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Env-Specific IgA from Viremic HIV-Infected Subjects Compromises Antibody-Dependent Cellular Cytotoxicity

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ABSTRACT

Elucidating the factors that modulate HIV-specific antibody-dependent cellular cytotoxicity (ADCC) will help in understanding its role in HIV immunity. The aim of this study was to determine whether IgA could modify the magnitude of ADCC in HIV infection, abrogating its protective role. Plasma samples from 20 HIV-positive (HIV^+) subjects enrolled during primary HIV infection (PHI), 10 chronically infected subjects (chronic), and 7 elite controllers (EC) were used. ADCC was determined by using a fluorometric ADCC assay, before and after removal of plasma IgA. Data were analyzed by using nonparametric statistics. ADCC was documented in 80% of PHI enrollment samples and in 100% of PHI 12-month, chronic, and EC samples; it peaked after acute infection, reached a plateau in chronic infection, and decreased after initiation of antiretroviral treatment (ART). Significant associations between ADCC and disease progression were found only after removal of plasma IgA from 12-month PHI samples: the magnitude of ADCC not only increased after IgA removal but also correlated with $CD4^+$ T-cell preservation. This work provides evidence that gp120-specific IgA was capable of modifying ADCC responses during natural HIV infection for the first time and adds to similar evidence provided in other settings. Furthermore, it underscores the complexity of the ADCC phenomenon and will help in an understanding of its underlying mechanisms.

IMPORTANCE

Although the induction of ADCC-mediating antibodies in HIV-infected subjects has been extensively documented, the association of these antibodies with protection from disease progression is poorly understood. Here, we demonstrate that plasma IgA is a factor capable of modifying the magnitude of IgG-mediated ADCC in HIV infection, mitigating its beneficial effect. These results help in understanding why previous studies failed to demonstrate correlations between ADCC and disease progression, and they also contribute to the notion that an HIV vaccine should stimulate the production of ADCC-mediating IgG antibodies but not IgA.

Despite the success of antiretroviral treatment (ART), human immunodeficiency virus (HIV) still represents a major public health concern (1), and a vaccine is urgently needed. One major advance of the RV144 trial was the achievement of an estimated efficacy of 31.2% (2). Subsequent correlate analyses showed that Env-specific IgG antibodies correlated inversely with infection risk (3). Moreover, antibody-dependent cellular cytotoxicity (ADCC), broadly induced by this vaccine regimen (4), was found to be a correlate of protection in vaccinees with low levels of Env-specific IgA (3). These findings suggested that the modest protection induced by the RV144 vaccine regimen might be attributed to humoral immunity and, more specifically, to ADCC.

Besides the RV144 trial, there are many other reasons to reexamine the mechanisms of ADCC during the natural course of HIV infection. The induction of ADCC-mediating antibodies in plasma (5–11), cervicovaginal fluids (12, 13), and breast milk (14) from HIV-infected subjects has been extensively documented. However, their association with protection from disease progression is less unequivocal. Cohort studies performed with elite controllers (ECs) showed that these individuals had higher ADCC than viremic subjects (9). One early report by Baum et al. (5) established that ADCC was associated with disease progression in terms of $CD4^+$ T-cell counts, but later, other studies on recently and chronically infected subjects failed to demonstrate definitive and conclusive associations (7, 8, 11, 15–20). More recently, passively acquired ADCC activity in infants born to HIV-infected mothers was not associated with protection but was associated with reduced mortality (21).

Many factors could have influenced the dissimilar results and precluded the drawing of definite conclusions, including the use of different models to assay ADCC, inclusion criteria to enroll

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TABLE 1 Characteristics of HIV$^+$ subjects enrolled per study group

Group and patient	Sex[i]	Age (yr)	Infection stage[a]	No. of days postinfection[b]	VL[c,d]		Viral set point[f] (log_{10} HIV-1 RNA copies/ml plasma)	CD4$^+$ T-cell count[cec,e] (cells/μl)	CD4 set point[f] (cells/μl)	Presence of <350 CD4 cells/μl during 1st yr[g]	Treatment with HAART[h]
					HIV-1 RNA copies/ml plasma	Log_{10} HIV-1 RNA copies/ml plasma					
PHI (n = 20)											
NP01	M	45	Stage V	60	2,707	3.4	3.9	902	600	No	No
NP02	M	40	Stage VI	60	8,613	3.9	4.3	851	758	No	No
NP03	M	47	Stage V	45	112	2.0	2.5	743	679	No	No
NP04	M	43	Stage V	45	19,522	4.3	4.3	525	442	No	No
NP05	F	37	Stage V	60	9,532	4.0	3.6	590	587	No	No
NP06	M	32	Stage VI	150	256,051	5.4	4.7	797	943	No	No
NP07	M	33	Stage V	45	21,986	4.3	3.3	609	658	No	No
NP08	F	26	Stage VI	60	17,420	4.2	4.1	685	402	No	No
NP09	F	34	Stage V	24	>500,000	>5.7	4.3	603	490	No	No
NP10	M	73	Stage VI	120	13,962	4.1	4.6	577	544	No	No
NP11	M	30	Stage V	45	455,417	5.7	5.2	698	685	No	No
P01	F	31	Stage V	30	>500,000	>5.7	5.4	374	342	Yes	No
P02	F	41	Stage VI	90	3,662	3.6	3.9	302	281	Yes	No
P03	M	43	Stage V	45	98,684	5.0	4.9	256	256	Yes	No
P04	M	40	Stage V	70	1,147	3.1	3.5	568	424	Yes	No
P05	M	25	Stage VI	60	242,199	5.4		161		Yes	Yes
P06	M	36	Stage VI	60	>500,000	5.7		475		Yes	Yes
P07	M	26	Stage VI	60	>500,000	5.7		446		Yes	Yes
P08	M	45	Stage V	60	>500,000	5.7		259		Yes	Yes
P09	F	28	Stage VI	30	>500,000	5.7		222		Yes	Yes
Chronic (n = 10)											
C01	F	21	Chronic		85,947	4.9		25			No
C02	M	22	Chronic		18,580	4.2		511			No
C03	M	49	Chronic		43,436	4.6		479			No
C04	M	28	Chronic		555	2.7		555			No
C05	M	36	Chronic		1,288	3.1		431			No
C06	F	27	Chronic		1,817	3.2		689			No
C07	M	47	Chronic		11,026	4.0		585			No
C08	F	38	Chronic		22,475	4.3		143			No
C09	F	29	Chronic		14,784	4.3		139			No
C10	F	24	Chronic		253,167	5.4		5			No
EC (n = 7)											
EC01	M	54	Chronic		<50	<1.7		566			No
EC02	F	41	Chronic		<50	<1.7		912			No
EC03	M	36	Chronic		<50	<1.7		456			No
EC04	F	60	Chronic		<50	<1.7		570			No
EC05	F	43	Chronic		<50	<1.7		817			No
EC06	M	39	Chronic		<50	<1.7		595			No
EC07	F	37	Chronic		<50	<1.7		344			No

[a] PHI subjects were stratified according to Fiebig stages (31).
[b] Number of days from the presumed date of infection to the date at which the enrollment sample was obtained.
[c] For PHI subjects, data correspond to enrollment samples. For chronically infected and EC subjects, data correspond to samples obtained at enrollment.
[d] Performed by using a Versant HIV-1 RNA 3.0 assay (Siemens). Lower and upper detection limits are 50 and 500,000 HIV RNA copies/ml, respectively (1.7 and 5.7 log_{10} HIV RNA copies/ml, respectively).
[e] Flow cytometry double platform (FACSCanto; BD Biosciences).
[f] Applicable only to PHI subjects who did not start highly active antiretroviral therapy during the first year postinfection (see Materials and Methods).
[g] Indicates whether the CD4$^+$ T-cell count dropped below 350 cells/μl at any time during the first year postinfection or not, thus defining subgroups where the PHI counts were <350 and >350 cells/μl (see Materials and Methods).
[h] Regarding PHI subjects, data illustrate if subjects started highly active antiretroviral therapy (HAART) during the first year postinfection. For those subjects who started highly active antiretroviral therapy, enrollment samples were obtained before treatment initiation. Data indicate whether chronically infected and EC subjects had ever been on highly active antiretroviral therapy.
[i] F, female; M, male.

TABLE 2 Summary of clinical data corresponding to HIV$^+$ subjects enrolled per study group[g]

Group	Median no. of days postinfection (IQ range)[a]	Viral load[c,d]		Viral set point[f] (mean $\log_{10}$ HIV RNA copies/ml ± SD)	Median CD4$^+$ T-cell count[d,e] (cells/μl) (IQ range)	Median CD4 set point[f] (cells/μl) (IQ range)
		Median HIV RNA copies/ml (IQ range)	Mean HIV RNA $\log_{10}$ copies/ml ± SD			
PHI						
All ($n = 20$)[b]	60 (45–60)	60,335 (8,843–488,854)	4.6 ± 1.07	4.1 ± 0.7	572 (320–695)	544 (402–679)
PHI>350 ($n = 11$)	60 (45–60)	17,420 (8,613–256,051)	4.2 ± 0.74	4.2 ± 1.7	685 (590–797)	600 (490–685)
PHI<350 ($n = 9$)	60 (37,50–65)	242,199 (51,173–500,000)	4.9 ± 0.9	4.4 ± 0.87	302 (239–460)	311 (262–403)
Chronic ($n = 10$)		16,682 (1,685–54,064)	4.1 ± 0.83		455 (110–562)	
EC ($n = 7$)		<50	<1.7		570 (456–817)	

[a] Number of days from the presumed date of infection to the date at which the enrollment sample was obtained.
[b] In this group, 5 subjects initiated highly active antiretroviral therapy during the first year postinfection.
[c] Versant HIV-1 RNA 3.0 assay (Siemens). Lower and upper detection limits are 50 and 500,000 HIV RNA copies/ml, respectively (1.7 and 5.7 $\log_{10}$ HIV RNA copies/ml, respectively).
[d] For PHI subjects, data correspond to enrollment samples. For chronic and elite controller subjects, data correspond to samples obtained at enrollment.
[e] Flow cytometry double platform (FACSCanto; BD Biosciences).
[f] Set points were not calculated for subjects who initiated highly active antiretroviral therapy during the first year postinfection ($n = 5$) (see Materials and Methods).
[g] IQ, interquartile.

study subjects, and definitions of progression, but also, the putative existence of mitigating plasma factors interfering with ADCC has been proposed as a factor. In other words, if the potential protective role of ADCC-mediating antibodies was mitigated by any factor during natural infection, it would not be surprising to find any associations between ADCC and progression. Remarkably, this has not been extensively studied yet, highlighting that the field deserves further research. The IgG1 and IgG3 subclasses were shown to be potent inducers of anti-HIV ADCC (19, 21–23). Conversely, the role of the IgA isotype is controversial (24). Correlate analysis from the RV144 trial suggested that vaccine-induced plasma IgA might block IgG binding, interfering with its effector function (23). However, whether such an effect might occur in HIV-infected subjects has not been elucidated yet.

The aim of this study was to determine if IgA was a factor capable of modifying the magnitude of IgG-mediated ADCC in HIV infection, abrogating its protective role. The results indicated that the magnitude of ADCC after removal of IgA was higher than that in nondepleted plasma and correlated directly with the percentage of CD4$^+$ T cells in viremic subjects, thus supporting the hypothesis presented. To our knowledge, this is the first study demonstrating that the beneficial effect of ADCC is mitigated by gp120-specific IgA during natural HIV infection.

MATERIALS AND METHODS

Study subjects. The following study groups were enrolled (Tables 1 and 2): 8 healthy HIV-seronegative donors (HIV^{neg}), 10 chronically infected subjects (chronic) (infected for >3 years, detectable viral load [VL], and ART naive), 7 ECs (infected for >5 years, undetectable VL [<50 HIV RNA copies/ml plasma], ART naive, and no record of opportunistic and/or AIDS-related diseases), and 20 subjects enrolled within 6 months from the presumed date of infection (primary HIV infection [PHI]). PHI subjects were enrolled by the Grupo Argentino de Seroconversión Study Group. Inclusion criteria, time from infection, and set point calculations were reported previously (25–27). Briefly, a PHI enrollment sample was obtained in the case of detection of HIV RNA or p24 antigen with a simultaneous negative or indeterminate Western blot assay result or a positive Western blot result with a negative test within the previous 6 months. Chronically infected individuals were defined as subjects infected for >3 years who had a detectable VL (>50 HIV RNA copies/ml plasma) and were ART naive, and ECs were defined as subjects infected for >5 years who had an undetectable VL (<50 HIV RNA copies/ml plasma), were ART naive, and had no record of opportunistic and/or AIDS-related diseases. The study was reviewed and approved by the Comité de Ética Humana, Facultad de Medicina, Universidad de Buenos Aires. All participants provided written informed consent.

Plasma VL (branched DNA) (Versant HIV-1 RNA 3.0 assay; Siemens Healthcare, United Kingdom), CD4$^+$ T-cell count (flow cytometry double platform) (BD FACSCanto; BD Biosciences, USA), cellular immune activation (HLA-DR and CD38 expression on peripheral blood mononuclear cells [PBMCs] as determined by flow cytometry [26]), and plasma soluble factors (39-plex Milliplex multi-analyte panel human cytokine/chemokine kit; Millipore, USA) were assessed as described previously (26–28).

Samples. Blood samples were collected and centrifuged to separate plasma. For ADCC assays, plasma was first diluted (10-fold in RPMI medium), passed through a 0.2-μm-pore-size filter, and heat inactivated (1 h at 56°C). PBMCs from one HIV-negative donor were isolated by Ficoll-Hypaque density centrifugation (Amersham, Sweden), cryopreserved, and used as effector cells in ADCC assays. Cells from the same donor were used in all assays to avoid bias from donor to donor.

RFADCC assay. A rapid fluorometric ADCC (RFADCC) assay was performed as described previously (29). Briefly, CEM-NK^R target cells (AIDS Research and Reference Reagent Program) (30) were double stained with PKH26 red fluorescent cell linker (Sigma-Aldrich, USA) and CFSE (carboxyfluorescein diacetate succinimidyl ester; Molecular Probes, USA) and coated with a recombinant gp120 protein derived from the HIV-1 BaL strain (recombinant $gp120_{BAL}$, obtained from the AIDS Research and Reference Reagent Program, DAIDS, NIAID). After 1 h, cells were dispensed in U-bottom 96-well plates (5,000 cells/well) together with different dilutions of inactivated plasma, in triplicates. After 15 min at room temperature, effector cells (PBMCs thawed and rested overnight) were added at an effector-to-target cell ratio of 50:1. Plates were centrifuged and incubated for 4 h at 37°C. Cells were washed, fixed, acquired on a FACSCanto flow cytometer (BD Biosciences, USA), and analyzed by using FACSDiva v6.1.3 software (BD Biosciences). Target cells were initially gated on a forward-scatter (FSC)-versus-side-scatter (SSC) plot and subsequently gated on an SSC-versus-PKH26 plot. Next, a PKH26-versus-CFSE plot was generated to determine the percent target cell killing (%ADCC killing), which was calculated as the proportion of cells that remained PKH26^{high} but that had lost the viability dye (CFSE^{neg}). Results are presented as the medians of data from experiments performed in

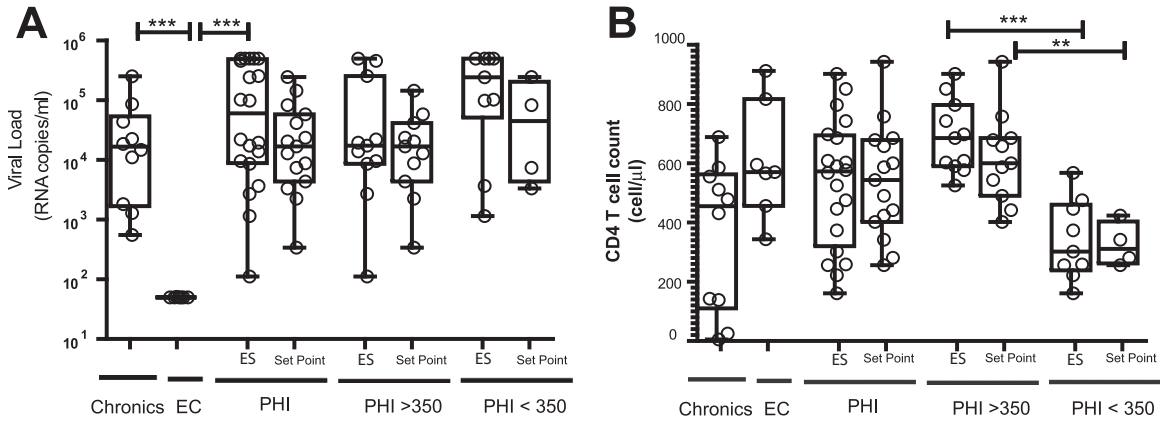

FIG 1 Viral loads (A) and CD4 T-cell counts (B) of enrolled HIV$^+$ subjects per study group. For PHI subjects, values corresponding to both baseline and the set point are shown. Also, PHI subjects are shown as a whole group (PHI) and split into subgroups, PHI>350 and PHI<350, dependent on whether their CD4$^+$ T-cell count dropped below 350 cells/μl at any time during the first year postinfection or not. For PHI subjects, viral and CD4 set points were calculated as the geometric means of determinations obtained between 6 and 12 months after the presumed date of infection. Set points were not calculated for those subjects who started ART during the first 12 months of infection ($n = 5$). Inclusion criteria for subjects and time from infection were reported previously (25–27). The 350-cells/μl endpoint was chosen based on national and international recommendations for initiation of ART from 2010, when most of these individuals were already enrolled (25). Horizontal lines stand for median values. ES, enrollment samples. *P* values were calculated by using a Mann-Whitney U test. Asterisks denote different *P* values: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

triplicate and with the background subtracted (%ADCC killing for uncoated target cells). The threshold for positive responses for a given plasma dilution was defined as the mean of the background plus 3 standard deviations (SD). The endpoint ADCC titer was defined as the highest plasma reciprocal dilution at which the %ADCC killing was greater than or equal to the cutoff value.

Coating and saturation of target cells were verified by flow cytometry with anti-gp120 monoclonal antibody 2G12 (AIDS Research and Reference Reagent Program) followed by staining with anti-human IgG-allophycocyanin (APC).

ELISA for Env-specific plasma IgG and IgA. The gp120-specific IgG concentration and gp120-specific IgA titers were determined by an enzyme-linked immunosorbent assay (ELISA). Ninety-six-well, flat-bottomed, half-area plates (GreinerBio-One, Germany) were coated with 25 ng/well of $gp120_{BAL}$. For gp120-specific IgG quantitation, 25 μl/well of plasma dilutions (initially 1/500 for PHI enrollment samples and 1/10,000 for the other groups) was dispensed in triplicates. A standard curve was constructed, consisting of 2-fold serial dilutions of anti-gp120 monoclonal antibody 2G12 starting at 24 ng/ml. IgG detection was performed by using an anti-human IgG antibody labeled with horseradish peroxidase (HRP; Sigma-Aldrich, USA) and developed with tetramethylbenzidine (TMB)-ELISA solution (BD Biosciences, USA). The absorption at 450 nm was read on a Multiskan EX microplate reader (Thermo/Labsystems). The IgG concentration was extrapolated from the standard curve and multiplied by the dilution factor. gp120-specific IgA levels were determined by endpoint titration. Twofold serial dilutions of plasma were prepared, starting at a 1:20 dilution. The secondary antibody was an anti-human IgA-HRP (Sigma-Aldrich, USA). Plates were developed and read as described above for IgG. The endpoint IgA titer was defined as the reciprocal of the highest plasma dilution at which the average optical density (OD) value was ≥2-fold the average OD value for control wells. Sera from HIV-negative subjects were tested as controls.

Depletion of plasma IgA. Bulk IgA was removed from plasma by using the Pierce immunoprecipitation kit (Thermo Scientific, USA). Briefly, 1 mg of goat anti-human IgA antibody (Sigma-Aldrich, USA) was immobilized onto columns. Plasma samples diluted 1/10, filtered, heat inactivated, and supplemented with protease inhibitors (Pierce) were added to the columns, incubated overnight at 4°C, and eluted by centrifugation. The flowthrough was collected and stored for further analysis. Plasma samples passed through columns coupled with an isotype-matched control antibody were used as controls (nondepleted plasma). To confirm IgA (but not IgG) depletion, dot blots developed by using either HRP-labeled goat anti-human IgA or IgG antibodies were performed. Additionally, levels of gp120-specific IgA and IgG were requantitated by an ELISA after IgA depletion, as described above for whole plasma. Additionally, IgG binding to gp120-coated cells in IgA-depleted and nondepleted plasma samples was evaluated by flow cytometry using anti-human IgG-APC as a secondary antibody. The mean fluorescence intensity (MFI) was recorded.

Data analysis. Statistical analyses were performed by using GraphPad Prism 5 software. Data are expressed as median values with interquartile ranges (25% to 75%) and were analyzed by nonparametric methods unless otherwise stated. Mann-Whitney or Kruskal-Wallis tests were used to compare two or multiple intergroup variables, respectively. A Wilcoxon test was used to compare paired variables. Correlations were determined by using Spearman's rank test. All tests were considered significant when the *P* value was <0.05.

RESULTS

Description of study subjects. Plasma samples from 37 HIV-infected subjects were used: 20 PHI subjects enrolled within 6 months of infection, 10 chronically infected subjects, and 7 ECs (shown in Table 1 and summarized in Table 2). Enrollment samples for PHI subjects were obtained at a median of 60 days from the presumed date of infection and corresponded to Fiebig stages V and VI (31). The median VL and CD4$^+$ T-cell counts for the PHI group at enrollment were 60,335 HIV RNA copies/ml and 572 cells/μl, respectively (Table 2 and Fig. 1). For the chronic group, median VL and CD4$^+$ T-cell counts were 16,682 HIV RNA copies/ml and 455 cells/μl, respectively. ECs had undetectable VL and a median of 570 CD4$^+$ T cells/μl. In the PHI group, 5 subjects initiated ART during the first year postinfection (median of 90 days postinfection) following medical indication (Table 1). However, enrollment samples were always obtained before ART instauration. Data obtained by using samples obtained when the subject was on ART were analyzed separately. For certain analyses, PHI subjects were further divided into two subgroups (Tables 1 and 2 and Fig. 1): those whose CD4$^+$ T-cell counts dropped below

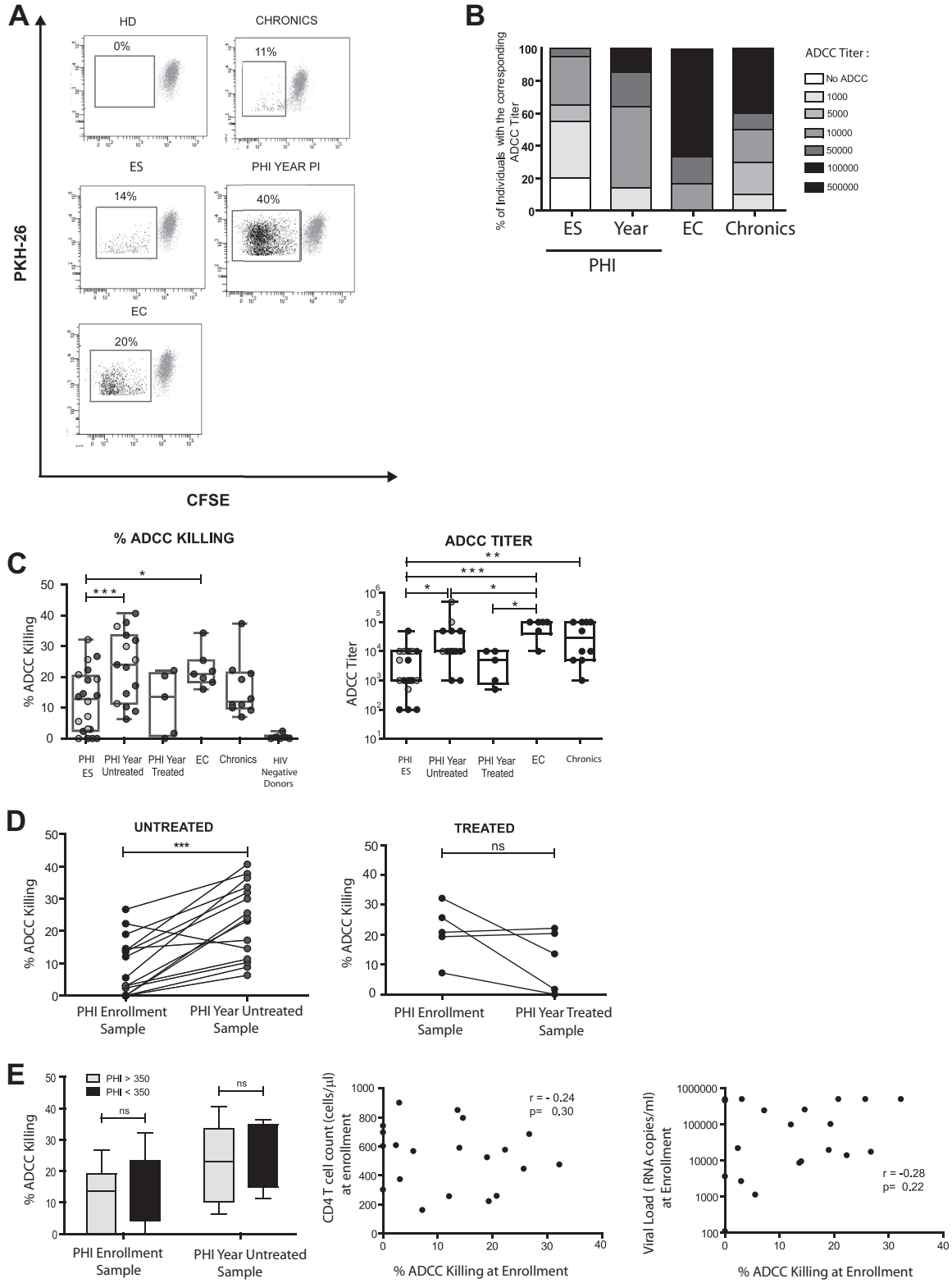

FIG 2 ADCC responses in PHI subjects, chronically infected individuals (chronics), and elite controllers (EC) measured by an RFADCC assay.(A) Dot plots for one representative subject per study group. A PKH26-versus-CFSE plot was generated to determine the percentage of ADCC killing, defined as the proportion of cells that remained PKH26^{high} but lost the viability dye (CFSE^{neg}). Percentages shown in each plot correspond to results obtained after calculating and subtracting the background (%ADCC killing for uncoated target cells) from the media of triplicate conditions. Initially, gating was performed with an FSC-versus-SSC plot. Target cells were gated on an SSC-versus-PKH26 plot. (B) Proportion of individuals with the corresponding ADCC titers. To calculate ADCC titers, the threshold for positive responses was defined as the mean background %ADCC killing plus 3 SD. The endpoint ADCC titer was defined as the reciprocal of the higher plasma dilution at which the %ADCC killing was greater than or equal to the threshold. (C)

350 cells/μl at any time during the first year postinfection (PHI<350) (rapid progressors) and those whose $CD4^+$ T-cell counts did not (PHI>350) (typical progressors). By doing this, we aimed to differentiate subjects with more rapid or aggressive progression of early infection and to investigate the association of this pattern with the magnitude of ADCC responses. Regarding chronically infected subjects, the individuals enrolled in this study included subjects with preserved immune status as well as subjects with advanced immune deterioration (Table 1), reflecting the natural heterogeneity of such an HIV-positive (HIV^+) population.

ADCC responses emerge early after infection and increase during the first year postinfection but not in subjects receiving ART. First, we aimed to revisit previously reported ADCC findings by determining gp120-specific antibodies capable of mediating ADCC in plasma samples from all study subjects (for PHI, enrollment and 12-month samples were used) by using an RFADCC assay. Representative dot plots are shown in Fig. 2A. The results indicated that 80% of PHI subjects had detectable ADCC responses (defined as a titer of ≥1,000) at enrollment, with this number increasing to 100% by 12 months postinfection. Chronically infected subjects had ADCC titers of ≥1,000, and all ECs had titers of ≥10,000 (Fig. 2B). When comparing PHI enrollment and 12-month samples from ART-naive subjects, both the %ADCC killing and ADCC titers significantly increased over time (median %ADCC killing of 12.85% versus 24% [$P = 0.0003$]; median titer of 1,000 versus 10,000 [$P = 0.0206$]) (Fig. 2C and D, left). Conversely, subjects who started ART during the course of the study showed a decrease or stability of both ADCC titers and %ADCC killing with the 12-month samples, compared to samples from untreated PHI subjects, chronically infected subjects, and ECs (Fig. 2C and D, right). The %ADCC killing and ADCC titers did not differ significantly between chronically infected and EC subjects. Only a minor, but still significant, difference was observed in comparisons of EC and PHI 12-month ADCC titers.

Overall, gp120-specific antibodies capable of mediating ADCC arise early after infection, and their levels increase over time. This was further noticed when PHI subjects were segregated according to their Fiebig stage at enrollment: samples corresponding to Fiebig stage VI had significantly higher ADCC than did Fiebig stage V samples by analysis of either %ADCC killing (medians of 3.1% and 19.33%, respectively; $P = 0.02$) or ADCC titers (medians of 1,000 and 10,000, respectively; $P = 0.01$), thus reinforcing the notion that the magnitude of the ADCC antibody response rapidly increases over time after infection. A plateau is then eventually reached, since no significant difference was observed between PHI 12-month samples and samples from the chronically infected group. Although ECs tended to have higher ADCC than chronically infected individuals, no statistically significant difference was observed, indicating that high titers of ADCC-mediating antibodies are maintained despite the absence of detectable viral replication in standard VL assays (50 HIV RNA copies/ml). The results also illustrated that initiation of ART during the first year postinfection modifies the kinetics of the ADCC response.

No association between the magnitude of the ADCC response and early disease progression. We then sought to investigate if the magnitude of the early ADCC response impacts subsequent disease progression. First, ADCC responses in samples from the PHI<350 and PHI>350 groups at both enrollment and 12 months were compared. No significant differences were observed between the PHI<350 and PHI>350 groups for either enrollment or 12 month samples (Fig. 2E, left [%ADCC killing]; ADCC titers not shown). No correlations were found between %ADCC killing at enrollment and enrollment VL (Fig. 2E, middle), $CD4^+$ T-cell counts (right), levels of cellular immune activation, or levels of soluble plasma biomarkers of immune activation (not shown). More relevant, no correlations were observed between ADCC at enrollment (evaluated as either %ADCC killing or ADCC titer) and viral set point, immune set point, $CD4^+$ T-cell decay slope, or 12-month VL and $CD4^+$ T-cell counts as alternative markers of disease progression (not shown). Finally, there were no observed correlations between 12-month ADCC and concurrent VL or CD4 T-cell count. Overall, these analyses showed that the magnitude of the early ADCC response has no impact on subsequent disease progression during the time frame investigated.

HIV-1 Env-specific IgG levels and IgG/IgA ratios (but not IgA levels) correlate with ADCC responses. Given the lack of observed correlations between ADCC and protection from disease progression, and considering previous reports attributing a protective role to ADCC-mediating antibodies in humans (5, 8, 9), we decided to investigate the presence of putative mitigating factors in the plasma of infected subjects by determining gp120-specific IgG and IgA levels and IgG/IgA ratios in subjects' plasma samples.

Levels of Env-specific IgG in PHI enrollment samples were significantly lower than those in PHI 12-month samples and in samples from the chronic and EC groups and increased over time in untreated infection but decreased in those subjects that initiated ART (Fig. 3A). No significant difference was observed between PHI 12-month, chronic, and EC samples. Conversely, gp120-specifc IgA titers declined over the course of infection except in ECs, who maintained significantly higher gp120-specifc IgA titers than those in 12-month PHI and chronic samples (Fig. 3B).

In order to exclude that declining gp120-specific IgA concur-

%ADCC killing corresponding to a plasma dilution of 1/1000 (left) and ADCC titers (right), corresponding to each group. %ADCC data correspond to background-subtracted values. Of note, the indicated differences in %ADCC killing were also statistically significant at all plasma dilutions tested. For HIV-negative donors, %ADCC killing corresponded to a 1/100 plasma dilution, which was the only dilution tested. (D) ADCC responses in PHI enrollment and 12-month postinfection samples from subjects who remained ART naive (left) ($n = 15$) or not (right) ($n = 5$) during the first year postinfection. Each line represents one subject. Results correspond to a plasma dilution of 1/1,000. (E) PHI subjects were further divided into two subgroups depending on whether their CD4 T-cell count dropped below 350 cells/μl at any time during the first year postinfection (PHI<350) or not (PHI>350). (Left) Comparison of %ADCC killing between the PHI<350 and PH>350 groups in both enrollment and 12-month samples (only ART-naive subjects). Results correspond to a 1/1,000 plasma dilution. (Middle and right) Correlations between ADCC responses evaluated in PHI subjects at enrollment versus enrollment $CD4^+$ T-cell counts (middle) and enrollment viral loads (right). For box-and-whisker plots, the horizontal line represents the median value, the boxes represent the interquartile range, and the whiskers extend from minimum to maximum values. Intra- and intergroup differences were analyzed by using Wilcoxon and Mann-Whitney tests, respectively. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. In panel B, black and gray dots denote PHI>350 and PHI<350 subjects, respectively. In correlation analyses, r and P values were determined by Spearman's test, and all P values were nonsignificant (ns). ES, enrollment samples; PI, postinfection; HD, healthy HIV-negative donors.

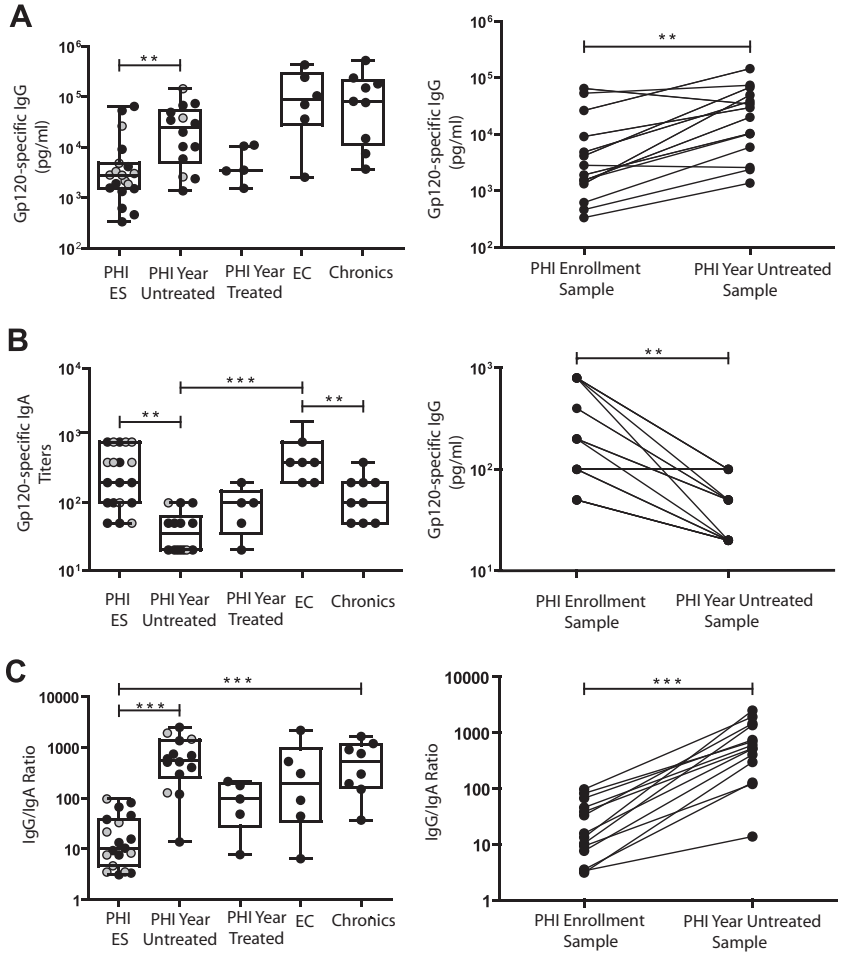

FIG 3 gp120-specific IgG concentrations, IgA titers, and IgG/IgA ratios among PHI, chronic, and EC subjects. To determine gp120-specific IgG antibodies and gp120-specific IgA titers, ELISAs were performed. (A) gp120-specific IgG antibodies (picograms per milliliter). (B and C) gp120-specifc IgA titers (B) and IgG/IgA ratios (C). (Left) Distribution of gp120-specific antibodies and IgG/IgA ratios in all study groups. (Right) Subject-by-subject comparison of gp120-specific antibodies in PHI enrollment versus 12-month samples (ART-naive subjects only). Each line represents one subject. In the left panels, the horizontal lines in box-and-whisker plots represent the median values, the boxes represent the interquartile ranges, and the whiskers extend from the minimum to the maximum values. Intra- and intergroup differences were analyzed by using Wilcoxon and Mann-Whitney tests, respectively. ES, enrollment sample. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. Black and gray dots denote PHI>350 and PHI<350 subjects, respectively.

rent with increasing gp120-specific IgG titers could be an artifact introduced by IgG outcompeting IgA, IgG was depleted in five plasma samples, and gp120-specific IgA was reanalyzed by an ELISA. Identical titers were obtained, excluding the above-mentioned possibility (not shown). IgG/IgA ratios increased over time during PHI, being significantly higher in chronic and in PHI 12-month samples than in PHI enrollment samples (Fig. 3C). No significant difference was observed among 12-month PHI, chronic, and EC samples.

Afterwards, correlations between ADCC and gp120-specific antibodies were studied. For this purpose and since no differences in IgG, IgA, and IgG/IgA ratios between 12-month PHI and chronic samples were observed, data from these groups were merged. To do this, we also took into account that samples from subjects with PHI at 12 months postinfection and from chronically infected subjects did not differ in their virological and immunological parameters (contrary to ECs) (Fig. 1). As reported previously (12, 14, 17, 20), gp120-specific IgG levels directly correlated with ADCC in all study groups ($r = 0.476$ and $P = 0.039$ for PHI enrollment samples [not shown] [shown in Fig. 4A for the rest of the groups]). Conversely, no correlations between gp120-specific IgA and ADCC responses were observed (Fig. 4B). Interestingly, gp120-specific IgG/IgA ratios showed strong direct correlations with ADCC magnitude in the group of 12-month PHI and chronic subjects and in the EC group (Fig. 4C), providing initial support to the notion that gp120-specific IgA antibodies might mitigate the capacity of specific IgG to mediate ADCC.

Depletion of IgA from plasma of viremic HIV-infected subjects enhances the magnitude of ADCC. According to our initial hypothesis, and based on the significant correlations between IgG/IgA ratios and ADCC, we depleted IgA from plasma samples of six randomly selected PHI subjects (12-month samples) and four ECs and reevaluated ADCC responses in both IgA-depleted and non-depleted (passed through an isotype control-coupled column) plasma samples. Figure 5A shows results from one representative PHI subject: IgA was successfully and specifically depleted, as shown by dot blotting (left). In all cases, the gp120-specific IgA titer was <10 after depletion. The ADCC measured in IgA-de-

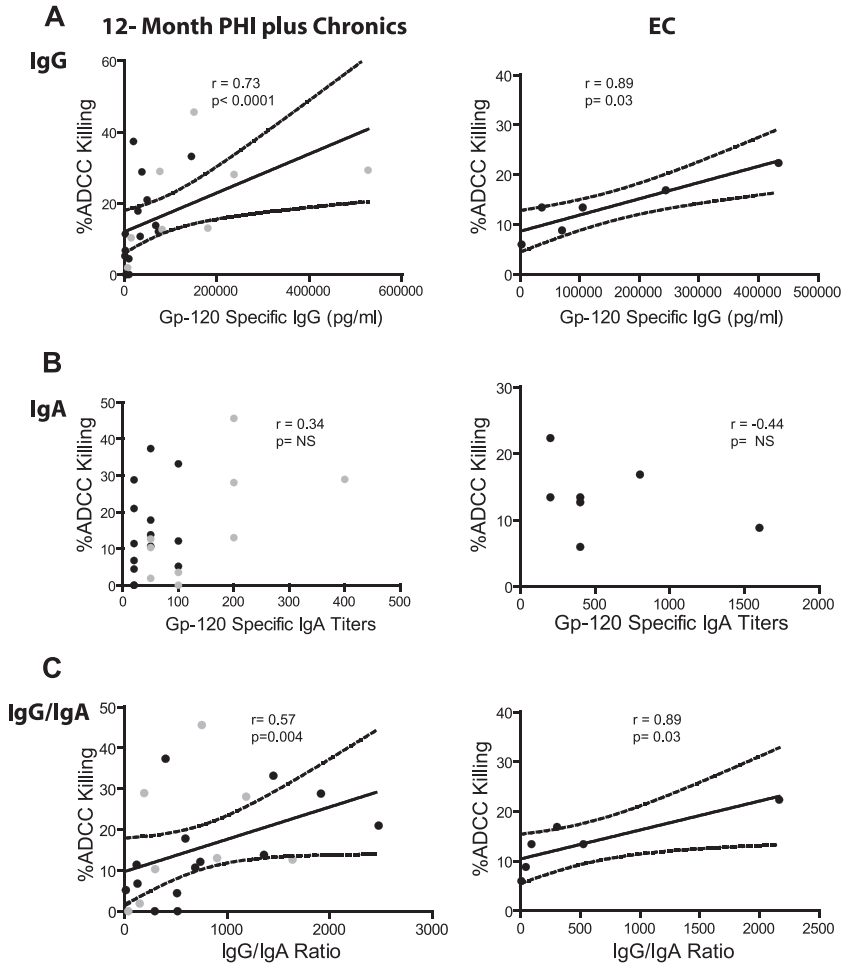

FIG 4 Magnitude of ADCC correlates with gp120-specific IgG and the IgG/IgA ratio but not with gp120-specific IgA. Shown are correlations between ADCC and plasma gp120-specific IgG concentrations (A), plasma gp120-specific IgA titers (B), and gp120-specific IgG/IgA ratios (C) in PHI 12-month plus chronic (left) (%ADCC killing tested at a 1/5,000 plasma dilution) and EC (right) (%ADCC killing tested at a 1/10,000 plasma dilution) samples. In the right panel, black and gray dots denote PHI and chronic subjects, respectively. *r* and *P* values were determined by Spearman's test. Regression lines and 95% confidence intervals are shown.

pleted plasma was significantly higher than that in nondepleted plasma from the same subject (Fig. 5A, right). In this particular example, the ADCC response was negative in nondepleted plasma but became positive after IgA depletion. Moreover, the increment in the magnitude of the ADCC response correlated with a higher level of IgG binding to cells coated with gp120 (Fig. 5, right). This finding was then extended to the other five PHI subjects tested, except for one (Fig. 5B, left). Moreover, the increment was statistically significant when grouped (Fig. 5B, right). ADCC was not modified by IgA removal in ECs (Fig. 5C). In line with this observation, the ADCC ratio of IgA-depleted over nondepleted plasma was significantly higher in subjects with PHI than in ECs (Fig. 5D). Altogether, these results show that plasma IgA modulates ADCC in the context of natural viremic infection, probably mitigating its protective function.

ADCC responses in IgA-depleted plasma correlate with the percentage of CD4$^+$ T cells. Finally, correlation analyses between clinical parameters and %ADCC killing observed in IgA-depleted or nondepleted plasma were performed. No correlations between ADCC evaluated in nondepleted plasma and CD4$^+$ T-cell percentages were observed (Fig. 6, left). Conversely, a strong, positive, nearly significant correlation between the percentage of CD4$^+$ T cells and the magnitude of ADCC obtained with IgA-depleted plasma was found (Fig. 6, right), indicating that eliminating IgA-mediated interference strengthens the association between ADCC and the percentage of CD4$^+$ T cells (higher *r* coefficient) and also the significance (lower *P* value) of this association. These results, together with those presented above, where higher ADCC values were obtained after IgA removal, indicate that IgA might be interfering with the ADCC mechanism, as measured under our experimental conditions. In turn, this would impede the finding of an association between ADCC and disease progression, which becomes evident after the removal of the IgA component.

DISCUSSION

Understanding the role of ADCC-mediating antibodies in the modulation of HIV disease progression is a field of ongoing intense research and renewed interest. In line with the description that vaccine-elicited Env-specific IgA antibodies block gp120 binding of ADCC-mediating IgG (23), evidence of a similar effect occurring in natural viremic HIV infection is

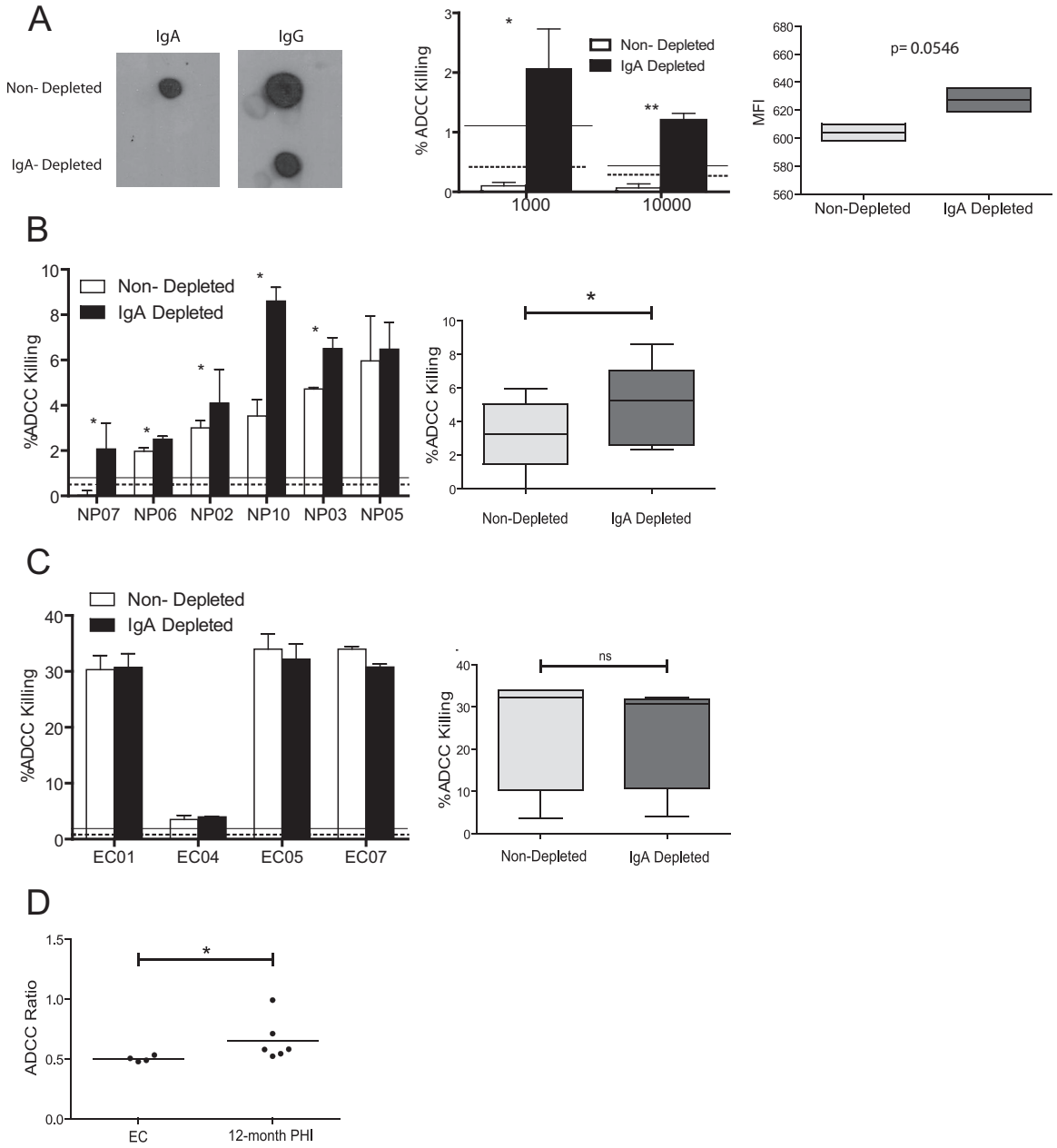

FIG 5 Evaluation of ADCC responses after IgA plasma depletion in HIV-infected subjects. An immunoprecipitation procedure was performed to deplete plasma IgA from 6 randomly selected PHI subjects (12-month samples) and 4 ECs. (A) Results corresponding to one representative subject. After confirming that IgA was successfully and specifically depleted (left), %ADCC killing was evaluated in IgA-depleted and nondepleted plasma samples at 1/1,000 and 1/10,000 dilutions (middle). Horizontal lines represent cutoff values (calculated as described in Materials and Methods) under nondepleted (dashed lines) and IgA-depleted (continuous lines) conditions. IgG binding to cells coated with gp120 was evaluated with a 1/1,000 dilution of IgA-depleted and nondepleted plasma (left) by flow cytometry. Mean fluorescence intensity (MFI) was recorded as a measure of IgG binding. (B) The magnitude of ADCC measured in IgA-depleted plasma is significantly higher than that in nondepleted plasma for 5 out of 6 12-month PHI samples (left), and this increment is statistically significant when grouped (right). (C) The magnitude of ADCC is not modified by IgA removal from plasma samples of ECs (D). The ADCC ratio [%ADCC killing in nondepleted plasma/(%ADCC killing in nondepleted + IgA-depleted plasma)] was significantly higher in 12-month PHI samples than in EC samples. Each assay was performed three times, each in triplicates. In panels B and C, horizontal lines represent mean cutoff values under nondepleted (dashed lines) and IgA-depleted (continuous lines) conditions. In these panels, all responses (except the response observed in nondepleted plasma from subject NP07) are positive according to the criteria described in Materials and Methods. NP, nonprogressor; EC, elite controller. For box-and-whisker plots, the horizontal line represents the median value, the boxes represent the interquartile range, and the whiskers extend from the minimum to the maximum values. Intra- and intergroup differences were analyzed by using Wilcoxon and Mann-Whitney tests, respectively. *, $P < 0.05$; **, $P < 0.01$.

presented here for the first time. Moreover, ADCC measured in IgA-depleted plasma correlated with $CD4^+$ T-cell preservation in viremic subjects.

One major achievement of this work was the simultaneous evaluation of ADCC in three groups of HIV-infected subjects at different disease stages (acute versus chronic) and with different clinical outcomes (controlled versus noncontrolled infection). Moreover, clinical follow-up of subjects identified during PHI

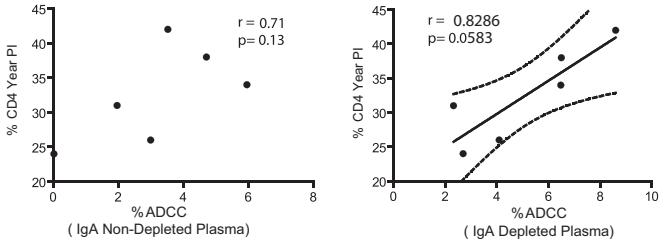

FIG 6 ADCC responses evaluated in IgA-depleted plasma are correlated with the percentage of $CD4^+$ T cells. Shown are correlations with the magnitude of ADCC evaluated in nondepleted plasma (left) and in IgA-depleted plasma (right) and the percentage of $CD4^+$ T cells at a year postinfection. *r* and *P* values were determined by Spearman's test. The regression line and 95% confidence intervals are shown in the right panel.

allowed us to correlate the magnitude of the ADCC response observed at enrollment with subsequent disease progression. We demonstrated that ADCC activity peaks very early after infection, reaches a plateau in chronic infection, and decreases after the instauration of ART, confirming data from previous reports (5–17, 19, 32). The results that we initially obtained using whole plasma supported the notion that ADCC has no beneficial role in slowing disease progression, despite ECs showing slightly higher ADCC than the other groups. This is because no association between ADCC and disease progression (defined by several means) after acute infection in terms of VL, $CD4^+$ T-cell count, soluble factors, and cellular immune activation was found, as shown in Fig. 2. Because results obtained between the PHI>350 and PHI<350 groups were so similar and because the correlations were so weak, we considered that the same results would be obtained even if the sample size was increased. Also, although PHI follow-up was at 1 year, we believe that if ADCC was truly a strong correlate of protection from progression, differences should be observed in the short term after infection, as in the case of cellular immunity (26, 27). The discrepancy between our data and the sizable evidence supporting the idea that ADCC responses could contribute to defense against HIV in the course of natural infection (5, 8, 9) made us reason that it was quite probable that a there is beneficial effect of this effector function in our study but that it is being mitigated by some plasma factors. Thus, we went one step further and provided evidence regarding gp120-specific IgA acting as such a mitigating factor in the context of natural HIV infection, as has been reported in the RV144 vaccine setting (3, 23).

We demonstrate that the level of gp120-specific IgG was associated with the capacity to mediate ADCC in all study groups, which is in agreement with data from previous studies (12, 14, 17, 19, 20), and that there was no correlation between gp120-specific IgA titers and ADCC responses, as also reported previously (12, 14). New to this study is the fact that gp120-specific IgG/IgA ratios are associated with ADCC activity, providing the first evidence of the effect of the IgG/IgA ratio on the magnitude of the ADCC response. One point to consider is that recombinant monomeric gp120 was used in both ADCC analyses and ELISAs. Although a correlation between the magnitude of ADCC and gp120-specific IgG binding in ELISAs was observed here and in other reports, as mentioned above, it is important to note that the gp120 epitopes exposed in each assay differ largely. It has been specifically demonstrated that gp120 epitopes exposed in the RFADCC assay as performed here are those exposed in the gp120 protein after adopting the CD4-bound conformation (33). Moreover, previous reports have shown that exposure of ADCC-specific epitopes in infected cells occurs only after conformational changes associated with CD4 binding occur and were mapped to the gp120 C1 region (34–36). This is particularly relevant in the context of our results: it indicates that antibodies that are able to recognize the gp120 protein bound to CD4 molecules and subsequently trigger ADCC are present in HIV-infected subjects. Thus, therapeutics taking advantage of this mechanism and enhancing this recognition are desirable, as proposed by Richard et al. (37).

Finally, direct evidence of IgA interference in the magnitude of ADCC in natural viremic infection is presented here for the first time, as the level of ADCC measured in IgA-depleted plasma was significantly higher than that in nondepleted plasma for 5 out of 6 subjects. Moreover, ADCC measured in IgA-depleted plasma correlated with $CD4^+$ T-cell preservation. It should be remarked that in the RFADCC assay, NK cells are the effector component (18), and although there are some controversies, it is accepted that NK cells are unable to perform IgA-mediated ADCC (38), highlighting that it is a proper assay to uncover the interfering role of IgA.

The presence of IgA mitigating the protective role of ADCC activity may be a suitable explanation for the discrepancy observed between studies where a correlation of ADCC with protection was reported and others (like ours) where it has been difficult to demonstrate such a correlation. The association of ADCC activity with protection reported previously was achieved by evaluating ADCC responses with the use of purified IgG instead of whole plasma (8, 19). Consequently, the role of the inhibitory effect of IgA in those cases was nonexistent. On the other hand, some early studies reported inhibition of ADCC activity at high plasma concentrations but reported a correlation with disease status using serial dilutions of serum (5, 13). By doing this, the inhibitory factor, in this case IgA, could have been diluted out. Finally, a recent report indicates that the relative ratios of the different IgG subclasses impact ADCC activity during the course of HIV infection. More specifically, the loss of ADCC activity was related to a loss of HIV-specific IgG3 levels and rising titers of subclasses with less functionality (19). Our work adds further evidence to the field demonstrating that IgA interferes with ADCC activity and, therefore, that higher gp120-specific IgG3 levels that are able to counteract the detrimental role of IgA would lead to protective ADCC responses.

Detrimental roles of IgA in other pathologies have also been described (discussed in references 23 and 38). Our results are limited to show that gp120-specific plasma IgA diminishes the magnitude of *in vitro* IgG-mediated ADCC in viremic HIV^+ subjects infected for 1 year, masking its association with the rate of disease progression. What happens in other body compartments, different from plasma, was unexplored, although it deserves consideration.

Finally, a particular picture is observed in ECs: a high magnitude of ADCC despite the absence of significant antigen stimulation, high plasma gp120-specific IgG levels but also high IgA levels, and a positive correlation of the gp120-specific IgG/IgA ratio with ADCC but no IgA interference in depletion experiments. Further insights into IgG and IgA epitope fine-mapping, structure (including glycosylation profiles), function, and affinity in ECs will be needed since dissecting the mechanisms governing HIV-specific IgG and IgA development in ECs, and differences from those in viremic subjects, will be instrumental for developing

strategies to elicit the best humoral immune profile. Specifically, we hypothesize that gp120-specific IgA is directed toward C1 conformational epitopes in subjects where IgA depletion has a considerable impact on ADCC activity (PHI viremic subjects), thus outcompeting C1-specific ADCC-mediating IgG (as reported in the RV144 vaccine setting by Tomaras et al. [23]). On the contrary, in ECs, where IgA depletion has no impact on ADCC activity, IgA specificity may be different from C1, so no IgA-versus-IgG competition occurs. Further investigations in this line will be needed to better understand the basis of ADCC mechanisms in viremic and aviremic subjects.

In summary, we consider that this study represents an important extension beyond the scope of previous reports dealing with ADCC in HIV infection. As initially suggested in the RV144 vaccine setting, gp120-specific IgA antibodies are now identified as factors interfering with the ADCC response in viremic infection, precluding its effect on slowing disease progression. This further emphasizes the notion that vaccine strategies may be more beneficial if they induce IgG-biased antibody responses. Also, much remains to be learned regarding the factors that shape the complex humoral responses in both typical HIV-infected progressors and ECs as well as about the complex interactions between the virus itself and host immunity. As development of an effective vaccine and novel immunotherapies progresses, the data presented here underscore the importance of continuously improving our understanding of the functionality of antibodies elicited either during natural infection or by candidate vaccines.

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