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Antibody Response to *Achromobacter xylosoxidans* during HIV Infection Is Associated with Lower CD4 Levels and Increased Lymphocyte Activation

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Inflammation during HIV infection is associated with worse disease outcomes and progression. Many mechanisms have been indicted, including HIV itself, coinfections, and gut microbial translocation. Concerning microbial translocation, we hypothesized that adaptive immune responses to a specific bacterial species known to be present in gut-associated lymphoid tissue are higher among HIV-infected individuals than among HIV-uninfected controls and are associated with T cell activation and lower CD4 T cell counts. By characterizing the IgG response to *Achromobacter xylosoxidans*, we found that HIV-infected participants who were immunoresponsive ($n = 48$) had significantly lower CD4 percentages ($P = 0.01$), greater CD4 activation (percentages of $RA^- CD38^+$) ($P = 0.03$), and higher soluble CD14 ($P = 0.01$). HIV-positive individuals had higher anti-*A. xylosoxidans* IgG titers than HIV-uninfected individuals ($P = 0.04$). The results suggest an abnormal adaptive immune activation to gut microflora during HIV infection.

Chronic immune activation contributes to viral persistence and worse disease outcomes in people with HIV infection (1, 2). An understanding of the drivers of this immune activation is important for designing new strategies to prevent HIV end-organ damage, especially in the setting of antiretroviral therapy (ART) (3). *Achromobacter xylosoxidans* is a Gram-negative aerobic proteobacterium in the *Alcaligenaceae* family that oxidizes xylose and glucose but not lactose as part of the normal human gut flora, is found in mesenteric lymph nodes and Peyer's patches, and is able to survive in water and soil (4, 5). During the severe immunodeficiency of HIV infection, *Achromobacter* can be an opportunistic pathogen, causing bacterial meningitis (6) and pulmonary abscesses (7). Here, we present the detection and characterization of immunoglobulin G (IgG) reactive to *A. xylosoxidans* in an untreated HIV-infected cohort compared to those in uninfected controls.

Recent studies detecting *A. xylosoxidans* in dendritic cells of mesenteric lymph nodes in humans and livers of innate and adaptive immune-deficient mice suggested a tightly controlled balance of systemic immune suppression and local microbial control under normal conditions. The compartmentalization of *A. xylosoxidans* and privilege from adaptive immunity are dependent on the innate lymphoid cells at the gut-immune interface, whose function is mediated through regulatory T cells (8), which are depleted early during the course of HIV infection. This bacterium was detected by *in situ* hybridization in Peyer's patches and mesenteric lymph nodes of healthy humans, nonhuman primates (4, 9), and mice (8), which shows local immune control. In Rag2 knockout mice depleted of interleukin 22 (IL-22)-producing innate lymphoid cells, *A. xylosoxidans* invaded the periphery (8). CD4 regulatory T cells in gut-associated lymphoid tissue are depleted during acute HIV infection (10, 11), a process that may alter microbial control balance. While *A. xylosoxidans* has been found in specific compartments in healthy humans, it has also been isolated from the lungs of cystic fibrosis patients (12) and thus can be pathogenic when not under control. Because of the elevated immune responses to this bacterium in two diseases marked by elevated inflammation in the gut, Crohn's disease and chronic hepatitis C virus (HCV) infection (8), we hypothesized that evidence for decompartmentalization and the IgG response to *A. xylosoxidans* may be found in untreated HIV-infected individuals.

Based on prior observations of IgG responsiveness to *A. xylosoxidans* and the known consequences of HIV infection for gut-associated lymphoid cells, we hypothesized that (i) the IgG response to *A. xylosoxidans* would be increased in HIV-infected participants compared to that in HIV-uninfected controls, (ii) the IgG response would correlate with evidence of immunosuppression (counts and percentages of CD4 and $RA^- CD38^+$ T cells), and (iii) the IgG response would correlate with measures of HIV persistence (HIV DNA levels in peripheral blood mononuclear cells [PBMC] and HIV RNA shed in semen). In order to address these hypotheses, we developed a custom assay to compare measurements of IgG titers from serial dilutions of low sample volumes of blood serum. We found that, as with HCV infection and Crohn's disease, HIV-positive individuals had higher anti-*A. xylosoxidans* titers. Additionally, CD4 percentages were lower and activated ($RA^- CD38^+$) CD4 T cell counts were higher in the HIV-positive individuals who were immunoresponsive to *A. xylosoxidans* than in those who were nonresponsive.

MATERIALS AND METHODS

All the participants signed informed consent forms, and the protocol was approved by the University of California San Diego Human Research

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TABLE 1 Summary of clinical data and multiple sampling time points of HIV-infected participants

Characteristic	Result (mean ± SD) for HIV-uninfected participants at time point 1 ($n = 12$)	Result (mean ± SD) for HIV-positive participants at time point:			
		1 ($n = 36$)	2 ($n = 13$)	3 ($n = 12$)	4 ($n = 6$)
Average EDI (days)[a]		129 ± 72	161 ± 68	346 ± 267	617 ± 396
CD4 cells/μl (%)[b]	1,313 ± 409 (54 ± 9)	727 ± 310 (33 ± 10)	804 ± 280 (34 ± 9)	738 ± 341 (30 ± 8)	684 ± 256 (29 ± 7)
CD8 cells/μl (%)[b]	694 ± 184 (29 ± 4)	1,282 ± 846 (55 ± 11)	1,246 ± 412 (53 ± 8)	1,508 ± 914 (59 ± 9)	1,334 ± 595 (58 ± 7)
Percent activated[c] CD4	10 ± 3	18 ± 7	17 ± 9	17 ± 7	16 ± 7
Percent activated[c] CD8[b]	4 ± 2	30 ± 20	31 ± 16	27 ± 10	20 ± 7
Blood viral RNA (ln[copies/μl])		11.01 ± 1.86	11.24 ± 1.52	10.97 ± 1.10	10.32 ± 1.16
Seminal fluid viral RNA (ln[copies/μl])		6.89 ± 2.49	6.77 ± 4.42	6.90 ± 3.98	7.76 ± 1.36
Peripheral blood HIV DNA (copies/10^6 cells)		98.6 ± 205	31.7 ± 69.1	55.5 ± 140	28.7 ± 27.2
Soluble CD14 (ng/ml)	1,007 ± 170	1,285 ± 297	1,197 ± 258	1,157 ± 176	1,096 ± 986

[a] Estimated duration of infection.
[b] $P < 0.001$ comparing HIV-positive versus controls by Student's *t* test.
[c] RA^- $CD38^+$ cells.

Protections Program. The cohort was previously described (13). From collected blood samples, $CD4^+$ T lymphocyte subsets were measured by flow cytometry (LabCorp, San Diego, CA), and HIV RNA was quantified in blood plasma (Amplicor HIV monitor test; Roche Molecular Systems, Inc., Pleasanton, CA). Seminal viral loads and PBMC HIV DNA levels were also measured by TaqMan PCR (Life Technologies, Carlsbad, CA), as described elsewhere (13). No subjects in the present study were receiving ART, and they were not appreciably immunosuppressed (mean CD4 T cell count, 727 ± 310 cells/μl). The estimated duration of infection (EDI) (13–15) was calculated at the time of intake. A total of 80 samples from 36 HIV-infected participants (13 with longitudinal sampling at up to 4 time points) and 12 HIV uninfected controls (at single time points) were tested. The clinical characteristics of the cohort are shown in Table 1; study participants in the present study were included in the study of Gianella et al. (13).

We developed a custom assay for measuring anti-*A. xylosoxidans* IgG levels. In brief, we developed this assay on the Meso Scale Discovery platform (16). Protein from suspension cultures of *A. xylosoxidans* (catalog no. 3444; American Type Culture Collection, Manassas, VA), sequenced for quality control, was used to coat bare Meso Scale plates (catalog no. L15XA; Meso Scale Discovery, Rockville, MD) at 100 μg/well in phosphate-buffered saline (PBS) and incubated at 37°C until the electrodes were dry. After being coated, washed in PBS (0.5% Tween 20), and blocked in a solution of 2.5% normal goat serum and 2.5% fetal bovine serum (empirically determined to be optimal) for 1 h, 3-fold serial dilutions of plasma samples starting at a 1/50 dilution were generated on the plate and incubated at room temperature for 1 h with shaking. We chose this approach rather than a single-point endpoint to conserve information about the quantity and affinity of the antibody binding, which would be lost if we analyzed only a single dilution (17). The detection antibody was prepared with 1.15 mg anti-human IgG, Fcγ specific (Jackson ImmunoResearch, West Grove, PA), and 26.1 nmol sulfo-*N*-hydroxysuccinimide ester (Meso Scale Discovery). The same batch of labeled antibody was used for all measurements. IgG binding to *A. xylosoxidans* protein antigens was detected and quantified by reading the plates on a Sector Imager 2400 (Meso Scale Discovery) after washing each well and adding 150 μl substrate solution to the wells. The electrochemiluminescence was plotted versus ln(dilution) and fitted using the standard least-squares method to determine the midpoint (MP) titer, the endpoint (EP) titer, and the *y* intercept (Y_0), unique to each sample.

For hypothesis testing for comparisons between groups, Student's *t* tests were used (e.g., IgG titers versus HIV status or *A. xylosoxidans* responsiveness versus CD4 cell count, percentage of RA^- $CD38^+$ cells, HIV DNA, HIV RNA, or EDI). For comparisons between the groups, only the first time point was used. For the HIV-infected group that was immunoresponsive to *A. xylosoxidans*, we tested for correlations between IgG titers and the immunologic data listed above by linear regression. When necessary, the data were natural log transformed to normalize distributions. We report the results as means ± standard deviations.

RESULTS

Overall, 42% of the subjects had detectable levels of IgG responsive to *A. xylosoxidans*. In comparing the HIV-infected versus -uninfected groups, we found no difference in the proportions of individuals with measurable immune responses to *A. xylosoxidans*. However, the anti-*A. xylosoxidans* EP titers were significantly higher in the HIV-infected individuals (18,958 ± 1,655) than in the HIV-uninfected individuals (3,463 ± 595; $P = 0.009$) (Table 2). Among the HIV-infected participants followed longitudinally, there was a stepwise increase in the proportion of subjects who were *A. xylosoxidans* responsive (Fig. 1A); however, this was not statistically significant ($P = 0.1$, Cochran-Armitage test for monotonic trend), likely because of the lower sample sizes at time points 3 and 4.

When we considered the markers of the HIV DNA reservoir, there was a significant negative correlation between the HIV DNA in PBMC (copies/10^6 cells) and the IgG titers ($R = 0.51$, $P = 0.008$) (Fig. 1B). We speculated that this observation may have been related to the durations of HIV infection; however, there were no significant associations between the IgG titers and the EDI or HIV RNA levels, nor was there a significant difference in EDI for *A. xylosoxidans* responsiveness. We therefore considered that this observation may have reflected the number of CD4 cells and hypothesized that the CD4 percentage would be lower in participants with detectable responsive IgG levels.

Several immunological markers of HIV disease were associated with *A. xylosoxidans* responsiveness, as summarized in Table 2. Specifically, the CD4 percentage was significantly lower in responsive individuals (28.5% ± 10.1%) than in nonresponsive individuals (36.7% ± 9.4%, $P = 0.01$). Additionally, the proportion of activated (RA^- $CD38^+$) CD4 T cells was higher in the responsive group (20.7% ± 6.9%) than in the nonresponsive group (16.1% ± 6.9%, $P = 0.03$).

TABLE 2 Summary statistics of HIV immunological data in *Achromobacter*-immunoresponsive and -nonresponsive groups and IgG titers in HIV-infected and -uninfected control groups from the first time point

Parameter	Result (mean ± SD) for the indicated group			Result (mean ± SD) for the indicated group		
	Nonresponsive ($n = 23$)	Responsive ($n = 13$)	P^a	HIV positive ($n = 13$)	HIV negative ($n = 7$)	P^a
Clinical (HIV-infected only)						
CD4 cells (%)	36.7 ± 9.4	28.5 ± 10.1	0.01			
CD4 count (cells/μl)	791 ± 311	618 ± 289	0.05			
RA^- $CD38^+$ CD4 (%)	16.1 ± 6.9	20.7 6.9	0.03			
RA^- $CD38^+$ CD8 (%)	27.1 ± 20.4	36.1 ± 17.6	0.08			
Peripheral blood HIV DNA (ln[copies/10^6 cells])	1.75 ± 2.85	3.12 ± 2.70	0.10			
EDI[b] (log[days])	16.1 ± 0.5	16.1 ± 0.7	0.50			
Blood HIV RNA (ln[copies/μl] in cell-free fluid)	10.9 ± 1.4	11.0 ± 1.4	0.39			
Seminal HIV RNA (ln[copies/μl] in cell-free fluid)	7.4 ± 2.8	5.9 ± 1.3	0.04			
Soluble CD14 (ng/ml)	1,189 ± 200	1,455 ± 366	0.01			
Achromobacter IgG titers (responsive only)						
Y_0 titer (signal)				21,375 ± 2,144	10,829 ± 1,515	0.14
Midpoint titer (dilution^{-1})				450 ± 95	183 ± 42	0.07
Endpoint titer (dilution^{-1})				18,958 ± 1,655	3,463 ± 595	0.009

[a] One-sided Student's *t* test comparing the two groups.
[b] Estimated duration of infection.

DISCUSSION

In a well-characterized cohort (13) of HIV-infected participants and matched HIV-uninfected controls, we found that, as with HCV infection and Crohn's disease (8), HIV-infected individuals had higher anti-*A. xylosoxidans* titers than HIV-uninfected controls. Additionally, the CD4 percentages were lower and the proportions of activated CD4 T cell counts were higher in the HIV-infected individuals who were immunoresponsive to *A. xylosoxidans* than in those who were nonresponsive. Although lower CD4 cell counts were observed in individuals with IgG responsive to *A. xylosoxidans* than in those with nonresponsive IgG, none of the subjects were significantly immunosuppressed. However, the mean CD4 percentage (28%) in the responsive group was below the normal healthy range (30 to 60%) (18, 19). Additionally, the significant increase in the percentage of RA^- $CD38^+$ CD4 T cells suggests that the adaptive immune response to commensal gut flora may be associated with immune activation of CD4 cells, which may correspond also to a higher CD4 cell death rate, as shown by lower CD4 levels in the responsive group (Table 2). There was a significant negative correlation between the anti-*A. xylosoxidans* IgG Y_0 titer and HIV DNA in PBMC (Fig. 1B).

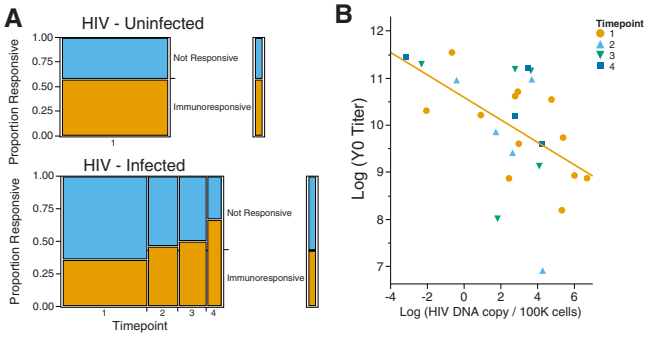

FIG 1 (A) Contingency plots showing the proportions of *A. xylosoxidans*-responsive individuals (*y* axis) in HIV-positive and -negative groups, separated by time points (*x* axis). The proportion of responsive individuals increases as the number of sampling visits increases in the HIV-positive group. (B) Anti-*A. xylosoxidans* Y_0 IgG titers versus peripheral blood mononuclear cell HIV DNA (copies/10^6 cells), which illustrates a significant negative correlation and linear fit (Pearson $R = -0.51$, $P = 0.008$), color coded by sampling time point and natural log transformation.

In this report, we have also described an assay that uses a low sample volume of human plasma to detect and characterize IgG binding to a common environmental and gut bacterium that can become an opportunistic pathogen in immunosuppressed individuals. Because this method assesses a broad range of concentrations, we derived information about the signal at high (Y_0) and low (EP titer) concentrations, informing on characteristics of both the number of antibodies and the affinity with which they bind. Since the EP titer, but not the Y_0 or MP titer, was significantly higher in the HIV-infected group, we hypothesize that the anti-*A. xylosoxidans* IgG levels in the HIV-infected group were of higher affinity but not of higher quantity (20), which is evidence for multiple exposure events.

As access to treatments that suppress HIV replication has improved immensely, there has been increased interest in determining the immunological factors associated with the HIV reservoir and immune status (21). In one study of untreated HIV-infected patients, levels of IgG responsive to other gut bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, and *Enterococcus faecalis*) were not increased (22). Here, we measured adaptive immune responses to a commensal bacterial species, *A. xylosoxidans*, which can be an opportunistic pathogen under severe immunocompromising conditions (4–7), as they relate to immune markers in HIV-infected

individuals. Under normal containment, the peripheral immune system does not come into contact with commensal bacteria, a process mediated through regulatory T cells and bacterium-loaded mesenteric lymph node dendritic cells (23). This species can survive in soil, has been found in drinking water (5), is increasing in incidence in the lungs of cystic fibrosis patients (18), and can be a nosocomial pathogen (24). Based on our findings and the bacterial translocation previously proposed in HIV infection (25), we suggest that the loss of containment via early CD4 cell loss in gut-associated lymphoid tissue (10) causes *A. xylosoxidans* to be one among many drivers of immune activation in HIV infection.

In conclusion, we found significant associations between immunological HIV markers and IgG responsiveness to *A. xylosoxidans*. The interesting paradox is that the HIV-infected individuals who were responsive, as determined by IgG binding, had lower CD4 percentages and higher numbers of activated CD4 T cells; despite lower CD4 cell counts, B cells were still able to mature and respond. However, there was a negative correlation between HIV DNA levels and Y_0 IgG titers. This signifies a complex relationship between immune activation, CD4 cell survival, microbial translocation, and the peripheral HIV DNA reservoir.

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