatis–stimulated cells did not result in decreased HIV-1 infectivity after 3 days of incubation (Figures 1a and b and 3f and g). However, similar to previous reports, our data also showed that the addition of exogenous recombinant CCL3L1 reduced HIV-1 infectivity through interaction with CCR5 receptor on CD4 T cells (Figure 5f and g). These contradictory observations suggest that CCR5 inhibition is not exclusively protective from HIV-1 infection without complete CCR5 blockade (Bhattacharya et al., 2009; Menten et al., 2002; Urban et al., 2009). It is well to reason that the expression threshold of CCL3L1 needed to cause significant inhibition of the CCR5 co-receptor on the cell surface cannot be achieved by C. trachomatis stimulation of the cells (Figure 5f and g). Thus, CCL3L1 may play contrasting roles within the cells where it promotes HIV-1 replication and viral release while causing inhibition of HIV-1 through interaction with the CCR5 co-receptor on the cell membrane (Figure 6).

In a recent report by Zahoor et al. (2015), they found that CCL3L1, CCL3L3, and CCL3 were upregulated by HIV-1 vpr,
indicating a role in HIV-1 infectivity. In addition, the epithelial cell line (A2EN) with Chlamydia trachomatis infection significantly increased the cell surface expression of galactosylceramide, an HIV-1 alternative primary receptor, and the most commonly studied HIV-1 coreceptors, CXCR4 and CCR5 (Buckner et al., 2016; Schust et al., 2012). Future investigations may identify what genes interact with CCL3L1 to enhance HIV-1 susceptibility in the presence of Chlamydia trachomatis. An interplay between CCL3L1 and other genetic factors may be responsible for the increased HIV-1 susceptibility during STI coinfections.

In summary, we found that Chlamydia trachomatis infection substantially enhanced HIV-1 susceptibility and identified the chemokine CCL3L1 as a major regulator of infection during Chlamydia trachomatis–HIV-1 coinfection. This study attempted to decipher the molecular role of Chlamydia trachomatis on the susceptibility of CD4 T cells and the infectivity of HIV-1. This study would serve as the basis for understanding the immunological changes and the regulation of pathways responsible for resistance or susceptibility to HIV-1 among Chlamydia trachomatis–infected patients. It would also contribute to the development and implementation of efficient HIV-1 preventive strategies and specific guidelines for the treatment of STIs.

**MATERIALS AND METHODS**

In this study, PBMCs were extracted from blood samples of healthy and Chlamydia trachomatis–infected human donors from the Guangzhou Blood Bank (Guangzhou, China) and the Dermatology Hospital of Southern Medical University (Guangzhou, China), respectively. The study was approved as nonhuman subject research by the institutional review board protocols of the Dermatology Hospital of Southern Medical University (2018015). Written informed consent was obtained from all subjects or, if subjects are aged <18 years, from a parent and/or legal guardian.

Methods for PBMC purification, CD4+ T-cell isolation, cell culture, HIV-1 infection, RNA extraction, and normalization and analysis of differentially expressed genes are provided in Supplementary Materials and Methods. A full description of each experiment is provided in Supplementary Materials and Methods.

**C. trachomatis stimulation of PBMC**

PBMCs from healthy subjects were infected with Chlamydia trachomatis at a multiplicity of infection of one at a high cell concentration of $1 \times 10^7$ cells per ml for 12–24 hours and then diluted to a final cell concentration of $2 \times 10^6$ cells per ml for normal cell culture for 3 days to stimulate antigen-specific T-cell activation. Cells were then infected with HIV-1, followed by staining with aqua blue and antibody cocktails to surface antigens.

**In vitro HIV-1 infection**

Three days after initial Chlamydia trachomatis stimulation, PBMCs were infected with pretitrated HIV-1 R5 and X4 strains (50 ng/ml of HIV-1 p24) obtained through the NIH AIDS reagent program. For replication of HIV-1 in PBMCs of healthy and Chlamydia trachomatis–infected patients, $5 \times 10^5$ cells per well were inoculated with pretitrated HIV-1 (50 ng/ml p24) for 2 hours and replaced with 3 ml fresh culture medium containing 50 U of human IL-2 (Beijing, China). For infection of wild-type and CCL3L1/CCL3L3 sequence variation Jurkat cell lines, $5 \times 10^5$ cells per well were infected with pretitrated HIV-1 (50 ng/ml p24). Cells or supernatants were harvested on designated days to measure the titers of HIV-1 with p24 ELISA or RNA extraction.

**Data availability statement**

RNA sequencing data generated in this study have been deposited in the Gene Expression Omnibus under Gene Expression Omnibus accession number GSE164264.

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**CONFLICT OF INTEREST**

The authors state no conflict of interest.

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**AUTHOR CONTRIBUTIONS**

Conceptualization: EED, ST; Formal Analysis: EED, JZ, LH, HW; Investigation: EED, ZW, LW, WK, RX, FR, SC; Project Administration: HW, LY, ST; Writing - Original Draft Preparation: EED, FK, KD, ST; Writing - Review and Editing: EED, FK, ST

**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2021.09.020.

**REFERENCES**


Hegele A, Joas S, Engels C, Kirchhoff F. Increased susceptibility of CD4+ T cells from elderly individuals to HIV-1 infection and apoptosis is associated with reduced CD4 and enhanced CXCR4 and FAS surface expression levels. Retrovirology 2015;12:86.


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SUPPLEMENTARY MATERIALS AND METHODS

Cells, bacteria, and viruses

293T, HeLa, and TZM-bi cells were cultured in DMEM (HyClone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum (Gibco, Waltham, MA), 1% penicillin-streptomycin (Gibco), and 1% L-Glutamine.Proviral pNL4-3 Bal (R5) and pNL4-3 (X4) plasmids were obtained through the National Institutes of Health AIDS reagent program and used for in vitro HIV infection.

Production of HIV-1 virus

293T cells were plated in six-well plates at 5 × 10^5 cells per well. Live HIV-1 virus was produced by transfecting 293 T cells with 2.5 µg of proviral pNL4-3 Bal (R5) and pNL4-3 (X4) plasmids using Lipofectamine 3000 (Invitrogen, Waltham, MA), according to the manufacturer's instructions. The medium was changed after 6 hours, and the cells were cultured for additional 48 hours. The cell supernatant containing the live virus was collected and stored at −80 °C until infection. For titration of HIV-1, the cell supernatant was treated with Triton X-100 (0.5% v/v) and analyzed for viral content using an HIV-1 p24 ELISA, according to the manufacturer's instruction.

Establishment of Chlamydia trachomatis cultures

Chlamydia trachomatis infection of HeLa cells was carried out as previously described (Dzakah et al., 2021). HeLa cells were plated at 1 × 10^5 cells per well using six-well plates for 12 hours. Cells were washed with PBS, and then 500 µl of DMEM containing 30 µg/l of diethylaminoethyl-dextran were added, and plates were incubated at 37 °C for 10 minutes. Diethylaminoethyl-dextran-containing medium was removed, and the C. trachomatis samples were diluted in 1 ml of serum-free DMEM before adding into each well. The plates were kept at 37 °C for 30 minutes, followed by centrifugation at 1,000 r.p.m. for 1 hour at room temperature. The culture medium was then replaced by a complete medium containing 10% fetal bovine serum, and the cells were incubated at 37 °C for 40 hours. The cells were harvested by scraping the plate in sucrose-phosphate-glutamate buffer and then sonicated on ice for 10 minutes with 5 seconds on and 5 seconds off. The tubes were then centrifuged at 1,000 r.p.m. for 10 minutes. Aliquots of the C. trachomatis elementary bodies were stored at −80 °C.

PBMC isolation

Buffy coat fractions of 25 ml blood were layered over Ficoll-Paque Plus (GE Healthcare, Chicago, IL) and centrifuged at 400g for 30 minutes. The PBMC portion was gently transferred to a clean tube containing 25 ml PBS and then centrifuged at 350g for 10 minutes at room temperature. PBMCs were either suspended in RPMI 1640 complete medium or processed for CD4 isolation.

Antigen stimulation of PBMC

PBMCs from healthy subjects were infected with C. trachomatis at a multiplicity of infection of one at a high cell concentration of 1 × 10^7 cells per ml for 12–24 hours and then diluted to a final cell concentration of 2 × 10^6 cells per ml for normal cell culture in culture plate for 3 days to stimulate antigen-specific T-cell activation. At the same time, unstimulated PBMCs were used as a control. After 3 days of culture, cells were then infected with HIV-1 followed by staining with aqua blue and antibody cocktails to follow antigen.

In vitro HIV-1 infection

Three days after initial C. trachomatis stimulation, PBMCs were infected with pretitrated HIV-1 R5 and X4 strains (50 ng/ml of HIV-1 p24). For determination of infectivity, cells were maintained for additional 3 days, followed by fixation and antibody staining. Flow cytometry based on intracellular HIV-1 p24 expression was performed to determine the percentage of infected cells. To determine the copy number of HIV-1, PBMCs from healthy subjects were first stimulated with C. trachomatis for 3 days, followed by infection with HIV-1 for 4 hours. Cells were washed, and efavirenz was added to block the spread of HIV-1 to new cells. Cells were harvested after 3 days, and HIV-1 DNA copy number was determined by RT-qPCR using HIV-1 Gag and GAPDH plasmids to generate the standard curve. For replication of HIV-1 in PBMCs of healthy and C. trachomatis–infected patients, 5 × 10^6 cells per well were inoculated with pretitrated HIV-1 (50 ng/ml of HIV-1 p24) for 2 hours. Cells were washed thrice with PBS and then completely replaced with a 3 ml fresh culture medium containing 50 U of human IL-2. For infection of wild-type and CCL3L1/CCL3L3 mutant Jurkat cell lines, 5 × 10^5 cells per well were infected with pretitrated HIV-1 (50 ng/ml p24). Cells or supernatants were harvested on designated days to measure the titers of HIV-1 with p24 ELISA or RNA extraction. Virus entry was monitored by the measurement of luciferase activity in TZM-bi cells. After C. trachomatis stimulation for 3 days, both stimulated and unstimulated cells were infected with equal amounts of HIV-1 pseudotyped with VSV-G for 4 hours. Cells were washed thrice, and a fresh medium was added for 48 hours before measuring the luciferase activity described previously (Martins et al., 2015).

HIV-1 p24 ELISA

Cell supernatants were collected and treated with Triton X-100 (0.5% v/v), and the concentration of p24 in cell supernatant was analyzed using a commercially available HIV-1 p24 ELISA test kit (Beijing Key-Bio Biotech, Beijing, China) following the manufacturer’s instructions. Prettitrated p24 antigens provided by the manufacturer were used for the generation of a standard concentration curve.

Flow cytometry

Cells were first stained for viability with LIVE/DEAD Fixable Aqua Blue (Life Technologies, Carlsbad, CA), followed by intracellular and surface marker staining, including p24-phycoerythrin, CD4-phycoerythrin-Cy5, CD8-BV785, CD3-BV421, CD69-APC, CCR5-PerCP-Cy5.5, and HLA-DR-FITC (BD Biosciences, Franklin Lakes, NJ). Flow cytometry was performed, and the data were analyzed using FlowJo software, version 9.2 (FlowJo, Ashland, OR).

CD4 T-cell isolation

Antigen-specific sorting of CD4 T cells was carried out using the EasySep Human CD4+ T-cells isolation kit (Stemcell Technologies, Vancouver, Canada) according to the manufacturer’s instructions. Isolated cells were then used for the quantification of cell-associated HIV-1 DNA and gene
Expression analysis in both HIV-infected and healthy subjects.

HIV-1 DNA copy number quantification

HIV-1–infected C. trachomatis–stimulated and normal PBMCs were lysed in lysis buffer (10 mM Tris, 5 mM EDTA, 1% SDS, pH 8.0) for 1 hour. DNA extraction was performed using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). Plasmids for HIV-1 Gag (200 base pair, GGGACAGCTACCAACCATCCC for forward primer and TTGGCTGACCTGATTGCTGT for reverse primer) and control GAPDH (300 base pair, GAGTCAACGGGTGAGTTCG for forward primer and CGCGAAAGGAAAGAAAGCGT for reverse primer) were constructed using the pNLF1-N vector and used to generate standard curves. Genomic DNA from each sample was used for HIV-1 DNA quantification and extrapolation from the standard curves.

RNA extraction, sequencing, and RT-qPCR

Total RNAs from antigen-specific CD4 T cells and unstimulated CD4 T cells were extracted using a Qiagen RNA extraction kit following the manufacturer’s instructions. RNAs were precipitated and resuspended in water and then treated with DNaseI for 30 minutes (Promega, Madison, WI). mRNA sequencing was carried out at the Beijing Genome Institute (Shenzhen, China). The first-strand cDNA was then synthesized with 500 ng total RNA using TransScript All-in-One First-Strand cDNA Synthesis SuperMix (Transgen Biotech, Beijing, China).

Small interfering RNA design and knockdown of target genes

Small interfering RNAs (siRNAs) were designed to target either the 5’ untranslated region, 3’ untranslated region, or open reading frame of the gene of interest. siRNA transfection was done using Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer’s instruction. Briefly, Jurkat cells were plated at 2.5 x 10^5 cells per well in six-well plates. About 125 µl of Opti-MEM were used to dilute 5 µl of Lipofectamine 3000 followed by the dilution of 75 pmol of siRNA. The siRNA mixture was then added to the cells dropwise. The culture medium was changed after 6–8 hours, and the cells were harvested after 48 hours for RNA extraction and qPCR to check for knockdown efficiency of the siRNA.

CRISPR/Cas9 editing of CCL3L1/CCL3L3

Single guide RNAs were designed against various target genes that have been selected after siRNA screening. Three single guide RNAs were cloned into the pCRISPR-LvSG03 vector (GeneCopoeia, Rockville, MD) and were used for transfection into Jurkat/Cas9/Hyg cells followed by the addition of 1.0 µg/ml puromycin after 24 hours for the selection of positive clones. The mutant cell lines were examined by PCR and western blot analyses.

Western blotting

Cell lysates or immunoprecipitated products were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with mouse anti-CCL3L1 primary antibodies (GeneTex, Irvine, CA) overnight at 4 °C. Blots were washed and incubated with horseradish peroxidase–conjugated goat anti-mouse secondary antibodies for 1 hour at room temperature. Finally, blots were washed and visualized with ECL substrate (Pierce, Appleton, WI).

Statistical analysis

All RNA sequencing data are from two biological replicates. Quantitative data were analyzed by Students’ t-test in GraphPad Prism 8.0 (GraphPad Software, San Diego, CA). Western blot images were analyzed using ImageJ software, version 1.41o (National Institutes of Health, Bethesda, MD).

Supplementary references


**Supplementary Figure S1. HIV-1 infection and determination of HIV-1 copy number.** (a) Infection of healthy CT-stimulated PBMCs (n = 5) with the X4 strain of HIV-1. Data are expressed as the mean ± SEM. Student’s t-test, *** P < 0.001. (b) Standard curve for GAPDH. (c) Standard curve for HIV-1 Gag. CT, *Chlamydia trachomatis*. 

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Supplementary Figure S2. Expression levels of activation markers among CT-infected patients. (a) Expression level of CCR5 among CT-infected patients and healthy donors (n = 7). (b) Expression of CD69 among CT-infected patients and healthy donors (n = 7). (c) Analysis of HLA-DR expression. Healthy PBMCs were stimulated for 3 days, followed by HLA-DR staining and flow cytometry. Student’s *t*-test. CT, *Chlamydia trachomatis*; n.s., not significant; SSC-A, side scatter area.
Supplementary Figure S3. siRNA knockdown of IL6 and SOX5. (a) Representative flow cytometry analysis of HIV-1 infectivity after siRNA knockdown of IL6 and SOX5. (b) Analysis of the siRNA knockdown of IL6 in Jurkat cell line. (c) Analysis of the siRNA knockdown of SOX5 in Jurkat cell line. Student’s t-test, * P < 0.05, ** P < 0.01, and *** P < 0.001. Data are presented as the mean ± SEM from three independent experiments. CT, Chlamydia trachomatis; ctrl, control; FSC-A, forward scatter area; n.s., not significant; siRNA, small interfering RNA.
Supplementary Table S1. Characteristics of *Chlamydia trachomatis*–Infected Subjects

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Abbreviations: F, female; M, male; NAAT, nucleic acid amplification test.

1Antibody against PGP3 antigen of *C. trachomatis* was detected by our in-house luciferase immunosorbent assay (Shui et al., 2021).