Virus-driven Inflammation Is Associated With the Development of bNAbs in Spontaneous Controllers of HIV

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>As Published</td>
<td><a href="https://doi.org/10.1093/cid/cix057">https://doi.org/10.1093/cid/cix057</a></td>
</tr>
<tr>
<td>Publisher</td>
<td>Oxford University Press</td>
</tr>
<tr>
<td>Version</td>
<td>Original manuscript</td>
</tr>
<tr>
<td>Accessed</td>
<td>Wed Mar 13 01:52:14 EDT 2019</td>
</tr>
<tr>
<td>Citable Link</td>
<td><a href="http://hdl.handle.net/1721.1/118388">http://hdl.handle.net/1721.1/118388</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>Creative Commons Attribution-Noncommercial-Share Alike</td>
</tr>
<tr>
<td>Detailed Terms</td>
<td><a href="http://creativecommons.org/licenses/by-nc-sa/4.0/">http://creativecommons.org/licenses/by-nc-sa/4.0/</a></td>
</tr>
</tbody>
</table>
Virus-driven inflammation associates with the development of bNAbss in spontaneous controllers of HIV

Anne-Sophie Dugast*, Kelly Arnold*, Giuseppe Lofano, Sarah Moore, Michelle Hoffner, Melissa Simek, Pascal Poignard, Michael Seaman, Todd Suscovich, Florencia Pereyra, Bruce Walker, Doug Lauffenburger, Doug Kwon, Brandon Keele and Galit Alter

* These authors contributed equally to this work.

1 Ragon Institute of Massachusetts General Hospital, Harvard University and Massachusetts Institute of Technology, Cambridge, MA, 02139.

2 Department of Biological Engineering, MIT, Cambridge, MA, 02139.

3 Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, CA.

4 IAVI Neutralizing Antibody Center, The Scripps Research Institute, La Jolla, CA.

5 International AIDS Vaccine Initiative, New York, NY.

6 Beth Israel Deaconess Medical Center, Boston, MA

7 NCI-Frederick, MD.

Short title: Virus-driven inflammation and bNAbs in controllers
Abstract

Understanding the mechanism(s) by which neutralizing antibodies (NAbs) emerge naturally following infection is crucial for the development of a protective vaccine against HIV. While previous studies have implicated high viremia and associated immune activation as potential drivers for the development of bNAbs, here we sought to unlink the effect of these 2 parameters via the evaluation of predictors of NAb breadth evolution among a group of spontaneous controllers of HIV that exhibit low level viremia. A unique inflammatory profile, including high plasma levels of CXCL13, sCD40L, IP10, RANTES and TNFa, was observed among controllers who evolved bNAbs. Interestingly, viral loads and tissue viremia, but not intermittent blips, were associated with these cytokine profiles and NAb breadth. However, despite this association with elevated viremia, viral diversity was not significantly associated with increased breadth in controllers who develop bNAbs, suggesting that persistent exposure to higher levels of viremia, rather than more viral variants may be essential for the evolution of NAb breadth. Given that controllers naturally exhibit less antigenic variation than progressive patients, these results highlight the critical nature of antigenic persistence, in the setting of a unique inflammatory signature that may draw, retain, and activate B cells more effectively as a natural mechanism for the generation of protective humoral immune responses against HIV.
Introduction

The development of a protective vaccine against HIV will likely require the induction of cross-reactive broadly neutralizing antibodies (bNAb). Over the past decade, there has been a rapid increase in the discovery of a large number of novel bNAb (1-3) that have highlighted that only a limited set of sites of neutralizing vulnerability exist on the surface of the HIV viral envelope. However, despite the knowledge of the key immunological targets on the virus, the mechanism(s) by which these bNAb emerge naturally is unknown, but may hold the key for the rational design of immunogens that may elicit bNAb through vaccination.

Roughly 10-30% of chronically infected individuals develop neutralizing antibody breadth, but only years after infection (4-9). Key to the evolution of bNAb is the selection of highly mutated and unusual B cell receptors (BCR) (10, 11). Recent studies point to a critical role for rapid viral diversification in driving the evolution of these unusual BCR clonal repertoires (12, 13). In contrast, population based studies, using cross-sectional cohorts, pointed to an association between high-viremia (4), low CD4+ T cell counts (14), and infection associated immune activation (5) as drivers of the development of cross-neutralizing activity. Thus while it is plausible that a rapidly evolving antigen is critical for B cell diversification, it is also plausible that changes in CD4-help and/or the adjuvanting activity of specific inflammatory cytokine profiles may be equally critical for the maturation of the most effective B cell responses. However, because viral load, CD4-counts, and
inflammation are tightly linked, to date, resolution of the role of individual parameters on the evolution of bNAbs is incompletely understood.

While the majority of the most potent bNAbs have been isolated from untreated patients with high viremia, more recent studies in patients who spontaneously (15, 16) or in the absence of antiretroviral therapy (Elite suppressors) (17) control viremia, also develop remarkable neutralizing activity. Moreover, a number of recent potent bNAbs have been isolated from these patients that spontaneously control HIV to low levels, otherwise known as controllers (2) (7, 18) suggesting a role for additional factors, that beyond viremia, may be crucial for the development of neutralizing antibody breadth.

Thus, here we speculated that immunological signatures, beyond viremia alone, could be defined in spontaneous controllers and may provide critical insights for the specific immunological signals that are required for the evolution of these potent humoral immune responses. This study highlights the existence of a unique B cell adjuvanting inflammatory signature in controllers, in the setting of persisting, rather than highly diverse, antigenemia that if co-administered during immunization may potentially promote the evolution of bNAbs through vaccination.

Results
Controllers evolve neutralizing antibody breadth.

Previous studies have linked high viremia, low CD4-counts, and inflammation to the evolution of neutralizing antibody breadth (4, 5, 14). However, given that these features are all highly inter-related, separating out viral replication effects from unique inflammatory signatures, that may also be key to B cell activation, to precisely define the key drivers of neutralizing antibody activity have been difficult to resolve. Interestingly, many of the most potent bNAbs have been cloned from individuals with low viremia, arguing that neutralizing antibody activity may evolve even in the setting of low viral replication. Thus, because, a fraction of HIV-infected patients spontaneously control HIV viral replication in the absence of therapy, also known as controllers (19), we first aimed to determine whether high viremia was a requisite for the evolution of neutralizing antibody breadth resulting in only rare cases of neutralizing antibody activity among controllers. The breadth of NAbs was screened in 334 controllers using a panel of tier 2 and 3 viruses. Approximately, 25% of controllers neutralized at least 5 tier 2 viruses (Fig 1A). Specifically, 8% of the controllers neutralized 80% of viruses and 17% neutralized 45%-80% of the viruses tested. Conversely, 37% of controllers could not neutralize any tier 2 or 3 viruses, and were therefore classified as non-neutralizers (Fig 1A). These data illustrate that despite low to undetectable plasma viremia, a significant proportion of controllers generate neutralizing antibody breadth at the same frequencies as those observed in cohorts of patients with progressive disease (4).

A unique inflammatory signature associates with the development of neutralizing breadth in controllers.
Beyond viral loads, the evolution of neutralizing antibody activity has been linked to enhanced immune activation. Thus we next aimed to examine whether specific inflammatory profiles existed among controllers with neutralizing antibody breadth in the absence of high antigenemia. The levels of 18 distinct serum analytes including cytokines, chemokines, and secreted ligands all involved in T/B cell communication, were analyzed among 73 controllers that neutralized more than 45% of viruses and 85 controllers who did not evolve any appreciable bNAb. High plasma levels of sCD40L, IP10, CXCL13, RANTES and TNFα were individually associated with the emergence of neutralizing antibody breadth in controllers in univariate analyses (Fig 1B), suggestive of a unique inflammatory profile associated with the development of bNAb. Conversely, cytokines typically involved in T or B cell activation (IL4, IL6 IL13, etc), were instead associated with a lower likelihood to induce bNAb (Fig 1B). Furthermore, given that cytokines are induced in a coordinated manner, we next sought to define the multivariate biomarker profiles that best distinguished controllers who develop bNAb versus those who do not. Partial least squares discriminant analysis (PLSDA) revealed that a profile involving soluble CD40L, RANTES, TNF-α, IP-10 and CXCL13 classified controllers who develop bNAb versus those who do not with 69% classification accuracy and 66% cross-validation accuracy (Fig 1C-D). Though this profile was unable to perfectly classify individuals that exhibited a range of neutralization breadth profiles, these data indicate higher levels of specific combinations of cytokines that are more likely to be produced among individuals with bNAb activity. Moreover, multivariate and univariate analysis identified similar
cytokines that were associated with the evolution of breadth (Fig 1D). Importantly, the classification accuracy (into subjects with neutralizing breadth versus those without) was enhanced using the multivariate aggregate cytokine signature, which was not enhanced by the inclusion of viral load levels (data not shown). These data suggest that controllers who elicit neutralizing breadth exhibit a unique plasma cytokine inflammatory signature.

An overlapping, but narrower, inflammatory signature tracks with the development of bNAbs starting in acute HIV infection.

To further explore whether this unique inflammatory signature associated with bNAb responses in controllers, also emerged early in acute infection, we next analyzed the same panel of cytokines in a subset of 11 acutely infected individuals, half of which evolved neutralizing antibody breadth and half that did not (4). Though we only had access to a limited number of patients, a PLSDA model of all 18 cytokines measured at early time points pointed to a clear separation between acutely infected subjects that went on to generate neutralizing breadth compared to those that did not following infection. The model performed with 82% classification accuracy and 82% cross validation accuracy (Fig 2A). This profile was marked by high plasma level of CXCL13 and low level of MIP1a, IL1b, GMCSF, IL12p70 and IL4 (Fig 2B). Similarly, at later time points (>1 year post infection), we observed that the PLSDA modeling also classified patients reliably who went on to develop bNAbs or not with 83% classification accuracy and 83% cross validation accuracy (Fig 2C). At later time points, the multivariate profile was additionally marked by high plasma levels of IP10 and CXCL13, and low levels of IL10 and IL6.
in addition to the early low levels of IL1b, GMCSF, IL12p70, and IL4 (Fig 2D), suggesting that the cytokine signature becomes more complex with persisting infection.

Because previous reports in chronically HIV infected patients have linked CXCL13 levels to viral load levels (20), we next examined the relationship of viral load on cytokine levels. While the correlations between CXCL13 and IP10 with viral loads at early time points were not significant (Fig 2E and 2F respectively), positive correlations between these 2 cytokines and viral loads were significant at one year post-infection (r=0.6 and p=0.05 in Fig 2G; r=0.68 and p=0.018 in Fig 2H). As previously described (20-22), these data suggest an intimate link between CXCL13 and IP10 and viremia after the resolution of acute disease in patients unable to spontaneously control HIV viral replication in the absence of therapy (Fig 2G and 2H).

Viremia in blood and tissues associate with the development of neutralizing breadth.

Given the evolving association between viremia in acutely infected patients and cytokines associated with the bNAb activity, we next examined the relationship of viremia and the overall cytokine signature among our controllers. Specifically, controllers can be classified as those with detectable viremia (Viremic Controllers – VC> >50-2000 copies of RNA/ml) and those with undetectable viremia (Elite Controllers – EC< <50 copies of RNA/ml). Thus our initial analysis focused on VCs, that exhibit detectable viral loads. Viral loads in VCs correlated significantly with
plasma levels of CXCL13, IP10 and sCD40L ($r=0.27$ and $p=0.0094$, $r=0.22$ and $p=0.0318$, $r=0.27$ and $p=0.0084$ respectively; Fig 3A) suggesting that even low antigenemia may drive persistent levels of cytokines that may contribute to the development of bNAb.

Given that ECs exhibit undetectable viremia, no relationships with cytokines could be defined. However, 21% of ECs evolved bNAb activity in our cohort (data not shown), strongly arguing that despite their very low antigenemia, ECs still have the capacity to evolve this activity. Interestingly, CD4 counts were inversely correlated with the generation of neutralizing antibody breadth in ECs (Fig 3B) and elevated levels of IP10, TNFα and RANTES classified ECs with breadth from those that exhibited no bNAb activity (Fig 3C), suggesting the potential persistence of viremia outside of the blood (23). While the number of blips in viremia did not differ between the ECs who developed breadth and those who do not (data not shown), detectable viremia in at least one of the sampled tissues (PBMCs, duodenum, ileum and colon) was observed among ECs with bNAb activity compared to ECs that did not evolve breadth (Fig 3D). These data strongly argue that persistent antigenemia, in tissues, may constantly drive a unique cytokine profile, observable in the blood that together may play a critical role in driving the continued evolution of neutralizing antibody breadth.

Viral diversity is not associated with the evolution of neutralizing antibody breadth.
Key events in viral diversification (12, 24) have been linked to the evolution of bNAb activity. Similarly, high viremia, associated with greater viral diversity, has also been linked to the evolution of bNAbs (13). Thus we finally aimed to determine whether viral diversity, in addition to elevated antigen levels, was key to the evolution of bNAbs within the controllers. Thus viral envelopes were sequenced from the plasma of 10 VCs who developed neutralizing antibody breadth versus 10 VCs who did not, that were matched for viral loads (fig 4A and fig 4B). Interestingly, while equivalent numbers of viral envelopes were sampled (supplemental figure), only a marginal level of increased mean envelope diversity was observed among the VCs who developed bNAb-activity compared to those that did not (Fig 4C and 4D). Moreover, viral diversity was not correlated with neutralizing antibody breadth, suggesting that the presence of persistent antigenemia, perhaps in the setting of a unique inflammatory profile, rather than viral diversity, may be essential for the evolution of neutralizing antibody breadth.
Discussion

Understanding the mechanism(s) by which bNAbs arise naturally may provide the key for the rational development of a vaccine able to provide protection from HIV infection. The data presented here demonstrate that HIV controllers evolve neutralizing antibody breadth to a similar extent as patients that progress normally to HIV (4-9) despite the presence of lower viral set-points. Moreover, controller breadth appears to evolve in the setting of low-but persistent viral replication, the absence of significant viral diversity, but in the setting of a unique cytokine profiles. These data suggest that vaccine efforts that result in the expression of persisting antigenemia, in the setting of targeted cytokine profiles (ie. via select adjuvant usage) may contribute to the evolution of bNAb activity.

Whether the observed cytokine profiles contribute to the evolution of neutralization breadth or is a simple biomarker of persisting viral replication within specific tissues is unclear. However, the cytokine signature observed in controllers who evolve bNAbs represents a unique set of biomarkers that have been initially centrally linked to B cell activation and germinal center (GC) formation (25-27) that is associated with the development of bNAbs. Yet, these cytokines may also mark the persistence of viral replication within the lymphnode, a newly established sanctuary for the viral reservoir (23), where CXCL13 is readily produced to recruit cells to the germinal center (28), potentially highlighting the critical nature of persisting antigen at the site of antigen-presentation and selection of B cells. Conversely, the combined persistence of virus within the sanctuary, linked to the production of key activating B cell signals may be essential for the evolution of
neutralizing antibody breadth in the absence of high antigen diversity. Thus our data suggest that vaccine strategies able to “depot” antigen within GCs, linked to the induction of specific cytokine profiles via the use of specific adjuvants, may lead to the evolution of protective antibodies. Recently, TLR7/8 ligation has been linked to the production of CXCL13, suggesting that lymphnode targeting vaccine strategies, such amphiphile based approaches (29), may represent a critical means to induce the evolution effective B cell clonal responses.

Contrary to previous studies highlighting the critical nature of viral diversity in the evolution of neutralizing antibody breadth, here we observed only a marginal difference in mean viral diversity across VCs who develop significant neutralizing antibody breadth and those who do not. While diversification may be essential for the rapid evolution of breadth (12, 13), persistent antigenemia, in the setting of inflammatory profiles, may also lead to the evolution of breadth. However, whether the controllers that evolve breadth were infected with unique viral sequences that enabled the recruitment of particular germline BCRs or diversified rapidly (12, 13) is unclear. Importantly, the envelope sequences sampled among the neutralizer and non-neutralizer VCs did not show any unique features, even among the acutely infected patients, yet it is plausible that other viruses may persist within tissues that may exhibit unique properties that are able to induce bNAbbs. However, data from the acutely infected subjects clearly illustrate that elevated levels of CXCL13 soon after infection may be a key predictor for the evolution of breadth. Thus whether this cytokine marks the deposition and high viral replication of virus, again, within the GC, or a key to the evolution of more functional GCs soon after
infection is unclear but highlights the critical nature of continued GC activation, even by less diverse viral envelopes, among subjects that go on to elicit neutralizing antibody breadth. Thus these data raise the possibility that low concentration of a limited pool of antigens may be sufficient, in the setting of a strong and persistent GC response, to ultimately drive the generation of a protective humoral immune response.

Collectively, our results provide evidence that neutralizing antibody breadth is achievable in the absence of high levels of viremia and viral diversity. Instead, low concentration of a limited pool of antigens and strong and persistent GC stimulation may be required. While viremia may be necessary for the production of the cytokines/chemokines associated with B cell selection and GC formation, adjuvants alone or in combination, that drive similar inflammatory profiles may promote the development of NAbs in the setting of persistent vaccine immunogens, providing a novel means by which next generation vaccines may elicit bNAbs.

Material and Methods

Study subjects
A total of 163 controllers, 74 chronically infected individuals (2-5.8 log copies RNA/mL), and 14 untreated longitudinally tracked acutely infected individuals (3.8-5.6 log copies RNA/mL) (4) were recruited for this study. Controllers included both elite controllers (ECs), who spontaneously control viral replication to undetectable levels (<75 copies/mL with CD4=353-1813 cells/mm³) and viremic controllers (VCs) with detectable but low viral loads (~20-1658 copies of RNA/mL and CD4=172-1794 cells/mm³). Additionally, HIV-negative healthy control was obtained from the MGH blood bank. All subjects signed informed consent and the study was approved by the MGH Institutional Review Board.

**Neutralization assay**

HIV-1 neutralization breadth was assessed using the Tzm-bl cell–based pseudovirus neutralization assay, as described (30) against a panel of Env-pseudoviruses derived from 9 Clade B Tier 2 and two Tier 3 neutralization sensitivities: AC10.0.29*, RHPA4259.7*, THRO4156.18*, REJO4541.67*, WITO4160.33*, TRO.11*, SC422661.8*, QH0692.42*, CAAN5342.A2# and Tier 3: PVO.4* and TRJO4551.58*. Neutralization was defined as at least 50% inhibition of infection at a 1:20 dilution. The neutralization breadth was defined as the percentage of the 11 isolates neutralized by each plasma sample. All samples that showed reactivity to the murine leukemia virus–pseudotyped virion controls were excluded.

**Cytokine levels**
Plasma levels of 17 cytokines including IP10, sCD40L, TNFa, MIP1a, MIP1b, RANTES, IL4, IL5, IL6, IL8, IL10, IL13, IL12p70, IL17, IFNg, GMCSF and IL1b were measured by luminex (Millipore) and analyzed on a Bio-plex 200 (Bio-Rad Laboratories). CXCL13 plasma levels were determined by ELISA (R and D systems).

Detecting and quantifying HIV Viral Load by qRT-PCR

Pinch biopsies of intestinal tissue were mechanically homogenized. RNA was extracted (Qiagen) and quantitative reverse transcription-PCR (qRT-PCR) was performed (Agilent Technologies) using the HIV-1 gag SK462 (AGTTGGAGGACATCAAGCAGCCATGCAAAT) and SK431 (TGCTATGTCACTTCCCCCTTGGTTCTCT) primers. Relative HIV RNA copy numbers (viral load, VL) were normalized to levels of ribosomal S9 (ribs9) protein by qRT-PCR (forward: AAGGCCGCCGGGAACTGCTGAC, reverse: ACCACCTGCTTTGGAGGACCTGATA). Average relative gut VL was calculated as the mean VL across each measured gut compartment (transverse colon, terminal ileum, and duodenum). The limit of detection (10-7 relative copies) was used for compartments in which no HIV RNA was detected.

Brandon_Viral diversity

Statistical Analysis and Partial Least Squares Discriminant Analysis
An ANOVA with a post-hoc Tukey’s test was used to analyse cytokines/chemokines differences between groups. Spearman’s correlations were used to examine the inter-relatedness of different parameters. *p* values less than 0.05 were considered significant. Partial least squares discriminant analysis (PLSDA) (22, 31), was used to determine multivariate cytokine/chemokine profiles that best distinguished between HIV-infected individuals who developed bNAbs versus those who did not, as previously described (22). Cross-validation was performed by iteratively excluding subsets of data (in groups of 10%) before model generation, then testing model performance using excluded data. After initial generation of a model with all measured cytokines, the Variable Importance Projection (VIP) score of each cytokine was used to select cytokines that contributed most to cohort classification.
Acknowledgements

This work was supported by the Bill and Melinda Gates Foundation CAVD (OPP1066973: Development of broadly neutralizing antibodies in HIV infection and following immunization and OPP1032817: Leveraging Antibody Effector Function), the National Institute of Health (R01 AI080289, R01 A102660-01 and R37 AI080289-06A1), and the Ragon Institute of MGH, MIT and Harvard.
Fig 1. An inflammatory signature associates with the development of neutralizing antibody breadth in controllers.

(A) The pie chart represents the breadth of cross-neutralizing activity against a panel of 11 tier 2 virus isolates. (B) depicts the “relative risk” for controllers to develop or do not develop neutralizing activity based on the presence or absence of 18 different cytokines/chemokines for which the concentration (pg/mL) was measured in the plasma of controllers. (C) PLSDA reveals a multivariate cytokine signature that differentiates controllers who develop neutralizing activity (blue dots) and controllers who do not (red dots) with 31% and 33% error for calibration and cross-validation, respectively. (D) Latent variable 1 (LV1) represents the multivariate profile that differentiates controllers who develop bNAbs versus those who do not and is associated with high plasma levels of sCD40L, RANTES, TNFa, IP10 and CXCL13.

Fig 2. An early inflammatory signature associates with the development of bNAbs.

(A) PLSDA reveals an early cytokine signature (< 6months post infection) that differentiates acute infected individuals who go on to develop bNAbs (blue dots) versus those who do not (red dots) and characterized by high plasma levels of CXCL13 (B). (C) PLSDA reveals a unique cytokine signature late in infection (>1 year post infection) that is associated with the development of neutralizing activity
and that is characterized by high levels of CXCL13 and IP10 (D). (E-H) A Spearman rank correlation was used to analyze the relationship between the plasma levels of CXCL13 or IP10 with viral loads in early (E, F) or late (G, H) acutely infected individuals.

Fig 3. Viremia in blood and tissues associate with the development of neutralizing breadth.

(A) Spearman rank correlation was used to determine the relationship between the concentration level (pg/mL) of CXCL13, IP10, TNFa, RANTES and sCD40L in viremic controllers. (B) Spearman rank correlation was used to determine the relationship between the CD4 count levels and the neutralizing antibody breadth in elite controllers (full circles) and viremics (open circles). (C) Latent variable 1 (LV1) represents the multivariate profile that differentiates ECs who develop bNAbs versus those who do not and is associated with high plasma levels of IP10, TNFa, RANTES and IL4. (D) The level of viremia was measured by PCR in PBMCs, duodenum, ileum and colon in patients who develop bNAbs (N) versus those who do not (N) with each color dot representing one patient.

Fig 4. High viral diversity is not required for the development of bNAbs in controllers.
(A) depicts an evolutionary tree of the diversity of HIV viral envelope sequenced in the plasma of 10 viremic controllers who develop bNAbs and 10 viremic controllers who do not. (B) shows no differences in viral loads between the 20 viremic controllers (half of which that develop bNAbs and half that do not) in which the viral envelopes were sequenced. (C) represents the mean viral diversity in 10 viremic controllers who develop bNAbs (in blue) and 10 viremic controllers who do not (in red). (D) Spearman rank correlation was used to determine the relationship between the breadth of neutralization and the mean viral diversity in 20 viremic controllers, half of which develop bNAbs (blue circles) and half that do not (red circles).

**Supplemental Fig 1. Equivalent number of sequences was analyzed in each patients.**

The figure depicts a Spearman rank correlation between the mean viral diversity measurement and the number of viral sequences analyzed in the plasma of 10 viremic controllers who develop bNAbs (blue) and 10 viremic controllers who do not (in red).
References


**Figures**
Figure 4

A

B

C

D

Supplemental Figure

\[ r = -0.1035 \]
\[ p = 0.6829 \]