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Human Immunodeficiency Virus–Infected Immunological Nonresponders Have Colon-Restricted Gut Mucosal Immune Dysfunction

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Background. Human immunodeficiency virus (HIV)–infected immunological nonresponders (INRs) fail to reconstitute their $CD4^+$ T-cell pool after initiation of antiretroviral therapy, and their prognosis is inferior to that of immunological responders (IRs). A prevailing hypothesis is that the INR phenotype is caused by a persistently disrupted mucosal barrier, but assessments of gut mucosal immunology in different anatomical compartments are scarce.

Methods. We investigated circulating markers of mucosal dysfunction, immune activation, mucosal Th17 and Th22 cells, and mucosa-adherent microbiota signatures in gut mucosal specimens from sigmoid colon and terminal ileum of 19 INRs and 20 IRs in addition to 20 HIV-negative individuals.

Results. INRs had higher blood levels of the enterocyte damage marker intestinal fatty acid–binding protein than IRs. In gut mucosal biopsies, INRs had lower fractions of $CD4^+$ T cells, higher fractions of interleukin 22, and a tendency to higher fractions of interleukin 17–producing $CD4^+$ T cells. These findings were all restricted to the colon and correlated to circulating markers of enterocyte damage. There were no observed differences in gut microbial composition between INRs and IRs.

Conclusions. Restricted to the colon, enterocyte damage and mucosal immune dysfunction play a role for insufficient immune reconstitution in HIV infection independent of the gut microbiota.

Keywords. HIV; immunological nonresponders; mucosal immunology; gut microbiota.

A compromised mucosal barrier with damaged enterocytes accompanies the depletion of gut mucosal $CD4^+$ T-cell subsets T-helper (Th) 17 and Th22 in untreated human immunodeficiency virus (HIV) infection [1–3]. The damaged epithelial barrier allows luminal contents to enter the lamina propria in a process often referred to as microbial translocation [4]. Microbial translocation induces focal gut inflammation and is associated with systemic inflammation [5, 6], which predisposes to non-AIDS morbidity and mortality [7–9].

In parallel with the disruption of the gut mucosal barrier, there is a shift to an HIV-associated dysbiotic composition of the gut microbiota [10]. This dysbiosis is characterized by a lower microbial diversity, lower abundance of beneficial short chain fatty acid–producing bacteria, and higher abundance of Gammaproteobacteria with proinflammatory potential [10, 11]. The dysbiosis is associated with gut mucosal and systemic immune activation, inflammation, and metabolic syndrome and negatively associated with gut mucosal interleukin (IL) 17 and IL-22 secretion [11, 12].

In most patients, antiretroviral therapy (ART) stops disease progression and the circulating $CD4^+$ T-cell pool reconstitutes. Data indicate that ART does not fully reverse gut dysbiosis [12]. Still, gut mucosal $CD4^+$ T-cell populations, including the Th17 and Th22 subsets, are to a large extent restored and microbial translocation and systemic inflammation reduced [2, 4, 11, 13–15]

In 12%–30% of ART-treated people with HIV (PWH), the circulating $CD4^+$ T cells do not recover despite full viral suppression [16–19]. These patients, commonly termed immunological nonresponders (INRs), have increased risk of chronic inflammation, immune activation, immune failure, non-AIDS

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morbidity, and mortality [20–26]. The etiology of their incomplete immune recovery has remained enigmatic [27, 28]. Studies on mucosal barrier function have indicated an association between INR phenotype and alterations in tight junction proteins and intestinal epithelial cell turnover [6, 29]. However, there is a lack of studies on gut specimens designed to target the hypothesis that INRs have a dysfunctional mucosal immunity compared with PWH immunological responders (IRs), independent of factors known to predispose to their disease phenotype (ie, old age, long duration of HIV infection, and low nadir CD4 count) [27]. Increased understanding of the immune mechanisms behind the unfavorable prognosis of INR could identify potential therapeutic targets adjuvant to ART and improve the health of PWH.

We collected mucosal biopsies from both the sigmoid colon and the terminal ileum from a cohort of INRs and compared them with IRs to assess mucosal $CD4^+$ T-cell function focusing on IL-17 and IL-22 response. Furthermore, we assessed if alterations in mucosal T-cell function were associated with a mucosal-adherent gut microbial signature.

MATERIALS AND METHODS

Study Design and Participants

PWH were identified in the outpatient clinic of the Department of Infectious Diseases, Oslo University Hospital's patient registry and checked for eligibility. Inclusion criteria were white males, aged 25–65 years, >4 years HIV seropositive, on continuous ART, and with HIV RNA <50 copies/mL continuously for >3.5 years. INRs were defined with $CD4^+$ T-cell count <400 cells/μL continuously for >3.5 years, and IRs were defined with $CD4^+$ T-cell count >600 cells/μL continuously for >3.5 years. HIV-negative controls were recruited from males aged 25–65 years referred to the Department of Gastroenterology outpatient colonoscopy service for control of polyps and who were confirmed HIV seronegative. All participants were age matched (± 5 years), and enrolled INRs and IRs were matched on nadir $CD4^+$ T-cell count (± 20 cells/mL when INR nadir was <100 cells/mL, ± 50 cells/mL when INR nadir was >100 cells/mL). See Supplementary Figure 1 for the recruitment procedure scheme and the Supplementary Methods for exclusion criteria. Nineteen INRs, 20 IRs, and 20 HIV-negative controls were included in the analyses. Study participants' key characteristics are presented in Table 1.

Lamina Propria Mononuclear Cell Preparation, Mitogen Stimulation, and Flow Cytometry

All study participants underwent colonoscopy with pinch biopsies. The biopsies were enzymatically digested with Collagenase blend type H (1 mg/mL final concentration, Sigma-Aldrich, Darmstadt, Germany) and DNase I (20 U/mL final concentration, Invitrogen, Carlsbad, California). Dissolved cells were frozen and stored at –150°C until use.

Thawed and rested lamina propria mononuclear cells were stimulated for 12 hours with phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Saint Louis, Missouri) and ionomycin (Sigma-Aldrich) and after 1 hour in the presence of BD Golgiplug (BD Biosciences, San Jose, California). The cells

Table 1. Characteristics of Study Participants

Characteristic	INR (n = 19)	IR (n = 20)	HIV Negative (n = 20)	*P* Value
Age, y, median (IQR)	49.6 (43.9–58.9)	52.5 (48.2–59.3)	54.8 (50.7–59.2)	.36[a] .55[b]
Nadir $CD4^+$ T-cell count, cells/μL, median (IQR)	90 (22–157)	101 (31–178)	N/A	.53[b]
BMI, kg/m^2, median (IQR)	25.5 (23.5–27.1)	25.6 (23.2–27.2)	25.7 (24.5–27.4)	.68[a] .97[b]
Time since HIV seroconversion, y, median (IQR)	10.2 (7.3–21.8)	18.2 (11.8–24.9)	N/A	.054[b]
$CD4^+$ T-cell count at enrollment, cells/μL, median (IQR)	327 (269–374)	777 (690–867)	N/A	<.001[b]
CD8+ T-cell count at enrollment, cells/μL, median (IQR)	574 (439–954)	781 (565–933)	N/A	.28[b]
CD4/CD8 ratio at enrollment, median (IQR)	0.48 (0.34–0.74)	1.00 (0.74–1.21)	N/A	<.001[b]
Risk group, frequency	16 MSM 2 MSW 1 unknown	18 MSM 2 unknown	20 unknown	N/A[c]
ART regimen at enrollment, frequency	2 INSTI 11 NNRTI 5 PI 1 PI + INSTI	12 INSTI 5 NNRTI 2 PI 1 PI + NNRTI	N/A	N/A[c]

Abbreviations: ART, antiretroviral therapy; BMI, body mass index; HIV, human immunodeficiency virus; INR, immunological nonresponder; INSTI, integrase strand transfer inhibitor; IQR, interquartile range; IR, immunological responder; MSM, men who have sex with men; MSW, men who have sex with women; NA, Institute of Clinical Medicine; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor; y, years.

[a]Kruskal–Wallis test.

[b]Mann–Whitney *U* test, INR vs IR.

[c]Assumptions for χ^2 test not met.

were then stained and assessed on a BD FACSCanto II (BD Biosciences) flow cytometer. All flow cytometry data were analyzed with FlowJo Software (version 10.5.3, Ashland, Oregon). Th22, Th17, and Th1 cells were identified as $CD45^+$live lymphoid singlets $CD3^+\gamma\delta TCR^-CD8^-IL\text{-}22^+/IL\text{-}17^+/IFN\text{-}\gamma^+$, respectively. The gate was set on the unstimulated control and applied on the stimulated sample for each study subject. The percentage of cytokine-positive cells was reported as net values with unstimulated control values removed. See the Supplementary Methods for detailed description of procedures.

Lymphocyte Activation Assessments

Lamina propria mononuclear cells and peripheral blood mononuclear cells (PBMCs) were thawed and rested as described in the Supplementary Methods. Next, 5.0×10^5 cells were stained with surface markers before acquisition on a BD LSR Fortessa (BD Biosciences) flow cytometer. See the Supplementary Methods for antibodies and fluorochromes applied and representative flow cytometry gating for activation markers on PBMCs (Supplementary Figure 2) and lamina propria mononuclear cells (Supplementary Figure 3).

Soluble Markers

Enzyme-linked immunosorbent assays were used for analysis of ethylenediaminetetraacetic acid plasma for CD14, lipopolysaccharide binding protein, lipopolysaccharide, and regenerating islet-derived protein 3 alpha (REG3α), of serum for IL-6, C-reactive protein, CD163, Zonulin, IL-18, and intestinal fatty acid–binding protein (I-FABP), and citrated plasma for D-dimer. See the Supplementary Methods for details.

16S Ribosomal RNA Gene Sequencing

Libraries were generated by amplification of the V3–V4 region of the 16S ribosomal RNA gene of DNA extracted from gut mucosal biopsies and from stool samples, and sequenced on the Illumina MiSeq platform (Illumina, San Diego, California). Reads were processed with Quantitative Insights Into Microbial Ecology (QIIME) 1.9.1 using SortMeRNA 2.0 and mapping to the Silva 128 database databases. For detailed description, see the Supplementary Methods.

Statistical Analysis

Data were assumed not to be normally distributed. According to the project's protocol, analyses with 3 experimental groups were performed by Kruskal–Wallis test. Mann–Whitney *U* tests between INRs and IRs were performed in parallel and independent of the Kruskal–Wallis test, whereas no direct comparisons between INRs or IRs and HIV-negative controls were performed. Analyses of differences in relative abundance between phylogenetic phenotypes were performed in LEfSe [30]. Subsequent Benjamini–Hochberg calculation of false-discovery rate and permutational multivariate analysis of variance test for comparison of microbial β-diversity were done in R (https://www.r-project.org/). All other statistical analyses were performed using Prism 8 software (GraphPad, La Jolla, California). All test values are printed in relevant tables or figures.

Ethical Consideration

The study was approved by Regional Committee for Medical and Health Research Ethics (approval identifier 2015/2125) and Oslo University Hospital's data protection officer and conducted in accordance with the Declaration of Helsinki and International Conference on Harmonisation/Good Clinical Practice. All participants received verbal and written information about the study and signed informed consent prior to enrollment.

RESULTS

Immunologic Nonresponders Have Signs of Enterocyte Damage, but No Evidence of Systemic Inflammation or T-Cell Activation

I-FABP levels were higher in INRs than IRs (2089 vs 1279 pg/mL, $P = .014$; Figure 1A). In addition, levels of REG3α, a novel marker of enterocyte damage [31], tended to be higher in INRs than IRs (7196 vs 4811 pg/mL, $P = .11$; Figure 1B). Levels of I-FABP and REG3α correlated within PWH ($r = 0.49$, $P < .01$; Figure 1C), but not within the HIV-negative controls (Figure 1D). Assessment of other markers of inflammation and microbial translocation did not reveal significant differences between INRs and IRs (Supplementary Table). Assessed by the flow cytometric expression of CD38 and HLA-DR on PBMCs, this cohort of PWH showed no differences in $CD4^+$ or $CD8^+$ T-cell activation between INRs and IRs (Supplementary Figure 4).

Mucosal T-Cell Fractions and Activation Is Altered in the Colon of Immunlogical Nonresponders

We next examined lamina propria mononuclear cells and evaluated the fraction of $CD4^+$ T cells of all mucosal T cells by flow cytometry. In the sigmoid colon, but not in the terminal ileum, INRs had a significantly lower fraction of mucosal $CD4^+$ cells compared to the IRs (Figure 2A and 2B), indicating a colon-specific difference between the 2 HIV-infected groups. This notion was supported by a significant correlation between mucosal $CD4^+$ T-cell fractions in sigmoid colon and terminal ileum among the HIV-negative controls ($r = 0.56$, $P = .021$) that was not found in PWH (Figure 2C and 2D). Among INRs there were no positive correlations between blood CD4 count and mucosal $CD4^+$ T-cell fractions (Supplementary Figure 5). Moreover, in the sigmoid colon of PWH, but not in the terminal ileum, there was an inverse correlation between mucosal $CD4^+$ T-cell fractions and I-FABP levels ($r = -0.38$, $P = .023$; Supplementary Figure 6).

In a separate flow cytometry analysis of lamina propria mononuclear cells to determine if INRs had altered mucosal T-cell activation compared to IRs in sigmoid colon or terminal ileum,

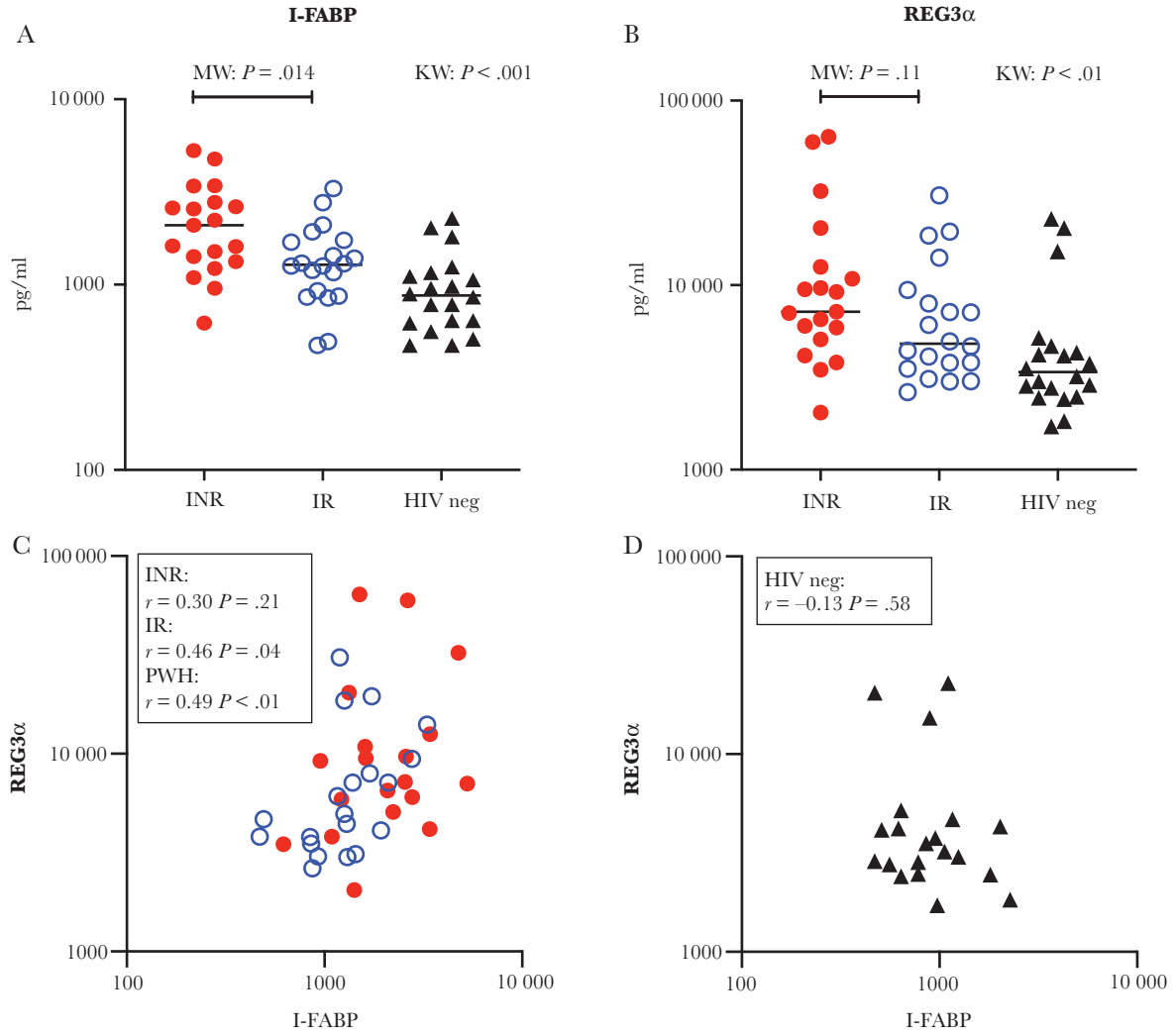

Figure 1. Higher level of enterocyte damage in immunological nonresponders (INR). *A*, Serum concentration of intestinal fatty acid–binding protein (I-FABP). *B*, Plasma concentration of regenerating islet-derived protein 3 alpha (REG3α) analyzed by enzyme-linked immunosorbent assay in INR compared to immunological responders (IR); human immunodeficiency virus (HIV)–negative individuals (HIV neg) served as controls. Horizontal bars represent median values. Kruskal–Wallis (KW) and Mann–Whitney (MW) test values. Spearman correlation between I-FABP and REG3α in people living with HIV (INR: dots, IR: open circles) (*C*) and HIV-negative individuals (triangles) (*D*).

we found that INRs had a higher fraction of mucosal $CD38^+$ $CD8^+$ T cells in sigmoid colon than IRs, but no difference in $CD38^+HLA\text{-}DR^+CD8^+$ T cells (Supplementary Figure 7). In contrast, there were no difference in $CD38^+CD8^+$ T cells between INRs and IRs in terminal ileum. Within mucosal $CD4^+$ T cells, there were no detectable differences in immune activation assessed by CD38 or CD38/HLA-DR double expression between INRs and IRs in either anatomical compartment (Supplementary Figure 7).

Mucosal Cytokine Responses Differ Between Immunological Nonresponders and Immunological Responders in Sigmoid Colon but Not in Terminal Ileum

We then assessed the mucosal T cells' capacity to secrete IL-17 and IL-22. Lamina propria mononuclear cells from sigmoid colon and terminal ileum were stimulated with PMA and ionomycin and analyzed by flow cytometry with regard to IL-17 and IL-22 response (Figure 3A). In the sigmoid colon, the median fractions of IL-22^+ and IL-17^+ $CD4^+$ T cells were about 1.5 and 2 times higher in INRs compared to IRs, respectively (IL-22: 16.2% vs 10.3 %, $P = .04$; IL-17: 13.9% vs 6.8%, $P = .06$) (Figure 3B). There was no significant difference between INRs and IRs in interferon-gamma (IFN-γ) response, but both groups had lower median IFN-γ response than the HIV-negative controls. Moreover, in the sigmoid colon of INRs, $CD4^+$ T cells' IL-17 and IL-22 responses correlated with the $CD4^+$ T-cell fractions (IL-17: $r = 0.49$, $P = .058$; IL-22: $r = 0.58$, $P = .019$). The same correlations were not observed in IRs (Figure 3C).

In the terminal ileum, there were no signs of a different response between INRs and IRs with respect to IL-17, IL-22, or

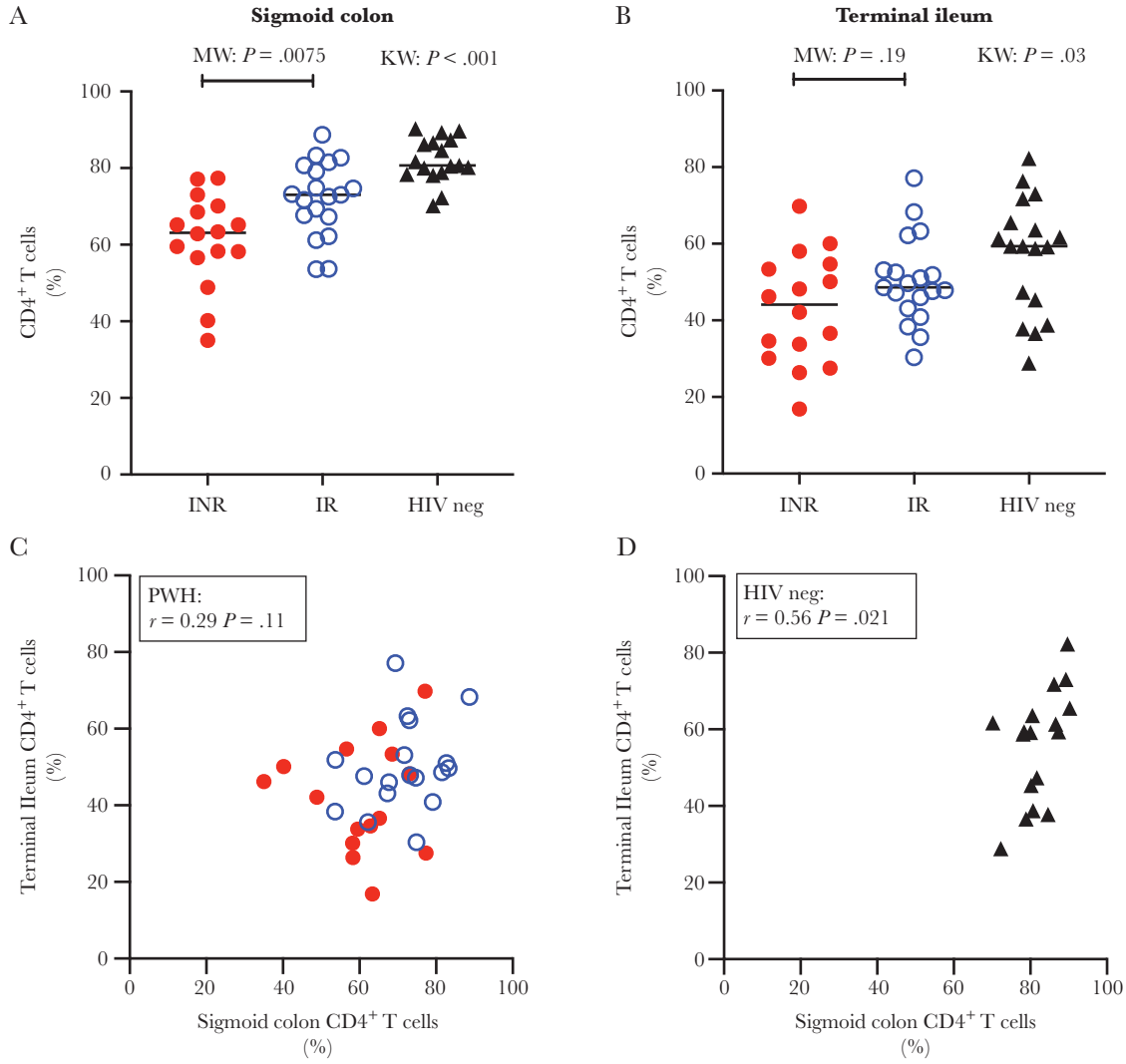

Figure 2. Immunologic nonresponders (INR) have lower sigmoid colon CD4$^+$ T-cell fractions that correlate with enterocyte damage. Fractions of CD4$^+$ T cells of total T cells analyzed by flow cytometry on lamina propria mononuclear cells in sigmoid colon (*A*) and terminal ileum (*B*) of INR compared to immunological responders (IR); human immunodeficiency virus (HIV)–negative individuals (HIV neg) served as controls. Horizontal bars represent median values. Kruskal–Wallis (KW) and Mann–Whitney (MW) test values. Spearman correlation between CD4$^+$ T-cell fractions of total T cells in sigmoid colon and terminal ileum in people living with HIV (INR: dots, IR: open circles) (*C*) and HIV-negative individuals (triangles) (*D*).

IFN-γ (Figure 3B). In mucosal CD8$^+$ T cells, the IL-17 and IL-22 responses were generally low and with no differences between INRs and IRs for any of the cytokines (data not shown).

Mucosa-Associated and Fecal Microbial Composition Is Similar in Immunological Nonresponders and Immunological Responders

We hypothesized that the gut microbiota may be linked to the observed enterocyte damage and altered CD4$^+$ T-cell function in INRs. There were no differences between INRs and IRs in any of the compartments for either intra-individual (α-diversity) (Figure 4A) or global microbiota composition (β-diversity) (Figure 4B). Furthermore, there were no differences in relative abundance between INRs or IRs at the phylum, family (Figure 4C), or genus level (data not shown), in any of the anatomical compartments. However, both groups differed from the HIV-negative controls, as evaluated by β-diversity measures (Figure 4B). Within PWH, there were individuals who were characterized by enrichment of Brachyspiraceae (Spirochaetae phylum) in sigmoid colon, but there were no significant differences between INRs and IRs with regard to these taxonomic units (Figure 4C).

In Immunological Nonresponders, I-FABP Correlates Inversely With Sigmoid Colon CD4$^+$ T-Cell Cytokine Response and Activation

As INRs were characterized by higher blood I-FABP levels and altered sigmoid colon mucosal T-cell function, we wanted to investigate if there was a connection between these findings (Figure 5A). Among INRs, but not among IRs, and restricted to the sigmoid colon, there was an inverse correlation between I-FABP and IL-17-response ($r = -0.57$, $P = .02$) and between I-FABP and

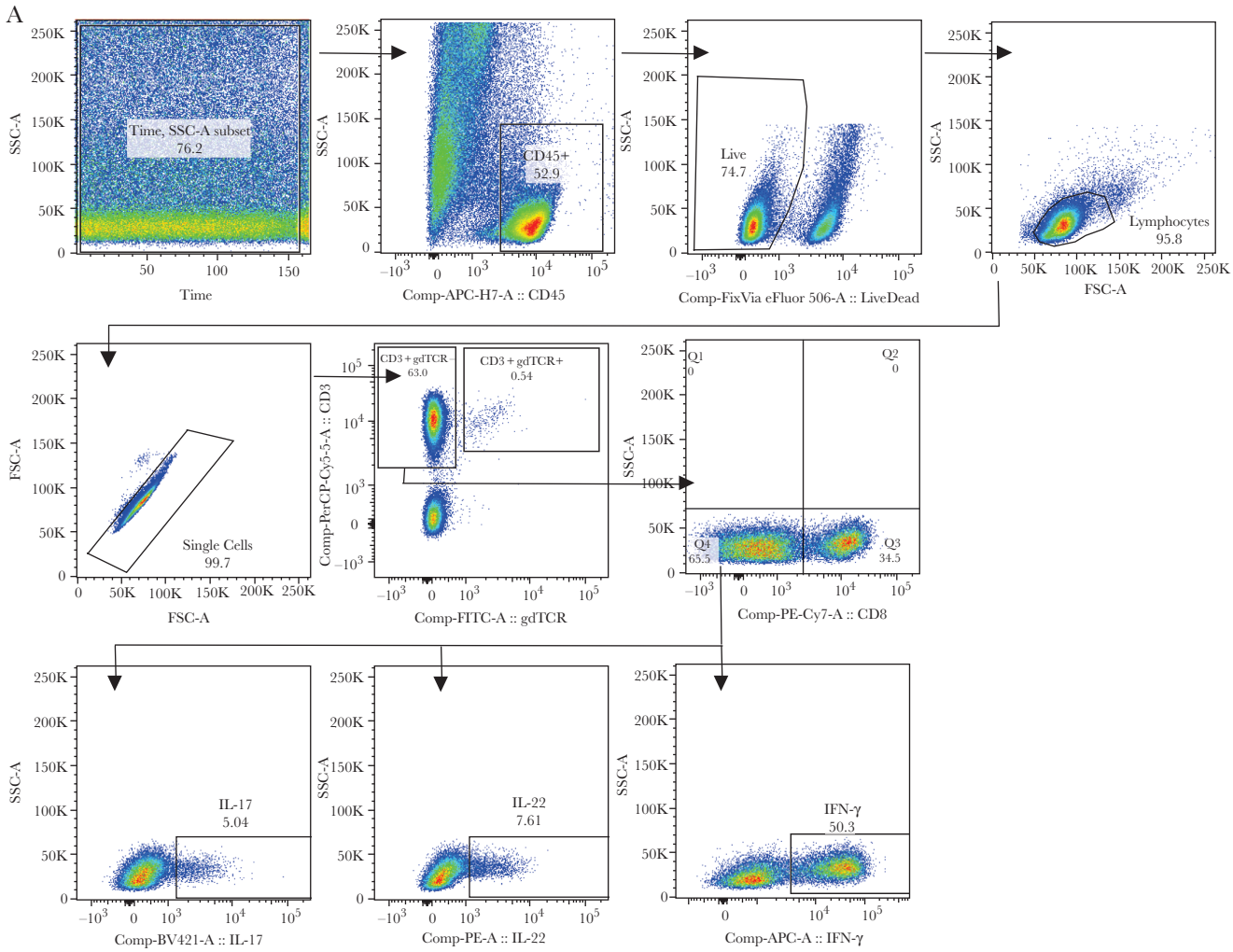

Figure 3. Higher interleukin (IL) 22 production in sigmoid colon CD4$^+$ T cells in immunological nonresponders (INR). *A*, Representative flow cytometry analysis of mitogen-stimulated lamina propria mononuclear cells. *B*, Sigmoid colon fractions of IL-22$^+$, IL-17$^+$, and interferon (IFN)–γ$^+$ CD4$^+$ T cells in INR compared to immunological responders (IR); human immunodeficiency virus (HIV)–negative individuals (HIV neg) served as controls. Terminal ileum fractions of IL-22$^+$, IL-17$^+$, and IFN-γ$^+$ CD4$^+$ T cells in INR compared to IR, with HIV-negative individuals as controls. All analyses by flow cytometry on lamina propria mononuclear cells. Horizontal bars represent median values. Kruskal–Wallis (KW) and Mann–Whitney (MW) test values. *C*, Spearman correlation between sigmoid colon CD4$^+$ T-cell fractions and sigmoid colon IL-17$^+$ CD4$^+$ T cells and sigmoid colon IL-22$^+$ CD4$^+$ T cells in people living with HIV (INR: dots, IR: open circles).

CD4$^+$ T-cell activation (CD38$^+$CD4$^+$ T cells: $r = -0.75$, $P < .001$; HLA-DR$^+$CD38$^+$CD4$^+$ T cells: $r = -0.50$, $P = .03$) (Figure 5B and 5C). Moreover, within INRs' sigmoid colon, the CD4$^+$ T-cell IL-17 response correlated positively with sigmoid colon CD38$^+$CD4$^+$ T cells ($r = 0.63$, $P = .01$) (Figure 5D).

DISCUSSION

We here present novel data demonstrating that INRs have higher levels of enterocyte damage, lower level of mucosal CD4$^+$ T cells, higher level of mucosal CD8$^+$ T-cell activation, and augmented Th17 and Th22 responses in sigmoid colon compared to IRs. None of the observed differences could be detected in terminal ileum and they were not related to mucosa-associated microbiota.

IL-17 and IL-22 are important for the function of intestinal epithelia cells as they induce tight junction protein expression, stimulate secretion of antimicrobial peptides and mucus production, regulate cell cycling, and enhance mucosal inflammatory reaction against invading pathogens [32]. Th17 development is in part regulated by gut resident microbes whereas microbes' relevance to Th22 function is less characterized [33]. We found a higher IL-22 response, and a strong trend toward a higher IL-17 response, in the sigmoid colon mucosa of INRs. IFN-γ response was included in the analysis to confirm the effectiveness of the PMA/ionomycin stimulation. Our finding of higher IL-22 and IL-17 cytokine responses should be considered as cytokine specific and not an INR-related ubiquitous responsiveness to mitogen

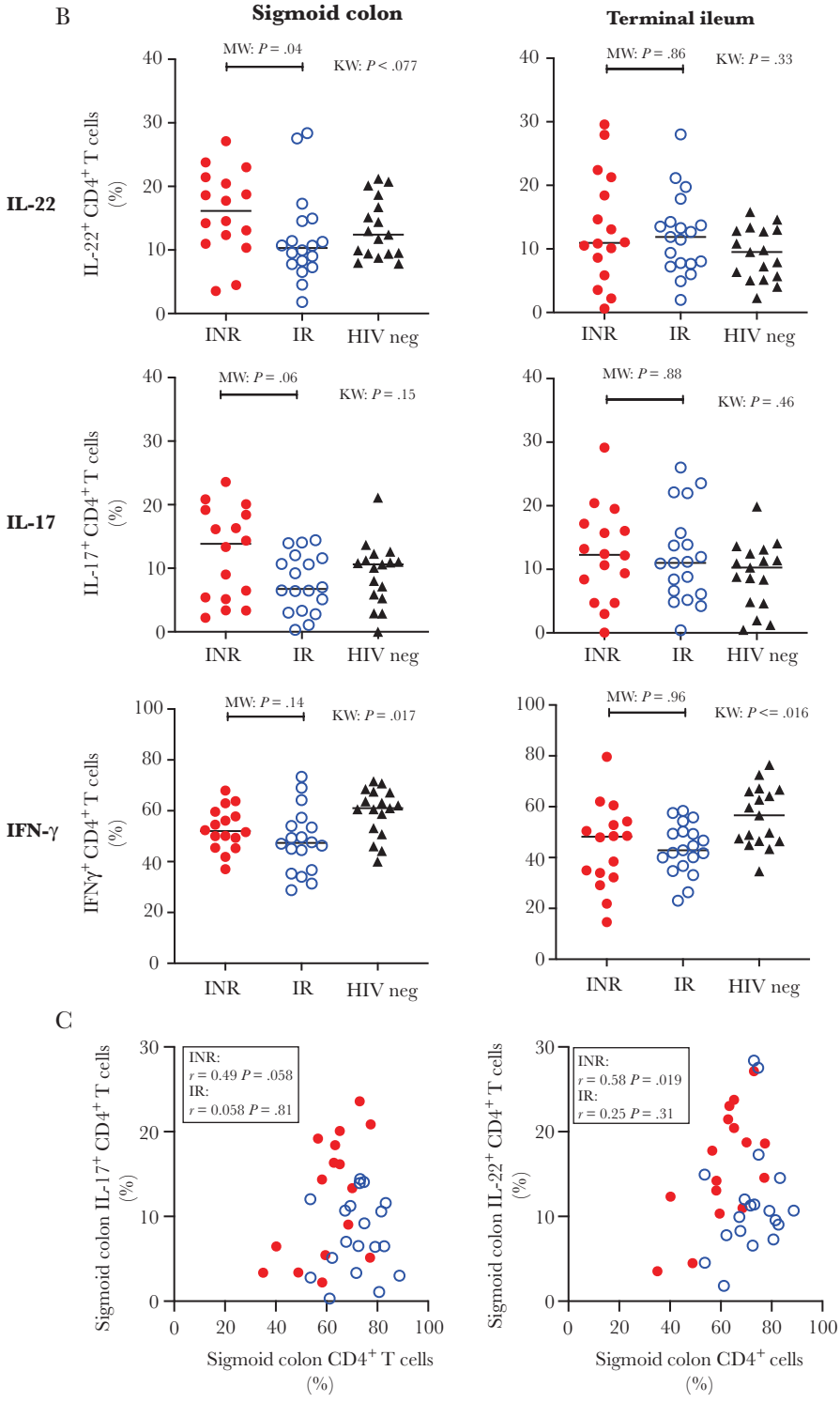

Figure 3. Continued.

stimulation, as there was no difference between INRs and IRs in IFN-γ response. The lower IFN-γ response in mucosal $CD4^+$ T cells of PWH compared with HIV-negative controls could have accentuated the IL-22 and IL-17 responses in PWH as literature report inhibition by interferons on IL-17 expression [34]. However, this interaction cannot explain the observed differences between INRs and IRs in IL-22 and IL-17 $CD4^+$ T-cell responses.

Our hypothesis was that INR patients would have fewer IL-17– and IL-22–producing T cells, and that this would

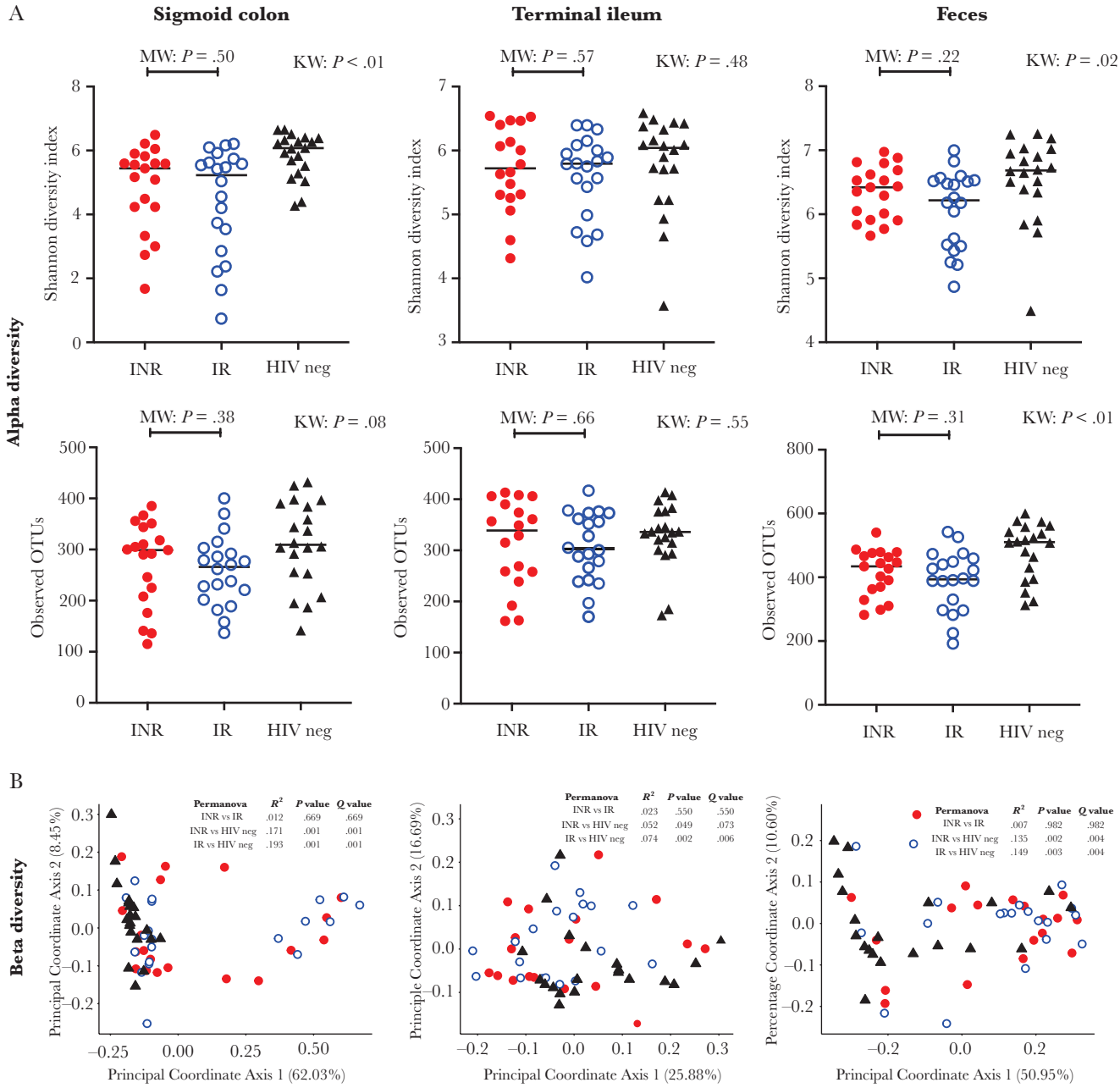

Figure 4. No differences in gut microbiota between immunological nonresponders (INR) and immunological responders (IR). *A*, Alpha diversity analyzed by Shannon index and observed operational taxonomic units (OTUs) for differences between INR, IR, and human immunodeficiency virus (HIV)–negative individuals (HIV neg) in sigmoid colon, terminal ileum, and feces. Horizontal bars represent median values. Kruskal–Wallis (KW) and Mann–Whitney (MW) test values. *B*, β-diversity analyzed by weighted UniFrac analyses of differences between INR, IR, and HIV-negative controls in sigmoid colon, terminal ileum, and feces. Permutational multivariate analysis of variance test values (INR: dots, IR: open circles, HIV negative: triangles). *C*, Relative abundance of taxonomic phylum (top 3 rows) and family (lower 3 rows) in sigmoid colon, terminal ileum, and feces of INR, IR, and HIV-negative controls.

contribute to an impaired mucosal barrier. In the colon, we observed that the INR individuals with the lowest IL-17 response and the lowest fraction of $CD4^+$ T cells had the highest level of enterocyte damage as measured by I-FABP levels. These results indicate that within INRs, IL-22^+ and IL-17^+ $CD4^+$ T cells are indeed crucial for maintaining a healthy gut barrier. Moreover, our data are in line with previous reports demonstrating that gut mucosal IL-17– and IL-22–producing T cells are to a large extent restored in PWH with successful immune reconstitution after ART initiation, and that restoring the function of these cells is crucial for mucosal integrity [2, 14]. Although no studies have previously assessed mucosal Th17 or Th22 in INRs, 2 recent articles report that INRs have higher fractions of Th17 cells in PBMCs [35, 36].

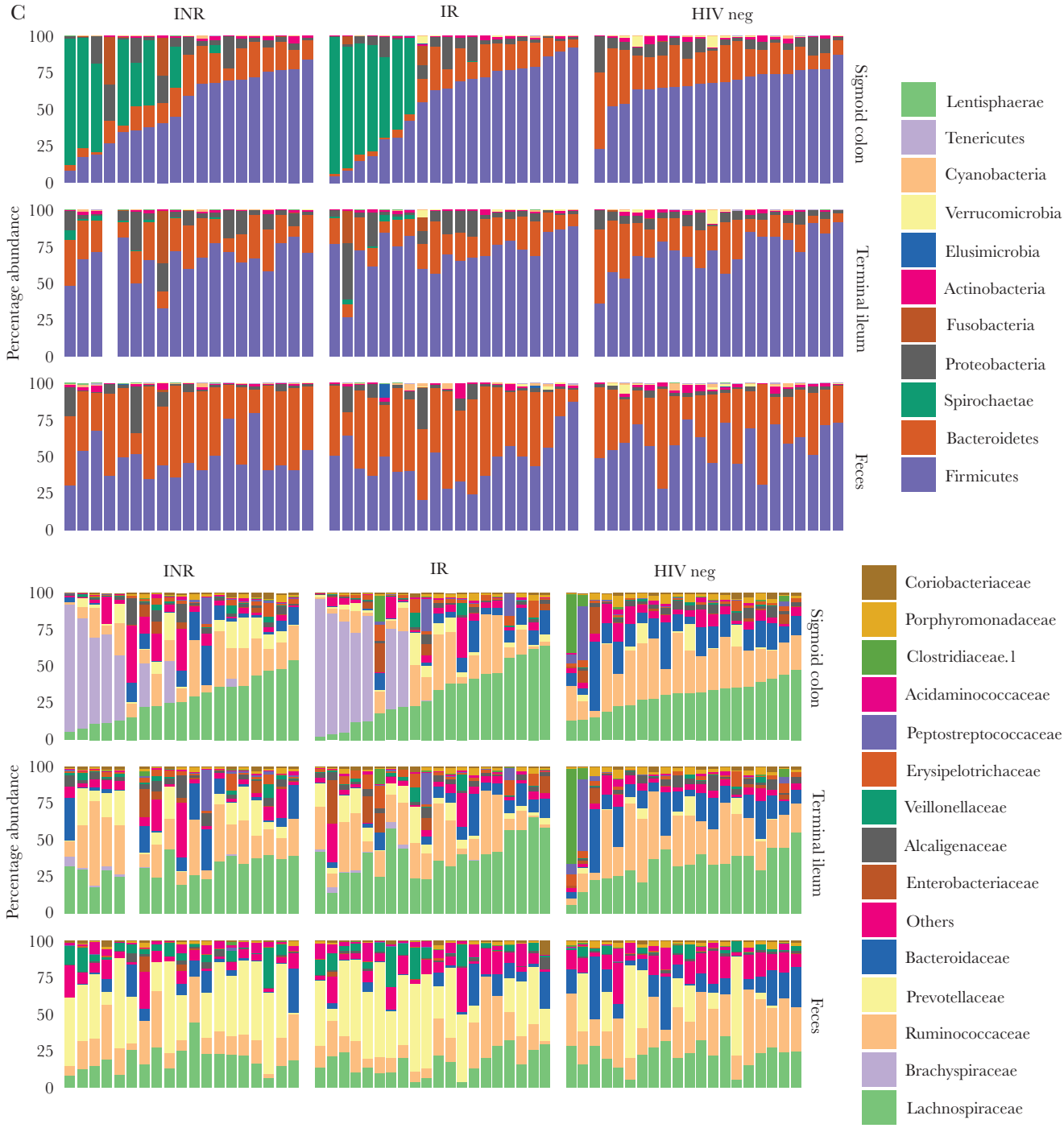

Figure 4. Continued.

A striking finding in this study was the anatomical compartment-specific differences. Differences between INRs and IRs in $CD4^+$ T-cell fraction, $CD4^+$ T-cell IL-22 response, and $CD8^+$ T-cell activation was only observed in the colon, as were the inverse correlation between I-FABP and IL-17 response and mucosal $CD4^+$ T-cell activation. Basic mucosal immunology studies have hypothesized that, compared to the colon, a more pronounced immune regulation takes place in the terminal ileum with its greater epithelium–lumen interface area, higher density of lymphoid follicles, and higher concentration of dietary immune-regulating substances such as retinoic acid aryl hydrocarbon receptor ligands [37]. Our findings indicate that the mucosal immunology of the distal colon may be clinically relevant in HIV infection.

Our analyses of the mucosal microbiota indicate that the sigmoid colon T-cell alterations in INRs are not driven by

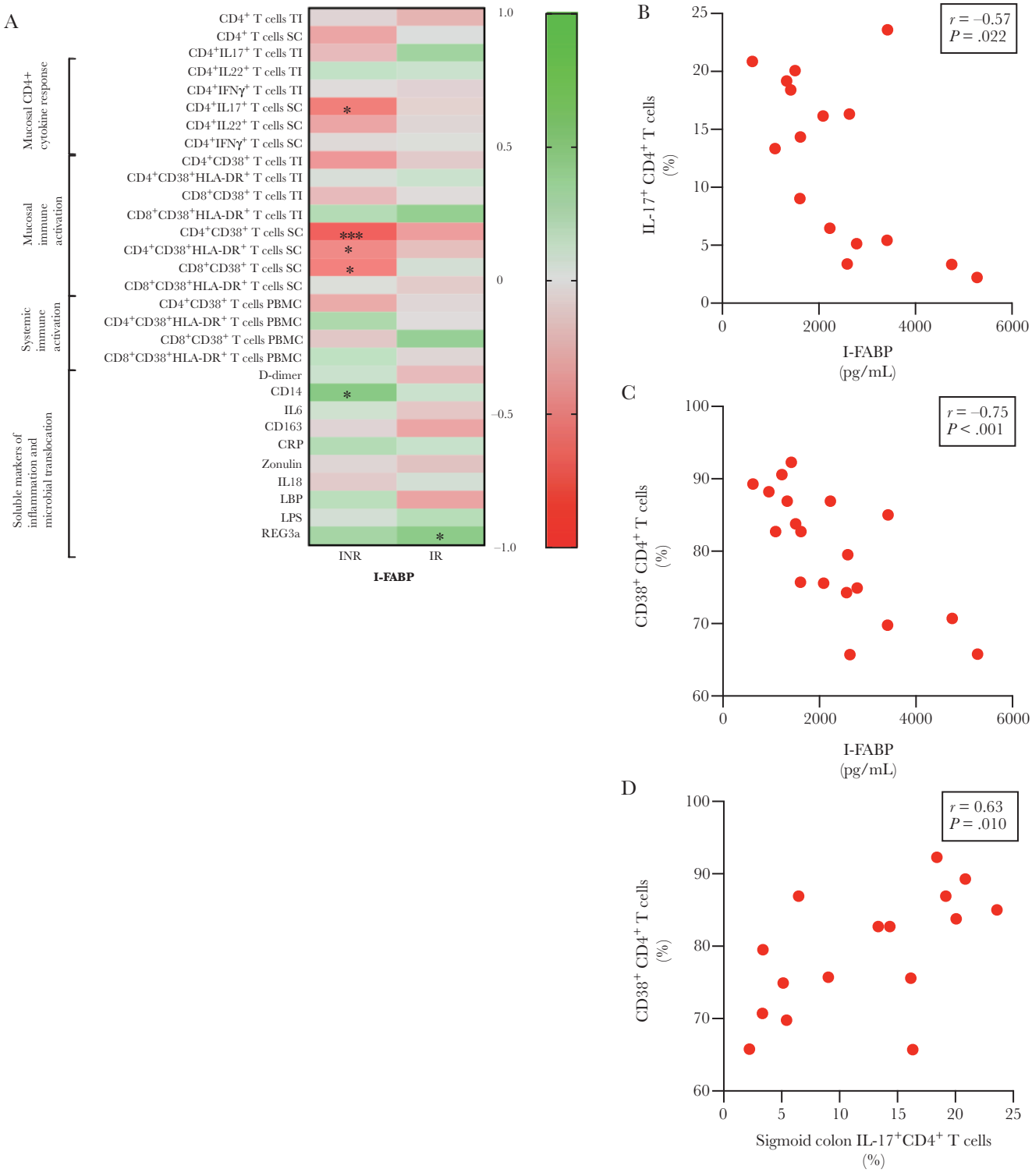

Figure 5. Intestinal fatty acid–binding protein (I-FABP) correlates inversely with sigmoid colon T-cell cytokine response and activation in immunological nonresponders (INR). *A*, Heat map illustrating Spearman correlations between I-FABP and listed parameters in INR and immunological responders (IR) in terminal ileum (TI), sigmoid colon (SC), and peripheral blood mononuclear cells (PBMCs). *R* values are shown on the scale. *P* values lower than the defined level of significance are marked as **P* < .05 and ****P* < .001. Spearman correlations between I-FABP and SC interleukin (IL) 17$^+$ CD4$^+$ T cells (*B*) and SC CD38$^+$CD$^+$4 T cells (*C*), and between SC IL-17$^+$ CD4$^+$ T cells and CD38$^+$CD4$^+$ T cells (*D*) within INR (dots).

microbial dysbiosis, as there were no overall differences between INRs and IRs in microbial composition, either in the sigmoid colon or in the terminal ileum and feces. This finding is supported by 2 recent reports comparing fecal samples from INRs and IRs. [38, 39]. A previous study detected reduced fecal α-diversity in PWH with CD4 count <200 cells/μL

compared with patients with CD4 count >200 cells/μL [40]. We emphasize that our study included an analysis of mucosal specimens from 2 separate gut compartments and hence assessed mucosa-associated microbes with intimate relation to the mucosal T cells. Most studies on gut microbiota in HIV infection have been performed on fecal samples [10], and the validity of fecal samples as a substitute for mucosa specimens in determining mucosa adherent microbiota has been questioned [41].

Although we did not detect a difference in microbial composition between INRs and IRs, analysis of β-diversity indicated a significant difference between both the PWH groups and the HIV-negative control individuals. First described by Noguera-Julian et al, sexual preference is a significant determinant of gut microbial composition [42]. Of the 39 INRs and IRs in our study, there were 5 individuals who were not men who have sex with men, and these 5 were evenly distributed between INRs and IRs. We therefore considered the gut microbiota comparisons between these 2 groups as valid. The results do not allow us to draw conclusions on PWH-specific gut microbial alterations as the sexual preferences of the HIV-negative controls were not recorded.

Analyses of soluble markers revealed that INRs had significantly higher levels of circulating I-FABP and recorded 1.5 times higher median level of REG3α than IRs. The latter variable was not significantly different between INRs and IRs, but correlated significantly with I-FABP. I-FABP is an established marker of enterocyte damage in many conditions, including HIV infection [8], while REG3α has emerged as a novel marker. A recent report found REG3α to be a relevant marker of intestinal damage in untreated HIV infection [31]. The other soluble markers of inflammation and microbial translocation did not demonstrate relevant differences between INRs and IRs. Previous reports on these markers in INRs vary in conclusions, but many studies are, as ours, of limited statistical power [24, 26, 29, 43]. The largest study reported higher levels of soluble CD14 and IL-6 in INRs [23]. Our study did not reproduce previous reports on increased immune activation in INRs [23, 26, 44–46]. Methodological differences in definitions of INRs and IRs between these reports and our study, along with the limited number of patients enrolled, are possible explanations as to why we did not replicate these results. With the exception of a subtle increase in $CD38^+CD8^+$ T cells in sigmoid colon of INRs compared with IRs, we detected no other significant alterations in $CD38^+$ or $HLA\text{-}DR^+CD38^+$ T cells in either the terminal ileum or the sigmoid colon, indicating no grossly increased mucosal immune activation in INRs. To conclude, in our study, INRs display enterocyte damage as a more prominent feature than systemic inflammation and immune activation.

We believe the major strengths of this study are the well-defined, clinically valid, human study groups and the extensive mucosal sampling. The definition of immunological nonresponse in this study was <400 cells/μL. Throughout the history of HIV research, the CD4 cell counts defining immunological nonresponse have varied. As the CD4 count threshold for initiating ART has been raised over time, we argue that a CD4 count of 400 cells/μL has a high future validity for defining INRs. A cutoff of 400 cells/μL is also reported to be the preferred value to separate INRs from IRs [47]. Compared with IRs, INRs in our study had a significantly lower CD4/CD8 ratio, which has emerged as a strong predictor of non-AIDS morbidity [48]. We believe that our study sample group is representative for PWH with an incomplete immune recovery and a clinical prognosis inferior to those with a complete immune response to ART. Finally, it should be pointed out that the INRs and IRs of our study were matched for age and nadir CD4 count, suggesting that the mucosal differences found between the 2 groups should not be attributed to known confounders for the INRs phenotype.

The study has some limitations. The statistical power of the study is limited and some of the variables (eg, REG3α) did not reach statistical significance in analysis between INRs and IRs, even though the difference in median values were visually discernible. As the main objective of the study was to identify mucosal immunopathology that distinguished INRs from IRs, and due to the limited power, we decided to downplay statistical tests between PWH subgroups and HIV negative controls that would require multiple-comparison corrections and risk of type II errors. Also, the applied methods did not allow us to determine absolute numbers of lamina propria mononuclear cell populations. Pilot studies had demonstrated that weighing the biopsies was not feasible, so the flow cytometry counts could not be normalized to a fixed measure. A potential skewing of ART regimen between INRs and IRs could affect the results, but the scientific evidence supporting such a hypothesis is limited and inconsistent [49, 50]. Finally, as this is a cross-sectional study, we cannot conclude on causality or the mechanisms of the observed differences.

To conclude, this study supports the hypothesis that PWH with an incomplete immune recovery to ART have a disrupted mucosal function that is related to IL-17– and IL-22–producing $CD4^+$ T cells. The study provides new knowledge of the mucosal disruption in INRs, which primarily takes place in the colon. Scientific studies that could eventually allow enhancement of Th17 and Th22 in HIV infection should be encouraged.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Author contributions. D. H. R. designed the study in collaboration with A. W. M., B. S., M. T., J. R. H., D. K., and A. M. D. H. R.; M. H. M.-M., and A. W. M. included and examined all study participants. M. H. M.-M., K. B. L., I. S., and M. K. performed laboratory analyses. M. H. M.-M., K. B. L., S. L. H., K. H., M. K., and D. H. R. analyzed the data. M. H. M.-M. and D. H. R. drafted the manuscript. All authors contributed in editing and finalizing the manuscript.

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