Increase of CXCR3^{+} T cells impairs Th17 cells recruitment in the small intestine mucosa through IFN-γ and IL-18 during treated HIV-1 infection

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Summary:

Increase in Th1-type cells in the duodenal mucosa of HIV-1-infected individuals under antiretroviral therapy could contribute to a reduction of CCL20 and CCL25 expression by enterocytes through IL-18 and IFN-γ, while inducing CXCR3-ligands, thus perpetuating Th17/Th1 imbalance.

Abstract

The restoration of $CD4^+$ T cells, especially of Th17 cells, remains incomplete in the gut mucosa of most HIV-1-infected individuals despite sustained antiretroviral therapy (ART). Herein, we report an increase in absolute number of $CXCR3^+$ T cells in the duodenal mucosa on ART. The frequencies of Th1 and $CXCR3^+$ $CD8^+$ T cells were increased and negatively correlated with CCL20 and CCL25 expression in the mucosa. *Ex-vivo*, we showed that IFN-γ, the main cytokine produced by Th1 and effector $CD8^+$ T cells, down-regulates the expression of CCL20 and CCL25 by small intestine enterocytes, while it increases the expression of CXCL9/10/11, the ligands of CXCR3. IL-18, a pro-Th1 cytokine produced by enterocytes also contributes to down-regulate CCL20 expression, and increases IFN-γ production by Th1 cells. This could perpetuate an amplification loop for CXCR3-driven Th1-type cells recruitment to the gut, while impairing Th17 cells homing through the CCR6-CCL20 axis in treated HIV-1-infected individuals.

Key words: HIV-1; gut; CXCR3; Th1; CD8; IFN-γ; IL-18; CCL20; CCL25

INTRODUCTION

During HIV-1 infection, the integrity of the intestinal immune barrier is disrupted due to a deep depletion of gut $CD4^+$ T cells [1, 2]. Among them, Th17 cells, which have a major role in antimicrobial mucosal immunity, are particularly targeted [3]. Th17 cell loss has been linked to microbial translocation from the gut lumen into the bloodstream, resulting in deleterious systemic inflammation [4, 5].

Despite a prolonged and effective antiretroviral therapy (ART), the restoration of $CD4^+$ T cells remains incomplete in the gut mucosa (particularly in effector sites, *i.e. lamina propria*) of most HIV-1-infected individuals. This contrasts with the significant restoration of $CD4^+$ T cells in their peripheral blood and organized lymphoid tissues [1, 6-8]. We previously reported that a lack of recruitment of $CD4^+$ T cells to the gut could contribute to this impaired immune reconstitution [9].

The $\alpha4\beta7$-MAdCAM-1, CCR9-CCL25, and CCR6-CCL20 chemotactic axes play critical roles in the homing of immune cells to the small intestine. The $\alpha4\beta7$ integrin binds MAdCAM-1, which is expressed on gut endothelial cells [10]. CCL25 and CCL20 chemokines, which are produced by enterocytes, drive the homing of CCR9- and CCR6-expressing immune cells, respectively [11-13].

We previously described alterations in the CCR9-CCL25 and CCR6-CCL20 chemotactic axes that could contribute to the poor restoration of $CD4^+$ T cell in the small intestine mucosa of treated HIV-1-infected individuals who initiated ART during the chronic phase of infection [9, 14]. This appears to be linked to a reduced production of both CCL20 and CCL25 by small intestine enterocytes. CCL20 down-regulation specifically impacts the mucosal recruitment of $CCR6^+$ $CD4^+$ T cells, notably Th17 cells [14].

We found that the frequency of Th1 and $CD8^+$ T cells is increased in the duodenal mucosa of HIV-1-infected individuals on ART, contrasting with the global depletion of gut $CD4^+$ T cells. Th1 and effector $CD8^+$ T cells share the expression of CXCR3, suggesting that the CXCR3 chemotactic axis

could be functional in this setting. We thus assessed whether $CXCR3^+$ T cells could play a role in the disequilibrium of the gut mucosa homeostasis observed in treated HIV-1-infected individuals. We examined the impact of Th1-type inflammation on the production of chemokines by the enterocytes and how it drives Th1 and Th17 cells recruitment to the gut mucosa.

METHODS

Individuals and samples. The ANRS EP44 study group was previously described [9]. Briefly, 20 HIV-1-infected individuals were studied. They received effective ART for a median of 5 years (IQR, [4–5.5]), initiated during the chronic stage of infection. All had sustained plasma HIV-1 RNA <20 copies/mL. Their median blood $CD4^+$ T cell count was 668 cells/µL (IQR [451–849]) on ART at the time of sampling. Patient demographic information is available in supplemental Table S1. Twenty uninfected individuals were used as controls. Biopsies of duodeno-jejunal mucosa and blood samples were collected from each patient. Additional information is available in supplemental methods.

Ethics statement. The study was approved by the Institutional Review Board CPP Sud-Ouest et Outre-Mer II. All participants provided informed consent (trial registration number NCT01038401).

Isolation and phenotyping of small intestine mucosal lymphocytes. Duodeno-jejunal biopsies (n=5) were digested with 0.5 mg/mL collagenase type II-S (Sigma-Aldrich). T cells were isolated by positive selection (EasySep Human CD3-Positive Selection Kit, Stemcell). Flow cytometry analyses were performed on a BD LSRFortessa (BD Biosciences). Th1 and Th17 cells were defined as $CD3^+CD4^+CXCR3^+CCR4^-CCR6^-$, and $CD3^+CD4^+CXCR3^-CCR4^+CCR6^+CD161^+$ cells, respectively. $CD8^+$ T cells were defined as $CD3^+CD4^-CD8^+$ cells. Additional information is available in supplemental methods.

Immunohistochemistry. Fresh tissues were fixed in 4% neutral buffered formalin and embedded in paraffin. Immunohistochemistry was performed on 3 µm sections using anti-CXCR3 (R&D Systems) mAb. Quantification was performed using LAS v3.7 (Leica microsystems) and NIS-element (Nikon), in a blinded fashion regarding HIV-1 infection status.

Isolation, culture, and differentiation of primary small intestine epithelial cells (IEC). IEC were isolated from upper small bowel surgical resections obtained from individuals free of HIV and inflammatory bowel diseases. Subsequent culture and enterocytes differentiation were performed as described in supplemental methods.

***Ex-vivo* stimulation of primary IEC by cytokines.** Primary IEC cultured on transwell inserts were stimulated for 15 hours with 0.5, 5, or 50 ng/mL of human IFN-γ, IL-2, TNF-α, IL-18 (ThermoFisher), IL-17A, IL-1ß (eBiosciences) added into the bottom chamber.

Th1 and Th17 cell sorting and coculture with primary IEC. Th1 and Th17 cells were sorted by flow cytometry on a FACSARIA II (BD Biosciences) from PBMCs of healthy donors. Gating strategy is shown in supplemental Figure S1. Sorted Th1 and Th17 cells were cultured in CTS OpTmizer medium (Life Technologies) and activated with PMA/ionomycin before being added into the bottom chamber for coculture with IEC on transwell inserts during 15 hours. Anti-IFN-γR1 mAb (10 µg/mL, Universal Biologicals) and IL-18BP (1 µg/mL, R&D systems) were used to antagonize the biological effect of IFN-γ or IL-18, respectively. Additional information is available in supplemental methods.

RT-PCR quantification of chemokine mRNAs. RNA was extracted from primary IEC using the RNeasy minikit (Qiagen). CCL20 and CCL25 mRNA quantification was performed on a LightCycler 480 (Roche). CXCL9, CXCL10, CXCL11, pro-IL-18, IL-18RAP, IL-18BP, and Caspase-1 mRNAs were quantified with the QuantiGene-Plex 2.0 Assay (Affymetrix). Additional information is available in supplemental methods.

Quantification of chemokines. CCL20, CXCL9, CXCL10, and CXCL11 were quantified by ELISA (R&D Systems) in the bottom chamber of transwells.

Cell migration assay. Sorted Th1 or Th17 cells were loaded in the upper chamber and control medium or conditioned medium from Th1:IEC or Th17:IEC cocultures was loaded in the bottom chamber of transwells (Corning). The number of cells trafficking from the upper to the bottom chamber through the 5 µm-pore membrane in 15 hours was counted. Anti-CCL20 mAb, and a mix of anti-CXCL9/10/11 Abs was used to antagonize chemotaxis. Additional information is available in supplemental methods.

Statistical analyses. Quantitative variables were compared using the Wilcoxon's rank-sum test. Paired Wilcoxon's test was used to compare chemokine expression between the different conditions in IEC cultures from each donor. Correlations were estimated by calculating Spearman's rank correlation coefficients. All tests were 2-sided, and *P* values <0.05 were considered statistically significant. Statistical analyses were performed with Stata 10.0.

RESULTS

Increase of CXCR3$^+$ T cells in the duodenal mucosa of treated HIV-1-infected individuals suggests effective cell recruitment through this axis.

We and others previously showed that the frequency and absolute number of CD4$^+$ T cells in the *lamina propria* of the intestine mucosa of HIV-1-infected individuals do not normalize, despite sustained effective ART [1, 6-9]. However, the restoration is not uniform among different CD4$^+$ T cell subsets. In contrast to gut Th17 cells, whose frequency remained lower in the duodenal mucosa of treated HIV-1-infected individuals than in uninfected controls (median frequency of 4.3% vs. 7.6%, respectively, *P*=0.031), we found that the frequency of gut Th1 in CD4$^+$ T cells was increased in treated HIV-1-infected individuals compared to uninfected controls (median frequency of 9.0% vs. 4.7%, respectively, *P*=0.001; Figure 1A).

The increased frequency of Th1 cells suggests effective recruitment of this cell subset to the gut mucosa. Th1 are characterized by the expression of CXCR3, a chemokine receptor involved in T cell trafficking to peripheral sites of Th1-type inflammation. Effector CD8$^+$ T cells share expression of CXCR3 with Th1 cells. Similarly, the frequency of CXCR3$^+$ in CD8$^+$ T cells was increased (median frequency of 27.4% vs. 18.1%, *P*=0.012, Figure 1B) in the duodenal mucosa of treated HIV-1-infected individuals compared to uninfected controls.

Flow cytometry data were confirmed by *in-situ* detection of CXCR3$^+$ cells, the absolute number of CXCR3$^+$ cells was increased in treated HIV-1-infected individuals compared to uninfected controls (quantification of CXCR3$^+$ cells per unit surface area, *P*=0.020; Figure 1C). Immunochemistry analysis showed that CXCR3$^+$ cells were mainly localized in the *lamina propria* of the duodenal mucosa (Figure 1D).

This suggests that CXCR3-mediated cell recruitment, involved in the homing of Th1 and effector CD8$^+$

T cells to the gut, is functional in this setting.

***In-vivo*, the frequencies of Th1 and CXCR3$^+$ in CD8$^+$ T cells negatively correlate with CCL20 and CCL25 expression in the duodenal mucosa.**

We previously linked the lack of immune reconstitution of gut CD4$^+$ T cells, notably of CCR6$^+$ cells such as Th17 cells, to a decreased expression of CCL20 and CCL25 in the duodenal enterocytes of treated HIV-1-infected individuals [9, 14]. However, the factors influencing CCL20 and CCL25 expression in HIV-1-infected individuals remain unclear. Herein, we found that the frequency of gut Th1 cells was negatively correlated with the expression of CCL20 (ρ=-0.51, P=0.007, Figure 2A) and CCL25 (ρ=-0.81, P<0.0001, Figure 2B) in the duodenal mucosa. Similarly, the frequency of gut CXCR3$^+$ in CD8$^+$ T cells was negatively correlated with the expression of CCL20 (ρ=-0.47, P=0.013, Figure 2C) and CCL25 (ρ=-0.48, P=0.043, Figure 2D). It should be noted that these correlations were statistically non-significant when analyzed separately in HIV-1 infected individuals and uninfected controls. However, the frequency of total duodenal CD8$^+$ T cells (most of which were effector memory cells, data not shown) of HIV-1-infected individuals, with an increased subject number, was also negatively correlated with the expression of CCL20 (ρ=-0.55, P=0.022) and CCL25 (ρ=-0.53, P=0.024, supplemental Figure S2).

These findings prompted us to investigate whether Th1 and effector CD8$^+$ T cells could play a functional role in reducing the expression of CCL20 and CCL25 chemokines by enterocytes.

***Ex-vivo*, IFN-γ blunts CCL20 and CCL25 expression by enterocytes.**

The functional impact of IFN-γ, the main cytokine produced by Th1 and effector CD8$^+$ T cells, on enterocyte chemokine expression was investigated using an *ex-vivo* model of human primary IEC

isolated from the upper small intestine and cultured as monolayers of differentiated enterocytes on transwell inserts. To mimic the epithelium-*lamina propria* interaction, the enterocytes were stimulated at their basal side by cytokines. mRNA expression was quantified by qRT-PCR in the enterocytes.

The quantification of CCL20 and CCL25 mRNA revealed that IFN-γ induced a decreased expression of both chemokines by enterocytes, with a stronger effect on CCL20 expression (up to 6-fold decrease, *P*=0.005) than on CCL25 (up to 3-fold decrease, *P*=0.002) (Figure 3A, left panel).

In addition to IFN-γ, poly-functional Th1 and effector $CD8^+$ T cells can also produce IL-2 and TNF-α. We thus assessed the impact of these cytokines on enterocyte expression of CCL20 and CCL25. IL-2 slightly decreased CCL20 expression (up to 2-fold decrease, *P*=0.027) while it had no consistent effect on CCL25 expression (Figure 3A, center panel). By contrast, TNF-α importantly increased CCL20 expression (up to 3-log increase, *P*=0.001) while it reduced CCL25 expression (up to 3-fold decrease, *P*=0.001) (Figure 3A, right panel).

IL-17A, the main Th17 cytokine, moderately increased CCL20 expression (up to 3-fold, *P*=0.002), while it had no consistent effect on CCL25 expression (Figure 3B).

***Ex-vivo*, IL-18 and IL-1ß had opposite effects on CCL20 and CCL25 expression by enterocytes.**

IL-18 is a pro-Th1 cytokine which is released by the enterocytes through activation of the epithelial inflammasome [15]. As IFN-γ, IL-18 induced a marked decrease of CCL20 expression (up to 6-fold decrease, *P*=0.008), and a non-significant increase of CCL25 expression (less than 2-fold increase, *P*=0.26) (Figure 3C, left panel).

IL-1ß, a pro-Th17 cytokine, is produced, as IL-18, through activation of the inflammasome. However, contrary to IL-18, the enterocytes are not a significant source of IL-1ß which is mainly produced by

gut macrophages in inflammatory conditions [15]. IL-1ß strongly induced CCL20 expression (more than 3-log increase, *P*=0.008) by enterocytes while it reduced CCL25 expression (up to 4-fold, *P*=0.008) (Figure 3C, right panel).

IL-18 and IL-1ß thus have strong opposite effects on CCL20 expression. Both are released upon activation of the inflammasome but producing cells and triggers are not the same in the intestine mucosa [15].

Herein, we found that IL-18 level was increased in the plasma of treated HIV-1-infected individuals compared to uninfected controls (*P*=0.033, supplemental Figure S3). By contrast, IL-1ß level was not different (*P*=0.67, supplemental Figure S3). Increased IL-18 level may thus contribute to a pro-Th1 environment in treated HIV-1-infected individuals.

Gene expression analyses were confirmed by the quantification of CCL20 protein by ELISA (Figure 3D). CCL25 protein could not be quantified as the levels produced *ex-vivo* were below the detection limit.

Interactions between Th1 cells and enterocytes blunt CCL20 and CCL25 expression through IL-18 and IFN-γ.

To mimic the epithelium-*lamina propria* interactions in a Th1- or Th17-microenvironment and, to globally assess the combined effect of cytokines, the primary IEC were co-cultured with Th1 or Th17 cells added into the bottom chamber of transwells. mRNA expression was quantified by qRT-PCR in the enterocytes. We found that a Th1-microenvironment lead to a 5-fold decrease in CCL20 (*P*=0.017) and a 2-fold decrease in CCL25 expression (*P*=0.041, Figure 4A) by the enterocytes relatively to a Th17-microenvironment. These results were confirmed by ELISA with a 2-fold decrease of CCL20 protein in Th1:IEC compared to Th17:IEC cocultures (*P*=0.040, Figure 4B). CCL25 protein could not be quantified as the levels produced were below the detection limit.

The decrease of CCL25 expression in Th1:IEC coculture was mainly due to IFN-γ as blocking IFN-γ biological activity through IFN-γR1 significantly restored CCL25 expression (*P*=0.043, Figure 4C), while blocking IL-18 biological activity by IL-18BP did not have any significant impact. By contrast, blocking IFN-γR1 did not change CCL20 expression, while it was restored when blocking IL-18 (*P*=0.025, Figure 4D).

These results suggest that IFN-γ and IL-18 cooperate in reducing CCL20 and CCL25 expression by enterocytes in a Th1-microenvironment compared to a Th17-microenvironment.

IFN-γ and IL-18 synergize to amplify the Th1 response in the gut mucosa.

Stimulation of the enterocytes by IFN-γ increased their expression of pro-IL-18 mRNA (up to 3-log increase, *P*=0.011, Figure 5A). It also increased the expression of the IL-18 receptor accessory protein (IL-18RAP) (more than 2-log increase, *P*=0.021, Figure 5B). IL-18RAP has been shown to increase IL-18 binding and signaling [18]. But IFN-γ also induced the expression of the IL-18 antagonist IL-18BP by enterocytes (data not shown), allowing for tight regulation of the IL-18 biological effect in the gut mucosa.

Production of mature IL-18 by the enterocytes requests activation of the epithelial inflammasome, leading to the cleavage of pro-IL-18 into mature IL-18 by caspase-1 [15]. In coculture experiments, caspase-1 expression was increased by 2-fold in Th1:IEC but not in Th17:IEC cocultures (*P*=0.011, Figure 5C).

IL-18 production by the enterocytes amplified IFN-γ production by Th1 cells as IFN-γ production was 4-fold increased if Th1 cells were cultured in presence of IL-18 (*P*= 0.006, Figure 5D).

These results suggest that a pro-Th1 amplification loop could occur between IFN-γ, produced by Th1 cells, and IL-18, produced by the enterocytes, in the intestinal mucosa.

***Ex-vivo,* IFN-γ increases CXCR3 ligands expression by enterocytes.**

We found increased frequencies of $CXCR3^+$ T cells, notably Th1 and effector $CD8^+$ T cells, in the duodenal mucosa of treated HIV-1-infected individuals compared to uninfected controls. The recruitment of $CXCR3^+$ T cells to the gut mainly depends on the production of CXCR3-ligands CXCL9, CXCL10, and CXCL11. Quantification of CXCL9, CXCL10, and CXCL11 mRNA in primary IEC stimulated with IFN-γ demonstrated a highly-upregulated expression (up to 5-log increase) for the three chemokines, albeit less pronounced for CXCL11 (*P*=0.008, Figure 6A, left panel). It should be noted that basal CXCL11 expression by enterocytes in unstimulated conditions was much higher (16- to 64-fold) than CXCL9 and CXCL10 expression, both of which rather appeared to be highly inducible (data not shown). Gene expression analyses were confirmed by the quantification of CXCL9, CXCL10, and CXCL11 proteins by ELISA (*P*=0.028 for CXCL9 and CXCL10, Figure 6A, right panel).

IL-18 had no impact on CXCL9, CXCL10, and CXCL11 expression by enterocytes at mRNA and protein levels (data not shown), by opposition to its effect on CCL20 expression.

These results were confirmed by comparing Th1:IEC vs. Th17:IEC cocultures; a 10- to 40-fold increase in CXCL9, CXCL10, and CXCL11 expression was observed in a Th1-microenvironment relatively to a Th17-microenvironment (*P*=0.008, Figure 6C). These results were confirmed by ELISA with an increase production of CXCL9 (*P*=0.007) and CXCL10 (*P*=0.013) chemokines in Th1:IEC compared to Th17:IEC cocultures (Figure 6C). This effect is mainly driven by IFN-γ as blocking its biological activity through IFN-γR1 significantly reduced CXCL9, CXCL10, and CXCL11 expression (*P*=0.008, Figure 6D).

Interactions between Th1 cells and enterocytes amplify the recruitment of Th1 over Th17 cells.

Finally, we assessed the global effect of the interactions between Th1 cells and enterocytes on the recruitment of Th1 and Th17 cells. We performed cell migration assay using conditioned medium from Th1:IEC or Th17:IEC cocultures to assess chemotaxis of Th1 and Th17 cells. Conditioned medium from Th1:IEC coculture induced the recruitment of twice more Th1 than Th17 cells, and this effect was dependent on CXCR3 ligands as blocking CXCL9, CXCL10, and CXCL11 chemokines blunted Th1 cells recruitment (P=0.023, Figure 7A). Conversely, Th17 cells recruitment mainly depended on CCL20 and is favored over Th1 cells by a Th17 microenvironment (P=0.038, Figure 7B).

Taken together, these data suggest that a gut microenvironment enriched with IFN-γ-producing $CXCR3^+$ cells, such as Th1 and effector $CD8^+$ T cells, stimulates the expression of CXCR3-ligands CXCL9, CXCL10, and CXCL11 in response to IFN-γ, but decreases the expression of CCL20 and CCL25 through IFN-γ and IL-18. This perpetuates a pro-Th1 amplification loop for cell recruitment to the gut at the expense of other $CD4^+$ T cell subsets, notably Th17 cells that mainly depend on the CCR6-CCL20 axis.

DISCUSSION

Herein, we showed that the frequencies of Th1 and CXCR3$^+$ CD8$^+$ T cells are increased in the duodenal mucosa of HIV-1-infected individuals on ART, contrasting with the impaired homing of CCR6$^+$ CD4$^+$ T cells, including Th17 cells [14]. Th1 and effector CD8$^+$ T cells share the expression of CXCR3, suggesting efficient homing through this chemotactic axis in this setting, while the CCR6-CCL20 and CCR9-CCL25 axes appear to be impaired, as previously shown [9, 14].

Our results also suggest a functional role of Th1 and effector CD8$^+$ T cells, mediated by IFN-γ and IL-18, in the down-regulation of CCL20 and CCL25 expression by enterocytes. By contrast, IFN-γ strongly increases the expression of CXCR3 ligands, creating an amplification loop that promotes Th1-type cells recruitment to sites of inflammation, in agreement with previous reports [19, 20]. In addition to CD4$^+$ and CD8$^+$ T cells, the recruitment of CXCR3$^+$ cells in the gut mucosa could also concern ILC1, NK, and NKT cells, that share IFN-γ production [21].

CCL20 is usually considered as an inducible inflammatory chemokine, with increased expression reported in inflammatory bowel diseases and some enteric infections [22], contrasting with an increase of CXCR3 ligands in some other infections [23]. Thus, the pathways involved in mucosal immune responses differ between the various settings, favouring either Th17 or Th1 responses. *In-vivo*, we reported that treated HIV-1-infection was associated with a reduced expression of CCL20 by small intestine enterocytes [14]. *Ex-vivo*, the regulatory T cell (Treg) cytokines TGF-β and IL-10, and the Th1 cytokine IFN-γ down-regulate CCL20 expression, while the Th17 cytokine IL-17A increases it [14]. The down-regulated expression of CCL20 thus matches with the imbalance in the Th17/Treg and Th17/Th1 ratios that we observed in the duodenal mucosa of treated HIV-1-infected individuals. The inflammasome cytokines IL-18 and IL-1β have strong opposite effects on CCL20 expression by enterocytes. IL-18, which is a pro-Th1 cytokine produced by enterocytes, decreases CCL20

expression, while IL-1β, a pro-Th17 cytokine produced by inflammatory macrophages in the gut mucosa increases CCL20 expression. IFN-γ and IL-18 thus appear to cooperate in reducing CCL20 expression and creating an amplification loop for Th1-type cells recruitment.

The nature of the inflammatory stimuli that could drive Th1-type inflammation in the gut mucosa of treated HIV-1-infected individuals remains unclear. HIV-1 DNA persists at higher levels in gut than in blood $CD4^+$ T cells, and HIV-1 RNA can be detected in the gut mucosa despite sustained effective ART [9, 24]. Residual HIV-1 RNA could be due to low-level virus replication that continues despite ART and/or the sporadic release of viruses from latently infected cells when being activated. Even low-level production of virus particles or proteins could provide enough antigenic stimuli for driving HIV-specific T cell responses.

Mucosal reactivation of CMV in the gut could also be an important driver of T cell activation [25, 26]. Repeated microbial translocation events, through breaches of the gut epithelium, could also provide inflammatory stimuli driving the recruitment of effector immune cells to the gut mucosa [27]. The inflammasome IL-1β and IL-18 cytokines could play an important role in orientating the mucosal immune response [28-30]. IL-18 seems predominant in the setting of HIV-1 infection [16, 17]. We found increased plasma levels of IL-18, but not of IL-1β, in the treated HIV-1-infected individuals included in the present study.

In conclusion, we have found increased frequencies of Th1 in $CD4^+$ and $CXCR3^+$ in $CD8^+$ T cells in the duodenal mucosa of HIV-1-infected individuals on ART, in contrast with the reduced frequency of Th17 cells. Absolute numbers of mucosal $CXCR3^+$ cells were increased, suggesting efficient cell recruitment through this chemotactic axis in this setting. We showed that the interactions between Th1 and enterocytes lead to a decrease in CCL20 and CCL25 expression but to an increase in CXCR3-ligands production by enterocytes, creating a positive feedback loop for Th1-type cells recruitment to the gut, while impairing Th17 cells homing through the CCR6-CCL20 axis.

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FIGURE LEGENDS

Figure 1. Increased frequency of Th1 in $CD4^+$ and $CXCR3^+$ in $CD8^+$ T cells contrasts with Th17 cells depletion in the duodenal mucosa of treated HIV-1-infected individuals.

A. Frequency of Th17 cells ($CD3^+$ $CD4^+$ $CXCR3^-$ $CCR4^+$ $CCR6^+$ $CD161^+$) and Th1 cells ($CD3^+$ $CD4^+$ $CXCR3^+$ $CCR4^-$ $CCR6^-$) in $CD4^+$ T cells in the duodenal mucosa of treated HIV-1-infected (n=11) and uninfected individuals (n=19 for Th17, n=20 for Th1 cells). Percentage of cells were determined by flow cytometry. **B.** Frequency of $CXCR3^+$ in $CD8^+$ T cells in the duodenal mucosa of treated HIV-1-infected (n=11) and uninfected individuals (n=20). Percentage of cells were determined by flow cytometry. **C.** Absolute numbers of $CXCR3^+$ cells per unit surface area in treated HIV-1-infected (n=11) and uninfected individuals (n=10). Quantification was performed by immunohistochemistry using NIS-element (Nikon). **D.** Preferential localization of $CXCR3^+$ cells in the *lamina propria* of the duodenal mucosa. Representative treated HIV-1-infected and uninfected individuals are shown. $CXCR3^+$ cells (brown) were stained by immunohistochemistry. Original magnification × 400. Groups were compared with the Wilcoxon's rank-sum test. Median bars are shown.

Figure 2. Correlations between the frequencies of Th1 in $CD4^+$ and $CXCR3^+$ in $CD8^+$ T cells and CCL20 and CCL25 expression in the duodenal mucosa.

A. Correlation between the frequency of Th1 in $CD4^+$ T cells and CCL20 mRNA expression (n=11 treated HIV-1-infected and n=16 uninfected individuals). **B.** Correlation between the frequency of Th1 in $CD4^+$ T cells and CCL25 mRNA expression (n=10 treated HIV-1-infected and n=9 uninfected individuals). **C.** Correlation between the frequency of $CXCR3^+$ in $CD8^+$ T cells and CCL20 mRNA expression (n=11 treated HIV-1-infected and n=16 uninfected individuals). **D.** Correlation between

the frequency of $CXCR3^+$ in $CD8^+$ T cells and CCL25 mRNA expression (n=10 treated HIV-1-infected and n=8 uninfected individuals). Th1 in $CD4^+$ and $CXCR3^+$ in $CD8^+$ T cell frequencies were assessed by flow cytometry; CCL20 and CCL25 mRNA was quantified by qRT-PCR in epithelial cells isolated from biopsies of the duodenal mucosa. Spearman's rank correlation was performed.

A.U., arbitrary unit. Uninfected individuals, white symbols; ART-treated HIV-1-infected individuals, gray symbols.

Figure 3. Functional impact of Th1- and Th17-type cytokines on CCL20 and CCL25 expression by enterocytes.

A. Effect of Th1-type cytokines IFN-γ (**left panel**), IL-2 (**center panel**), and TNF-α (**right panel**) on CCL20 and CCL25 expression by enterocytes. **B.** Effect of the Th17 cytokine IL-17A on CCL20 and CCL25 expression by enterocytes. **C.** Effect of inflammasome cytokines IL-18 (**left panel**) and IL-1ß (**right panel**) on CCL20 and CCL25 expression by enterocytes. Monolayers of differentiated human primary enterocytes on transwell inserts were stimulated by the different cytokines at 0.5, 5, or 50 ng/mL. CCL20 and CCL25 mRNA was quantified in the enterocytes by qRT-PCR and expressed as fold-change compared to unstimulated enterocytes. **D.** Production of CCL20 protein by enterocytes at baseline and following cytokine stimulation. CCL20 was quantified in the bottom chamber by ELISA. Concentrations below the limit of detection (LOD) were assigned a value that was half of the LOD (LOD/2=3.9 pg/mL). Presented data were obtained from at least three independent experiments performed with different donors. Paired Wilcoxon's test was used; chemokine expression upon stimulation was compared to unstimulated condition in all panels; *, $P<0.05$; **, $P<0.01$.

A.U., arbitrary unit. Means and SEM are shown.

Figure 4. Impact of the interactions between Th1 or Th17 cells and enterocytes on CCL20 and CCL25 expression.

A. CCL20 and CCL25 fold-change expression in Th1:IEC relatively to Th17:IEC coculture. Enterocytes were cocultured with FACS-sorted activated Th1 or Th17 cells, added in the bottom chamber for 15 hours. CCL20 and CCL25 mRNA were quantified in the enterocytes by qRT-PCR. **B**. Production of CCL20 protein by enterocytes in Th1:IEC and Th17:IEC cocultures. CCL20 was quantified in the bottom chamber by ELISA. Impact of antagonizing IFN-γ (by an anti-IFNγR1 mAb) or IL-18 (by IL-18BP) biological activity on CCL25 (**C**) and CCL20 (**D**) expression in Th1:IEC relatively to Th17:IEC coculture. CCL20 and CCL25 mRNA were quantified in the enterocytes by qRT-PCR. Presented data were obtained from at least six independent experiments performed with different donors. Paired Wilcoxon's test was used; *, $P<0.05$.

A.U., arbitrary unit; IEC, intestine epithelial cells. Means and SEM are shown.

Figure 5. IFN-γ and IL-18 synergy between Th1 cells and enterocytes.

Effect of IFN-γ on IL-18 (**A**) and IL-18RAP (**B**) expression by enterocytes. Monolayers of differentiated human primary enterocytes on transwell inserts were stimulated at 0.5, 5, or 50 ng/mL of IFN-γ. IL-18 and IL-18RAP mRNA were quantified in the enterocytes by Luminex and expressed as fold-change compared to unstimulated enterocytes. **C.** Expression of Caspase-1 in Th1:IEC and Th17:IEC cocultures. Enterocytes were cocultured with FACS-sorted activated Th1 or Th17 cells, added in the bottom chamber for 15 hours. Caspase-1 mRNA was quantified in the enterocytes by Luminex and expressed as fold-change compared to unstimulated enterocytes. **D**. Production of IFN-γ by Th1 cells in the absence or presence of IL-18. FACS-sorted Th1 cells were cultured with or without IL-18 (10 ng/mL) for 15 hours. IFN-γ was quantified in the supernatant by ELISA. Presented data were obtained from at least six independent experiments performed with different donors. Paired Wilcoxon's test was used; IL-18 and IL-18RAP expression upon stimulation was compared to

unstimulated condition (panels A and B);*, *P*<0.05.

A.U., arbitrary unit; IEC, intestine epithelial cells. Means and SEM are shown.

Figure 6. Functional impact of IFN-γ and Th1 or Th17 cells on CXCL9, CXCL10, and CXCL11 expression by enterocytes.

A. Effect of IFN-γ on CXCL9, CXCL10, and CXCL11 mRNA expression (**left panel**) and chemokine production (**right panel**). Monolayers of differentiated human primary enterocytes on transwell inserts were stimulated by IFN-γ at 0.5, 5, or 50 ng/mL. CXCL9, CXCL10, and CXCL11 mRNA were quantified in the enterocytes by Luminex and expressed as fold-change compared to unstimulated enterocytes. CXCL9, CXCL10, and CXCL11 chemokines were quantified in the bottom chamber by ELISA. **B.** CXCL9, CXCL10, and CXCL11 fold-change expression in Th1:IEC relatively to Th17:IEC coculture. Enterocytes were cocultured with FACS-sorted activated Th1 or Th17 cells, added in the bottom chamber for 15 hours. CXCL9, CXCL10, and CXCL11 mRNA were quantified in the enterocytes by Luminex. **C.** CXCL9, CXCL10, and CXCL11 chemokine production in Th1:IEC and Th17:IEC cocultures. CXCL9, CXCL10, and CXCL11 chemokines were quantified in the supernantant collected from the bottom chamber by ELISA. **D**. Impact of antagonizing IFN-γ (by an anti-IFNγR1 mAb) biological activity on CXCL9, CXCL10, and CXCL11 expression in Th1:IEC relative to Th17:IEC coculture. CXCL9, CXCL10, and CXCL11 mRNA were quantified in the enterocytes by Luminex. Presented data were obtained from at least six independent experiments performed with different donors. A.U., arbitrary unit; IEC, intestine epithelial cells. Paired Wilcoxon's test was used; chemokine expression upon stimulation was compared to unstimulated condition (panels A and B); *, *P*<0.05; **, *P*<0.01.

Means and SEM are shown.

Figure 7. Chemotaxis of Th1 and Th17 cells in response to Th cells/IEC microenvironment.

Percentage of Th1 and Th17 cells migrating in response to a conditioned medium from Th1:IEC (**A**) or Th17:IEC (**B**) cocultures or a control medium from unstimulated enterocytes. The number of cells migrating through the 5 µm-pore membrane in 15 hours was counted. Th1 and Th17 cell chemotaxis was antagonized by anti-CXCL9/10/11 Ab or an anti-CCL20 mAb, respectively. Experiments were repeated three times with different donors.

Paired Wilcoxon's test was used; *, $P<0.05$.

IEC, intestine epithelial cells. Means and SEM are shown.

Figure 1

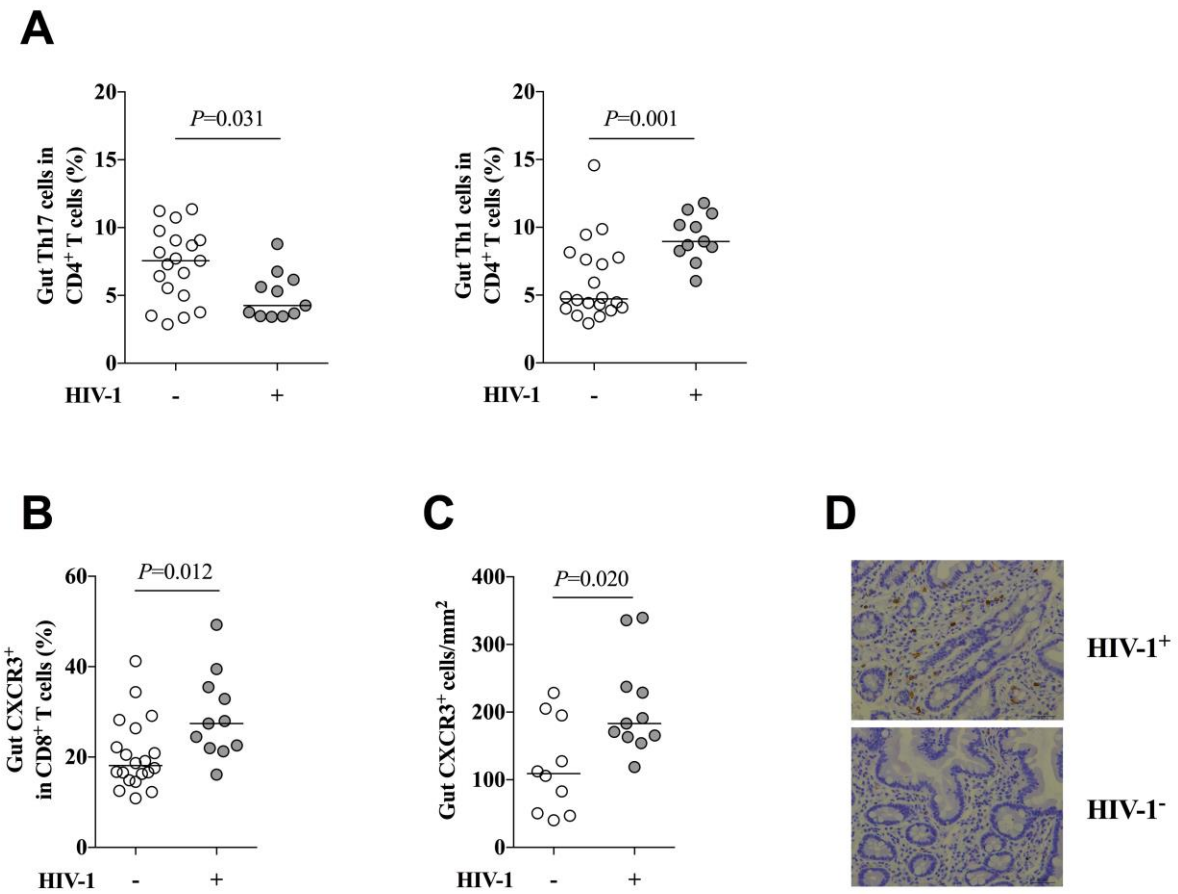
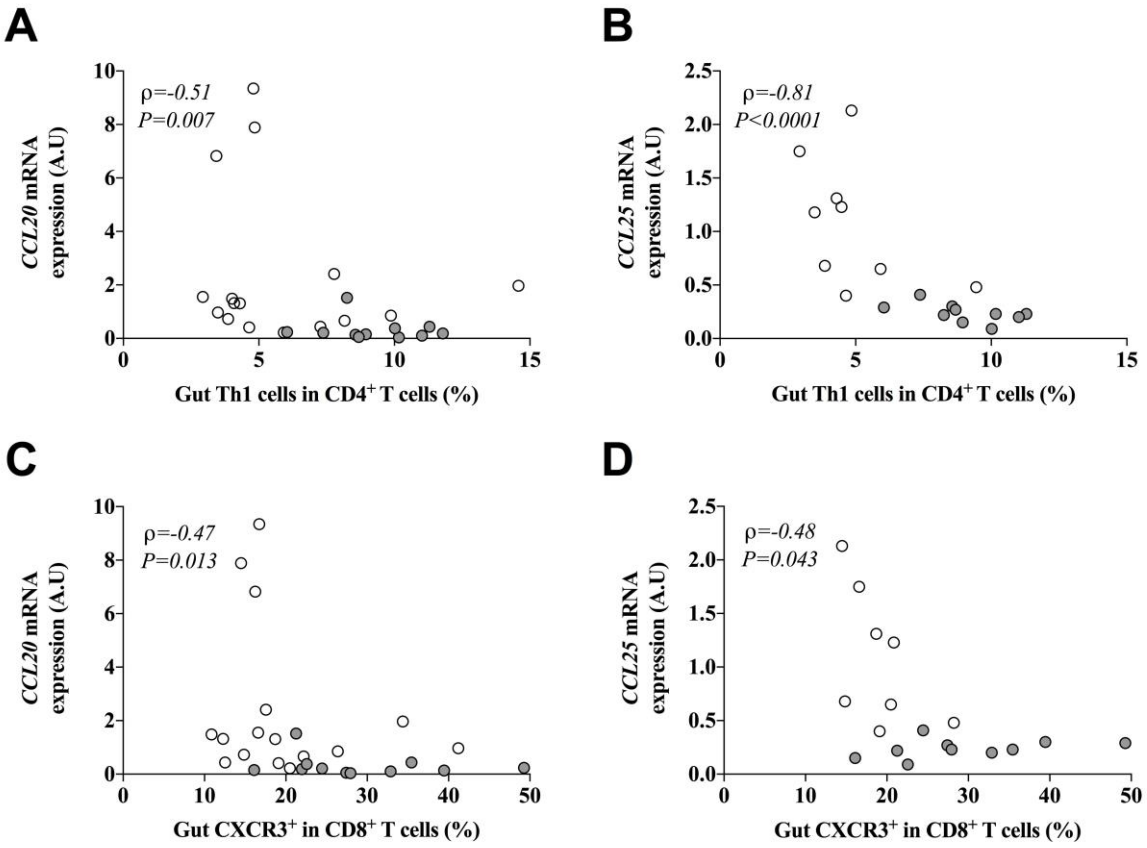


Figure 2



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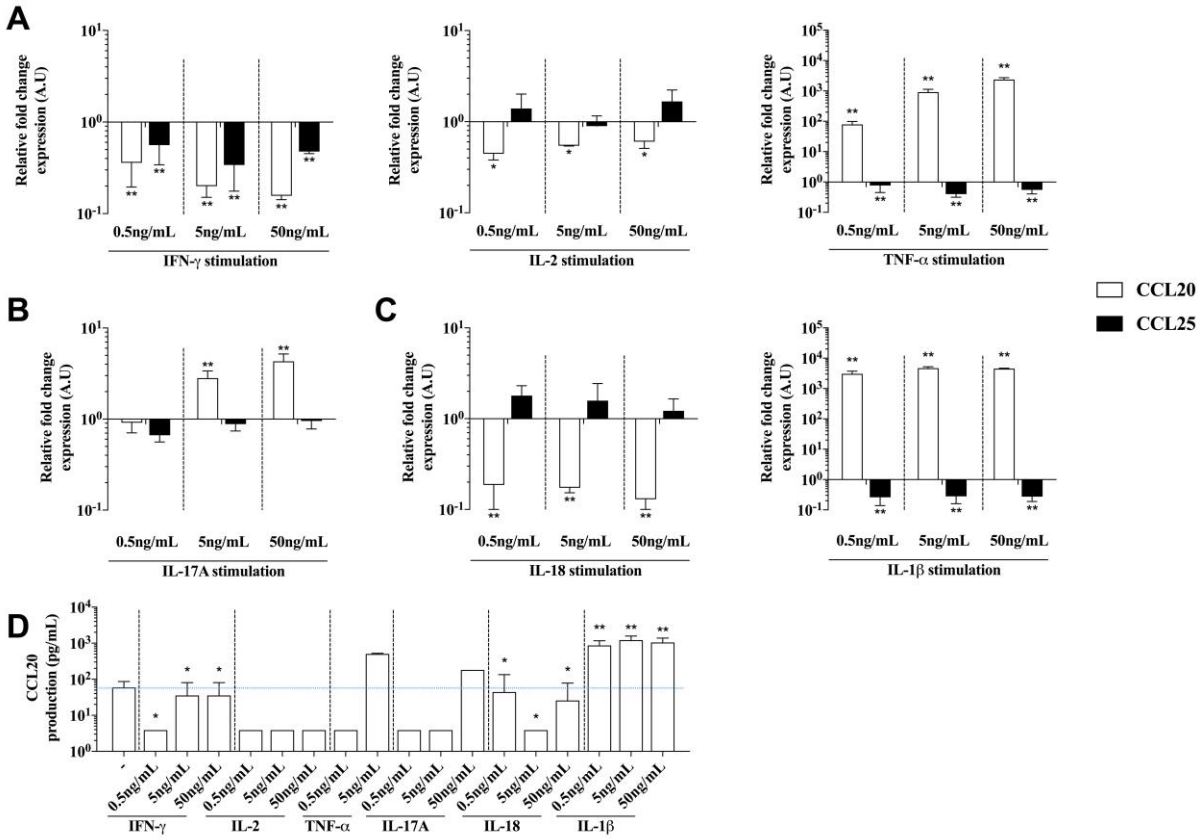

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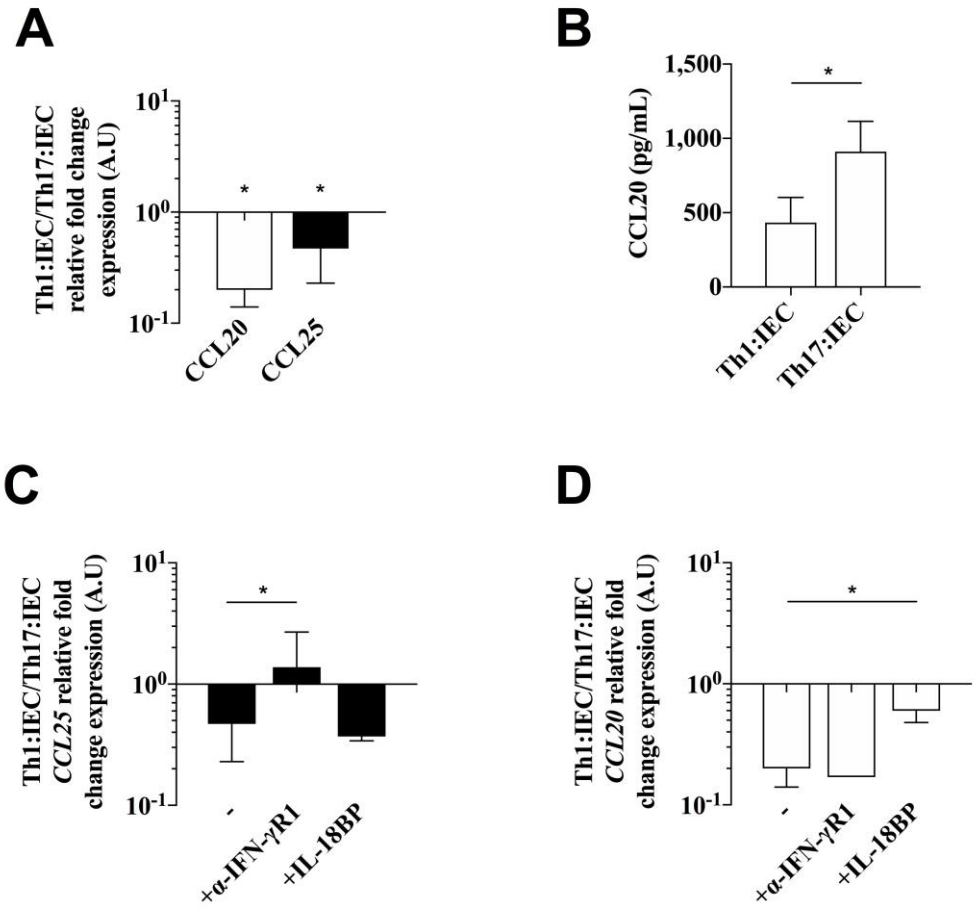

Figure 5

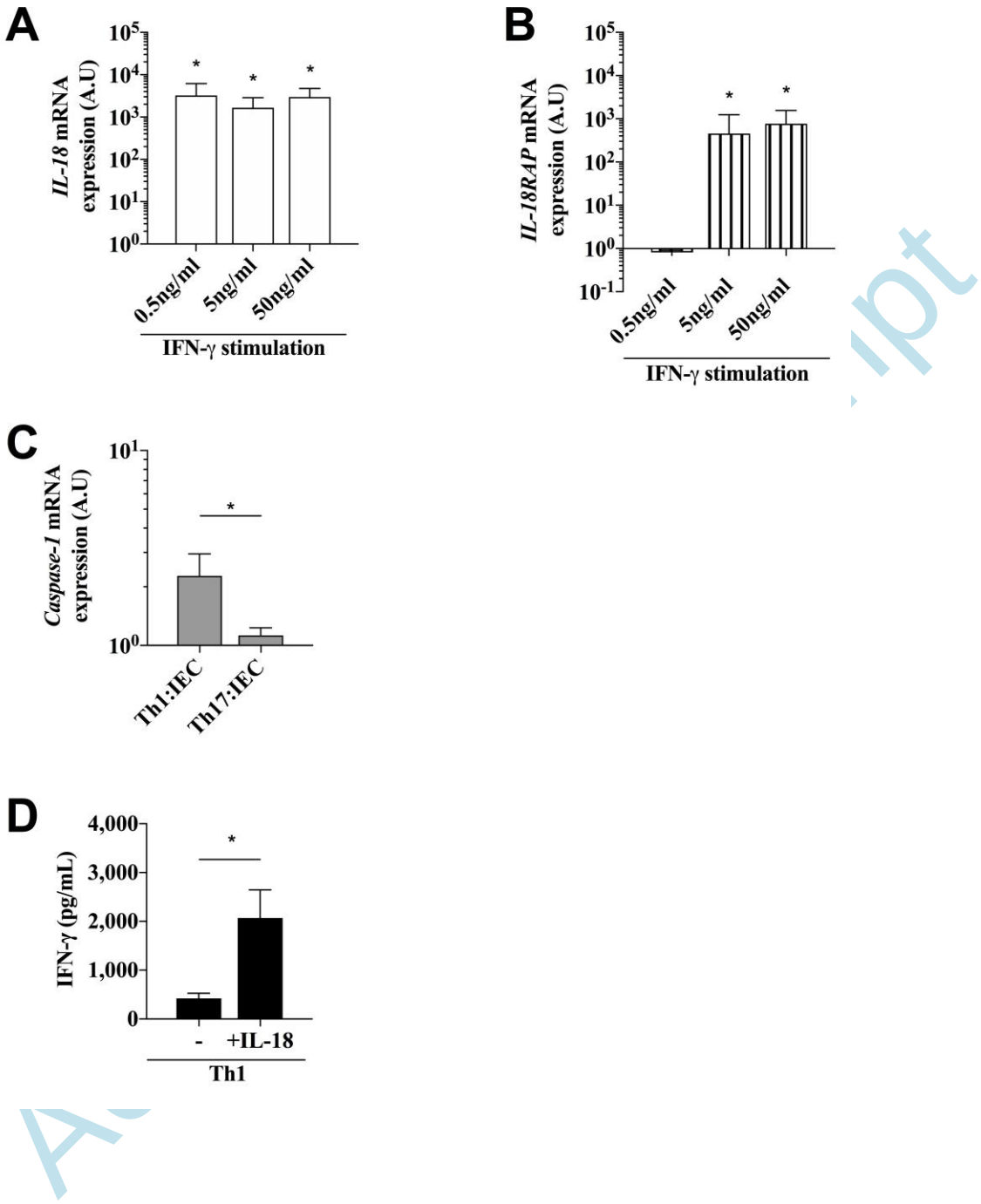

Figure 6

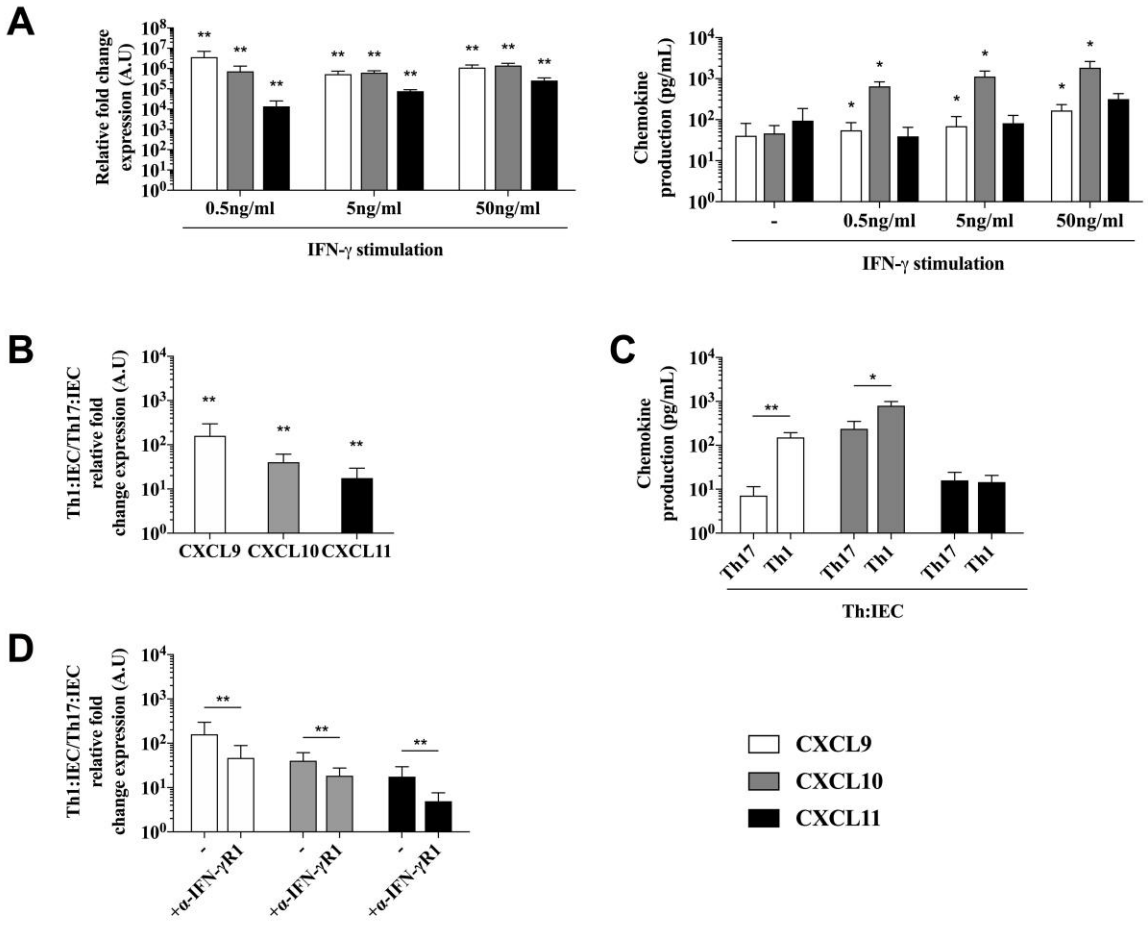

Figure 7