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Divergent Antibody Subclass and Specificity Profiles but Not Protective HLA-B Alleles Are Associated with Variable Antibody Effector Function among HIV-1 Controllers

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ABSTRACT

Understanding the coordination between humoral and cellular immune responses may be the key to developing protective vaccines, and because genetic studies of long-term HIV-1 nonprogressors have associated specific HLA-B alleles with spontaneous control of viral replication, this subject group presents an opportunity to investigate relationships between arms of the adaptive immune system. Given evidence suggesting that cellular immunity may play a role in viral suppression, we sought to determine whether and how the humoral immune response might vary among controllers. Significantly, Fc-mediated antibody effector functions have likewise been associated with durable viral control. In this study, we compared the effector function and biophysical features of HIV-specific antibodies in a cohort of controllers with and without protective HLA-B alleles in order to investigate whether there was evidence for multiple paths to HIV-1 control, or whether cellular and humoral arms of immunity might exhibit coordinated profiles. However, with the exception of IgG2 antibodies to gp41, HLA status was not associated with divergent humoral responses. This finding did not result from uniform antibody responses across subjects, as controllers could be regrouped according to strong differences in their HIV-specific antibody subclass specificity profiles. These divergent antibody profiles were further associated with significant differences in nonneutralizing antibody effector function, with levels of HIV-specific IgG1 acting as the major distinguishing factor. Thus, while HLA background among controllers was associated with minimal differences in humoral function, antibody subclass and specificity profiles were associated with divergent effector function, suggesting that these features could be used to make functional predictions. Because these nonneutralizing antibody activities have been associated with spontaneous viral control, reduced viral load, and nonprogression in infected subjects and protection in vaccinated subjects, understanding the specific features of IgGs with potentiated effector function may be critical to vaccine and therapeutic antibody development.

IMPORTANCE

In this study, we investigated whether the humoral and cellular arms of adaptive immunity exhibit coordinated or compensatory activity by studying the antibody response among HIV-1 controllers with different genetic backgrounds.

Elite controllers (ECs) are individuals able to spontaneously suppress viral replication to below the limit of detection (<50 viral RNA copies/ml of blood), and as such, they represent a promising opportunity to study protective immune responses to human immunodeficiency virus (HIV) infection (1). While the mechanisms behind control of viral replication remain unclear, genetic studies of ECs have pointed to a CD8⁺ T cell-mediated mechanism of control. A genome-wide association study (GWAS) revealed associations between HLA-B alleles and control, with the *HLA-B*5701* allele providing the strongest independent association (2, 3) and other alleles demonstrating association in an additive manner (4). Additional evidence implicates altered major histocompatibility complex (MHC) class I peptide binding resulting in superior viral recognition; specific amino acid residues within the MHC class I peptide-binding groove were associated with control (5), and an *in silico* model of thymic selection with the *HLA-B*5701* allele resulted in a higher proportion of naive T cells able to recognize viral epitopes and cross-react with mutants of targeted epitopes (6). Other studies have also shown evidence for a CD8⁺ T cell-based mechanism of control at the cellular level (7, 8). Together, this evidence suggests differential peptide presenta-

tion as a possible mechanism of viral control associated with HLA-B alleles: presentation of viral peptides that promote superior CD8⁺ T cell activation or that are crucial to viral fitness may ultimately result in reduced viral immune evasion and effective suppression.

Although there is compelling evidence for a T cell-mediated mechanism of control, it does not paint a comprehensive picture: while protective HLA alleles B57 and B27 are highly enriched among ECs (observed in 44 and 15%, respectively), the majority of ECs do

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not carry a protective HLA allele, many progressors possess these alleles, and immune responses in ECs are highly heterogeneous and may involve multiple mechanisms (7). Spontaneous control of HIV infection is thus complex: differential T cell activation may alternatively supplant a weaker or drive a stronger B cell response. While significant differences in the humoral immune response between controllers and progressors have been noted (9–15), the role that humoral immunity may play in viral control in the context of protective HLA alleles is incompletely resolved (16, 17).

Notably, antibodies can function as molecular beacons to recruit effector cells of the innate immune response, such as NK cells, macrophages, and dendritic cells. These effector functions include antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and antibody-dependent complement-dependent cytotoxicity (ADCC). The sum of these and other functions can be measured more generally as antibody-dependent cellular viral inhibition (ADCVI), and a number of studies have suggested that these extraneutralizing effector functions of antibodies play a role in an effective immune response to HIV (summarized in reference 18). In both humans and macaques, ADCC activity has correlated with delayed progression to AIDS (12, 13, 19, 20), and ADCVI has been reported in early stages of simian immunodeficiency virus (SIV) infection, coincident with the appearance of anti-gp140 antibodies. Viruses that had mutated to escape neutralization remained susceptible to ADCVI during later infection, and ADCVI was correlated with control of viral replication, showing that at least in this model, ADCVI played a substantial role in a protective immune response (21). Furthermore, in a French cohort, ADCC antibodies were detected at higher levels in nonprogressors than in viremic patients. Notably, ADCC responses were present in some patients with low CD8⁺ T cell activity, suggesting ADCC as an alternative route to control of viral replication (15). These associations with nonprogressive infection may mirror protective immune responses in the setting of vaccination, as in monkeys, vaccine-elicited antibodies with ADCC activity have correlated with protection or reduced viral loads (22–24), and in humans, antibodies with ADCC or ADCVI activity correlated with reduced risk of infection in the VAX004 trial and were readily induced in the RV144 trial, where in the absence of IgA, they correlated with reduced risk of infection (25–27).

Given both cellular and humoral associations with control of viral replication in ECs, we were motivated to investigate a possible relationship between these arms of adaptive immunity. More specifically, we sought to determine if the presence or absence of a protective HLA allele was associated with divergent humoral responses, supporting either the hypothesis that there are multiple paths to HIV type 1 (HIV-1) control, and that humoral immunity may be potentiated in controllers without protective HLA alleles, or the hypothesis that the humoral and cellular arms of immunity may be coordinately regulated in ECs. Our study was based on a cohort of 40 ECs, 20 with and 20 without a protective HLA allele. To probe the humoral response, we assayed antibody effector functions, including ADCC and ADCP, in cellular assays. Because these extraneutralizing effector functions are dependent on the interactions between the antibody's Fc region and the Fcγ receptors on effector cells, and vary between different IgG subclasses (IgG1, IgG2, IgG3 and IgG4), Fc glycoforms, and among the set of Fcγ receptors (28), we utilized a multiplexed assay to simultane-

ously assess antibody subclass and antigen specificity and further characterized bulk plasma IgG binding to FcγR and lectins.

With the exception of IgG2 responses to gp41, we saw that the presence or absence of a protective HLA allele was not associated with divergent antibody responses among controllers. However, regardless of HLA status, using antibody subclass specificity data, subjects could be classified into two groups with distinct humoral profiles. Whereas the HLA-defined groups exhibited no differences in antibody effector functions, the groups defined by features of their HIV-specific antibody responses, hereafter called array-defined groups, exhibited differential ADCP and ADCC activities, mannose-binding lectin (MBL) binding, and FcγR2A binding. Array-defined groups were primarily differentiated by the magnitude of the IgG1 response to HIV antigens. Thus, while the HLA background provided limited impact on humoral function, antibody subclass and specificity profiles were associated with divergent effector function. The ability of the antibody subclass specificity array and naive clustering methods to predict effector function both represents a biophysical means to predict activity in cell-based assays and points toward specific features of the humoral immune response as associated with these protective activities; it may therefore provide valuable insights into Fc-mediated antibody activities and immune control.

MATERIALS AND METHODS

Subject antibodies. ECs were chosen from a cohort of HIV-1-infected individuals that has been described previously (7). ECs were defined as subjects with plasma HIV RNA levels of <50 or <75 copies/ml based on a minimum of three determinations of plasma HIV RNA spanning at least a 12-month period in the absence of antiretroviral therapy. For comparison, 20 ECs containing protective HLA alleles, defined as B5701, B5703, or B2705, and 20 ECs without protective HLA alleles were selected at random. Subject characteristics are presented in Table S1 in the supplemental material. IgG was purified from plasma samples using the Melon Gel IgG spin purification kit (Thermo Scientific; 45206). The study was reviewed and approved by the Massachusetts General Hospital Institutional Review Board, and each subject gave written informed consent.

Cellular assays. ADCP activity was determined using a flow cytometric assay that measures the uptake of fluorescent gp120-coated beads by monocytic THP-1 cells in the presence of subject antibody. The antibody-dependent phagocytosis assay was performed as previously described, with subject antibodies tested at a concentration of 6.25 μg/ml (29, 30). ADCC activity was characterized using the rapid fluorometric ADCC (RFADCC) assay with NK cells as effectors, as previously described (31). A ratio of effectors to target cells of 10:1 was used, and subject antibodies were tested at a concentration of 50 μg/ml. HIV immunoglobulin (HIV-Ig; obtained from NABI and NHLBI, catalog number 3957; NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) and pooled human IgG (HuIgG) from HIV-negative donors (I2511; Sigma) were used as positive and negative controls. Both functional assays exhibit high reproducibility ($r > 0.85$) and coefficients of variation (CV) in the range of 5 to 15%.

Bulk and HIV-specific IgG isotype detection. Purified IgG samples were characterized for their bulk IgG and bulk IgG1, IgG2, IgG3, and IgG4 subtypes using a Milliplex MAP human immunoglobulin isotype kit (HGAM-301; Millipore) as recommended by the manufacturer (CV < 12%).

A customized multivariate Luminex assay was utilized to characterize the subclass of HIV-specific antibodies to gp120, gp140, gp41, and p24 (IT-001-0027p, IT-001-0021p, and IT-001-005p IT-001-017p; Immune-Technology Corp.), as previously described (32). Briefly, antigens were covalently conjugated to microspheres coded with internal fluorescent dyes, allowing for multiplexing. Prepared microspheres were incubated

with subjects' plasma IgG, resulting in affinity purification of HIV-specific IgG. After washing, microspheres were incubated with phycoerythrin (PE) detection antibodies, specific for IgG subclass type. Antigen specificity (microsphere identification [ID]) and subclass (PE signal) data were acquired on a FlexMap 3D Luminex cytometer. Background signal, defined as the fluorescence signal of microspheres and PE detection antibody in the absence of sample, was subtracted from the mean fluorescence intensity (MFI) for each measurement, and replicate samples exhibited coefficients of variation of less than 10%. HIV-Ig and pooled HuIgG from HIV-negative donors were used as positive and negative controls.

FcγR enzyme-linked immunosorbent assays (ELISAs). Following dilution in phosphate-buffered saline (PBS), His-tagged Fcγ receptors (recombinant human Fcγ RI/CD64, RIIA/CD32a, RIIB/C [CD32b/c], or RIIIA/CD16a; R&D Systems) were immobilized on nickel-nitrilotriacetic acid (Ni-NTA) HisSorb plates (Qiagen) at 0.5 μg/well overnight at 4°C. Plates were washed three times with PBS-Tween (PBST) and blocked with 100 μl/well of 1% bovine serum albumin (BSA) in PBS for 2 h at room temperature. Subsequent incubations were performed at room temperature, and washes were performed between steps as described above. Purified IgG samples were added at 100 μg/ml in 3-fold serial dilutions and incubated for 1 h before washing. Then, 2 μg/ml of horseradish peroxidase (HRP)-conjugated secondary detection antibody with the Fc domain removed [anti-HuIgG (γ) antibody F(ab')₂ fragment, HRP labeled; KPL] was added at 100 μl/well and incubated for 1 h before washing. Signal was developed by adding 50 μl/well of 0.04% *o*-phenylenediamine dihydrochloride (OPD; Sigma) diluted in 0.05 M phosphate citrate buffer, pH 5.0, and 30% H₂O₂ (Sigma). The reaction was quenched with 50 μl/well of 2.5 N H₂SO₄, and the optical density (OD) was read at 492 nm with a 650-nm reference wavelength on a Sunrise microplate absorbance reader (Tecan, Switzerland, with Magellan, version 6.5, software). Native and deglycosylated human IgG and a panel of Fc domain point mutated variants of the broadly neutralizing antibody b12 were used as controls (33), kindly provided by Dennis Burton. In replicated samples, coefficients of variation did not exceed 20%.

Lectin ELISAs. The mannose-binding lectin (MBL) and *Aleuria aurantia* lectin (AAL) ELISAs for measuring binding to mannose and fucose residues on purified patient IgGs were modified from a previously described method (34). Purified IgG samples were added at 30 μg/ml in 3-fold serial dilutions with 80 μl/well on 96-well plates (Maxisorp; Nunc, Thermo Scientific). Plates were incubated overnight at 4°C and then washed six times with PBST and blocked with 1% BSA in PBS at 250 μl/well for 2 h at room temperature. For MBL assays, the IgG-coated plates were first acid treated with 0.1 M Gly HCl, pH 2.0, and 0.15 M NaCl at 100 μl/well for 1 h at 37°C and then washed and reblocked for 1 h at 37°C. Biotinylated MBL (10405-H07H [Sino Biological] and 21327 [Thermo Scientific]) or biotinylated AAL (B-1395; Vector Laboratories) was dissolved in a Ca²⁺ buffer (20 mM Tris-HCl [pH 7.4], 0.15 M NaCl, 2 mM CaCl₂, 0.1% BSA, 0.1% Tween 20) and added at 2 μg/ml with 100 μl/well and incubated for 2 h at room temperature before washing. HRP-streptavidin (SA-5004; Vector Laboratories) diluted in PBS was added at 2 μg/ml with 100 μl/well before washing. Substrate development and OD readings were performed as described above. Native and deglycosylated human IgG served as positive and negative controls. In replicated samples, coefficients of variation did not exceed 20%.

Statistical analyses. Comparative analyses of antibody properties between patient groups were performed using unpaired two-tailed *t* tests with 95% confidence intervals and GraphPad Prism software. Because posttest corrections can introduce errors and rest on debatable assumptions, *P* values below 0.05 are reported alongside the corresponding Bonferroni adjusted significance threshold (α) to allow for correction based on family-wise errors associated with multiple comparisons. Correlation analyses were also performed in GraphPad Prism. Pearson correlation coefficients were calculated with two-tailed *P* values with 95% confidence intervals.

Clustering analysis was performed using the R software package.

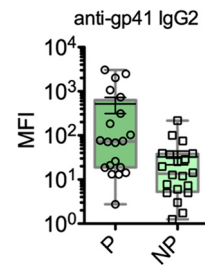


FIG 1 IgG2 responses to gp41. A comparison of antibody properties between elite controllers with (P) and without (NP) protective HLA-B alleles revealed only a significant difference in IgG2 antibodies against gp41 ($P = 0.03$, uncorrected; significance threshold of 0.0018 following Bonferroni correction is unmet). All other antibody properties tested ($n = 27$) showed no significant differences between these HLA-defined groups.

Antibody features were scaled and normalized according to their standard deviation (SD) from the mean, and truncated at ± 6 SDs. Pearson correlation coefficients were calculated for HIV-specific IgG isotype measurements between subjects. Subjects were then clustered using $1 - r$ dissimilarity and the Ward Linkage algorithm. The function heatmap.2 in the R gplots library was used to hierarchically cluster and visualize the array, grouping and reordering subjects and antibody features by similarity.

RESULTS

Protective HLA alleles are not associated with divergent humoral profiles. To determine whether ECs with or without protective HLA alleles have characteristically different HIV-specific antibody responses, we measured a variety of antibody properties, including levels of IgG1 to -4 in plasma, IgG galactosylation and fucosylation levels, FcγR binding, antibody-dependent cellular phagocytosis (ADCP) activity, and antibody-dependent cellular cytotoxicity (ADCC) activity, as listed in Table S2 in the supplemental material. We also determined the subclass composition of HIV-specific antibodies recognizing gp120, gp41, gp140, and p24 using a customized microsphere array. Unpaired *t* test analyses of antibody properties between HLA-defined groups revealed limited differences in these measurements (see Fig. S1 to S3 in the supplemental material). Though there was substantial variation among subjects in all assays, only gp41-specific IgG2 differed between HLA-defined patient groups ($P = 0.03$) (Fig. 1). Though complemented by trends toward increased levels of IgG2 to other HIV antigens among controllers with protective HLA-B alleles (see Fig. S1 in the supplemental material), in the absence of posttest correction, a statistically weak difference such as this may be expected as an artifact of multiple comparisons. Indeed, given the number of comparisons made, Bonferroni correction yields an adjusted significance threshold (α) of 0.0018, which is unmet. Thus, among this set of 28 antibody assays, the presence or absence of a protective HLA allele was not associated with remarkably divergent antibody responses, and the hypothesis that humoral immunity may be potentiated in subjects according to the absence or presence of protective HLA alleles was not supported.

IgG subclass specificity profiles define new subject groups. Given the variability in antibody features and functions observed between subjects, we next asked if HIV-specific antibody signatures could stratify controllers regardless of HLA status. To approach this question, we considered only the antigen-specific IgG

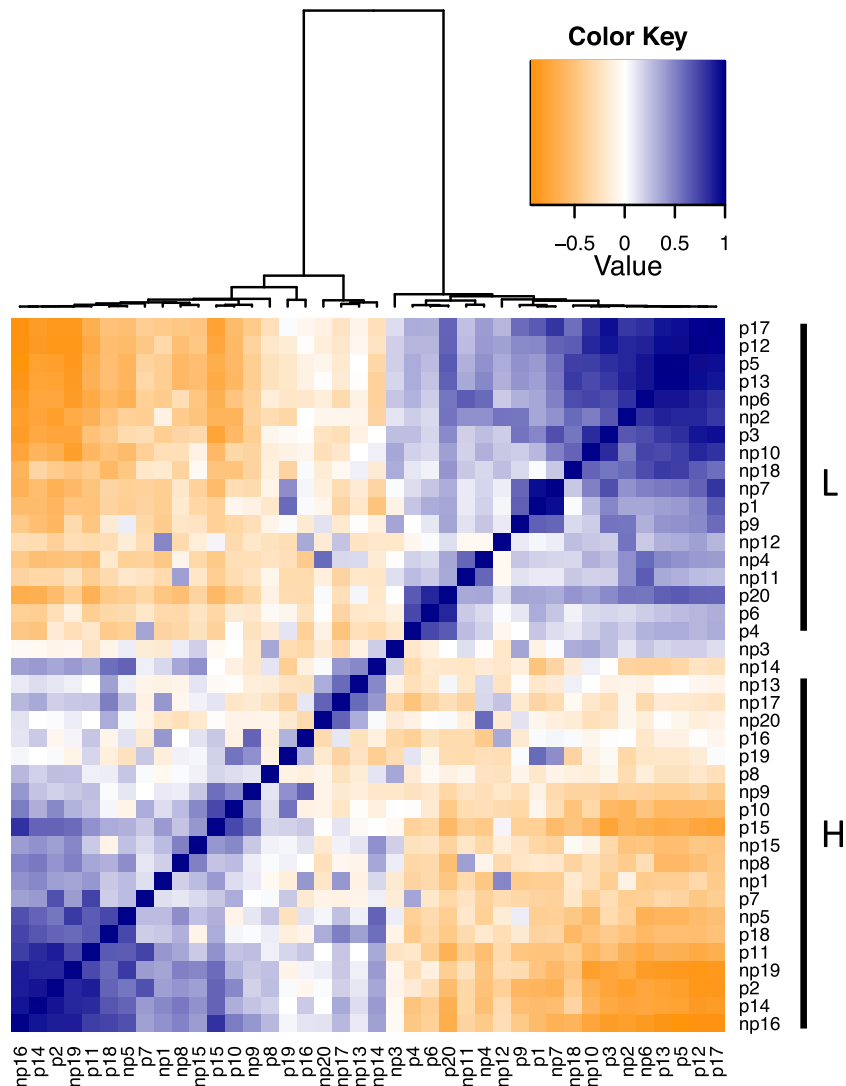


FIG 2 Antibody subclass specificity correlation matrix defines new subject clusters. Correlations between subjects were calculated based on the 16 antibody subclass specificity features measured on the customized microsphere array. Clustering patients according to their Pearson correlation coefficients, performed by Ward linkage, revealed two well-differentiated groups of subjects marked by the two branches of the dendrogram (labeled H and L). Subjects with (p) or without (np) protective alleles were evenly distributed between branches.

subclass measurements ($n = 16$, IgG1 to -4 specific to gp120, gp41, gp140, and p24), as measured by the microsphere array. Using this data, we calculated Pearson correlation coefficients between subjects, who were then clustered using Ward linkage. Clustering using array data revealed two new and well-defined groups (designated H and L), which were unrelated to the groups defined by HLA status (Fig. 2).

Array-defined groups exhibit differential functional activity. Unpaired t test analysis of antibody properties and function between array-defined groups (designated H and L) resulted in significant differences in both ADCP activity (Fig. 3A) ($P = 0.0047$) and ADCC activity (Fig. 3B) ($P = 0.0183$). Furthermore, subjects in array-defined groups differed with respect to MBL binding of plasma IgG (Fig. 3C) ($P = 0.0093$), suggesting differential antibody galactosylation. Similarly, differential binding to Fc γ R2A was observed (Fig. 3D) ($P = 0.044$), consistent with the variation in phagocytic activity observed. However, the differences in

RFADCC and Fc γ R2A do not meet the significance threshold of 0.0125 following Bonferroni correction. To reconfirm that these differences were unique to the array-defined groups and not present in the HLA-defined groups, comparisons of the same measurements between HLA-defined subject groups (designated P and NP) were done, and they did not result in any significant differences (Fig. 3E to H).

Array-defined groups are distinguished by antigen-specific IgG1 levels. To give better resolution with respect to the antibody characteristics that distinguished the array-defined groups, we examined the subjects' antibody signatures more closely. The correlation matrix analysis (Fig. 2) compressed the 16 antibody signature data points into single correlation coefficients. Therefore, we also took an alternative clustering approach that grouped subjects and antibody properties based on individual antigen subclass measurements ($n = 16$, IgG1 to -4 specific to gp120, gp41, gp140, and p24). Each antibody feature was centered and scaled accord-

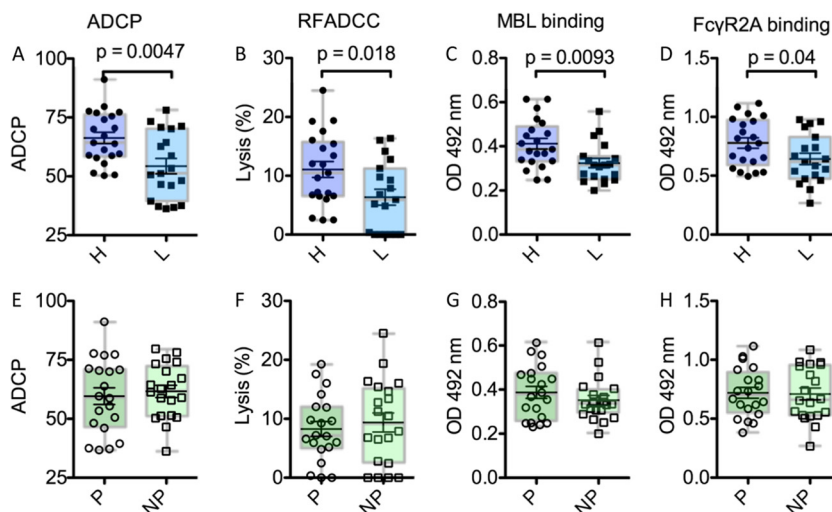


FIG 3 Array-defined subject groups distinguish antibody function. Comparison of functional measurements showed that the array-defined subject groups (blue, designated H and L) distinguish patients by phagocytic activity (A), NK cell-mediated cytotoxicity (B), and MBL (C) and FcγR2a (D) binding. (E to H) No differences in these functions are observed comparing patients based on HLA status (protective [P] and nonprotective [NP]; in green). The Bonferroni-corrected significance threshold given multiple comparisons is 0.0125.

ing to standard deviations from the mean value across all subjects. Features and subjects were then clustered according to the similarity of their antibody signatures, resulting in a heat map in which both similar features and similar subjects are grouped (Fig. 4A). The most striking structural feature of the heat map clustering was the division of subjects based on antigen-specific IgG1 levels. Subjects were split into two groups, as represented by the two branches of the dendrogram on the vertical axis, and these groups were largely structured by high (red) or low (blue) HIV-specific IgG1 levels. Though they were not identical, there was excellent agreement between the subject structure of the correlation matrix (Fig. 2) and heat map (Fig. 4A), suggesting that the correlation group assignments may have also been driven by differences in the prevalence of HIV-specific IgG1 antibodies.

Thus, to confirm that HIV-specific IgG1 levels were the indeed the salient features in the correlation-based clustering, we compared antigen-specific IgG1 levels between array-defined groups. Again, an unpaired *t* test analysis of antigen-specific IgG1 measurements between correlation-based array-defined groups resulted in significant differences for all HIV antigens (Fig. 4B to E) ($P < 0.0001$; the Bonferroni-corrected significance threshold given multiple comparisons is 0.0125.), while there were no significant differences between HLA-defined groups (Fig. 4F to I). Thus, while HLA status was not associated with divergent humoral signatures or functions, antibody profiles were not uniform across subjects, and ECs could be classified into two groups defined by antibody specificity and subclass responses and associated with distinct effector function profiles.

HIV-specific IgG1 correlates with effector function. We next sought to determine whether specific antibody features were associated with antibody effector function and determined whether there were any correlations between individual HIV-specific antibody features and functional data. Considering HIV-specific IgG subclass measurements individually, correlation analyses showed significant positive correlations between all HIV-specific IgG1 measurements and ADCP activity and between IgG1 specific to gp41 and p24 and ADCC activity (Fig. 5A). Representative correlation plots for gp120-

specific IgG1 levels and ADCP and RFADCC activity are provided (Fig. 5B and C). No other relationships between antigen-specific IgG subclasses and function were significant.

We also considered relationships between the subjects' HIV-specific antibody signatures and their antibody profiles more broadly by examining the relative prevalence of each IgG subclass in bulk plasma. Correlation analyses showed strong positive correlations between all antigen-specific IgG1 and non-specific plasma IgG1 levels (Fig. 5A and D). Thus, a subject's HIV-specific antibody signature, and HIV-specific IgG1 in particular, was associated with differences in the overall properties of plasma IgG.

Relationships among bulk plasma IgG properties. Finally, we examined the correlative relationships between patients' bulk plasma antibody profiles and antibody function. A summary of these relationships is represented in Fig. 6. A number of relationships regarding plasma IgG subclass composition were identified (Fig. 6, green boxed region): the relative plasma compositions of IgG1 and IgG2, IgG subclasses with divergent physiological functional activities, were strongly negatively correlated; in contrast, IgG1 and IgG3, subclasses with potent cytotoxicity, were strongly positively correlated. These relationships suggest coordination among subclass levels toward either a more (IgG1/3) or less (IgG2) activated state. Whether these associations are driven genetically or modulated by immune status is unknown.

Additionally, FcγR binding among low-affinity receptors was strongly associated, consistent with common IgG binding preferences, whereas binding to the high-affinity FcγR1 receptor was not correlated with binding to low-affinity receptors, consistent with known differences in antibody subclass and glycospecificity relative to FcγR2A, -2B, and -3A (Fig. 6, red boxed region). As would be expected, IgG1 levels correlate positively and IgG2 levels correlate negatively with FcγR binding, and surprisingly, despite comprising a small fraction of total plasma antibody, IgG3 levels were positively and significantly correlated with binding to all Fc receptors tested (subclass composition versus FcγR binding relationships are boxed in blue in Fig. 6).

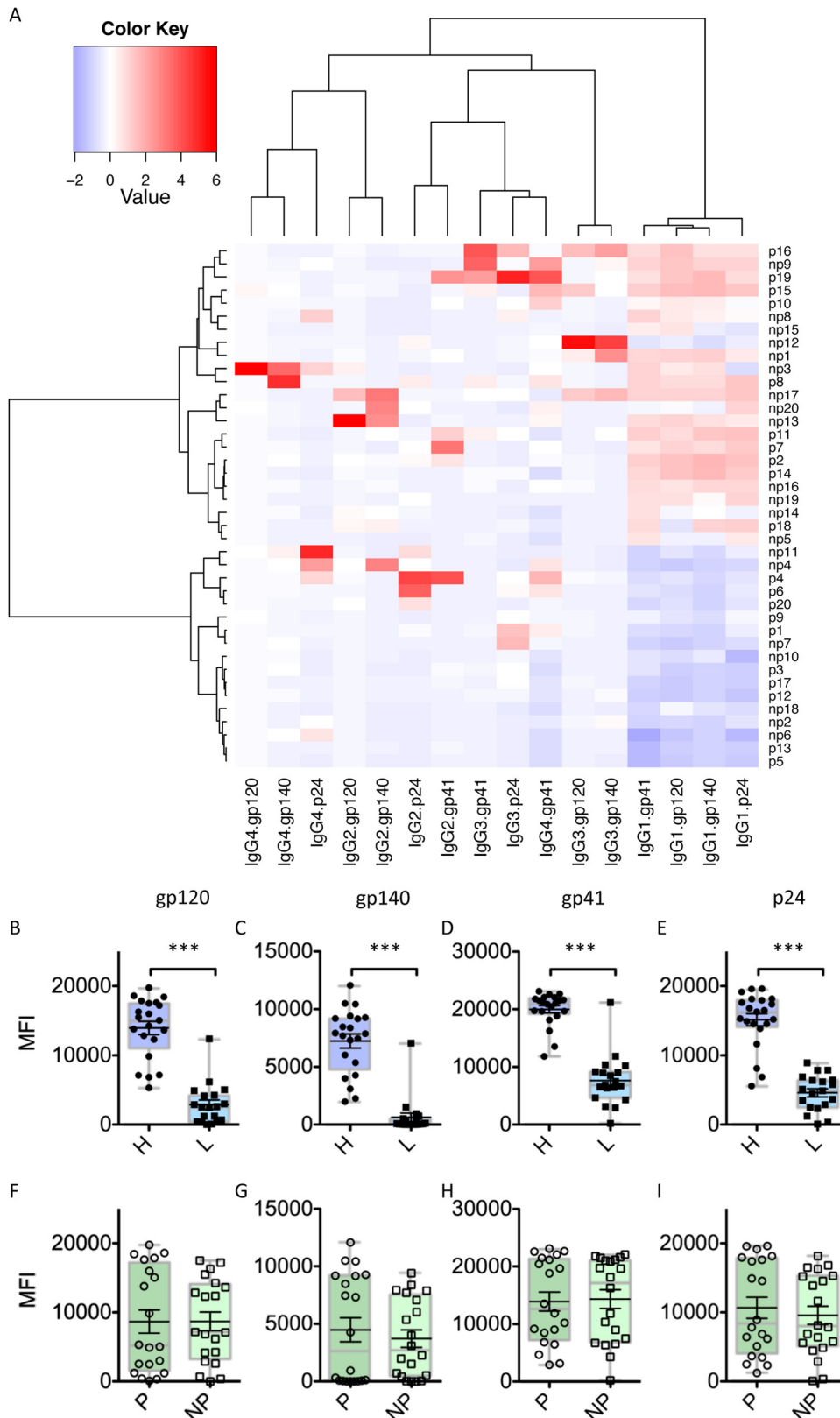


FIG 4 Array-defined groups are distinguished by HIV-specific IgG1 levels. (A) A clustering analysis of patients and features based on scaled and centered antibody subclass specificity measurements is represented by a heat map. Notably, all antigen-specific IgG1 features clustered together, with patients grouped consistently with differences in the levels of HIV-specific IgG1 antibodies. (B to E) Closer inspection of the array-defined groups showed that groups were differentiated by high (H) and low (L) IgG1 levels of antibodies to gp120 (B), gp140 (C), gp41 (D), and p24 (E). No difference was observed between HLA-defined groups of ECs with (P) or without (NP) protective alleles (F to I) or among other antibody subclasses (***, $P < 0.0001$). The Bonferroni-corrected significance threshold given multiple comparisons is 0.0125.

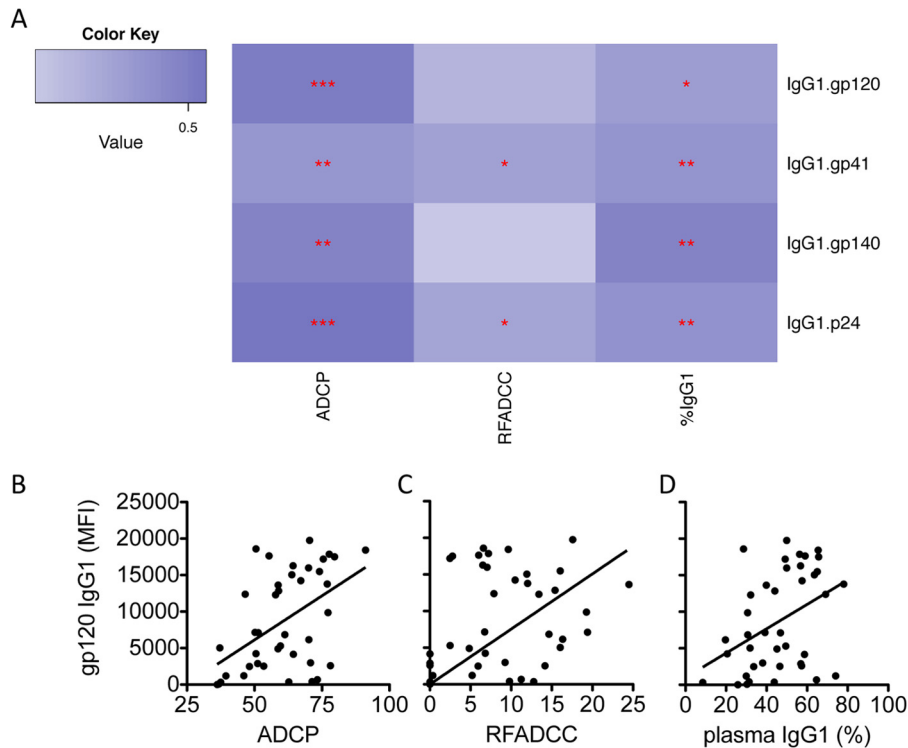


FIG 5 HIV-specific IgG1 levels correlate with enhanced effector function and elevation of total plasma IgG1. (A) Correlation coefficient plot of HIV-specific IgG1 versus phagocytosis (ADCC), cytotoxic activity (RFADCC), and the percentage of bulk plasma IgG of the IgG1 subclass (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (B to D) Scatterplots of gp120-specific IgG1 with ADCC (B), RFADCC (C), and plasma IgG1 levels as a percentage of total plasma IgG (D).

Lastly, a number of measurements made on total plasma IgG demonstrated significant correlations with functional measurements of HIV-specific antibodies (ADCC and RFADCC), though these antibodies are assumed to represent a minor species of total plasma IgG. Of particular note, fucosylation of plasma IgG, as measured by AAL ELISA, was inversely correlated with RFADCC activity. This relationship is consistent with the known ability of afucosylated antibodies to enhance ADCC activity but, again, surprising with respect to the observation that total plasma IgG exhibits properties consistent with the functional activity of a small subpopulation (~2%). Additionally, plasma IgG reactivity to MBL, a plasma protein that can initiate the complement cascade, was associated with phagocytic activity. Furthermore, MBL demonstrated strong associative relationships with plasma IgG subclass measurements, indicating that each subclass may exhibit inherent differences in ability to drive complement deposition via this pathway. Whether such differences are driven by their divergent protein backbones or differences in the glycosylation state among subclasses is unclear.

Thus, overall, a number of relationships consistent with known IgG-FcγR biology were captured using this unbiased exploration of correlative relationships, and consistent patterns were often observed between total plasma IgG and the HIV-specific fraction. Whether these results imply that preexisting differences in IgG characteristics impact the function of HIV-specific antibodies produced by each subject or whether the inflammatory state present during infection drives similar modifications to both HIV-specific and total plasma IgG remains to be determined.

DISCUSSION

ECs represent a promising opportunity to determine immunophenotypic markers of protective responses to HIV infection (1). While it has been speculated that there may be multiple mechanisms by which viral suppression is mediated and there have been many associations with nonprogression, translating these associations into a mechanistic understanding of viral control has been remarkably challenging. At the outset of this study, we hypothesized that a CD8⁺ T cell-dependent mechanism for immune control of HIV replication in ECs with a protective HLA allele might alter the characteristics of the humoral response. However, our results indicate that antibody responses between ECs with and without a protective HLA allele are not remarkably different. Among a set of 28 measurements, the level of IgG2 antibodies to gp41 was the only significant difference observed between the two HLA-defined groups, providing little support for the hypothesis that humoral immunity is potentiated in the setting of either protective or nonprotective HLA alleles.

This result has two potential explanations: either antibody responses were relatively uniform across subjects or protective HLA alleles were not associated with divergent humoral responses. To rule out the first explanation, we analyzed the subclass specificity profiles of HIV-specific antibodies. Correlation-based clustering analysis of the antibody array data grouped patients into two distinct groups, which were predictive of antibody effector function, including ADCC and ADCC activities. Closer inspection of array-defined groups showed that levels of HIV-specific IgG1 antibodies were the main structural feature distinguishing array-defined

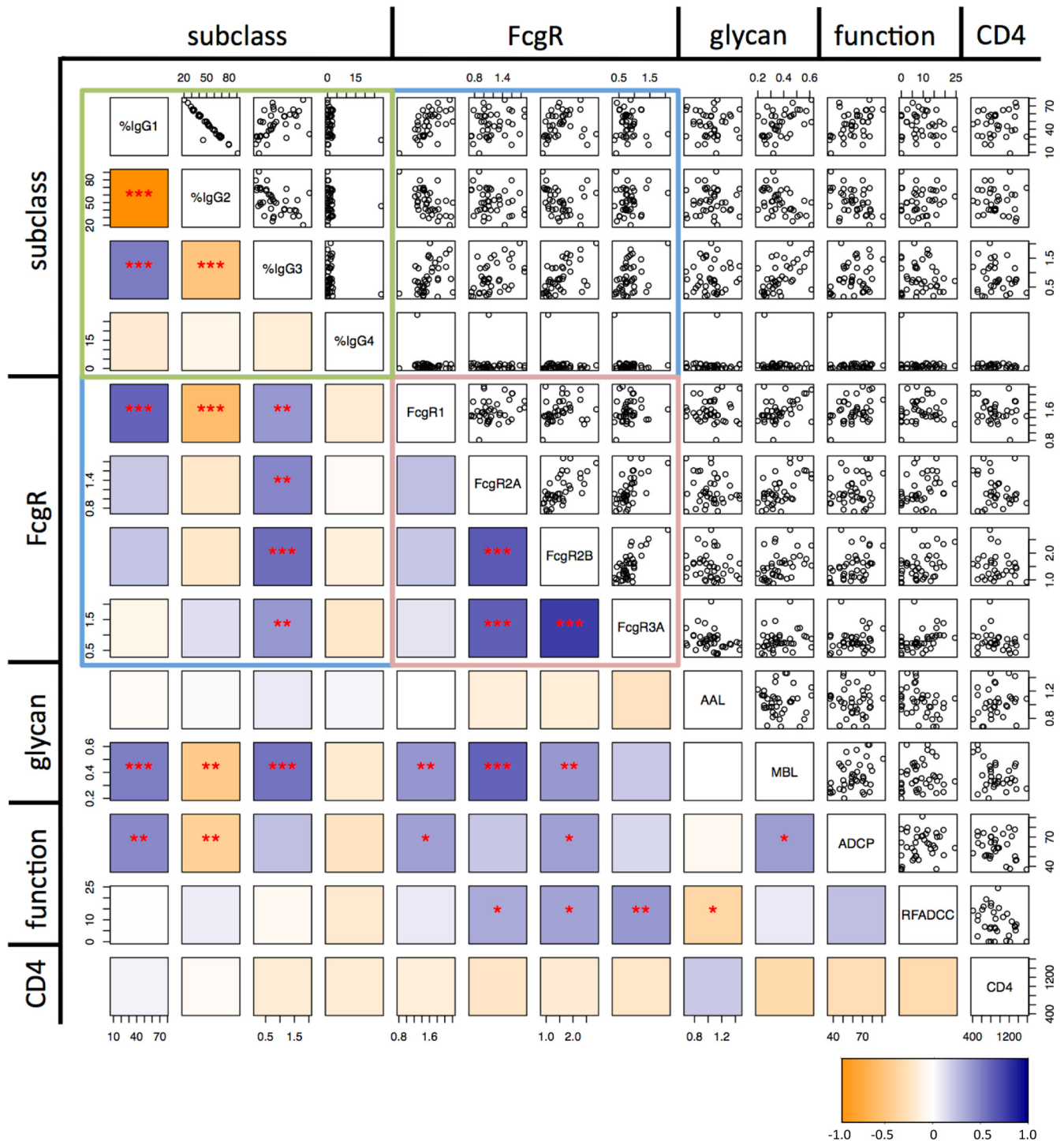


FIG 6 Correlations between bulk IgG subclass measurements and functional measurements. Shown are correlation coefficients and scatterplots of bulk plasma IgG composition, bulk plasma IgG FcγR binding, IgG fucosylation (AAL binding), IgG agalactosylation (MBL binding), ADCP and RFADCC activity, and subject CD4 count. Strength of correlation is represented with color, with orange representing strong negative correlations and blue representing strong positive correlations. Relationships between bulk plasma IgG subclasses are boxed in green; %IgG1 and %IgG3 showed strong positive correlation, while %IgG1 and %IgG2 showed strong negative correlation. Relationships between FcγR binding profiles are boxed in red; binding to FcγR2A, FcγR2B, and FcγR3A showed strong positive correlation and no relationship with the high-affinity FcγR1 receptor. Relationships between bulk plasma IgG subclasses and FcγR binding are boxed in blue; %IgG3 showed strong positive correlation with binding to all FcγRs despite its low serum abundance. %IgG1 showed strong correlation with binding to FcγR1, while %IgG2 showed strong negative correlation to FcγR1 binding. Phagocytic activity (ADCP) was positively associated with IgG1 and IgG3 levels and binding to FcγR1 and FcγR2B. Cytolytic activity (RFADCC) was positively associated most strongly with binding to FcγR3A and inversely with antibody fucosylation as measured by binding to the fucose-sensitive lectin AAL (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

groups. That HIV-specific IgG1 antibodies positively correlate with ADCC and ADCP activities suggests that these antibodies may be mechanistically relevant to the differences in functional measurements between array-defined groups. Therefore, while there is no strong associative relationship between HLA status and antibody responses, we demonstrate divergent antibody responses within this cohort of ECs and that these divergent responses lead to divergent antibody effector function activity.

Dissection of the antibody responses in this EC cohort yielded other relationships that are consistent with the biology of antibody effector function. For example, positive correlations between IgG3 levels and Fc receptor binding were observed (Fig. 6, blue box). This result is not surprising given that IgG3 has the strongest affinity for all Fc receptors, but it was unexpected given its relative rarity in plasma (less than 10% of total IgG). Fc γ R2A, Fc γ R2B, and Fc γ R3A binding were also positively correlated, reflecting their higher sequence and structural similarity relative to Fc γ R1 (Fig. 6, red box). Antibody fucosylation, as determined by AAL binding, and Fc γ R3A binding were correlated with ADCC activity (Fig. 6): these results are unsurprising given the expression of Fc γ R3A on NK cells and its known dependence on fucose. However, it was surprising that these properties of bulk plasma IgG were correlated with the function of the HIV-specific subspecies. Thus, several results of this study imply that global bulk IgG cues may be representative of the composition of the antibody compartment in a way that conserves Fc domain features across multiple antibody specificities. Indeed, the correlations between bulk and HIV-specific IgG1 levels observed in this study and the correlations between the subclass profiles of antibodies to different HIV antigens observed previously (32) are consistent with this possibility.

Additionally, the divergence of antibody responses within the EC patient cohort highlights the need to place these antibody effector functions in the context of viral control. First, it is not clear why divergent antibody responses might have arisen. Answering this question may provide fundamental insight into the signals required to elicit antibodies with potentiated effector function via vaccination. Second, it is unknown if these HIV-specific IgG1 antibodies are involved in control of viral replication. While effector function has correlated with nonprogression in numerous studies and in both humans and macaques (12, 13, 20), whether this association is causative remains to be determined.

Beyond HIV-specific IgG1, previous studies have described some correlative relationships between IgG subclasses and disease progression, yet there is limited consensus. Two studies from the same French cohort linked gp41-specific IgG2 and CD4⁺ Th1 responses with long-term nonprogression (35, 36), and Lal et al. found an association between the absence of IgG2 antibodies to gp41 and clinical disease (14). These results are consistent with our observation that IgG2 antibodies to gp41 are enriched among ECs with protective HLA alleles. However, another study highlighted the heterogeneity of antibody responses and reported elevated IgG1 specific to p24 and gp120 and IgG3 specific to gp120 in controllers compared to progressors. The same study also noted an absence of indications of differential Th1 and Th2 polarization between ECs and progressors (11). Two previous studies have considered the role of protective HLA alleles in ECs. The first reported that IgG2 antibodies against gag antigens were enriched among ECs without protective alleles relative to those with *HLA-B*57* (16). This finding was not confirmed in our study, and

though we observed that levels of IgG2 antibodies against gp41 differed between controllers according to HLA status, they exhibited the opposite trend and were enriched in ECs with protective alleles. The second study found that B*57 carriers exhibited a modest decrease in ADCC activity relative to subjects without this allele (17). While not confirmed, this finding is consistent with the trend observed between these subject groups in the data collected here. Therefore, the landscape of IgG subclass responses to HIV remains unresolved, and further study, both to link antibody profiles with mechanisms of antibody activity and to identify immune signals associated with divergent humoral responses, is necessary. While we only considered ECs in this study, our results further highlight the heterogeneity of humoral immune responses to HIV within ECs. Stratification of ECs, perhaps by antibody profile as demonstrated here, may diminish confounding differences between ECs and allow for more illuminating comparisons between subgroups of ECs and progressors to be made.

One intriguing result of this study is the correlation between HIV-specific IgG1 and total plasma IgG1. It is plausible that subjects with higher HIV-specific IgG1 levels had higher total IgG1 levels prior to infection, as genetic factors such as Gm allotypes can be responsible for variations in baseline IgG subclass concentrations (37). An underlying variation in IgG subclass concentration could explain the divergence in HIV-specific subclass response after infection, and knowledge of patients' Gm allotypes would rule out this possibility. Alternatively, the higher HIV-specific IgG1 response could be driven by the prevalence of HIV-specific IgG1 antibodies or other genetic factors. Elevated IgG1 levels could, alternatively, be indicative of different levels of inflammatory signals in these subjects, as HIV infection is known to drive IgG1-biased hypergammaglobulinemia. Further study is necessary to determine the direction of causality in this correlative relationship, which could provide a key to selective induction of antibodies with potentiated effector function.

In conclusion, we show that the presence or absence of protective HLA alleles is not associated with strikingly divergent antibody responses, suggesting that potential CD8⁺ T cell responses driven by these alleles do not dramatically alter the characteristics of the humoral immune response. Because some controllers experience transient viremia, longitudinal studies of the antibody features among subjects experiencing temporary loss of viral control would be of significant interest in further determining the role that antibody-mediated activities might play in the setting of spontaneous control.

However, regardless of HLA status, controllers could be grouped based on their HIV-specific antibody signatures, with HIV-specific IgG1 acting as the main distinguishing factor. These array-defined groups were associated with divergent antibody function in assays of NK cell-mediated cytotoxic and monocyte-mediated phagocytic activities. While it is unclear if these differences in antibody profiles underpin differences in mechanisms of control or nonprogression, our study further highlights the complexity of immune control in ECs and supports the hypothesis that altered antibody subclass distribution may play a mechanistic role in potentiated effector function. Because these nonneutralizing antibody activities have been associated with control, reduced viral load, nonprogression, and protection in vaccine studies, understanding the specific features of IgGs with potentiated effector function may be critical to vaccine development.

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