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Discordance between peripheral and colonic markers of inflammation during suppressive ART

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Abstract

Objective—Persistent systemic inflammation is associated with the inability of some HIV-infected patients to normalize circulating CD4+ T-cell levels after years of suppressive antiretroviral therapy (ART). In this work, we sought to understand whether such systemic inflammation is also associated with detectable signs of inflammation in biopsies from the rectosigmoid colon.

Design—Immunologic and virologic parameters were studied in in the peripheral blood and in rectosigmoid colon biopsies from individuals with viral suppression for at least two years and with peripheral CD4+ T-cell levels <350 cell/mm^3 (immunologic nonresponders, n=18) or >500 cell/mm^3 (immunologic responders, n=16).

Methods—Peripheral blood and rectosigmoid colon biopsies were analyzed by flow cytometry, ELISA, and quantitative PCR.

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Results—Non-responders had elevated T-cell activation and inflammatory cytokines in the circulation, but inflammatory gene expression in colon biopsies was not different as compared to responders and there was little relationship between blood and colon markers of inflammation. Blood inflammatory markers were positively associated with sCD14 levels indicative of monocyte activation.

Conclusions—These findings demonstrate that, in the context of treated HIV disease, it is easier to detect parameters of inflammation (including blood monocyte activation) in the peripheral blood than in isolated rectosigmoid colon biopsies. Accordingly, interventions to block such inflammation in this population might be most conveniently and accurately assessed in blood.

Keywords

HIV; ART; CD4 T-cell recovery; inflammation; mucosal biopsy; microbial translocation

Introduction

Although HIV replication can be suppressed by antiretroviral therapy (ART) and most individuals on ART recover peripheral blood CD4+ T-cells and regain immunocompetence to some degree, the expected lifespan of treated individuals is shorter than that of the general population[1]. Moreover, among ART-treated, HIV-infected subjects, those with limited CD4+ T-cell recovery (immunologic nonresponders) have lifespans that are even shorter[2] and an increased incidence of non-AIDS-related complications (e.g., cardiovascular disease and neurocognitive decline)[3] compared to those with more complete CD4+ T-cell recovery (immunologic responders).

T-cell activation is associated with HIV disease and is recognized as a more accurate predictor of disease progression than is CD4+ T-cell count or viral load alone[4-6]. T-cell activation does not fully normalize after the initiation of ART and the extent of residual elevation is associated with poor CD4+ T-cell recovery[7,8]. In non-responders, T-cell activation is elevated in association with a lack of peripheral blood CD4+ T-cell recovery[9-11]. Such T-cell activation may be induced by inflammatory cytokines such as type 1 interferon (IFN)[12,13], tumor necrosis factor (TNF)[6], and/or interleukin 6 (IL-6)[11,14], which are elevated during untreated and treated HIV infection[10,11,14-20]. These inflammatory cytokines are elevated concomitantly with T-cell activation[11], consistent with the hypothesis that the lack of CD4+ T-cell recovery during ART may be caused by persistent inflammation. If so, it would be important to understand which inflammatory pathways are active in which sites during ART to facilitate the design of interventions to dampen inflammation and to thereby decrease the incidence of inflammation-associated non-AIDS complications.

Many of the inflammatory pathways associated with HIV disease are induced upon stimulation of innate immune cells by viral or bacterial products. Previous reports have conflicted as to whether residual virus burden is elevated in non-responders[19,21,22] while other reports have demonstrated that markers of microbial translocation, thought to arise as a result of HIV-induced intestinal immune dysfunction, are associated both with limited CD4+ T-cell gains on therapy and with T-cell activation[11,23-25]. These previous studies, however,

have presented only limited data from the gastrointestinal tract. Understanding what takes place in the intestinal mucosal tissues is essential to understanding persistent inflammation, as this is a site where HIV replicates during untreated infection[26,27], and where HIV RNA and DNA (and possibly virus replication) may persist during otherwise suppressive ART[28,29].

To understand whether gastrointestinal inflammation or HIV reservoirs are associated with the lack of CD4+ T cell repopulation or persistent T-cell activation during ART, we studied plasma, peripheral blood mononuclear cells (PBMC), and rectosigmoid colon biopsies from immunologic responders and non-responders, where CD4+ T cell levels and T cell activation levels are widely different in peripheral blood. Here, we confirmed prior observations that there are higher levels of T-cell activation, systemic inflammation, and sCD14 in non-responders than in responders[9-11], and analyzed rectosigmoid colon biopsies for evidence of inflammation and virus load. While T-cell activation tended to be higher in the mucosal tissue of non-responders, we found little evidence for a difference in colon inflammatory gene expression or HIV RNA or DNA levels between non-responders and responders. Rather, the inflammatory signature differentiating these two groups is strongest in the peripheral blood and is associated with plasma sCD14 levels. This observation, due either to non-intestinal determinants of inflammation or to the limited and possibly non-representative sampling afforded by isolated colonic biopsies, may be instructive in the design and implementation of future attempts to intervene against inflammation in non-responders.

Methods

Subjects

Subjects were recruited from the SCOPE[30] or OPTIONS cohorts at SFGH. All subjects provided informed consent and the protocol was approved by the UCSF Committee on Human Research. Subjects on ART had at least two years of plasma viral load <40 copies/mL and were assigned as immunologic non-responders or responders based on CD4+ T-cell levels (<350 cells/mm^3 or >500 cells/mm^3, respectively). Untreated subjects with viremia (>1000 copies/mL) or who were HIV seronegative were recruited as comparator groups. Subjects receiving immunomodulatory or immunosuppressive therapies and/or those with a recent acute illness were excluded.

Tissue collection

Peripheral blood was collected by venipuncture and mononuclear cells were isolated using ficoll-hypaque (GE Healthcare). Mucosal biopsies (26-30) from the rectosigmoid colon were obtained during sigmoidoscopy using jumbo biopsy forceps. Biopsies were preserved in RNAlater (Qiagen, Valencia, CA) for mRNA measurements (n=2-4), snap frozen for virologic analysis (n=6), or placed in RPMI (Life Technologies, Carlsbad, CA) for cell isolation (n=12-16). Cells were prepared from biopsies in RPMI by collagenase digestion (Sigma, St. Louis, MO) followed by mechanical disruption and filtration without density enrichment[31].

Gene and virus quantification

RNA was extracted from whole blood using the PaxGene Blood RNA Kit (Qiagen, Valencia, CA) or from biopsies frozen in RNAlater (Life Technologies). Biopsies were homogenized in Trizol (Life Technologies, Carlsbad, CA), and RNA purified using the RNeasy mini-kit (Qiagen). cDNA was prepared using Omniscript RT Kit (Qiagen, Valencia, CA). qPCR for blood-derived cDNA was carried out using TaqMan Gene Expression MasterMix (Applied Biosystems) in a StepOne Plus Real Time PCR system (Applied Biosystems). Commercially available primer-probe sets from Applied Biosystems were used: GBP1 Hs00977005_m1, IFI27 HSs00271467_m1, MX1 Hs00895601_m1, OAS1 Hs00973637_m1. In the case of biopsy specimens, gene expression was measured by Fluidigm (Fluidigm, South San Francisco, CA) with gene-specific pre-amplification using computationally designed primers (DELTAgene). Relative expression was calculated using the ΔCT method, using HPRT as housekeeping gene. HIV RNA and DNA levels were measured in Trizol extracts from snap-frozen biopsies, using quantitative PCR for the LTR[32,33].

Flow cytometry

Freshly isolated cells were stained with an antibody cocktail using standard procedures. Antibodies used were (target, {fluorophore, clone, dilution}): CD3 {QDot655, S4.1, 1:2500}; CD4 {QDot605, S3.5, 1:1000}; CD8 {QDot 705, 3B5, 1:1000} from Life Technologies, Green Island, NY; CD45RO {ECD, UCHL1, 1:50} from Beckman Coulter, Brea, CA; CD27 {PerCP-Cy5.5, M-T271, 1:50}; HLA-DR {FITC, L243, 1:25}, and CD38 {PE, HB7, 1:25} from BD Bioscience (San Jose, CA). Dead cells were stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life Technologies). Stained cells were acquired on an LSRII using FACSDiVa software (BD Biosciences) and analyzed using FlowJo software (Treestar, Ashland, OR). An example gating strategy is shown in Supplemental Figure 1.

Soluble biomarkers

Plasma levels of IL-6, TNF-RI, TNF-RII and sCD14 were measured by ELISA using commercially available kits (R&D Systems, Minneapolis MN).

Statistical analysis

Two sample comparisons were made using the Mann-Whitney U test while multi-sample comparisons were made using the Kruskal-Wallis test and Dunn's posts-tests, both performed in the Prism software package (GraphPad, San Diego, CA). Correlations were measured using the Spearman correlation coefficient in the JMP software package (SAS, Cary, NC).

Results

CD4+ T-cell levels in peripheral blood are not strongly related to CD4+ T cell levels in colon biopsies

Patients on ART with undetectable viremia for at least two years and who were either non-responders (<350 CD4+ T cells/mm^3, n=18) or responders (>500 CD4+ T cells/mm^3, n=16) were studied. For comparison, viremic untreated subjects (n=9) and HIV-uninfected subjects (n=23) were enrolled. From each of these subjects, peripheral blood and rectosigmoid colon biopsies were obtained, and assays were performed to quantify CD4+ T-cell levels, T-cell activation, inflammation, and HIV burden. Some assays were phased in over the course of the study, such that it was not possible to perform all measurements in all subjects. The number of subjects studied with each assay is indicated in the text and figure legends.

Confirming previous reports[34], non-responders had lower CD4+ T-cell nadirs than did responders (Supplemental Table 1) and had significantly lower blood CD4+ T-cell levels than did responders or uninfected subjects (Figure 1A). The differences in current CD4+ T-cell count reflect fewer cells gained over time during ART (responders: 71.1 cells/year; non-responders: 11.7 cells/year; p<0.01) rather than shorter followup (responders-median 9.3 years; non-responders 5.2 years; p=ns)(Supplemental Table 1). In contrast, and as previously demonstrated[35-38], both responders and nonresponders had lower levels of CD4+ T cells in the colonic mucosa than uninfected subjects, as measured by flow cytometry and related to either total recovered cells (Fig. 1B) or CD3+ T cells (Fig.1C). However, responders and non-responders had similar rectal CD4+ T cell frequencies. Responders, compared to non-responders, had significantly more CD8+ T cells in peripheral blood (Fig.1D) but significantly lower CD8+ T cell frequency among all live cells in colon (Fig.1E). CD8+ T cell frequency among CD3+ cells in the colon was not different between responders and non-responders (Fig.1F). Among ART-suppressed participants, there was no evidence for a relationship between peripheral blood CD4+ T-cell count or frequency and colon CD4+ T-cell frequencies as a fraction of all extracted live cells (Fig. 1G) or of CD3+ T cells (Fig. 1H) (blood CD4+ T cell frequency data not shown).

Peripheral and colon T-cell activation are correlated and inversely related to peripheral CD4+ T cell levels

The expression of CD38 and HLA-DR was measured on memory (excluding CD45ROCD27+ naïve cells) CD4+ and CD8+ T cells from PBMC and from colon in a subset of patients. In PBMC, the fraction of CD38+HLA-DR+ CD4+ and CD8+ T cells from nonresponders (n=12) was higher than that found in responders (n=7) (Figs. 2A and 2B, respectively), confirming previous reports[34]. In colon, the fraction of CD38+HLA-DR+ CD4+ T cells, but not of CD8+ T cells, was also elevated in non-responders compared to responders (Fig. 2C and 2D). Higher levels of PBMC (Fig. 2E) or colon (Fig. 2G) CD4+ T-cell activation correlated with lower peripheral blood CD4+ T cell counts. While higher peripheral blood and rectal CD8+ T-cell activation also tended to be associated with lower peripheral blood CD4+ T cell counts, these correlations did not achieve statistical significance (Figs. 2F, 2H, respectively). No relationship was observed between blood and

colon T-cell activation levels (Fig. 2I, CD4+ T cells p=0.11; Fig. 2J, rho=0.37, CD8+ T cells p=0.25, rho=0.28).

Systemic, but not colonic, inflammation is elevated in non-responders in correspondence with peripheral CD4+ T cell levels

To investigate the activity of the major inflammatory pathways that are increased during HIV infection, markers characteristic of the type 1 interferon[12,13], TNF[6], and/or IL-6[14] activity were examined in blood and rectosigmoid colon biopsy specimens. The type 1 interferon pathway was assessed by qRT-PCR analysis of the relative expression of a panel of interferon-stimulated genes (ISG), including myxovirus resistance gene-1 (MX1), 2′-5′-oligoadenylate synthetase-1 (OAS1), interferon alpha-inducible protein-27 (IFI27), and guanylate binding protein-1 (GBP1). Confirming another report using different ISG[10], the expression of each of these transcripts in peripheral blood was elevated in non-responders as compared to responders (Fig. 3A). The geometric mean of each of the four ISG relative expression values for each subject was calculated (ISG Geomean) to consolidate ISG expression into an index with the intent to minimize variability and derive a more consistent metric.[39] Blood ISG Geomean was higher in non-responders and untreated subjects than in either responders or uninfected subjects (Fig. 3A). ISG expression in the colon biopsies was significantly elevated in each HIV-infected group when compared to uninfected subjects, but was surprisingly not different between non-responders and responders (Fig. 3B). While ISG levels in colon were not different between the groups, there was a significant, direct correlation between blood and colon ISG Geomean levels (Fig. 3E, p= 0.037, rho=0.45).

Soluble TNF receptors (i.e., sTNF-RI and sTNF-RII, which are shed upon TNF binding) were measured in plasma as biomarkers of TNF activity. The difference in sTNF-RI and –RII levels did not reach statistical significance between responders and nonresponders (Fig. 3C). Levels of sTNF-RII in non-responders were, however, higher than those found in uninfected individuals and were more comparable to those observed in viremic subjects (Fig. 3C). TNF transcript levels were measured in colon and no significant differences were observed between responders and non-responders (Fig. 3C). TNF transcript levels in colon also had no correlation with sTNF-RI or sTNF-RII levels in blood (Fig. 3E, p=0.79, rho=0.06; p=0.63, rho=0.11; respectively, data not shown).

Finally, levels of plasma IL-6 protein and of IL-6 mRNA in colon were assessed and, while levels in responders were higher and levels in non-responders trended higher than those found in uninfected subjects, they were not statistically different between responders and non-responders (Fig.3D), nor was there a correlation between blood IL-6 levels and colon IL-6 mRNA expression levels (Fig. 3E, p=0.31, rho=-0.22, data not shown).

Relationships between the blood and colon inflammatory markers and CD4+ T-cell levels or T-cell activation levels in responders and non-responders were then studied (Fig. 3F). Plasma inflammatory markers were generally found to be inversely related to blood and colon CD4+ T-cell levels, with statistical significance reached in the inverse relationship between PBMC ISG expression and blood or colon CD4+ T-cell levels, and between plasma sTNF-RII and blood CD4+ T-cell levels (Fig. 3F). Conversely, colon IL-6 mRNA levels correlated directly with the percentage of CD4+ T cells among CD3+ T cells in colon. There

was also a general trend for plasma inflammatory markers to correlate directly with levels of PBMC and colon CD4+ T-cell activation, with statistical significance reached in the direct relationship between plasma IL-6 and colon CD4+ and CD8+ T-cell activation, and between colon ISG expression and colon CD8+ T-cell activation. Blood ISG expression levels approached significant correlations with PBMC CD4+ and CD8+ T-cell activation (p=0.057 and p=0.052, respectively). However, and surprisingly, there was no evidence for a relationship between colon inflammatory cytokine gene expression and blood CD4+ T-cell levels or activation (Fig. 2E).

No relationship was observed between colon virus levels and CD4+ T cell levels or inflammation

To understand whether HIV reservoirs or residual virus production in the intestine may contribute to systemic inflammation and the lack of CD4+ T-cell recovery on ART, HIV RNA and DNA were assessed in colon biopsies. HIV RNA and DNA copies were measured in whole biopsies (two biopsies per subject) and normalized to cell equivalents by nucleic acid input. As expected, untreated subjects (n=9) had higher levels of HIV RNA than did either responders (n=7) or non-responders (n=16); however, no evidence for a difference in HIV RNA levels was observed between responders and non-responders (Fig. 4A). Likewise, no difference was observed between responders and non-responders in HIV DNA levels (Fig. 4B) or the ratio of HIV RNA copies to DNA copies (Fig. 4C). No associations were observed between HIV levels and CD4+ T-cell levels or T-cell activation in colon or PBMC, plasma inflammatory markers, or inflammatory gene expression in PBMC or in the colon (Fig. 4D).

Plasma soluble CD14 is elevated in non-responders in association with inflammation and colon T-cell activation

Since microbial translocation has been linked to systemic inflammation during chronic HIV infection[24], plasma sCD14 (shed by monocytes and macrophages in response to LPS[40]) was measured as an indirect marker of microbial translocation. Plasma sCD14 levels from non-responders were significantly higher than levels in uninfected individuals, but the difference between responders and non-responders did not reach statistical significance (Fig. 5A). However, sCD14 levels did correlate inversely with peripheral CD4+ T-cell levels (Fig. 5B), a relationship that was found to be stronger when non-responders were considered separately (non-responders p=0.0014; responders p=0.75). Plasma sCD14 levels correlated directly with markers of colon T-cell activation and systemic inflammation, but a relationship was not observed with colon inflammatory gene expression (Fig. 5B).

Discussion

Despite long-term suppression of HIV replication, non-responders are more susceptible to non-AIDS complications and have a shorter lifespan than uninfected individuals[2]. This clinical state is associated with higher levels of immune activation and inflammation[3], potentially due to a fundamental immunologic defect acquired before the initiation of therapy.[41] A proposed cause of this systemic inflammation is microbial translocation across a persistently damaged intestinal barrier[24], thought to be related to inflammation in the

intestine. To understand whether blood immune activation and inflammation are associated with inflammation or residual HIV in the intestinal mucosa in the context of treated HIV disease, blood and rectosigmoid colon biopsy specimens were obtained from HIV-infected subjects on suppressive ART who met criteria as nonresponders and responders, as well as from viremic, untreated HIV-infected subjects and uninfected subjects. T-cell activation and peripheral inflammatory markers were confirmed to be elevated in non-responders and PBMC ISG expression was found to be one of the strongest signatures differentiating non-responders from responders. Colonic HIV RNA and DNA levels or expression of inflammatory cytokines, on the other hand, did not appear to differ between non-responders and responders in this small study. Importantly, colon levels of HIV or inflammation also do not appear to correlate with levels of CD4+ T cells, T-cell activation in the peripheral blood, or with plasma inflammatory mediators. Despite the lack of colonic inflammatory or virologic differences measured here, monocyte activation (as measured by plasma sCD14, potentially indicative of microbial translocation) was inversely correlated with blood CD4+ T-cell levels and directly related to colon T-cell activation and plasma inflammatory cytokine levels,[11,25] markers that were elevated in non-responders.

This study has several limitations that will be important to address in future studies. First, its design did not control for CD4 nadir, a parameter that was found to be different between responders and non-responders at baseline (Supplemental Table 1), leaving open the possibility that the immune system was more damaged in non-responders prior to the initiation of ART and that such damage alone limited CD4+ T-cell recovery. In addition, the study was performed over the course of three years, during which time assays and concepts evolved, and some measurements were made only in subgroups of patients. The colon immunologic and virologic parameters were also measured in small pinch biopsies, which may not be representative of the tissue as a whole, introducing potentially significant sampling error, particularly since lymphoid aggregates likely have very different cell phenotypes and concentrations of virus than the surrounding lamina propria. Similarly, biopsies were taken only from the distal colon, which is likely not representative of the entire intestine.[28] Finally, this study was relatively small and the findings herein, particularly those negative findings related to colon inflammation and HIV levels, should be considered with caution due to limited statistical power.

Notwithstanding these shortcomings, our findings raise several points. First, we found that elevated levels of inflammation were more evident in the peripheral blood than in the rectosigmoid colon biopsies of non-responders compared to responders, despite the finding that colon ISG and IL-6 expression levels were higher in both groups relative to uninfected subjects. This observation is - at least on the surface - inconsistent with the hypothesis that local inflammation in the intestinal mucosa is proximally related to (and potentially causative of) microbial translocation and systemic inflammation. The finding, however, has a number of potential non-mutually exclusive interpretations and implications: 1) the rectosigmoid colon may not be a prominent location of breaches permitting microbial translocation across the intestinal epithelium as compared to other regions of the intestine;[28] 2) breaches may be present in the distal colon, but are highly focal and difficult to sample by a limited set of biopsies; 3) translocated microbial products are not immunostimulatory in the intestine itself, but only when they reach more systemic sites such as the mesenteric

lymph node, liver, or systemic lymphoid tissues; and/or 4) the discordance between blood and colon inflammation may indicate that microbial translocation into the lamina propria is ongoing in both responders and non-responders but is contained, e.g., by a “firewall” of meseteric lymph nodes,[42] in responders, preventing the systemic spread of microbial products and inflammation to systemic sites that is seen in non-responders. Detailed investigation of the dynamics of virus production, microbial products, and inflammatory mediators in various tissue compartments would pinpoint the source and cause of systemic inflammation during treated and untreated lentiviral infection.

Secondly, systemic levels of activation (e.g., elevated CD38 and HLA-DR expression on circulating CD4+ and CD8+ T cells) are more readily correlated with circulating sCD14 levels than with the levels of HIV RNA or DNA found in rectosigmoid colon biopsies, suggesting that systemic T-cell activation may be driven more by microbial translocation than by HIV expression in the rectosigmoid colon. This observation could, on the other hand, be attributed to the likely production of HIV in other regions of the intestine[28] or in non-intestinal sites such as lymph nodes, spleen, or blood, and that such virus may be more related to systemic inflammation than virus in the distal colon; alternatively, it may simply reflect the non-representative nature of sampling that attends collection of biopsies from circumscribed areas of intestine that have variable cellular composition. It also remains possible that responders and non-responders are fundamentally different in the relationship between virus and inflammation, and that different results may be obtained when considering a larger group of non-responders alone. This study was particularly underpowered to detect relationships within the subgroups. Future studies that measure intestinal viral reservoir on extracted and sort-purified HIV target cells -and in a larger number of subjects with longitudinal design - will be important to confirm the present findings.

In sum, our findings support the hypothesis that systemic inflammation and monocyte activation are linked to limited CD4+ T-cell recovery during ART. This hypothesis indicates that interventions that diminish inflammation may improve CD4+ T-cell recovery during ART, limit the incidence of non-AIDS disease, and improve the expected lifespan among treated patients. Not least, the ability to measure such markers in the circulation provides a convenient sampling point to use when testing the efficacy of interventions to block inflammation *in vivo*. Finally, since differential inflammation or virus levels in rectosigmoid colon biopsies were not observed between responders and non-responders, these results also demonstrate that larger studies with systematic investigation of multiple tissues to identify the source of inflammation and studies with a longitudinal design may lead to enhanced understanding of the mechanisms responsible for AIDS immunopathogenesis and to novel strategies for clinical management.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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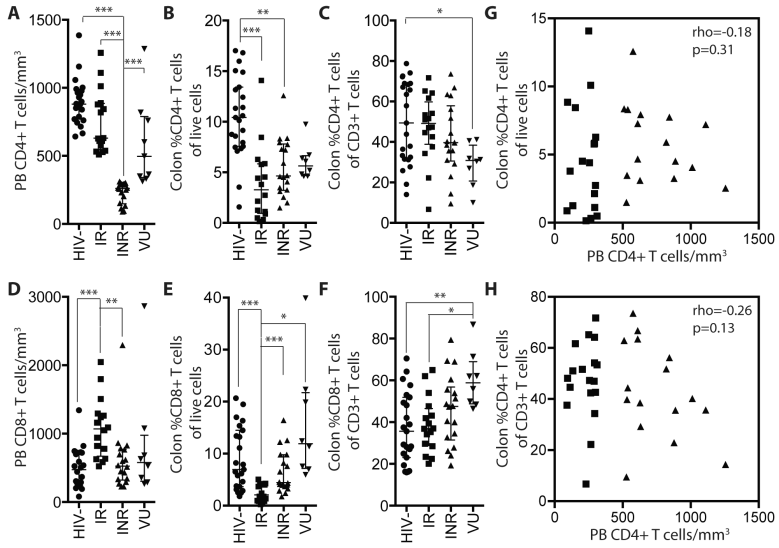

Figure 1. CD4+ T-cell levels in peripheral blood are not strongly related to CD4+ T-cell levels in colon biopsies

CD4+ (A) or CD8+ (D) T-cell counts per mm^3 in peripheral blood (PB) were measured by flow cytometry. CD4+ (B, C) or CD8+ (E, F) T-cell levels in colon were measured by flow cytometry and expressed as the percentage of CD4+ T cells among all live cells (B, E) or among CD3+ T cells (C, F). Correlations were tested between blood and either the percentage of colon CD4+ T cells among live cells (G) or among CD3+ T cells (H), using the Spearman correlation coefficient. For blood and tissue CD4+ T-cell measurements, the following number of subjects was studied: uninfected (HIV-, n=23), responder (IR, n=16), non-responder (INR, n=18), and viremic (VU, n=9) subjects. Bars indicate median and whiskers indicate inter-quartile range. *p<0.05, **p<0.01, ***p<0.001

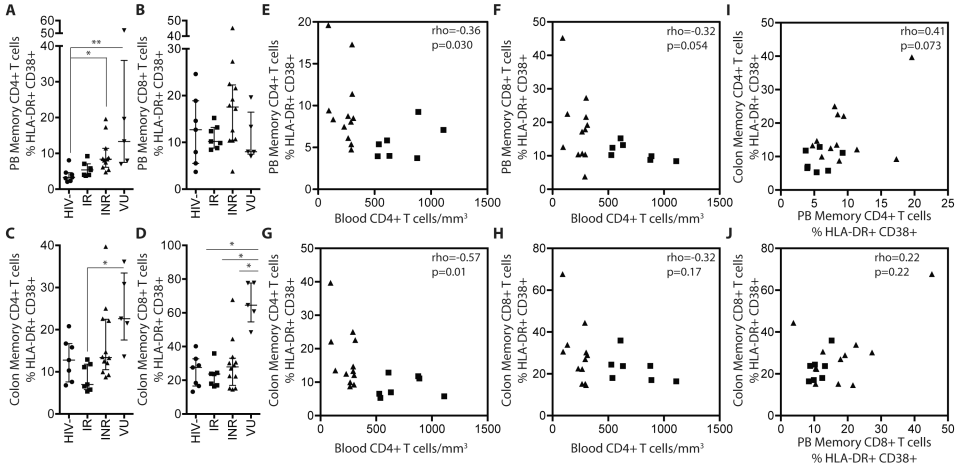

Figure 2. Non-responders have elevated levels of peripheral and colon T-cell activation

PBMC (A, B, E, F) and rectosigmoid colon cells (C, D, G, H) were stained with a panel of fluorescent monoclonal antibodies to identify T-cell memory subsets (CD45RO, CD27) and T-cell activation (HLA-DR, CD38). Naïve T cells (CD45RO-CD27+) were excluded and the fraction of memory CD4+ (A, C, E, G) or CD8+ (B, D, F, H) T cells co-expressing HLA-DR and CD38 was measured. T-cell activation levels were compared between responders and non-responders using the Mann-Whitney U test (A, B, C, D) or were tested for correlations with blood CD4+ T-cell levels (E, F, G, H), using the Spearman correlation coefficient. CD4+ (I) or CD8+ (J) T cell activation levels in blood versis colon were tested for correlations using the Spearman correlation coefficient. For T-cell activation measurements, the following number of subjects were studied: nonresponders (INR, n=12) and responders (IR, n=7). Bars indicate median and whiskers indicate inter-quartile range. (p=ns indicates p value greater than 0.05).

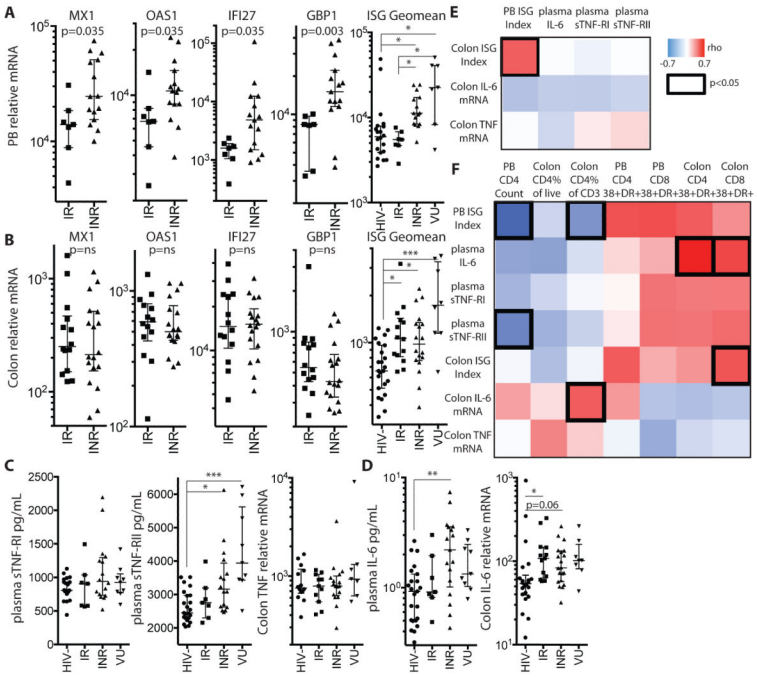

Figure 3. Markers of systemic inflammation are elevated in non-responders

mRNA for canonical interferon-stimulated genes (MX1, OAS1, IFI27, and GBP1) was measured in whole blood (A) or colon (B), and expression of each gene or the geometric mean expression of all four genes together (ISG Geomean) was compared between responders and non-responders using the Mann-Whitney U test. Plasma sTNF-RI and sTNF-RII levels as well as colon TNF mRNA levels (C) or plasma IL-6 and colon IL-6 mRNA levels (D) were compared between responders and non-responders using the Mann-Whitney U test. Correlations between markers of systemic inflammation versus colon inflammation (E) or between all inflammatory markers and blood or colon CD4+ T-cell levels or T-cell activation (F) were tested using the Spearman correlation coefficient, and the results summarized in a graphic where the rho value for the correlation between the indicated column parameter and row parameter is indicated by blue (negative) to red (positive) shading in the box representing the intersection of these labels and correlations with $p<0.05$ indicated by a heavy box. Blood gene expression values are from responders (IR, n=7) and non-responders (INR, n=15); colon gene expression values from responders (IR, n=14) and non-responders (INR, n=18); and plasma biomarker values from responders (IR, n=7) and non-responders (INR, n=16). Correlations in E and F include any subjects with values for both parameters listed: responders (n=7) and non-responders (n=12). Where not indicated, p-values for Dunn's post-tests are greater than 0.05. Bars indicate median and whiskers indicate inter-quartile range. (p=ns indicates p value greater than 0.05)

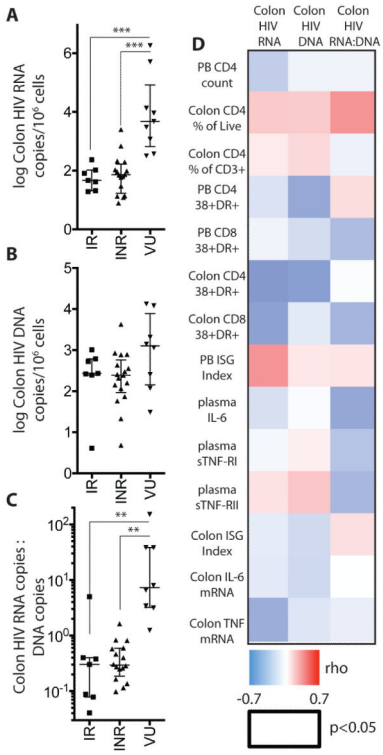

Figure 4. No difference was observed between colon virus levels in responders and non-responders

Virus RNA (A), DNA (B), or the ratio of RNA to DNA (C) were measured in colon by quantitative PCR and compared between the groups using the Kruskal-Wallis test and Dunn's post-tests. Virus measurements were tested for correlations with indicated parameters (D) using the Spearman correlation coefficient, and the results summarized in a graphic format where the rho value is indicated by blue (negative) to red (positive) shading and correlations with $p<0.05$ indicated by a heavy box. Virus measurements are from viremic (VU, n=9), responder (IR, n=7), and non-responder (INR, n=16) subjects. Correlations in D include all responders or non-responders where values are present for both parameters indicated (typically n=7 IR and n=10 INR). Where not indicated, p-values for Dunn's post-tests are greater than 0.05. Bars indicate median and whiskers indicate inter-quartile range. **p<01, *** p<0.001

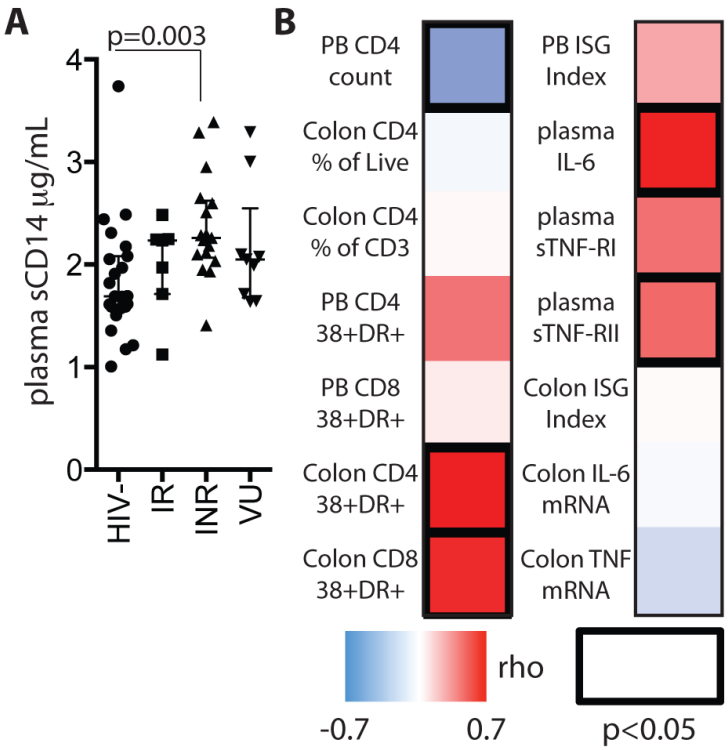

Figure 5. Monocyte activation is elevated in non-responders in association with inflammation and colon T-cell activation

Plasma sCD14 was measured in cryopreserved plasma using a commercially available ELISA kit and compared between uninfected (HIV-), viremic (VU), responders (IR), and non-responders (INR) using the Kruskal-Wallis test and Dunn's post-tests (A). Plasma sCD14 levels were tested for correlations with the indicated parameters (B) using the Spearman correlation coefficient, and the results summarized in a graphic format where the rho value is indicated by blue (negative) to red (positive) shading and correlations with $p<0.05$ indicated by a heavy box. Plasma sCD14 was measured in uninfected (HIV-, n=23), viremic (VU, n=9), responder (IR, n=7), and non-responder (INR, n=17) subjects. Correlations in B include all responders or non-responders where values are present for both parameters indicated. Where not indicated, p-values for Dunn's post-tests are greater than 0.05. Bars indicate median and whiskers indicate inter-quartile range.