Title: Antibody response to *Achromobacter xylosoxidans* during HIV infection is associated with lower CD4 levels and increased lymphocyte activation

Running: IgG response to commensal bacteria in HIV

Authors: Erick T. Tatro*, Intan Purnajo¹, Douglas D. Richman², Davey M. Smith², and Sara Gianella²

Affiliations: *Department of Psychiatry, †VA San Diego Healthcare System and Department of Medicine, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0603

*To Whom Correspondence Should be Addressed:
Erick T. Tatro
Email: etatro@ucsd.edu
Abstract

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 response to a specific bacterial species known to be present in gut associated lymphoid tissue would be higher among HIV infected individuals versus HIV uninfected controls and associated with T cell activation and lower CD4 T cell count. By characterizing the IgG response to *Achromobacter xylosoxidans*, we found that HIV-infected participants who were immunoresponsive \( (N = 48) \) had significantly lower CD4% \( (P = 0.01) \) and higher CD4 activation \( (%\text{RA} \cdot \text{CD}38^+) \) \( (P = 0.03) \). HIV-positive individuals had higher anti-*A. xylosoxidans* IgG titer than HIV uninfected \( (P = 0.04) \). The results suggest abnormal adaptive immune activation to gut microflora during HIV infection.

**Key words:** human immunodeficiency virus; CD4; CD8; IgG; antibody titer; Achromobacter *xylosoxidans*; opportunistic pathogen
Introduction

Chronic immune activation contributes to viral persistence and worse disease outcomes in human immunodeficiency virus (HIV) infection \((1, 2)\). Understanding the drivers of this immune activation is important to 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, oxidizing xylose and glucose but not lactose as part of the normal human gut flora, found in mesenteric lymph nodes and Peyer’s patches, and which can survive in water and soil \((4, 5)\). During severe immunodeficiency of HIV infection, *Achromobacter* can be an opportunistic pathogen, causing bacterial meningitis \((6)\) and pulmonary abscess in HIV disease \((7)\). Here, we present detection and characterization of immunoglobulin G (IgG) reactive to *A. xylosoxidans* in an untreated HIV infected cohort compared to uninfected controls.

Recent studies detecting *A. xylosoxidans* in dendritic cells of mesenteric lymph nodes in humans as well as liver of innate- and adaptive- immune deficient mice suggest a tightly controlled balance of systemic immune suppression and local microbial control under normal conditions. Compartmentalization of *A. xylosoxidans* and privilege from adaptive immunity are dependent on 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, non-human primates \((4, 9)\), and mice \((8)\), showing local immune control. In Rag2 knockout mice depleted of 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 the immune privilege and
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 so can be pathogenic when not under control. Because of elevated immune response to this bacteria in two diseases marked by elevated inflammation in the gut, Crohn’s disease and chronic hepatitis C infection (HCV) (8), we hypothesized that evidence for decompartmentalization and IgG response to *A. xylosoxidans* may be found in untreated HIV-infected individuals.

Based on the prior observations of IgG responsiveness to *A. xylosoxidans* and known consequences of HIV-infection on gut-associated lymphoid cells, we hypothesized the following: 1) IgG response to *A. xylosoxidans* would be increased in HIV-infected participants compared to HIV uninfected controls, 2) IgG response would correlate with evidence of immunosuppression (counts and percentages of CD4 and RA-CD38+ T-cells), and 3) 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 this, we developed a custom assay to compare measurements of IgG titer from serial dilutions of a low sample volume of blood serum. We found that, as with HCV and Crohn’s disease, HIV+ individuals had higher anti-*A. xylosoxidans* titers. Additionally, CD4% was lower and activated (RA-CD38+) CD4 T-cell count was higher in those HIV+ individuals immunoresponsive to *A. xylosoxidans* than those non-responsive.

**Materials and Methods**

All participants signed informed consent and the protocol was approved by the University of California San Diego Human Research Protections Program and the cohort was previously described (13). From collected blood, CD4+ T lymphocyte subsets were
measured by flow cytometry (LabCorp, San Diego, CA, USA) and HIV RNA was quantified in blood plasma (Amplicor HIV Monitor Test, Roche Molecular Systems, Inc., Pleasanton, CA, USA). Seminal viral load and PBMC HIV DNA levels were also measured by Taqman polymerase chain reaction (Life Technologies, Carlsbad, CA, USA), 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) and estimated duration of infection (EDI) (13-15) was calculated at the time of intake. A total of 80 samples were tested from 36 HIV infected participants (13 with longitudinal sampling, up to 4 timepoints) and single time-points for 12 HIV uninfected controls. Clinical characteristics of the cohort are shown in Table 1, study participants in the present study were included in Gianella (2012) (13).

We developed a custom assay for measuring anti-*A. xylosoxidans* IgG. In brief, we developed this assay on the Mesoscale Discovery platform (16). Protein from suspension cultures of *A. xylosoxidans* (American Type Tissue Culture #3444, Manassas, VA, USA), and sequenced for quality control, was coated on Mesoscale bare plates (Mesoscale Discovery #L15XA, Rockville, MD, USA) at 100 µg / well in phosphate buffered saline (PBS) at 37°C until the electrode was dry.. After coating, washing in PBS (0.5% Tween-20), and blocking in a solution of 2.5% normal goat serum and 2.5% fetal bovine serum (empirically determined optimal) for 1 h, threefold serial dilutions of plasma samples starting at 1/50 dilution were generated on the plate and incubated at room temperature for 1 h with shaking. We chose this approach rather than single-point endpoint to conserve information about both the quantity and affinity of antibody binding, which would be lost if analyzing only at a single dilution (17). Detection antibody was prepared with 1.15 mg anti human IgG Fcγ-specific (Jackson
Immunoresearch, West Grove, PA, USA) and 26.1 nmol SULFO n-hydroxsuccinimide ester (Mesoscale Discovery). The same batch of labeled antibody was used for all measurements. IgG binding to *A. xylosoxidans* protein antigens were detected and quantified by reading the plates on a Sector Imager 2400 (Mesoscale Discovery) after washing and adding 150 µL substrate solution. Electrochemiluminescence was plotted vs. ln(dilution) and fitted using standard least squares to determine the midpoint (MP) titer, the endpoint (EP) titer, and the y-intercept (*Y*₀), unique to each sample.

For hypothesis-testing comparing between groups, Student’s *t*-tests were used (e.g., IgG titers vs. HIV status; or *A. xylosoxidans* responsiveness vs. CD4 count, %RA-CD38⁺, HIV DNA, HIV RNA, and EDI). For comparison between groups, only the first timepoint was used. For the HIV-infected group immunoresponsive to *A. xylosoxidans*, we tested for correlation between IgG titer and those immunologic data listed above by linear regression. If necessary, data were natural log-transformed to normalize distributions. We report means ± standard deviations.

**Results**

Overall, 42% of subjects had detectable IgG responsive to *A. xylosoxidans*. In comparing HIV infected versus uninfected groups, there was no difference in the proportion of individuals with a measurable immune response to *A. xylosoxidans*. However, the anti-*A. xylosoxidans* EP titers were significantly higher in HIV infected (18,958 ± 1,655) compared to 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 (Figure 1A); however, this was not statistically significant (*P* = 0.1, Cochran-Armitage test for monotonic trend) likely because of lower sample size at timepoints 3 and 4.
When considering markers of the HIV DNA reservoir, there was a significant negative correlation between HIV DNA in PBMC (copies per 10^6 cells) and IgG titer ($R = 0.51, P = 0.008$), Figure 1B. We speculated that this observation may have been related to duration of HIV infection; however, there were no significant associations between IgG titer and EDI or HIV RNA levels, nor a significant difference in EDI between $A. xylosoxidans$ responsiveness. We therefore considered that this observation may reflect the number of CD4 cells and hypothesized that CD4% would be lower in participants with detectable responsive IgG.

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

**Discussion**

In a well-characterized cohort of HIV-infected participants and matched HIV-uninfected controls, we found that, as with HCV and Crohn’s disease, HIV-infected individuals had higher anti-$A. xylosoxidans$ titers than HIV uninfected controls. Additionally, the CD4% was lower and the proportion of activated CD4 T-cells count was higher in those HIV infected individuals immunoresponsive to $A. xylosoxidans$ compared to those who were non-responsive. Although greater lower CD4 counts were observed in individuals with IgG responsive to $A. xylosoxidans$, none of the subjects were significantly immunosuppressed. However, the mean CD4%, at 28%, in the responsive group was below the normal healthy range (30-60%) (18, 19).
Additionally, the significant increase in %RA-CD38+ CD4 T-cells suggests that adaptive immune response to commensal gut flora may be associated with immune activation of CD4 cells, which may correspond also to greater cell death of CD4 cells, as shown by lower CD4 in the responsive group (Table 2). There was a significant negative correlation between anti-*A. xylosoxidans* IgG Y₀ titer and HIV DNA in PBMC (Figure 1B).

Also, in this report, we describe an assay using a low sample volume of human plasma to detect and characterize IgG binding to a common environmental and gut bacteria 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 (Y₀), and low concentrations (EP titer), informing on characteristics of both the number of antibodies and the affinity with which they bind. Since the EP titer was significantly higher in the HIV-infected group, but not Y₀ or MP titers, we hypothesize that anti-*A. xylosoxidans* IgG in HIV-infected group were higher affinity but not quantity (20), which would be evidence for multiple exposure events.

As access to care for treatments that suppress HIV replication has improved immensely, there is increased interest in determining immunological factors associated with HIV reservoir and immune status (21). In untreated HIV-infected patients, IgG responsive to other gut bacteria; *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterococcus faecalis*; and did not find increased IgG (22). Here, we measured adaptive immune response to a commensal bacteria species, *A. xylosoxidans*, which can be an opportunistic pathogen under severely immunocompromised conditions (4-7), as it relates 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 bacteria-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 lungs of cystic fibrosis patients (18), can be a nosocomial pathogen (24). Based on our findings and the bacterial translocation previously proposed in HIV infection (25), we suggest that 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.

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

Acknowledgements

This work was supported by the National Institutes of Health [DA031591 to E.T.T.; AI100665, MH097520, MH62512, and DA034978 to D.M.S.; AI036214 (CFAR) and AI096113 (the Collaboratory for AIDS Research on Eradication [CARE; U19]) to D.D.R.; MH094159 to Cristian L. Achim; DA026306 to Igor Grant; and AI74621 to Susan J. Little], the University of California San Diego Academic Senate [to E.T.T.], Swiss National Science Foundation [ASMP3-136983 to S.G.], and the U.S. Department of Veteran Affairs. These entities did not participate in the study design, interpretation, or decision to publish.
Declarations

E.T.T., I.P, and S.G. declare no potential conflicts of interests. D.D.R. declares funding from Bristol-Myers Squibb, Gilead Sciences, Merck & Co, Monogram Biosciences, Biota, Chimerix, Gen-Probe. D.M.S. declares grant support from ViiV Pharmaceuticals and consultant fees from Gen-Probe and Testing Talent Services. These entities did not participate in the study design, interpretation, or decision to publish.
**Figures**

**Figure 1.** Contingency plots showing the proportion of *A. xylosoxidans* - responsive individuals (y-axis) in HIV-positive and negative groups, separated by timepoint (x-axis) (A). The proportion of responsive individuals increases as the number of sampling visits increases in HIV-positive group. Anti-*A. xylosoxidans* Y0 IgG titer vs. peripheral blood mononuclear cell HIV DNA (copies / $10^6$ cells) (B), illustrating significant negative correlation and linear fit (Pearson $R = -0.51, P = 0.008$), color coded by sampling timepoint and *natural-log* transformed.
### Table 1. Summary of clinical data and multiple sampling timepoints of HIV-infected participants

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>HIV-uninfected</th>
<th>HIV-positive</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>n</td>
<td>12</td>
<td>36</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>Average EDI&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>129±12</td>
<td>161±68</td>
<td>346±267</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;c&lt;/sup&gt; (%)</td>
<td>1313±409 (54±9)</td>
<td>727±31 (33±10)</td>
<td>804±280 (34±9)</td>
<td>738±341 (30±8)</td>
</tr>
<tr>
<td>CDB&lt;sup&gt;c&lt;/sup&gt; (%)</td>
<td>694±184 (29±4)</td>
<td>1282±846 (55±11)</td>
<td>1246±412 (53±8)</td>
<td>1508±914 (59±9)</td>
</tr>
<tr>
<td>Percent Activated&lt;sup&gt;b&lt;/sup&gt; CD4</td>
<td>10±3</td>
<td>18±7</td>
<td>17±9</td>
<td>17±7</td>
</tr>
<tr>
<td>Percent Activated&lt;sup&gt;b&lt;/sup&gt; CD8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4±2</td>
<td>30±20</td>
<td>31±16</td>
<td>27±10</td>
</tr>
<tr>
<td>Blood Viral RNA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>11.01±1.86</td>
<td>11.24±1.52</td>
<td>10.97±1.10</td>
</tr>
<tr>
<td>Semen Viral RNA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>6.89±2.49</td>
<td>6.77±4.42</td>
<td>6.90±3.98</td>
</tr>
<tr>
<td>peripheral blood HIV DNA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-</td>
<td>98.6±205</td>
<td>31.7±69.1</td>
<td>55.5±140</td>
</tr>
<tr>
<td>soluble CD14&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1,007±170</td>
<td>1,285±297</td>
<td>1,197±258</td>
<td>1,157±176</td>
</tr>
</tbody>
</table>

Reported are mean±standard deviation.  
<sup>a</sup>EDI - Estimated duration of infection (days),  
<sup>b</sup>RA-CD38<sup>c</sup>,  
<sup>c</sup>Ln(Copies/µL),  
<sup>d</sup>Copies/10<sup>6</sup> cells.  
<sup>e</sup>ng/mL.  
<sup>P</sup><sup><i>b</i></sup> < 0.001 comparing HIV+ vs Controls by Student’s t.

### Table 2. Summary statistics of HIV immunological data in Achromobacter immunoresponsive and non-responsive groups and IgG titers in HIV infected and uninfected control groups from first timepoint.

<table>
<thead>
<tr>
<th>Clinical Parameters (HIV infected only)</th>
<th>Non-responsive&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Responsive&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4%</td>
<td>36.7±9.4</td>
<td>28.5±10.1</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>CD4 Count (cells/µL)</td>
<td>791±311</td>
<td>618±289</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>%RA CD38&lt;sup&gt;c&lt;/sup&gt; CD4</td>
<td>16.1±6.9</td>
<td>20.7±6.9</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>%RA CD38&lt;sup&gt;c&lt;/sup&gt; CD8</td>
<td>27.1±20.4</td>
<td>36.1±17.6</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>peripheral blood HIV DNA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.75±2.85</td>
<td>3.12±2.70</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>EDI&lt;sup&gt;d&lt;/sup&gt; (log(days))</td>
<td>16.1±0.5</td>
<td>16.1±0.7</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>blood HIV RNA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.9±1.4</td>
<td>11.0±1.4</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>seminal HIV RNA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.4±2.8</td>
<td>5.9±1.3</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>soluble CD14 (ng/mL)</td>
<td>1,189±200</td>
<td>1,455±366</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Achromobacter IgG Titers (Responsive only)</th>
<th>HIV-positive</th>
<th>HIV uninfected</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Y&lt;sub&gt;a&lt;/sub&gt; titer (Signal)</td>
<td>21,375±2,144</td>
<td>10,829±1,515</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>Midpoint titer (Dilution&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>450±95</td>
<td>183±42</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Endpoint titer (Dilution&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>18,958±1,655</td>
<td>3,463±595</td>
<td>0.009</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Reported are mean (standard deviation).  
<sup>b</sup>One-sided Student’s t-test comparing the two groups.  
<sup>c</sup>Ln(Copies/10<sup>6</sup> cells).  
<sup>d</sup>Estimated duration of infection.  
<sup>e</sup>Ln(Copy/µL) in cell-free fluid.  


percentage of CD4+ lymphocytes are independent predictors of disease progression in HIV-infected persons initiating highly active antiretroviral therapy. J Infect Dis 195:425-431.


