$P = 0.04$) sparked a large posthoc analysis in which HIV-specific antibody-dependent cellular cytotoxicity (ADCC) has emerged as a possible correlate of protection [5–8].

Some individuals control HIV progression without ART for many years. Such individuals (broadly called 'HIV controllers') have been variously termed long-term slow progressors (LTSP; often defined clinically as maintaining $CD4^+$ T-cell count >400 cells/μl more than 7 years after acquisition of HIV), viremic controllers (who maintain plasma viral loads <3000 copies/ml for at least 1 year) elite controllers (maintain undetectable virus in the plasma for at least 1 year) [9] and posttreatment controllers (PTCs: maintain undetectable viremia after an initial period of cART) [10]. HIV-infected individuals who progress in a typical time frame usually have viral loads of more than 10 000 copies/ml [11]. Although viral factors (such as *nef*-deleted viral mutations) may play a role in facilitating slow progression in some cases [12], host factors are thought to primarily contribute to containment of HIV infection in HIV controllers [13]. For example, potentially protective CTL responses preferentially targeting Gag have been noted in elite controller and viremic controller individuals [13,14]. HLA-B27 and HLA-B57 alleles are overrepresented in HIV controllers and their interaction with natural killer (NK) cells and killer immunoglobulin-like receptors (KIR) have been associated with slow progression to AIDS [13–15]. Although HIV-1 envelope protein (Env) has generally been considered an ideal target for a vaccine, Env-specific CTL responses and NAb levels have generally been found to be lower in elite controllers [14,16–18]. On the contrary, anti-HIV ADCC responses have been associated with slower immunodeficiency virus disease progression in humans and macaques in most studies [17,19–23]. Whether such ADCC responses can target a diverse range of Env strains, however, is largely unknown. Given the interest in ADCC-based HIV vaccines following the RV144 trial, it is important to define the breadth of ADCC immunity that facilitate control of HIV.

The diversity of HIV is a major problem for HIV vaccine design [24,25]. HIV-1 is catogorized into four groups: M (main), O (outlier), N (non-M, non-O) and the recently recognised P. The M group, which accounts for the vast majority of HIV infections, contains nine subtypes (A through J), approximately 58 circulating recombinant forms (CRFs) and multiple unique recombinant forms (URFs) [26]. In 2004–2007, global HIV infections were caused by subtype A (12% of infections), subtype B (11%), subtype C (48%), CRF01_AE (5%) and subtype D (2%) [27]. Genetic variation between subtypes is 25–35% and genetic variation within subtypes is 15–20% [26]. Immune responses capable of contending with the high genetic diversity of HIV are imperative in efforts to develop a globally relevant HIV vaccine. Glycosylation adds another level of complexity to HIV-1 Env and it may be beneficial if Env-specific immune responses recognize diverse glycosylation profiles. Protein glycosylation is often considered a hindrance to effective NAb responses, with the glycan moieties masking critical neutralization sites [28]. However, some more recently described broadly NAbs (bNAbs) specifically target epitopes that include conserved glycan moieties [29,30]. Another concern with NAb responses to Env is that responses directed against gp140 trimers are thought to be more efficacious than responses directed against gp120 [31]. However, ADCC responses directed against epitopes on the nonexposed surfaces of gp120 may be useful, as such epitopes may be expressed on the surface of HIV-infected cells [32]. The magnitude and breadth of ADCC responses to both gp120 and gp140 has not previously been studied.

The breadth of the anti-HIV immune response is likely to be important in controlling HIV and has been studied extensively for NAb and CTL-based immune responses. Much work has been undertaken to generate ideas about how to induce bNAbs [33] and attempts have been made to induce broad CTL responses [34] to achieve protection against HIV [35]. To identify immune factors associated with control of HIV infection, we studied the breadth and strength of Env-specific ADCC responses from 11 HIV 'controllers' and compared these responses with those from 11 HIV 'progressors'.

Materials and methods

Study participants

The clinical characteristics of the 22 HIV-1 infected individuals are summarized in Table 1. The HIV controller group included two elite controllers, one PTC, five viremic controllers and three LTSPs. Within the HIV controller group, three LTSP individuals had commenced cART (at a mean of 15.1 years after infection) having a $CD4^+$ cell count of more than 400 cells/μl and viral load of less than 5000 copies/ml 7 years after initial infection. In the HIV progressor group, we included four individuals who had commenced cART within 8 months of HIV diagnosis. HIV progressors all had a plasma viral load off cART of more than 10 000 copies/ml. All individuals were recruited and consented to donate sodium heparin anticoagulated plasma samples for use in ADCC assays; healthy HIV-negative volunteers provided the donor cells. Research was conducted under the auspices of the Alfred Human Health Research and Ethics Committee.

HIV-1 antigens

The 18 HIV-1 gp120 and gp140 proteins utilized in the ADCC assays are summarized in Table 2, representing four major HIV subtypes (A, B, C and E; subtype E Env is from CRF01_AE). The reagents were obtained through

Table 1. Clinical characteristics of the study cohort.

	Individual #	Type of HIV controller	Sex	Age (years)	HIV-1 subtype	Plasma VL (copies/ml)	$CD4^+$ count (cells/μl)	$CD4^+$%	Years infected	Years infected until cART
HIV controllers	1	EC	M	50	ND	<20	1936	52	17	NA
	2	EC	M	61	ND	<20	337	25	11	NA
	3	VC	F	41	ND	52	801	38	15	NA
	4	VC	M	46	B	607	469	19	19	NA
	5	VC	M	26	B	819	896	30	5	NA
	6	VC	M	40	B	1160	513	19	12	NA
	7	VC	M	46	B	2788	574	24	9	NA
	8	PTC	F	49	ND	<20	1034	57	15	[a]
HIV controllers on cART	9	LTSP	M	43	B	1720[b]	699	28	14	11
	10	LTSP	M	52	B	4000[b]	469	17	14	11
	11	LTSP	M	52	B	4300[b]	430	25	17	14
HIV progressors	12	NA	M	27	B	49 900	649	18	1	NA
	13	NA	M	61	B	22 1000	185	18	8	NA
	14	NA	M	35	B	53 500	690	26	1	NA
	15	NA	M	24	B	10 4000	797	26	3	NA
	16	NA	M	45	B	12 600	509	19	2	NA
	17	NA	M	24	B	45 900	323	14	2	NA
	18	NA	M	31	B	45 932	718	47	1	NA
HIV progressors on cART	19	NA	M	60	B	10 0000[c]	306	17	4	<1
	20	NA	M	54	B	20 700[c]	466	18	3	<1
	21	NA	F	35	AE	40 8000[c]	76	6	1	<1
	22	NA	M	39	B	86 700[c]	134	10	9	<1

cART, combination antiretroviral treatment; EC, elite controller; LTSP, long-term slow progressor; NA, not applicable; ND, not detected; PTC, posttreatment controller; VC, viremic controller; VL, viral load.
[a]Received cART for first 5 years after HIV acquisition.
[b]VL at 7 years after infection or earliest available after 7 years, currently on cART with VL ≤20 copies/ml.
[c]VL pretreatment shown, currently on cART with VL ≤37 copies/ml.

the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH and Immune Technology Corp, New York as indicated in Table 2.

To determine whether various glycosylation patterns affected ADCC recognition of Env, different glycosylated forms of HIV-1_{AD8} gp140 were prepared. The wild-type glycosylated form of HIV-1_{AD8} gp140 bearing both complex and mannose N-linked glycans was expressed and purified in HEK 293T cells as previously described ('Complex and Mannose') [36]. Wild-type glycosylated gp140 was treated with Endoglycosidase-H (NEB, Massachusetts, USA) overnight at 37°C under non-denaturing conditions to cleave mannose N-linked glycans, but not processed complex oligosaccharides from glycoproteins, resulting in gp140 with only complex carbohydrates ('Complex-only'). The 'Mannose-only' gp140 was produced in HEK 293S GnT1$^-$cells that cannot synthesize complex N-glycans [37]. The mannose-only gp140 produced in GnT1$^-$cells was treated

Table 2. HIV-1 antigens used in the study.

		HIV-1 subtype	Amino acid sequence identity to HIV-1_{AD8} gp120/140
HIV-1 gp120	KER2018[b]	A	87%
	AD8[c]	B#1	100%
	BaL[a]	B#2	86%
	JRCSF[b]	B#3	86%
	MN[b]	B#4	82%
	CN54[a]	C#1	73%
	96ZM651[a]	C#2	70%
	CM235[a]	E#1	71%
	A244[b]	E#2	71%
	93TH975[a]	E#3	73%
HIV-1 gp140	92UG037[a]	A	78%
	AD8[c]	B#1	100%
	BaL[b]	B#2	88%
	SF162[a]	B#3	89%
	CN54[a]	C#1	75%
	97CN54[b]	C#2	75%
	93TH966[c]	E#1	74%
	A244[b]	E#2	74%

Source: [a]NIH AIDS Reagent Program, NIH. [b]Immune Technology Corp. New York. [c]Prepared by Rob J Center, University of Melbourne, Australia.

with Endoglycosidase-H overnight at 37°C under nondenaturing conditions to cleave all N-linked glycans from the gp140 ('Nonglycosylated').

Antibody-dependent cellular cytotoxicity mediated natural killer cell activation assay

The intracellular cytokine staining (ICS)-based ADCC-mediated NK cell activation assay was used to analyse cytokine production and degranulation of ADCC-activated NK cells as described previously [38,39]. In brief, 150 μl of HIV-negative healthy donor whole blood (from a single healthy donor) and 50 μl HIV-positive plasma was incubated at 37°C with different HIV-1 Env (1 μg/ml) for 5 h in the presence of Brefeldin A (final conc. 10 μg/ml; Sigma-Aldrich, St. Louis, Missouri, USA), Monensin (final conc. 10 μg/ml; BD Biosciences, San Jose, California, USA) and APC-H7-conjugated anti-CD107a antibody (clone H4A3; Biolegend, San Diego, California, USA). Following incubation, cells were surface stained with Per-CP-conjugated anti-CD3 (clone SK7; Biolegend) and PE-Cy7-conjugated anti-CD56 (clone HCD56; Biolegend) antibodies. Next, whole blood was treated with lysing solution (BD Biosciences) to remove red blood cells and the remaining white blood cells were treated with permeabilization solution (BD Biosciences) and stained with APC-conjugated anti-IFN-γ antibody (clone B27; Biolegend). Flow cytometry data were collected using a FACS Canto II Flow cytometer (BD Biosciences) and were analysed using Flow Jo version 10.0.6 software. NK cells were identified as $CD3^{-}CD56^{+}$ and responses were considered positive if the response [%NK cells expressing interferon-gamma (IFN-γ) and/or CD107a] was more than three times that of unstimulated NK cells incubated with HIV-positive plasma. ADCC responses were measured as %NK cells expressing IFN-γ and CD107a and the magnitude of ADCC responses was categorized as low (<2%), medium (2–8%) and high (>8% NK cell activation). The experiments using different glycosylated forms of HIV-1 gp140 were supplemented with 5 ng/ml interleukin (IL)-15 (R&D systems, Minneapolis, Minnesota, USA) for the 5 h incubation to enhance the NK cell activation as previously published [40]. This allowed better comparisons of responses in NK cell activation.

Antibody-dependent cellular cytotoxicity mediated killing using the rapid fluorometric ADCC assay

The rapid fluorometric ADCC (RFADCC) assay was used as previously described [41,42]. Briefly, 10^6 CEM.NKr-CCR5 cells in 100 μl of RF10 medium (RPMI; Life Technologies, Carlsbad, California, USA, 10% foetal calf serum, 1% penicillin-streptomycin, 2% glutamine) were coated separately with 3 μg of purified HIV-1 gp140 subtype A, B, C and E for 1 h at room temperature. Uncoated CEM.NKr-CCR5 cells were treated identically in the absence of gp140 protein. Coated and uncoated CEM.NKr-CCR5 cells were initially labelled with PKH26 (Sigma-Aldrich) and CFSE (Sigma-Aldrich). Around 2×10^4 PKH26 and CFSE and labelled CEM.NKr-CCR5 target cells were incubated with human plasma (1 : 100 final dilution) for 30 min at 37°C followed by addition of 2×10^5 PBMCs (total volume of 100 μl) to achieve a target:effector cell ratio of 1 : 10. Cells were incubated for 4 h at 37°C and then stained with Per-CP-conjugated anti-CD3 (clone SK7; Biolegend) and APC-H7-conjugated anti-CD14 (clone MϕP9; BD Biosciences) antibodies. Flow cytometry data were collected using a FACS LSR II Flow cytometer (BD Biosciences). The data analysis was performed using Flow Jo version 10.0.6 software and gating strategy as previously described [41]. For all gp140 responses, the no antigen control (uncoated target cells) responses were subtracted.

Statistical analyses

Statistical analyses were performed using SPSS version 18 software (IBM, Armonk, New York, USA). Mann–Whitney *U*-tests or Wilcoxon Signed Ranks tests were used to compare groups as indicated in the results section and figure legends. Median and interquartile range (IQR) was used to describe the data for Mann–Whitney *U*-tests and Wilcoxon Signed Ranks tests. A P value of 0.05 was considered to indicate a significant difference. Fisher exact tests were performed using the http://www.vassarstats.net website.

Results

Broad antibody-dependent cellular cytotoxicity mediated natural killer cell activation to different HIV-1 Env subtypes

Given the high sequence diversity of HIV, for an HIV vaccine to be globally effective, immune responses need to be broad and able to recognize multiple subtypes of HIV [25]. Twenty-two individuals primarily infected with HIV-1 subtype B (Table 1) were recruited to study the ability of ADCC antibodies in the plasma of these individuals to recognize diverse range of HIV-1 gp120 and gp140 proteins from subtype A, B, C and E (Table 2). Eleven individuals were designated as HIV controllers, having had HIV infection for 5–19 years without cART, maintaining a $CD4^{+}$ T-cell count of more than 400 cells/μl and a low HIV viral load of less than 5000 copies/ml as per the definitions of the controller groups (Table 1). Eleven individuals were HIV progressors, having a high viral load prior to cART of more than 10 000 copies/ml (Table 1). We first used an ADCC assay that studied ADCC-mediated NK cell activation; Fig. 1a shows the gating strategy for this assay. We detected broad ADCC-mediated NK cell activation to different subtypes of both HIV-1 gp120 and gp140. Figure 1b shows an example of ADCC antibodies in the plasma from an HIV controller able to recognize HIV-1 Env subtype B as well as subtype

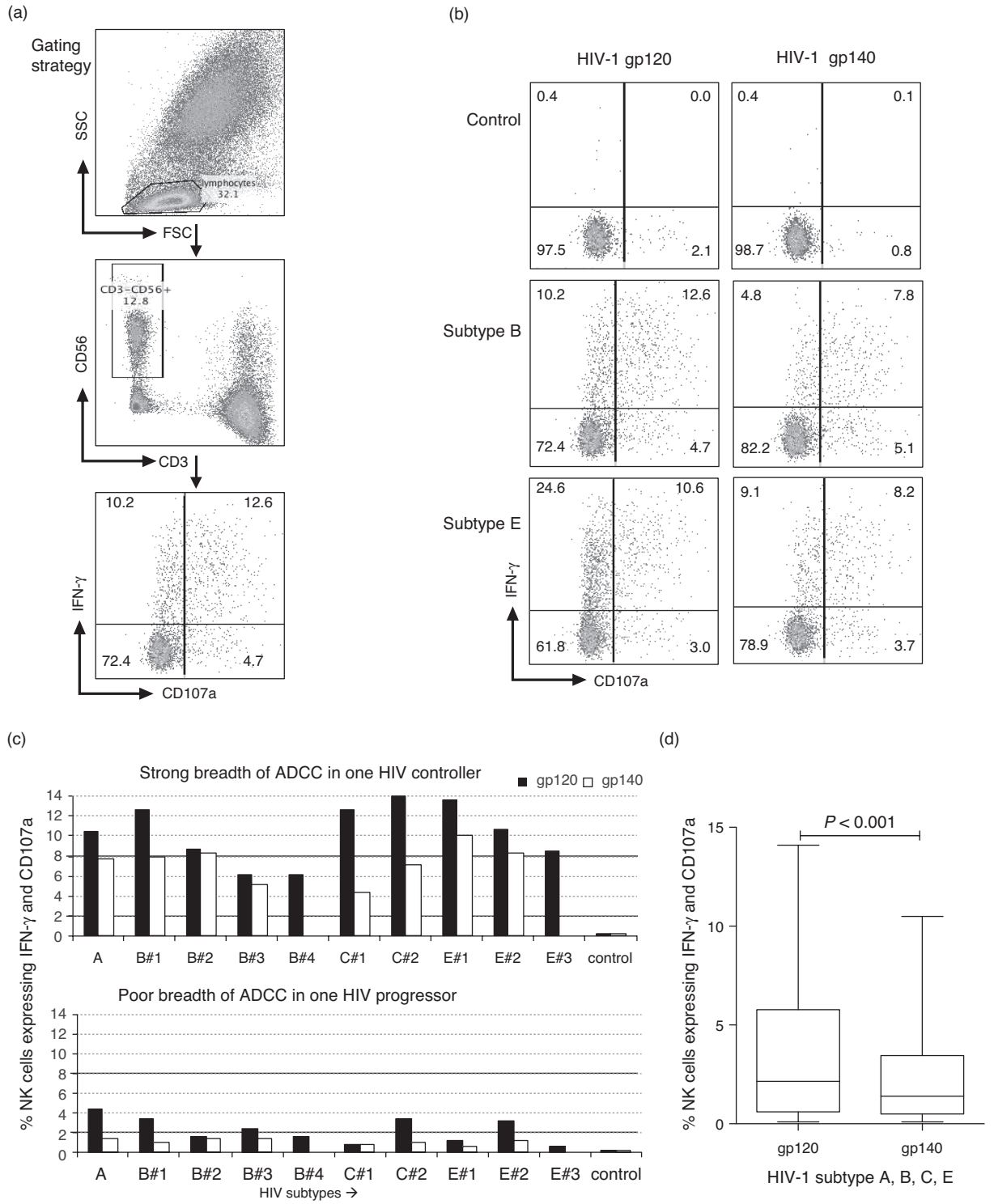

Fig. 1. Broad ADCC-mediated natural killer cell activation to different HIV-1 Env subtypes. (a) Gating strategy for the ADCC-mediated natural killer (NK) cell activation assay. First gate is on lymphocytes in the forward and side scatter (FSC/SSC) plot and then on $CD3^{-}CD56^{+}$ NK cells, which are analysed for dual intracellular interferon-γ (IFN-γ) and surface CD107a expression. (b) A flow cytometric example of ADCC responses using plasma from an HIV controller able to recognize HIV-1 Env subtype B as well as subtype E compared with unstimulated control in the presence of HIV controller plasma. ADCC responses to HIV-1 gp120 are compared with gp140. (c) The breadth of ADCC to a panel of 10 gp120 proteins and eight gp140 proteins across four HIV subtypes using plasma from one HIV controller (top plot) compared with one HIV progressor (bottom plot). Bars represent magnitude of NK cell activation. (d) The magnitude of ADCC to HIV-1 gp120 compared with gp140 across all 22 individuals (Wilcoxon-Signed Ranks test).

E gp120 and gp140 proteins. Figure 1c shows an example of ADCC responses in the plasma from one HIV controller (individual #6 in Table 1) in comparison to one HIV progressor (individual #12 in Table 1) against all Env (10 gp120 and 8 gp140 proteins) across four HIV subtypes. The HIV controller plasma demonstrated ADCC-mediated NK cell activation against all proteins tested, whereas the HIV progressor plasma recognized a much smaller subset of Env proteins with much lower magnitude of NK cell activation.

We noted that ADCC responses to the same concentrations of gp120 proteins were typically higher than to gp140 tested in our initial evaluations (seen in Fig. 1b, c). To formally evaluate this further, we tested ADCC responses in plasma from all 22 individuals against HIV-1 Env of four different subtypes for which we had both gp120 and gp140 proteins. We found that the magnitude of ADCC was significantly higher to HIV-1 gp120 than to gp140 across all individuals (median 2.1% NK cell activation, IQR 5.1% to HIV-1 gp120 vs. median 1.4% NK cell activation, IQR 2.8% to HIV-1 gp140; Wilcoxon-Signed Ranks test $P < 0.001$; Fig. 1d).

HIV controllers have broader antibody-dependent cellular cytotoxicity mediated natural killer cell activation than HIV progressors

HIV controllers are a model to study partial control of HIV [14,17,19,43,44]. When comparing plasma from 11 HIV controllers with 11 HIV progressors, we detected broader and more potent ADCC responses in HIV controllers (Fig. 2a and b). HIV controllers had significantly broader ADCC (10/11 individuals had cross-reactivity to one or more HIV-1 gp120 subtype) than HIV progressors (4/11; Fisher exact two-tailed $P = 0.02$). On average, HIV controllers displayed medium or high ADCC response (>2% NK cell activation) to a median of eight different HIV-1 gp120 proteins (IQR 4) compared with one for HIV progressors (IQR 5; Mann–Whitney *U*-test $P = 0.001$). Figure 2a (left-hand side heat map) shows that 11 of 11 HIV controllers generated more than 2% Env-specific ADCC responses to gp120 compared with five of 11 HIV progressors (Fisher exact two-tailed $P = 0.01$). The magnitude of HIV-1 gp120-specific ADCC responses of HIV controllers (median 4.3% NK cell activation, IQR 5.7%) was significantly higher than HIV progressors (median 0.6% NK cell activation, IQR 1.4%; Mann-Whitney U-test $P < 0.001$, Fig. 2a left-hand side heat map). A medium or high ADCC (>2% NK cell activation) to more than two subtypes of the gp120 subtypes was detected in nine of 11 HIV controllers compared with two of 11 HIV progressors (Fisher exact two-tailed $P = 0.008$). A similar pattern of ADCC responses between individual groups was noted to HIV-1 gp140 Envs (Mann–Whitney *U*-test $P < 0.001$, Fig. 2a right-hand side heat map).

In our cohort, three of the 11 HIV controllers and four of the 11 HIV progressors had commenced cART. The comparison of ADCC responses between cART-naive controllers (median 4.3% NK cell activation, IQR 6.2%) and cART-naive progressors (median 0.4% NK cell activation, IQR 0.8%) to HIV-1 gp120 was striking (Mann–Whitney *U*-test $P < 0.001$, Fig. 2a left-hand side heat map). This is in the setting of cART-naive controllers having significantly lower viral load (median = 329 co-copies/ml, IQR 1055) compared with cART-naive progressors (median 49900 copies/ml, IQR = 58100; Mann-Whitney U-test $P = 0.004$, Table 1), although their $CD4^+$ cell count was similar (median $CD4^+$ cell count = 687 cells/μl, IQR 519 in cART-naive controllers vs. median $CD4^+$ cell count = 649 cells/μl, IQR 395 in cART-naive progressors; Mann–Whitney *U*-test $P = 0.281$, Table 1).

Antibody-dependent cellular cytotoxicity mediated killing is higher in HIV controllers than in HIV progressors

To confirm the results above using the NK cell activation ADCC assay and to further assess killing of target cells by ADCC, we studied the ADCC-mediated killing using the RFADCC assay (Fig. 3a shows the gating strategy for this assay as previously described [41]). We studied plasma from a randomly selected subset of seven HIV controllers and seven HIV progressors against HIV-1 gp140 subtype A, B, C and E. Overall, we found good reactivity to HIV-1 gp140 subtype B (ADCC-mediated killing range 20–45%) and E (range 3–41%), less reactivity to subtype A (range 3–15%) and minimal to subtype C (range 0–4%) in all the individuals (Fig. 3b). This was in agreement with the findings of the NK cell activation ADCC assay wherein HIV-1 gp140 of subtype C overall elicited a weaker ADCC response than that of subtype B (median 1.35% NK cell activation, IQR 3.3% to subtype B #1 vs. 0.65% NK cell activation, IQR 1.3% to subtype C#1, Mann–Whitney *U*-test $P = 0.002$, Fig. 2a right-hand side heat map).

Comparing HIV controllers with HIV progressors in the RFADCC assay, we found a significantly higher magnitude of %ADCC-mediated killing in HIV controllers than in HIV progressors to both HIV-1 gp140 subtype B (median 38% killing, IQR 7.5% in HIV controllers vs. 24% killing, IQR 8.4% in HIV progressors; Mann-Whitney U-test $P = 0.001$) and the heterologous subtype E gp140 proteins (median 29% killing, IQR 8.5% in HIV controllers vs. 13.5% killing, IQR 8% in HIV progressors; Mann–Whitney *U*-test $P = 0.001$, Fig. 3b).

Higher antibody-dependent cellular cytotoxicity mediated natural killer cell activation to glycosylated HIV-1 gp140 than nonglycosylated form

The potential of the glycans added during HIV Env biosynthesis to affect HIV-specific immune responses *in*

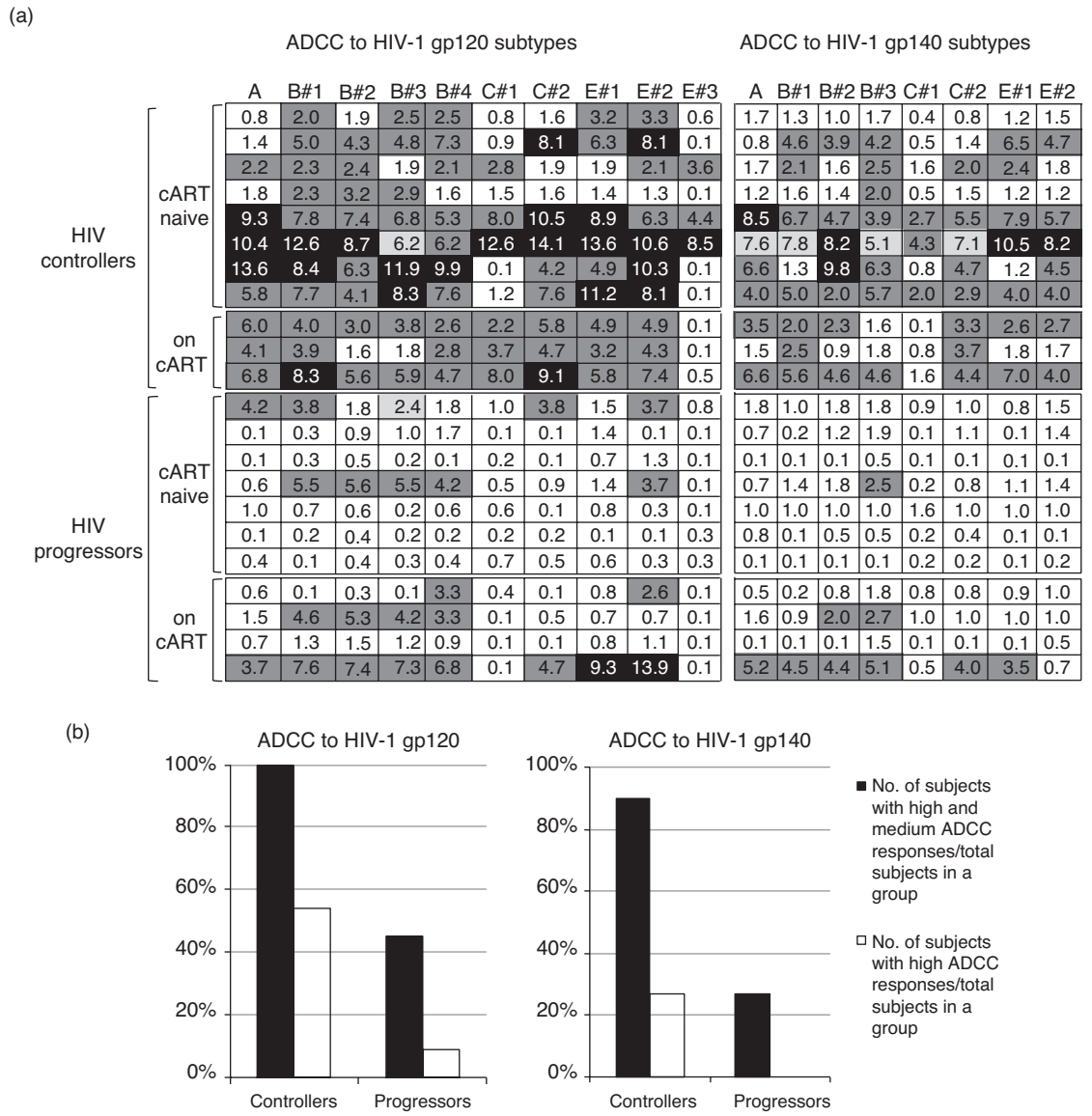

Fig. 2. HIV controllers have broader ADCC-mediated natural killer cell activation than HIV progressors. (a) ADCC responses to gp120 and gp140 of different HIV-1 subtypes in HIV controllers compared with HIV progressors, including individuals receiving cART in both the groups. The breadth of ADCC responses ($n = 22$) represented as heat maps (NK cell activation: low: <2% in white, medium: 2–8% in gray and high: >8% in black). (b) Comparison of the percentage of individuals (11 HIV controllers and 11 HIV progressors) with high or medium and high NK cell activation to HIV-1 gp120 (left graph) and gp140 (right graph).

vivo is debated. Glycosylation can result in both inhibition (e.g. by masking critical protein epitopes) and augmentation (e.g. glycan-dependent epitope) of an immune response, but the relative importance of glycosylation in ADCC recognition of Env is not clear [45,46]. We therefore compared ADCC responses with HIV-1_{AD8} gp140 with different glycosylation profiles purified from transfected 293T or 293S GnT1^{-} cells in a subset of 11 individuals with good ADCC responses. We found that the glycosylated form of HIV-1 gp140 triggered higher ADCC as opposed to the nonglycosylated form. Figure 4a (top) shows an example of the ADCC-mediated NK cell activation to glycosylated and nonglycosylated gp140 in the ADCC-mediated NK cell activation assay using plasma from an HIV controller. ADCC-mediated NK cell IFN-γ expression was significantly lower for the nonglycosylated gp140 ($P = 0.02$; Wilcoxon Signed Ranks test; Fig. 4b left-hand side plots). Given the lower magnitude of ADCC responses to gp140 than gp120 as previously mentioned, we amplified the magnitude of NK cell activation using IL-15 supplementation, a technique we have previously employed [40]. This allowed better comparisons of differences in NK activation. Figure 4a (lower) shows an example of the results obtained for the same individual using IL-15. Using IL-15 augmentation of ADCC

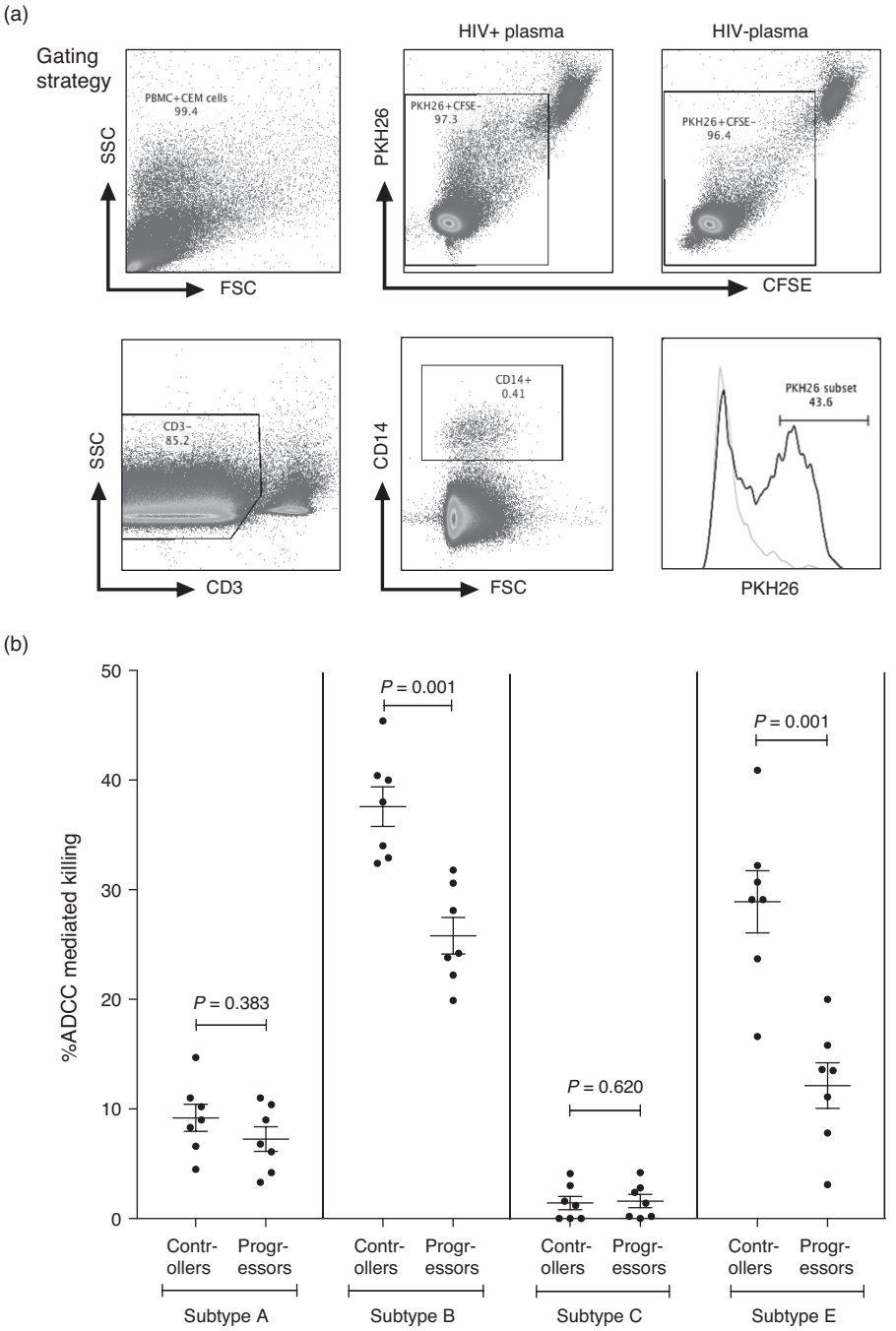

Fig. 3. ADCC-mediated killing is higher in HIV controllers than in HIV progressors in the RFADCC assay. (a) Gating on CFSE- cells followed by gating on CD3-CD14+ monocytes and analysis of PKH26 expression for these monocytes. A side-by-side comparison of PKH26 expression in the absence of HIV-positive plasma (grey histogram) and presence of HIV-positive plasma (black histogram). (b) ADCC-mediated killing performed on plasma from 7 HIV controllers and 7 HIV progressors to HIV-1 gp140 subtype A, B, C and E (Mann–Whitney *U*-test).

responses, we observed an increase in median NK cell IFN-γ expression from 12.6 to 21.1% and median CD107a degranulation from 2.9 to 4.5% to glycosylated gp140 (Fig. 4b). Using IL-15 treatment, we confirmed the significant drop in ADCC-mediated NK cell IFN-γ expression upon de-glycosylation of gp140 (median 21.1%, IQR 16.0% for glycosylated gp140 to median 12.7%, IQR 15.3% for nonglycosylated gp140; Wilcoxon-Signed Ranks test $P = 0.004$, Fig. 4b top right panel). We also saw a significant drop in NK cell CD107a degranulation upon de-glycosylation (median 4.5%, IQR 5.0% for glycosylated gp140 to median 2.3%, IQR 2.6% for nonglycosylated gp140; Wilcoxon-Signed ranks test $P = 0.015$, Fig. 4b bottom right panel) using IL-15

treatment. The IL-15 augmented ADCC responses of five individuals (median of triplicates) to HIV-1 gp140 with different glycan profiles show higher ADCC to HIV-1 gp140 with complex and/or mannose glycans than the nonglycosylated form (Fig. 4c). Taken together, our findings suggested that ADCC Abs produced during natural HIV infection recognize epitopes with a glycan component. This may also be due to glycans stabilizing Env

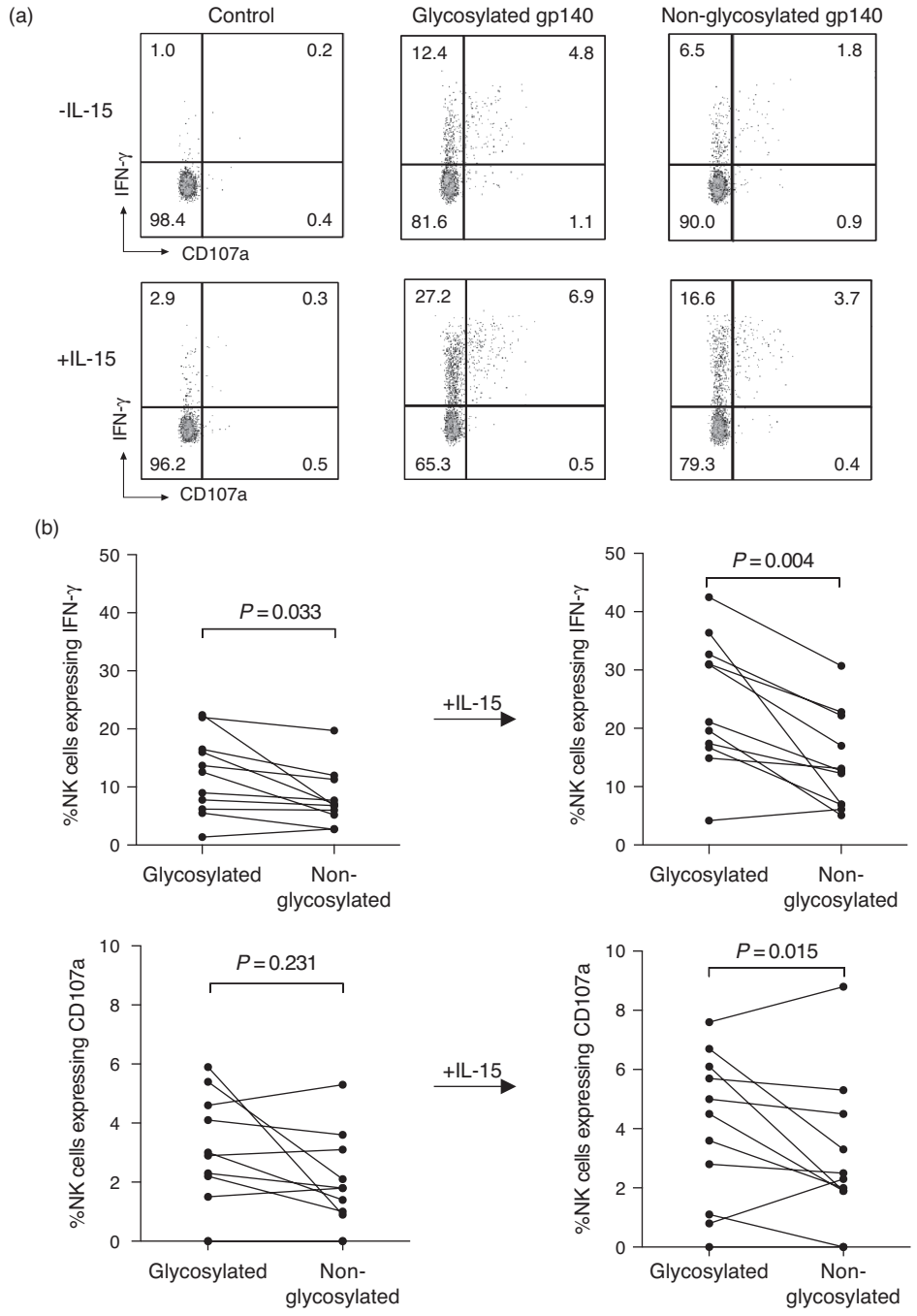

Fig. 4. Higher ADCC-mediated natural killer cell activation to glycosylated HIV-1 gp140 than nonglycosylated form. (a) ADCC responses to the glycosylated and nonglycosylated forms of HIV-1 gp140 compared to unstimulated control in the absence (top) and presence (lower) of IL-15. IL-15 supplementation for augmentation of ADCC responses to the glycosylated and nonglycosylated forms as shown in bottom panels. (b) Comparison of ADCC responses detected in plasma from 11 individuals to glycosylated and nonglycosylated forms of HIV-1 gp140, before and after IL-15 supplementation (Wilcoxon Signed ranks tests). (c) The IL-15 augmented ADCC responses of 5 individuals (median of triplicates) to different forms of glycosylated HIV-1 gp140.

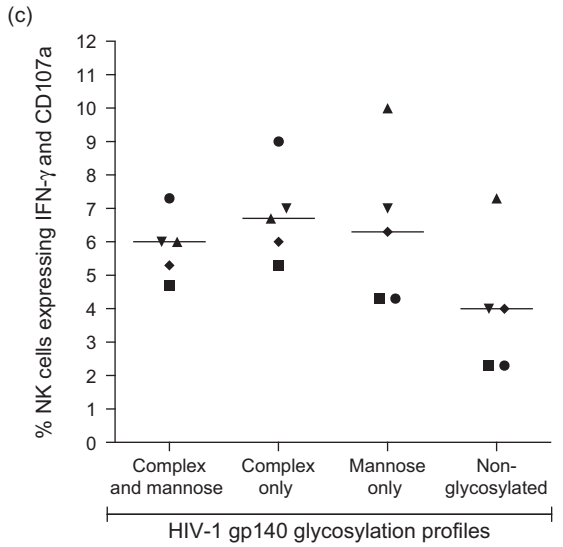

Fig. 4. (*Continued*).

structure for better presentation of epitopes to ADCC Abs or forming part of epitope itself.

Discussion

People who naturally control HIV infection can assist in our understanding of the immune correlates of protection from disease progression. Although CTL and NAb responses are associated with control of HIV infection, vaccines based on these principles have failed to provide protection in human efficacy trials [2,47]. The role of HIV-specific ADCC is gaining prominence following the posthoc analyses of the partially successful RV144 trial [6–8]. We studied Env-specific ADCC antibody responses in HIV controllers (elite controller, viremic controllers, long-term slow progressors and posttreatment controller) compared with HIV progressors. We found that HIV controllers have significantly broader and higher Env-specific ADCC responses than people with progressive HIV infection. We speculate that this may be due to HIV controllers targeting more conserved epitopes within Env across multiple HIV subtypes. Identifying conserved ADCC epitopes commonly targeted by HIV controllers could assist in designing improved HIV vaccine strategies. A limitation of our, and other, studies on HIV controllers is that HIV controllers are by definition infected for long periods of time without cART and it is difficult to adequately match duration of infection with HIV progression given recommendations to initiate cART expeditiously in HIV progressors. To address this potential confounder, we assessed whether duration of infection off cART correlated with breadth and magnitude of ADCC responses in this cohort and found no correlation ($P=0.084$, $r^2=0.212$; $P=0.532$, $r^2=0.031$, respectively, for breadth and magnitude vs. duration of infection). Thus, duration of infection does not solely account for the broad and robust responses observed in HIV controllers, suggesting that the broad ADCC responses observed may assist in the control of HIV. Inducing broad ADCC responses by preventive HIV vaccines should be pursued with novel HIV vaccine strategies.

Our results indicate that ADCC responses in HIV controllers commonly recognized most of the four major subtypes of HIV-1 spread across the globe. This contrasts with the limited development of bNAbs that develop in most HIV-infected people [48]. We found highest reactivity to subtype B and heterologous subtype E HIV-1 Envs, lower reactivity to subtype A Envs and only limited reactivity to subtype C Envs. This was consistent across both ADCC-mediated NK cell activation and ADCC-mediated killing assays. The robust cross-recognition of ADCC responses to B and E subtypes is interesting in the context of the partially successful RV144 trial, which used a bivalent B/E strategy and may point to an important common epitope shared across these subtypes. The modest recognition of subtypes A and C Envs in our primarily subtype B infected cohort suggests that, if ADCC-based vaccines are successful, a multivalent approach may be needed for a global HIV vaccine to cover all relevant subtypes effectively.

Glycosylation of HIV-1 Env is critical for folding of Env into functional form. Several bNAbs targeting Env epitopes with a glycan component have been reported [29], but no research has been reported for ADCC Abs. Our novel findings suggest that HIV-1 Env ADCC responses commonly target glycan-dependent epitopes. A potential limitation of these studies is that our gp140 glycosylation variants were produced in 293-derived cells and there will be at least subtle differences in glycosylation patterns of gp140 present on the outside of infected lymphocytes [49–52]. It is, however, difficult to generate purified glycosylation mutant gp140 proteins in infected lymphocytes and the striking reduction in ADCC recognition of de-glycosylated variants we observed strongly suggests that ADCC responses recognize a significant number of glycan-dependent epitopes. Further studies are needed to completely dissect the role of glycosylation and to identify conserved glycan-dependent epitopes.

In summary, broadly reactive ADCC responses in subtype B infected HIV controllers frequently target a range of HIV-1 subtype B and E Env and in some cases also subtype A and C Env. This indicates the potential utility of eliciting ADCC responses with novel HIV vaccines. Characterizing conserved ADCC epitopes should help in designing globally relevant ADCC-based HIV vaccines.

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V.M. performed and analysed ADCC assays. V.M., S.K. and I.S. conceived the study and wrote the manuscript. M.K., L.W. and M.P. provided guidance with the ADCC assays. R.C. and C.G. prepared HIV-1_{AD8}, HIV-$1_{93TH966}$ gp120/140 and glycosylation mutant proteins. W.W. and V.M. performed statistical analysis of the data. I.S. and S.K. recruited individuals. All authors read and approved the final manuscript. We are grateful to all the individuals who kindly provided blood samples.

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Conflicts of interest

We declare that we have no conflicts of interest.

References

1. Battegay M, Nuesch R, Hirschel B, Kaufmann GR. **Immunological recovery and antiretroviral therapy in HIV-1 infection.** *Lancet Infect Dis* 2006; **6**:280–287.
2. Buchbinder SP, Mehrotra DV, Duerr A, Fitzgerald DW, Mogg R, Li D, *et al*. **Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial.** *Lancet* 2008; **372**: 1881–1893.
3. Pitisuttithum P. **HIV-1 prophylactic vaccine trials in Thailand.** *Curr HIV Res* 2005; **3**:17–30.
4. Sanou MP, De Groot AS, Murphey-Corb M, Levy JA, Yamamoto JK. **HIV-1 vaccine trials: evolving concepts and designs.** *Open AIDS J* 2012; **6**:274–288.
5. Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, Paris R, *et al*. **Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand.** *N Engl J Med* 2009; **361**:2209–2220.
6. Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S, Tomaras GD, Alam SM, *et al*. **Immune-correlates analysis of an HIV-1 vaccine efficacy trial.** *N Engl J Med* 2012; **366**:1275–1286.
7. Bonsignori M, Pollara J, Moody MA, Alpert MD, Chen X, Hwang KK, *et al*. **Antibody-dependent cellular cytotoxicity-mediating antibodies from an HIV-1 vaccine efficacy trial target multiple epitopes and preferentially use the VH1 gene family.** *J Virol* 2012; **86**:11521–11532.
8. Tomaras GD, Ferrari G, Shen XY, Alam SM, Liao HX, Pollara J, *et al*. **Vaccine-induced plasma IgA specific for the C1 region of the HIV-1 envelope blocks binding and effector function of IgG.** *Proc Natl Acad Sci U S A* 2013; **110**:9019–9024.
9. Kamya P, Tallon B, Melendez-Pena C, Parsons MS, Migueles SA, Connors M, *et al*. **Inhibitory killer immunoglobulin-like receptors to self HLA-B and HLA-C ligands contribute differentially to natural killer cell functional potential in HIV infected slow progressors.** *Clin Immunol* 2012; **143**:246–255.
10. Sáez-Cirión A, Bacchus C, Hocqueloux L, Avettand-Fenoel V, Girault I, Lecuroux C, *et al*. **Post-treatment HIV-1 controllers with a long-term virological remission after the interruption of early initiated antiretroviral therapy ANRS VISCONTI study.** *PLoS Pathog* 2013; **9**:e1003211.
11. Mellors JW, Kingsley LA, Rinaldo CR Jr, Todd JA, Hoo BS, Kokka RP, *et al*. **Quantitation of HIV-1 RNA in plasma predicts outcome after seroconversion.** *Ann Intern Med* 1995; **122**:573–579.
12. Deacon NJ, Tsykin A, Solomon A, Smith K, Ludford-Menting M, Hooker DJ, *et al*. **Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients.** *Science* 1995; **270**:988–991.
13. Zaunders J, van Bockel D. **Innate and adaptive immunity in long-term non-progression in HIV disease.** *Front Immunol* 2013; **4**:95.
14. Walker BD. **Elite control of HIV infection: implications for vaccines and treatments.** *Top HIV Med* 2007; **15**:134–136.
15. Kamya P, Boulet S, Tsoukas CM, Routy JP, Thomas R, Cote P, *et al*. **Receptor-ligand requirements for increased NK cell polyfunctional potential in slow progressors infected with HIV-1 coexpressing KIR3DL1∗h/∗y and HLA-B∗57.** *J Virol* 2011; **85**:5949–5960.
16. Pereya F, Addo MM, Kaufmann DE, Liu Y, Miura T, Rathod A, *et al*. **Genetic and immunologic heterogeneity among persons who control HIV infection in the absence of therapy.** *J Infect Dis* 2008; **197**:563–571.
17. Lambotte O, Ferrari G, Moog C, Yates NL, Liao HX, Parks RJ, *et al*. **Heterogeneous neutralizing antibody and antibody-dependent cell cytotoxicity responses in HIV-1 elite controllers.** *AIDS* 2009; **23**:897–906.
18. Doria-Rose NA, Klein RM, Daniels MG, O'Dell S, Nason M, Lapedes A, *et al*. **Breadth of human immunodeficiency virus-specific neutralizing activity in sera: clustering analysis and association with clinical variables.** *J Virol* 2010; **84**:1631–1636.
19. Wren LH, Chung AW, Isitman G, Kelleher AD, Parsons MS, Amin J, *et al*. **Specific antibody-dependent cellular cytotoxicity responses associated with slow progression of HIV infection.** *Immunology* 2013; **138**:116–123.
20. Baum LL, Cassutt KJ, Knigge K, Khattri R, Margolick J, Rinaldo C, *et al*. **HIV-1 gp120-specific antibody-dependent cell-mediated cytotoxicity correlates with rate of disease progression.** *J Immunol* 1996; **157**:2168–2173.
21. Banks ND, Kinsey N, Clements J, Hildreth JE. **Sustained antibody-dependent cell-mediated cytotoxicity (ADCC) in SIV-infected macaques correlates with delayed progression to AIDS.** *AIDS Res Hum Retroviruses* 2002; **18**:1197–1205.
22. Jia M, Li D, He X, Zhao Y, Peng H, MA P, *et al*. **Impaired natural killer cell-induced antibody-dependent cell-mediated cytotoxicity is associated with human immunodefeciency virus-1 disease progression.** *Clin Exp Immunol* 2013; **171**:107–116.
23. Chung AW, Navis M, Isitman G, Wren L, Silvers J, Amin J, *et al*. **Activation of NK cells by ADCC antibodies and HIV disease progression.** *J Acquir Immune Defic Syndr* 2011; **58**:127–131.
24. Boutwell CL, Rolland MM, Herbeck JT, Mullins JI, Allen TM. **Viral evolution and escape during acute HIV-1 infection.** *J Infect Dis* 2010; **202**:S309–S314.
25. Ndung'u T, Weiss RA. **On HIV diversity.** *AIDS* 2012; **26**:1255–1260.
26. Santoro MM, Perno CF. **HIV-1 genetic variability and clinical implications.** *ISRN Microbiol* 2013; **2013**:481314.
27. Hemelaar J, Gouws E, Ghys PD, Osmanov S. **Global trends in molecular epidemiology of HIV-1 during 2000–2007.** *AIDS* 2011; **25**:679–689.
28. Koch M, Pancera M, Kwong PD, Kolchinsky P, Grundner C, Wang L, *et al*. **Structure-based, targeted deglycosylation of HIV-1 gp120 and effects on neutralization sensitivity and antibody recognition.** *Virology* 2003; **313**:387–400.
29. Julien JP, Sok D, Khayat R, Lee JH, Doores KJ, Walker LM, *et al*. **Broadly neutralizing antibody PGT121 allosterically modulates CD4 binding via recognition of the HIV-1 gp120 V3 base and multiple surrounding glycans.** *PLoS Pathog* 2013; **9**:e1003342.
30. Walker LM, Huber M, Doores KJ, Falkowska E, Pejchal R, Julien JP, *et al*. **Broad neutralization coverage of HIV by multiple highly potent antibodies.** *Nature* 2011; **477**:466–470.
31. Kovacs JM, Nkolola JP, Peng H, Cheung A, Perry J, Miller CA, *et al*. **HIV-1 envelope trimer elicits more potent neutralizing antibody responses than monomeric gp120.** *Proc Natl Acad Sci U S A* 2012; **109**:12111–12116.
32. Anderson DJ, Politch JA, Nadolski AM, Blaskewicz CD, Pudney J, Mayer KH. **Targeting Trojan Horse leukocytes for HIV prevention.** *AIDS* 2010; **24**:163–187.
33. Mascola JR, Haynes BF. **HIV-1 neutralizing antibodies: understanding nature's pathways.** *Immunol Rev* 2013; **254**:225–244.
34. Stephenson KE, SanMiguel A, Simmons NL, Smith K, Lewis MG, Szinger JJ, *et al*. **Full-length HIV-1 immunogens induce greater magnitude and comparable breadth of T lymphocyte responses to conserved HIV-1 regions compared with conserved-region-only HIV-1 immunogens in rhesus monkeys.** *J Virol* 2012; **86**:11434–11440.

35. Garber DA, Silverstri G, Feinberg MF. **Review: prospects for an AIDS vaccine: three big questions, no easy answers.** *Lancet Infect Dis* 2004; **4**:397–413.
36. Center RJ, Wheatley AK, Campbell SM, Gaeguta AJ, Peut V, Alcantara S, *et al*. **Induction of HIV-1 subtype B and AE-specific neutralizing antibodies in mice and macaques with DNA prime and recombinant gp140 protein boost regimens.** *Vaccine* 2009; **27**:6605–6612.
37. Reeves PJ, Callewaert N, Contreras R, Khorana HG. **Structure and function in rhodopsin: High-level expression of rhodopsin with restricted and homogeneous N-glycosylation by a tetracycline-inducible N-acetylglucosaminyltransferase I-negative HEK293S stable mammalian cell line.** *Proc Natl Acad Sci U S A* 2002; **99**:13419–13424.
38. Chung AW, Rollman E, Center RJ, Kent SJ, Stratov I. **Rapid degranulation of NK cells following activation by HIV-specific antibodies.** *J Immunol* 2009; **182**:1202–1210.
39. Stratov I, Chung A, Kent SJ. **Robust NK cell-mediated human immunodeficiency virus (HIV)-specific antibody-dependent responses in HIV-infected subjects.** *J Virol* 2008; **82**:5450–5459.
40. Wren L, Parsons MS, Isitman G, Center RJ, Kelleher AD, Stratov I, *et al*. **Influence of cytokines on HIV-specific antibody-dependent cellular cytotoxicity activation profile of natural killer cells.** *PLoS One* 2012; **7**:e38580.
41. Kramski M, Schorcht A, Johnston AP, Lichtfuss GF, Jegaskanda S, De Rose R, *et al*. **Role of monocytes in mediating HIV-specific antibody-dependent cellular cytotoxicity.** *J Immunol Methods* 2012; **384**:51–61.
42. Gomez-Roman VR, Florese RH, Patterson LJ, Peng B, Venzon D, Aldrich K, *et al*. **A simplified method for the rapid fluorometric assessment of antibody-dependent cell-mediated cytotoxicity.** *J Immunol Methods* 2006; **308**:53–67.
43. Genovese L, Nebuloni M, Alfano M. **Cell-mediated immunity in elite controllers naturally controlling HIV viral load.** *Front Immunol* 2013; **4**:86.
44. Mandalia S, Westrop SJ, Beck EJ, Nelson M, Gazzard BG, Imami N, *et al*. **Are long-term non-progressors very slow progressors? Insights from the Chelsea and Westminster HIV cohort, 1998-2010.** *PLos One* 2012; **7**:e29844.
45. Pejchal R, Doores KJ, Walker LM, Khayat R, Huang PS, Wang SK, *et al*. **A potent and broad neutralizing antibody recognizes and penetrates the HIV glycan shield.** *Science* 2011; **334**:1097–1103.
46. Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, *et al*. **Antibody neutralization and escape by HIV-1.** *Nature* 2003; **422**:307–312.
47. Pitisuttithum P, Gilbert P, Gurwith M, Heyward W, Martin M, van Griensven F, *et al*. **Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand.** *J Infect Dis* 2006; **194**:1661–1671.
48. Huber M, Trkola A. **Humoral immunity to HIV-1: neutralization and beyond.** *J Intern Med* 2007; **262**:5–25.
49. Raska M, Takahashi K, Czernekova L, Zachova K, Hall S, Moldoveanu Z, *et al*. **Glycosylation patterns of HIV-1 gp120 depend on the type of expressing cells and affect antibody recognition.** *J Biol Chem* 2010; **285**:20860–20869.
50. Bonomelli C, Doores KJ, Dunlop DC, Thaney V, Dwek RA, Burton DR, *et al*. **The glycan shield of HIV is predominantly oligomannose independently of production system or viral clade.** *PLoS One* 2011; **6**:e23521.
51. Doores KJ, Bonomelli C, Harvey DJ, Vasiljevic S, Dwek RA, Burton DR, *et al*. **Envelope glycans of immunodeficiency virions are almost entirely oligomannose antigens.** *Proc Natl Acad Sci U S A* 2010; **107**:13800–13805.
52. Willey RL, Shibata R, Freed EO, Cho MW, Martin MA. **Differential glycosylation, virion incorporation, and sensitivity to neutralizing antibodies of human immunodeficiency virus type 1 envelope produced from infected primary T-lymphocyte and macrophage cultures.** *J Virol* 1996; **70**:6431–6436.