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Role of intestinal myofibroblasts in HIV-associated intestinal collagen deposition and immune reconstitution following combination antiretroviral therapy

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Objective: To investigate the potential role of mucosal intestinal myofibroblasts (IMFs) in HIV and associated fibrosis in gut-associated lymphoid tissue.

Design: Profibrotic changes within the secondary lymphoid organs and mucosa have been implicated in failed immune reconstitution following effective combination antiretroviral therapy (cART). Microbial translocation is believed to be sustaining these systemic inflammatory pathways. IMFs are nonprofessional antigen-presenting cells with both immunoregulatory and mesenchymal functions that are ideally positioned to respond to translocating microbial antigen.

Methods: Duodenal biopsies, obtained from patients naive to cART, underwent trichrome staining and were examined for tissue growth factor-beta (TGF-β) expression. Combined immunostaining and second harmonic generation analysis were used to determine IMF activation and collagen deposition. Confocal microscopy was performed to examine IMF activation and Toll-like receptor (TLR)4 expression. Finally, primary IMF cultures were stimulated with lipopolysaccharide to demonstrate the expression of the inflammatory biomarkers.

Results: The expression of the fibrosis-promoting molecule, TGF-β1, is significantly increased in duodenal biopsies from HIV patients naïve to cART, and negatively correlated with subsequent peripheral $CD4^+$ recovery. The increase in TGF-β1 coincided with an increase in collagen deposition in the duodenal mucosa in the tissue area adjacent to the IMFs. We also observed that IMFs expressed TLR4 and had an activated phenotype since they were positive for fibroblast activation protein. Finally, stimulation of IMFs from HIV patients with TLR4 resulted in significantly increased expression of profibrotic molecules, TGF-β1, and interleukin-6.

Conclusion: Our data support the hypothesis that activated IMFs may be among the major cells contributing to the profibrotic changes, and thus, the establishment and maintenance of systemic inflammation interfering with immune reconstitution in HIV patients.

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Keywords: collagen A, gut-associated lymphoid tissue, HIV, immune reconstitution, intestinal fibrosis, intestinal myofibroblasts, lipopolysaccharide, tissue growth factor-beta, Toll-like receptor 4

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Introduction

Effective combination antiretroviral therapy (cART) has dramatically impacted the morbidity and mortality associated with HIV infection [1–3]. Among those who achieved $CD4^+$ T-cell counts in the normal range, HIV has become a manageable chronic disease with mortality approaching uninfected matched populations [4]. However, despite undetectable plasma HIV viral loads (pVLs), up to 20% of patients fail to realize complete immune reconstitution in the peripheral vascular compartment as measured by $CD4^+$ T-cell count [5–7]. This subgroup of patients is at a highest risk for residual morbidity from HIV infection [8–10], with increased frequency of AIDS and non-AIDS-defining malignancies, and complications of hepatic, metabolic, and cardiovascular diseases [10–14].

Incomplete immune reconstitution is an important challenge facing patients and providers with no effective interventions available. Treatment intensification with additional or more potent cART, cytokine therapy, and therapeutic vaccination has not had a clinically meaningful impact till the present date [15–19]. Age, especially over 40–45 years old, and lower naive $CD4^+$ T cells prior to initiating cART are the most reliable baseline characteristics associated with blunted $CD4^+$ T-cell rise [20,21]. Since most of the HIV viral replication occurs within the lymphoid tissue, reconstitution of the T cells within this compartment would be essential for the complete recovery from the disease. Thus, an understanding of the mechanisms preventing this process would be essential for the development of successful full immune reconstitution and functional cure. Recent studies point out that development of fibrosis due to the abnormal collagen deposition in the secondary lymphoid tissue is a major factor preventing successful immune restoration even during long-term cART [22,23]. The increase in the amount of collagen within the T zone of the secondary lymphoid tissue is suggested to be a prognostic marker for poor $CD4^+$ T-cell reconstitution and development of AIDS [24]. It has been proposed that collagen deposition in secondary lymph nodes perturbs the T cells and the fibroblastic reticular cell (FRC) network, leading to the disruption of $CD4^+$ T-cell migration and preventing T-cell access to the cytokines and growth factors [e.g. interleukin (IL)-7, lymphotoxin] essential for their homeostasis [24,25].

Reconstitution of $CD4^+$ T cells during cART is incomplete in the gut-associated lymphoid tissue (GALT), particularly within the mucosal lamina propria effector compartment [26–29]. Failure of GALT immune reconstitution not only contributes to the development of HIV-associated enteropathy, but might also be involved in the maintenance of the viral reservoir in the gut mucosa [26]. Further, this limited reconstitution of $CD4^+$ T cells in GALT correlates with an early and extensive collagen deposition in the terminal ileal lamina propria and Peyer patches to a greater extent than that which occurs in the lymph nodes [27]. Although the mechanisms of HIV-associated GALT fibrosis are unknown, attention has turned to the potential consequences of sustained immune activation driven by microbial antigen translocation [30–32]. Further, fibrosis in the setting of HIV/simian immunodeficiency virus-induced chronic immune responses may be due to the unbalanced activation of the gut innate immune system toward resident gut microbiota [24,33,34]. Despite the advance in the understanding of the influence of gut microbiota to the immune reconstitution in HIV, the role of the mucosal cell network in profibrotic changes within the GALT in HIV is understudied.

We, and others demonstrated that intestinal $CD90^+$ stromal cells (myofibroblasts and fibroblasts, IMFs) are nonprofessional antigen-presenting cells that are located beneath the epithelial basement membrane [35–38], and that play a major role in the innate gastrointestinal immune responses via signaling through Toll-like receptor (TLR)1–9 [39,40]. These cells are important contributors to the wound-healing process [41,42]. However, uncontrolled pro-inflammatory activation of IMFs leads to hardwired functional changes in these cells that may contribute to the gut fibrosis via activation of profibrogenic signaling [43]. This may result in increased collagen deposition in response to the pro-inflammatory cytokine milieu [e.g. IL-1β, IL-6, tissue growth factor-beta (TGF-β)] and also may contribute to the increase/modulation of these pro-inflammatory molecules [44,45]. Characterization of these processes has been well advanced in Crohn's disease, in which case chronic inflammation leads to the burst of profibrogenic stimuli from professional immune and stromal cells, leading to the downstream activation of collagen deposition by the IMFs [46]. Thus, in this present study, we have extended our investigations and have analyzed the profibrotic changes in the duodenum from the HIV-positive patients, and determined whether $CD90^+$ stromal cells are activated and thus may contribute to the profibrotic changes within the gastrointestinal mucosa in HIV.

Materials and methods

Clinical trial design and tissue processing

Treatment-naive HIV patients underwent upper endoscopy before and 9 months after starting cART. Duodenal specimens from HIV-negative volunteers were used as a control group. The negative control patients were of similar age and lifestyle as the HIV-infected patients enrolled in our GALT cohorts. All participants signed an informed consent form approved by the UC Davis Institutional Review Board prior to initiation of the study procedures. Duodenal tissue was snap-frozen for RNA

extraction, embedded for trichrome stain or immunofluorescence antibody assay (IFA), or digested to single-cell suspension for fluorescence-activated cell sorting (FACS) for T-cell subsets, lymphocyte activation levels, and IMF isolation. Trichrome stain slides were read as negative or positive (mild to severe) by a single blinded pathologist (R.S.). Peripheral blood mononuclear cells (PBMCs) had the same FACS analysis using a previously described 10-color panel and three-laser LSRII (Becton Dickinson Biosciences, San Jose, California, USA) flow cytometer [26]. Single-cell suspensions of 10^6 cells of duodenal tissue were pelleted for HIV proviral DNA quantification as previously described [47].

Real-time reverse transcription PCR

Real time reverse transcription PCR (RT-PCR) was selected as the most robust measure of chronic TGF-β tissue up-regulation [48]. Total cellular RNA was isolated from the duodenal cells using RNeasy RNA isolation kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. RT-PCR was performed according to the Applied Biosystem's two-step real-time RT-PCR protocol (Applied Biosystems, Foster City, California, USA). Human GAPDH was used as the housekeeping gene. The primers for human TGF-β1 and procollagen type I (α1) were reported previously [49]. The endpoint used in real-time PCR quantification – cycle threshold (CT) – was defined as the PCR cycle number that crossed the signal threshold. CT values ranged from 0 to 40, with the latter number assumed to represent no product formation.

Quantification of the induced cytokine gene expression from the primary IMF cultures was performed using the comparative CT method (Sequence Detector User Bulletin 2; Applied Biosystems) and reported as the fold-difference relative to the human housekeeping gene 18S rRNA. In order to calculate the fold change (increase or decrease), the CT value of 18S rRNA was subtracted from the CT value of the target cytokine gene to yield the CT. Change in the expression of the normalized target gene as a result of experimental conditions was expressed as 2-CT, where CT = CT experimental samples — CT biological control.

sCD14 measurement

Soluble CD14 levels in plasma samples were quantified by ELISA with the Quantikine Human sCD14 Immunoassay (R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's protocol in duplicate.

Primary intestinal myofibroblast cultures

Primary cultures of IMFs were generated as we have reported previously [50]. IMFs were passaged at least three times in a culture in order to eliminate residual macrophages and other adherent cells. Purity of the isolated cells was analyzed by flow cytometry analysis as we previously described [35].

Confocal microscopy

The immunostaining followed by confocal microscopy was performed as described previously [51]. Frozen tissue sections of the intestinal mucosa were fixed in 2% paraformaldehyde for 30 min, and were blocked with normal mouse and goat serum (1 : 10 in PBS) for 30 min at room temperature. The samples were then incubated with the mixture of the following antibodies: 1 μg/ml of antifibroblast marker ER-TR7 rat monoclonal antibodies (mAbs) (clone ER-TR7; Santa Cruz Biotech Inc., California, USA) conjugated to AF-546, anti-α-smooth muscle actin (SMA) monoclonal Abs (clone 1A4; Sigma), anti-CD90 (Clone 5E10; eBioscience, Inc., San Diego, California, USA) conjugated with AF-488, or TLR4 was detected using anti-TLR4 mAbs (clone HTA125; eBioscience) for 30 min at room temperature. Each staining step was followed by three washes with PBS. Zenon mouse IgG and Apex Antibody Labeling kits (Invitrogen, California, USA) were used to conjugate mouse and rat mAbs. Microscopy was performed with confocal and multiphoton microscopes Zeiss LSM510 META a (Carl Zeiss, Thornwood, New York, USA) and Olympus FV1000 MPE (Olympus, Center Valley, Pennsylvania, USA), as well as modified Zeiss LSM 410 confocal microscope, as described previously [52]. Combination of immunostaining and second harmonic generation (SHG) analysis followed by confocal and two-photon excitation and SHG microscopy was used to determine collagen A deposition within the intestinal mucosa as previously described by our group [52,53]. The physical origin of SHG within the connective and muscle tissues is attributed to the laser interaction with dipolar protein structures that is enhanced by the intrinsic chirality of the protein helices. Collagen has been shown to give rise to SHG [54]. The source of SHG signal in the mucosa arises mostly from fibrillar collagen, which is mostly located in the mucosal stroma [53].

Stimulation of cultured intestinal myofibroblasts with Toll-like receptor 4 agonists

Intestinal myofibroblast primary cultures were generated as described above. IMFs were stimulated with lipopolysaccharide (LPS), a TLR4 ligand (1 mg/ml), or buffered saline, for 24 h (InvivoGen, San Diego, California, USA). The RNA was extracted and the gene expression was measured using real-time RT-PCR as described above.

Statistical analysis

Nonparametric statistical methods were used throughout. Wilcoxon matched-pairs signed-rank test and Mann–Whitney test were used as appropriate. The Spearman rank-correlation coefficient was used to study correlations between parameters. All values are represented as the median (interquartile ranges). Analyses were performed with GraphPad Prism Software V6.01 (GraphPad Software, Inc., La Jolla, California, USA).

Results

Baseline characteristics and virologic/immunologic outcome after 9 months of antiretroviral therapy

The samples used for this pilot study were obtained during a randomized clinical trial in which HIV-positive volunteers naïve to ART underwent upper and lower endoscopy prior to and 9 months after being randomized to either a non-nucleoside reverse transcriptase inhibitor or raltegravir, both in combination with tenofovir/emtricitabine [26]. Overall, 16 HIV patients and five controls underwent upper endoscopy, and a subset underwent flexible sigmoidoscopy. This study focuses on the 14 study participants for whom the relevant samples were available for the assays reported herein at both baseline and at 9 months after cART, and negative control volunteers who underwent the same interventions. The control patients were family and friends of the HIV patients who were at a low risk for HIV infection. The demographic profiles and results from clinical responses are reported in Table 1, as well as the peripheral blood and duodenal tissue virologic and immunologic results. Importantly, the median $CD4^+$ T-cell count in the HIV patients rose from a median of 328 cells/μl (235–417) at baseline to 562 cells/μl (388–651) among those who completed 9 months of treatment ($n=14$). This represented a median increase of 181 cells/μl (145–295) in the peripheral blood absolute $CD4^+$ T-cell counts. All patients became undetectable after 9 months by standard commercial assays. The single-copy HIV RNA and proviral DNA assay results are listed in Table 1 for peripheral blood and tissue compartments. HIV proviral DNA declined from 208 (31–1189) to 110 (14–694) in the peripheral blood, and from 37 (3.9–193.4) to 15.4 (2.6–105.6) in the duodenal tissue single-cell suspensions of patients ($P=0.005$ and $P=0.32$, respectively). As expected, both plasma sCD14 concentration and $CD8^+$ T-cell percentage with the activated phenotype decreased after the introduction of cART ($P=0.01$ and $P=0.03$, respectively). Although we observed significant increases in the tissue $CD4^+$ T-cell count ($P=0.024$), they did not achieve the average level observed in the lamina propria (LP) of normal controls (data not shown).

Duodenal tissue tissue growth factor-beta expression is increased in HIV-positive patients and correlates with increases in collagen deposition

We analyzed the mRNA levels of TGF-β1 in duodenal LP and correlated it with the level of procollagen type I (α1, procollagen A) expression. TGF-β1 mRNA levels were higher in HIV patients [335.4 (187.6–621.7)] compared to controls [57.6 (2.1–134.6), $P=0.002$, respectively] (Fig. 1a). Procollagen A mRNA levels were also higher in patients [462.0 (189.0–805.5)], compared to controls [92.5 (49.7–258.3)] ($P=0.048$) (Fig. 1b). As would be expected and as an internal control, TGF-β1 and procollagen A expression levels were positively correlated ($r=0.65$, $P=0.0018$) (Fig. 1c). Conversely, tissue duodenal HIV proviral DNA did not correlate with TGF-β expression ($r=0.03$, $P=0.5$). Neither monocyte activation as measured by sCD14 levels, nor $CD8^+$ T-cell activation percentages correlated with peripheral blood or tissue HIV proviral DNA or HIV plasma viral load (both $P>0.5$). Interestingly, sCD14 and $CD8^+$ T-cell activation only correlated at the 9-month time-point ($r=0.63$, $P=0.01$), but not at baseline pretreatment, suggesting perhaps that several possible factors influence monocyte activation prior to suppression of HIV viremia, but less so once pVL is controlled by effective cART.

Profibrotic changes in duodenal tissue from HIV-positive patients negatively correlates with the reconstitution of $CD4^+$ T cells

In our next step, we analyzed whether increases in the fibrosis in duodenal lamina propria correlated with the immune reconstitution of the HIV-positive patients following initiation of ART, as has been reported in lymph node parenchyma [22]. Baseline duodenal tissue

Table 1. Demographics and summary immunologic and virologic data.

	Normal control	Baseline	cART 9 months	*P* value
n	5	14	–	
Male/female	3/2	9/5	–	
Race/ethnicity[a]	3/1/1	6/4/4	–	
Median age years (IQR)	42 (33–44)	37 (28–46)	–	
Peripheral blood $CD4^{+b}$	–	328 (235–417)	562 (388,651)	<0.0001
Plasma viral load (copies/ml)	–	25,600 (14,770–70,613)	11.5 (11.3, 750)	<0.0001
PBMC proviral DNA (copies/10^6 cells)	–	208 (31–1189)	110 14-694	0.005
Duodenal tissue proviral DNA (copies/10^6 cells)	–	37 (3.9–193.4)	15.4 (2.6–105.6)	0.32
sCD14 (μg/ml)	–	1.9 (1.7–2.4)	1.7 (1.2–1.8)	0.01
$CD8^+/CD38^+/HLA\text{-}DR^+$ PBMCs	–	44.2% (32.2–61.4)	27.7% (18.8–38.5)	0.03
Doudenal lamina propria $CD3^+/CD4^+$ (cells/μl)[c]	845 (665–1159)	105 (76.5–170.5)	151.5 (129–172)	0.02[b]
PBMC $\Delta CD4^b$ (IQR)	–	–	181 (145–295)	

cART, combination antiretroviral therapy; IQR, interquartile range; PBMC, peripheral blood mononuclear cell.
[a]Caucasian/African American/Hispanic.
[b]Baseline versus 9-month (Wilcoxon rank-sum test).
[c]Median cells/μl (interquartile range).

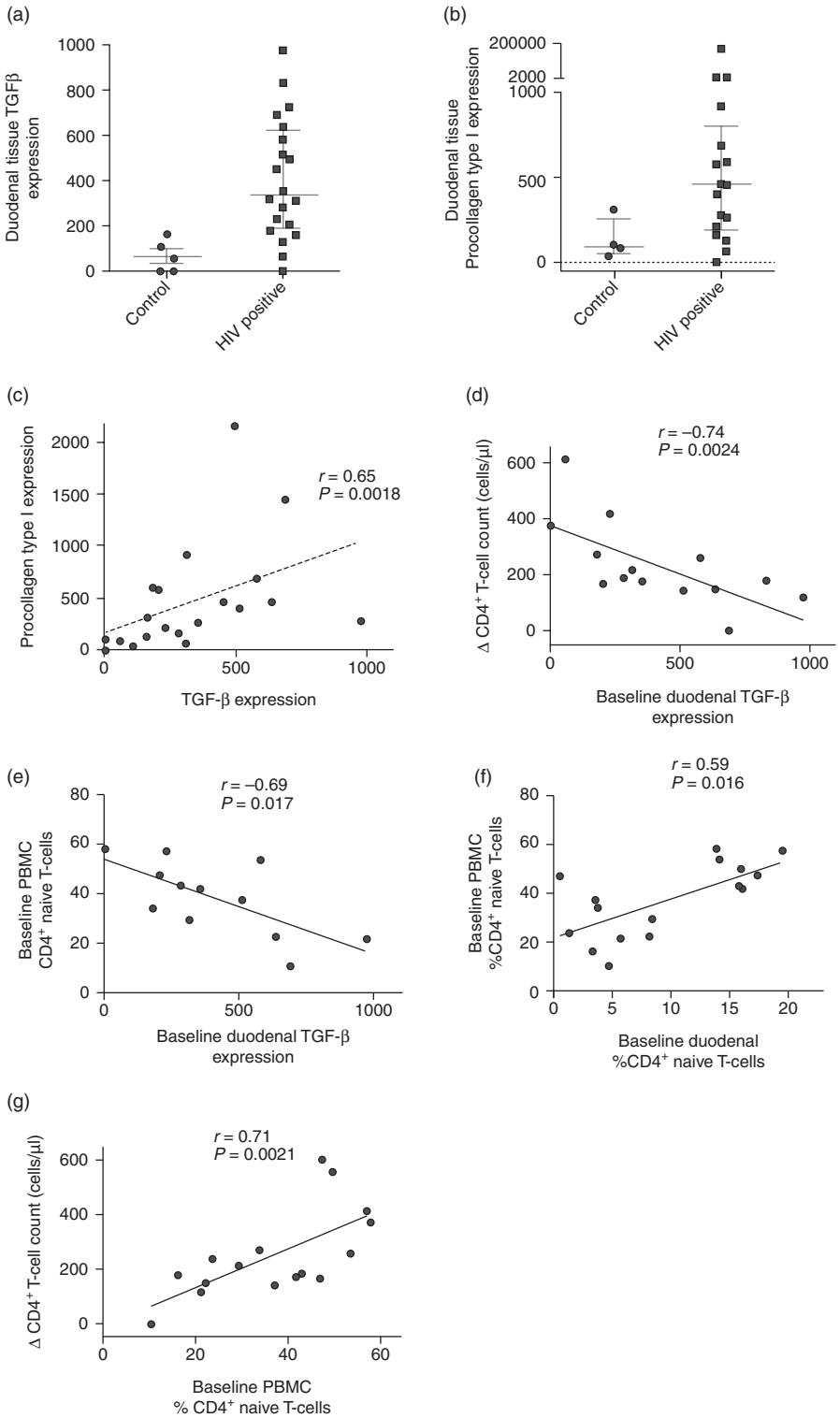

Fig. 1. Up-regulation of biomarkers of fibrosis is increased in duodenal tissue of untreated HIV-infected patients and predicts subsequent immunologic response to cART. Duodenal tissue mRNA levels of (a) TGF-β and (b) procollagen type I are increased in HIV-infected patients compared to controls by quantitative PCR ($P = 0.002$ and $P = 0.048$, respectively). (c) As expected, TGF-β and procollagen type I expression is positively correlated ($r = 0.65$, $P = 0.0018$). Immune reconstitution (rise in absolute PBMC T-cell counts) correlates (d) negatively with baseline tissue TGF-β expression and (e) negatively with peripheral naive $CD4^+$ T-cell proportions. Baseline PBMC-naive $CD4^+$ T cells correlate with the (f) naive $CD4^+$ T cells in the duodenal tissue compartment and (g) negatively with the TGF-β expression in the duodenal tissue. cART, combination antiretroviral therapy; PBMC, peripheral blood mononuclear cell; TGF-β, tissue growth factor-beta.

TGF-β, but not procollagen A expression, negatively correlated with subsequent rise in absolute T-cell counts ($r=-0.74$, $P=0.0024$) (Fig. 1d). Interestingly, the baseline PBMC-naive $CD4^+$ T-cell subset [median of 39.6% (22.1, 49.0)] was the only $CD4^+$ T-lymphocyte maturational subset to correlate with TGF-β expression at baseline ($r=-0.69$, $P=0.017$) (Fig. 1e). In parallel, the only subset of $CD4^+$ T lymphocytes to correlate between the two compartments of peripheral blood and duodenal tissue was the naïve $CD4^+$ T cells ($r=0.59$, $P=0.016$) (Fig. 1f). The absence of a correlation between the peripheral blood absolute $CD4^+$ T-cell count and the density of $CD4^+$ T cells in the duodenal tissue ($r=0.38$, $P=0.15$) suggests that the correlation between the two compartments relative to the naïve subsets is more likely due to systemic conditions for naive T-cell maturation as has been described in the lymph nodes previously [25]. Finally, and as others have reported regarding baseline immunologic parameters that best predict subsequent $CD4^+$ T-cell response to effective ART, baseline PBMC percentage of naive $CD4^+$ T cells correlated significantly with immune reconstitution ($r=0.71$, $P=0.002$) (Fig. 1g) [5,55,56]. These data strongly suggest that TGF-β expression in GALT correlates with subsequent immune reconstitution in the peripheral compartment.

Collagen deposition is increased in the duodenal lamina propria during HIV infection and is associated with the increase in the activation of the intestinal myofibroblasts

Next, we examined collagen deposition within the duodenal mucosa using trichrome stain followed by histological analysis. Hematoxylin and eosin (H&E) stain was included to identify inflammation, which was not seen (Fig. 2a). As detected by trichrome staining (Fig. 2b, collagen deposition is in blue), an increase in mucosal lamina propria collagen was observed in 10 out of 20 tested HIV-positive patients compared to one out of five control patients ($P=0.078$). Increased collagen deposition was confirmed by SHG analysis using two-photon microscopy in a subset of six out of 10 tested HIV-positive patients, but was not observed in a healthy control group (Fig. 2c). Taken together with our quantitative mRNA data analysis (Fig. 1a), these observations strongly support the conclusion that fibrotic changes occur in the gut during chronic HIV infection. No significant decrease in the collagen deposition was observed in the tissue from patients after 9 months of cART (data not shown).

Mucosal increase in collagen A deposition is concomitant with the increase in the activation of intestinal myofibroblasts

Intestinal fibrosis is classically viewed as an inflammatory process associated with the proliferation and activation of local IMFs, leading to abnormal deposition of collagen [44,57]. We observed that the increase in deposition of collagen A in the duodenal lamina propria of HIV-positive patients (Fig. 3, in blue) was accompanied with the disruption of normal mucosal fibroblastic network architecture and activation of IMFs, as determined by expression of α-SMA (Fig. 3, in green) and fibroblasts activation protein (FAP) (Fig. 3, in red). FAP is believed to be increased in fibroblasts in fibrotic tissue and cancer [58]. Thus, colocalization of the α-SMA (Fig. 3) and the FAP (formation of the yellow-orange color on merged images, Fig. 3) within IMFs from HIV-positive patients suggests that abnormal activation of the stromal cells in the intestinal mucosa may contribute to the fibrotic tissue remodeling in HIV-associated immunopathogenesis.

Stimulation of Toll-like receptor 4 on intestinal $CD90^+$ fibroblast/myofibroblasts derived from HIV-positive patients up-regulates the production of profibrotic mediators

The main driver of gut fibrogenesis is believed to be chronic inflammation, which leads to mesenchymal cell recruitment and activation. Recent findings suggest that microbial overgrowth and abnormality in microbial translocation play a critical role in gut fibrogenesis [34,57]. LPS is known to be a profibrotic agent [59]. Thus, we determined whether IMFs express TLR4, a receptor for LPS, in duodenal mucosa from HIV-positive patients. Using immunostaining followed by confocal microscopy of duodenal mucosa tissue sections, we were able to detect compartmentalized TLR4 expression in duodenal mucosa from HIV-positive patients (Fig. 4, showing in red). In duodenal LP, $CD90^+$ stromal cells (also known as IMFs) were among the major cell phenotypes expressing TLR4 (Fig. 4, showing in green). Next, we determined how TLR4 stimulation modulates expression of the cytokine and growth factors involved in the fibrosis. Our data demonstrated that stimulation of the HIV-derived IMF primary cultures with LPS (1 μg/ml) for 24 h resulted in significant up-regulation of TGF-β1 and IL-6 mRNA expression (Fig. 5). Taken together, these data suggest that during HIV enteropathy, the interaction of IMFs with LPS may contribute to the increase in the soluble mediators of fibrogenesis (IL-6, TGF-β1) and that may directly enhance collagen deposition by IMFs.

Discussion

Incomplete immune reconstitution following effective cART is associated with significant residual morbidity amongst HIV-infected patients. Its understanding and control is a significant unmet need in the management of HIV disease. Considerable debate exists in the literature regarding the underlying pathophysiology of failed immune reconstitution in the gut following effective cART. In this study, we sought to examine the role of events at the interface of the gut mucosa and the systemic immune system to explore which factors influence peripheral $CD4^+$ T-cell reconstitution following effective

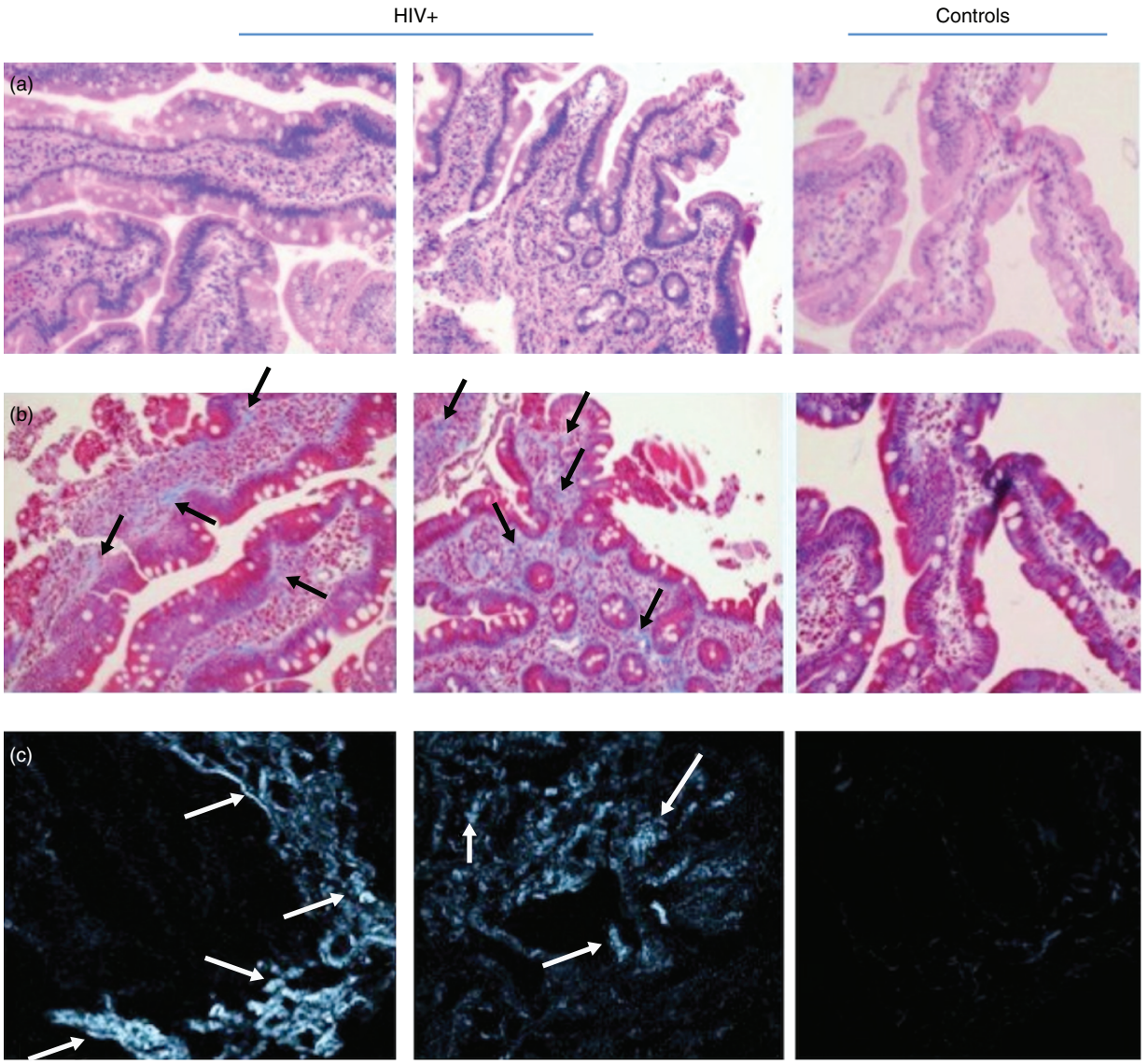

Fig. 2. Collagen deposition in duodenal lamina propria. Hematoxylin and eosin staining from representative two HIV-positive and an HIV-negative control reveals normal architecture (panel a). Trichrome stain (panel b) of duodenal mucosa was positive from 10/20 HIV-positive patients (representative examples from the same patients as panel a). (c) SHG analysis using two-photon microscopy was used to determine collagen A deposition (in gray), 40× objective. SHG analysis from duodenal mucosa from two additional HIV-positive and one HIV-negative control patients is shown. Arrows point out collagen deposition in panels b and c. Very little staining for collagen is evident for the HIV-negative sample, hence the faint image. SHG, second harmonic generation.

cART. We and others have previously demonstrated that higher percentages of pro-inflammatory bacterial taxa, including the Proteobacteria/Enterobacteriales, were associated with changes in the duodenal GALT T-cell subset proportions and T-cell activation [33,60]. Similarly, Dillon *et al.* [61] also observed a relationship between altered mucosal microbiome and blood T-cell activation. More recently, we reported the impact of oral serum-derived bovine immunoglobulin (which is broadly reactive against bacterial and viral antigen) on HIV enteropathy, gut mucosal repair and function, and systemic immune activation [62]. Most relevant to the work described herein, oral bovine immunoglobulin administration resulted in a significant increase in duodenal lamina propria $CD4^+$ T cells after only 8 weeks of treatment, supporting the hypothesis that bacterial products impact immune reconstitution locally [62]. Klatt *et al.* [63] observed similar results with the administration of probiotics/prebiotics in combination with ART in SIV-infected pigtail macaques, further supporting the hypothesis that microbial translocation is causing fibrosis.

The present study extends that work by exploring pathways of focal tissue inflammation by examining TGF-β expression as an effector molecule and procollagen type I expression as the downstream mediator of

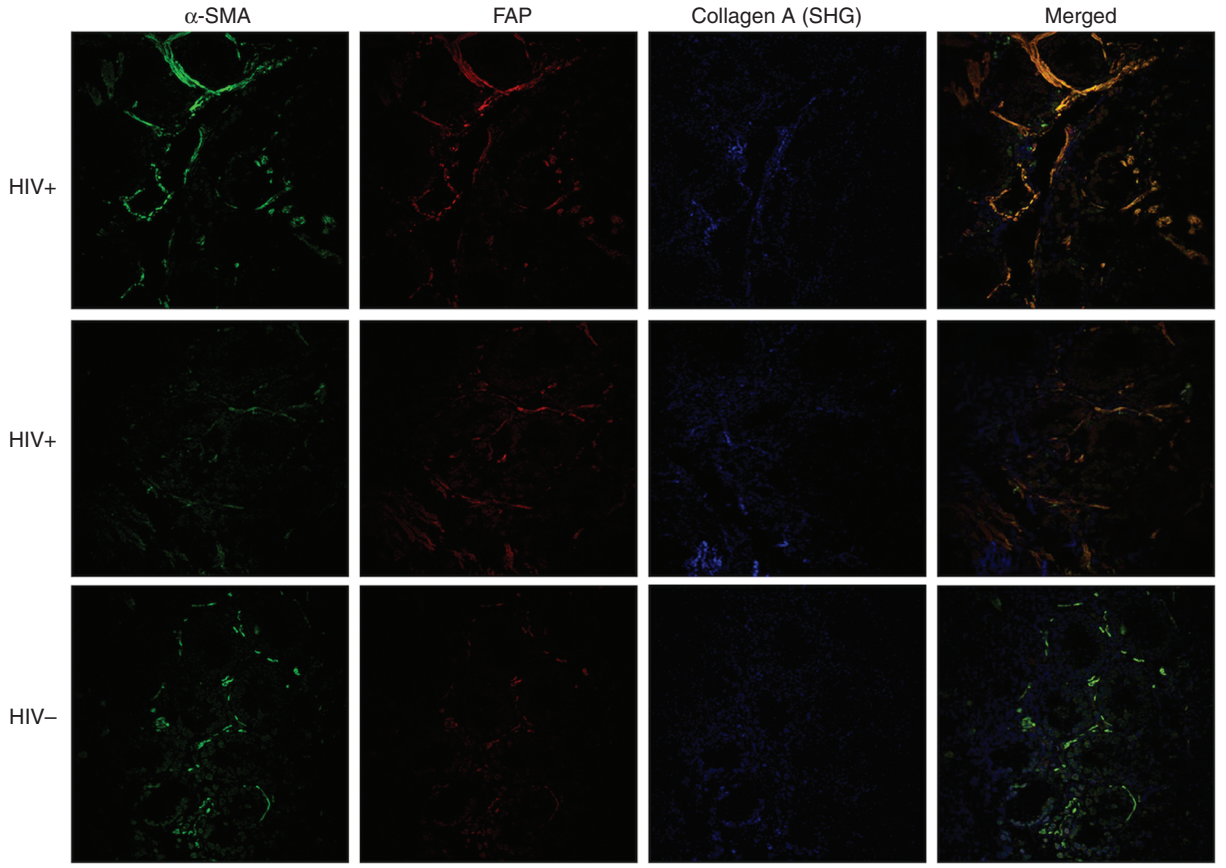

Fig. 3. Intestinal myofibroblasts are source of collagen in HIV disease. Anti-α-SMA+ mAbs (smooth muscle actin monoclonal antibodies) (clone 1A4) were used to detect myofibroblasts (shown in green), rabbit polyclonal fluorochrome conjugated anti-FAP Abs were used to analyze activation of IMFs (shown in red), collagen A deposition was detected by second harmonic generation (SHG) analysis (shown in blue) using two-photon microscopy. Increase in a 'filament' type fully formed collagen A in HIV-positive duodenal mucosa is adjacent to the α-SMA$^+$ IMFs (in green), which also express marker of activation, FAP, and result in formation of orange-yellow color on merged images. FAP, fibroblasts activation protein; IMF, intestinal myofibroblast.

fibrosis in duodenal tissue. We observed increased extracellular matrix deposition of collagen as detected by histopathologic measures using trichrome staining in a majority of duodenal samples and dual photon microscopy. Similar observations were previously reported by the Estes group for ileal lamina propria and Peyer patches [27]. Increased expression/production of procollagen and TGF-β1 in mucosal and lymph node tissue has been reported previously in the setting of both HIV-1 and macaque SIV infection in the cervix, lymph nodes, and GALT [64–68], demonstrating the systemic nature of HIV-associated mucosal inflammation. We were also able to demonstrate the impact of baseline profibrotic pathways on subsequent peripheral blood CD4$^+$ T-cell immune reconstitution. Our results, which focus on events at the GALT–gut lumen interface, identify a portal for systemic inflammation and by extension, immune activation in patients on effective cART.

Although previous studies have identified lymphoid T-regulatory and double-negative lymphocytes as a potential source for the TGF-β [25,66], we sought to pursue the potential role of widely distributed IMFs in the genesis of collagen deposition in GALT of HIV patients. This cell type is of particular interest in this regard as it is of the same lineage as the stellate cell in the liver and is known to play a key role in the fibrogenesis of other inflammatory bowel diseases such as Crohn's disease [46]. These stromal cells represent nonprofessional antigen-presenting cells which form a continuous interconnecting network under the epithelial layer of the entire gastrointestinal tract. Thus, they are ideally positioned to respond to microbial antigen translocation. When IMFs are stimulated with bacterial antigen, they increase in number and volume as demonstrated by α-SMA staining [69]. Indeed, we observed increased α-SMA staining in the duodenal tissues of HIV patients compared to control. We were also able to show co-localization of IMF and procollagen, supporting the hypothesis that these cells represent a source for profibrotic pathways and processes in GALT in HIV disease. To further pursue a sequence of events in the proposed pathway, we first identified whether an effector molecular receptor of bacterial antigen LPS signaling, TLR4, was present on

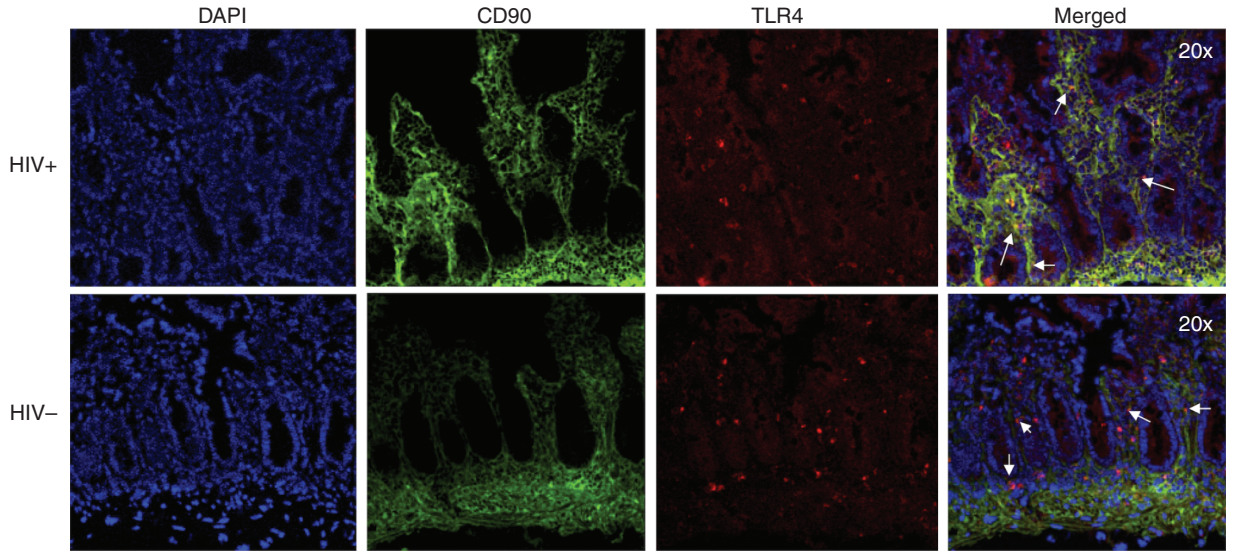

Fig. 4. Intestinal myofibroblasts are abundant in intestinal lamina propria of HIV-positive patients and express Toll-like receptor 4. Intestinal myofibroblasts (IMFs) are abundant in intestinal lamina propria of both HIV-negative and HIV-positive patients and express Toll-like receptor (TLR)4. Confocal microscopy analysis of longitudinally displayed duodenal sections. DAPI (4′,6-diamidino-2-phenylindole) was used to identify nuclei of cells (blue), antifibroblast marker CD90 mAbs (green), and fibroblasts activation protein (FAP) (red) was used to identify activated mesenchymal stromal fibroblasts/myofibroblasts in duodenal mucosa. TLR4 was detected using anti-TLR4 mAbs (clone HTA125). Faint staining for FAP and collagen A in HIV-negative tissue is typical for normal controls. Merged images demonstrated co-localization between CD90 and TLR4, yellow-orange coloring.

IMFs in the tissue samples from HIV patients. It is known from previous studies that there are increased plasma levels of LPS in HIV patients [30]. Although limited, the TLR4 staining was strongest on IMFs which are located in the subepithelial position – a location ideal for signaling microbial antigen translocation. Several pathogen-associated molecular patterns (PAMPs) recognition receptors have been detected previously on IMFs, and TLR2, 5, and 9 [45]. In the final set of experiments, we cultured IMFs from intestinal biopsy specimens and stimulated them with TLR4 agonists in order to assess whether TGF-β1 and IL-6 up-regulation could be detected. IL-6 is an important cytokine in the inflammation cascade and contributes to collagen deposition. It has been suggested to be a useful predictive marker for liver fibrosis in HIV-infected patients with alcohol addiction [70]. Consistent with animal models and other disease state reports [71,72], the IMFs had a significant increased expression of TGFβ-1 and IL-6, further supporting the proposed hypothesis. These data suggest that a complex of profibrotic molecules may be released by IMFs upon microbial stimulation in the HIV leaky gut which will require further investigation to verify.

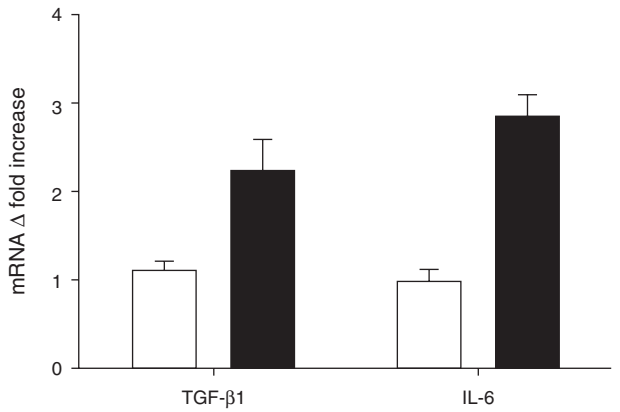

Fig. 5. Lipopolysaccharide stimulation of cultured intestinal myofibroblasts. Activation of primary culture of HIV-derived IMFs through TLR4 results in up-regulation of TGF-β1 and IL-6 expression. Open bars, IMF alone; solid bars, IMF stimulated with LPS, 1 μg/ml for 24 h; mRNA was analyzed using real-time RT-PCR. Relative fold increase was calculated using 18S as a housekeeping gene. The results represent mean of mRNA Δ fold increase of duplicates ± SE from three donors ($n = 3$). (*) $P < 0.05$. IL, interleukin; IMF, intestinal myofibroblast; TGF, tissue growth factor; TLR, Toll-like receptor.

A strong negative correlation was observed between baseline duodenal TGF-β expression and systemic immune reconstitution following 9 months of effective cART. A similar correlation was observed between baseline duodenal TGF-β expression and systemic naive $CD4^+$ T-cell percentages. Zeng *et al.* [25] have presented an important set of experiments demonstrating the impact of lymph node fibrosis and loss of normal architecture in the FRC network on IL-7 signaling which is necessary for naïve $CD4^+$ T-cell survival. The findings

reported herein provide a complimentary sequence of events that might be implicated in the incomplete immune reconstitution seen in up to 20% of patients receiving long-term successful cART. Recognizing that HIV is still detectable in tissue compartments [47], there does not appear to be a correlation between viral loads and failed immune reconstitution as might be expected if HIV-driven inflammation is the nexus for failed immune reconstitution [73]. Conversely, bacterial antigen present in high concentration adjacent to GALT may be altered in its composition favoring pro-inflammatory taxon [33,74,75]. These findings reveal a vital interaction between the gut microbiota and the mucosal immune system that calls for further investigation [75–77].

Intestinal myofibroblasts have been shown to impact T-regulatory cell activation and function [51], and are anatomically ideally situated to respond to microbial antigen translocation in the setting of HIV disease. Perhaps, these cells are what link failure of immune reconstitution despite virologic control. Although these present data do not prove, nor do they attempt to prove, a causal relationship between IMFs and up-regulated systemic profibrotic pathways, understanding the role of IMFs at the front gate of microbial antigen–mucosal immunity interactions may provide a rational strategy for restoring microbial communities and interrupting focal/systemic profibrogenic pathways. These novel findings in the small intestines of HIV patients may provide insights into alternative therapeutic opportunities to improve immune reconstitution and reduce associated morbidity.

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Conflicts of interest

The opinions expressed in this study are those of the authors and do not necessarily represent those of Merck & Co., Inc. The contents do not necessarily represent the views of the Department of Veterans Affairs or the United States Government.

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