Immunization with Clinical HIV-1 Env Proteins Induces Broad Antibody Dependent Cellular Cytotoxicity–Mediating Antibodies in a Rabbit Vaccination Model

Ingrid Karlsson,[1] Marie Borggren,[1] Sanne Skov Jensen,[1,2] Leo Heyndrickx,[3] Guillaume Stewart-Jones,[4] Gabriella Scarlatti,[5] and Anders Fomsgaard,[1,2] on behalf of the NGIN Consortium[*]

Abstract

The induction of both neutralizing antibodies and non-neutralizing antibodies with effector functions, for example, antibody-dependent cellular cytotoxicity (ADCC), is desired in the search for effective vaccines against HIV-1. In the pursuit of novel immunogens capable of inducing an efficient antibody response, rabbits were immunized with selected antigens using different prime–boost strategies. We immunized 35 different groups of rabbits with Env antigens from clinical HIV-1 subtypes A and B, including immunization with DNA alone, protein alone, and DNA prime with protein boost. The rabbit sera were screened for ADCC activity using a GranToxiLux-based assay with human peripheral blood mononuclear cells as effector cells and CEM.NKR$_{CCR5}$ cells coated with HIV-1 envelope as target cells. The groups with the highest ADCC activity were further characterized for cross-reactivity between HIV-1 subtypes. The immunogen inducing the most potent and broadest ADCC response was a trimeric gp140. The ADCC activity was highest against the HIV-1 subtype corresponding to the immunogen. The ADCC activity did not necessarily reflect neutralizing activity in the pseudovirus-TZMbl assay, but there was an overall correlation between the two antiviral activities. We present a rabbit vaccination model and an assay suitable for screening HIV-1 vaccine candidates for the induction of ADCC-mediating antibodies in addition to neutralizing antibodies. The antigens and/or immunization strategies capable of inducing antibodies with ADCC activity did not necessarily induce neutralizing activity and vice versa. Nevertheless, we identified vaccine candidates that were able to concurrently induce both types of responses and that had ADCC activity that was cross-reactive between different subtypes. When searching for an effective vaccine candidate, it is important to evaluate the antibody response using a model and an assay measuring the desired function.

Keywords: ADCC, vaccine development, neutralizing antibody, antibody mediated immunity

Introduction

THE INDUCTION OF ANTIBODIES mediating broad antibody-dependent cellular cytotoxicity (ADCC) in addition to neutralization is desired in the search for effective therapeutic and prophylactic vaccines against HIV-1. The partial efficacy of the RV144 trial displayed a possible correlation between protection and ADCC-mediating antibodies.[1] The importance of ADCC responses has also been shown in other HIV-1 vaccine studies[2–6] and passive-transfer experiments[7,8] performed in nonhuman primates. In chronically HIV-1-infected individuals, ADCC has been associated with the control of infection in slow progressors,[9–12] elite controllers,[13–16] and children.[17] A recent study has shown that HIV controllers have higher and broader ADCC-mediated natural killer (NK) cell activation compared to HIV progressors.[14] HIV-specific[18,19] and ADCC-mediating[20,21] antibodies decline during antiretroviral therapy, and a therapeutic vaccine during therapy may be an efficient approach to improve the antibody-mediated control of infection and the elimination of already-infected cells.

The ability of an HIV-1 vaccine to generate a broad functional anti-HIV-1 response is crucial. HIV-1 is extremely

[1]Department of Virology and Special Microbial Diagnostic, Statens Serum Institut, Copenhagen, Denmark.
[2]Infectious Disease Research Unit, Clinical Institute, University of Southern Denmark, Odense, Denmark.
[3]Biomedical Department, Virology Unit, Institute of Tropical Medicine, Antwerp, Belgium.
[4]Human Immunology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Oxford, United Kingdom.
[5]Viral Evolution and Transmission Unit, San Raffaele Scientific Institute, Milan, Italy.
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variable and has a high evolution rate. HIV-1 is divided into four groups: M, O, N, and P. The M group, which accounts for the vast majority of HIV-1 infections, contains nine subtypes (A through J), ∼79 circulating recombinant forms (CRFs), and multiple unique recombinant forms.[22] Moreover, HIV-1 infection is characterized by a high degree of genetic variability within the infected individual. Immune responses able to meet this high diversity of HIV-1 are crucial for any efficient HIV-1 vaccine.

The growing knowledge of conserved ADCC and neutralizing epitope structures has not yet translated into improved immunogens, emphasizing the importance of continuing empiric approaches in the search for relevant HIV-1 vaccine immunogens. There is an overlap between epitopes recognized by ADCC-mediating and neutralizing antibodies, with many ADCC-mediating antibodies also mediating neutralization, but there are also many ADCC-specific epitopes and probably many more yet to be defined.[23,24]

We focused on identifying and characterizing immunogens from clinical HIV-1 isolates that elicited broadly neutralizing antibodies in HIV-1-infected individuals. In this study, we screened their capacity to induce anti-HIV-1 antibodies in rabbits with ADCC function and compared the results with their IgG binding titers and neutralizing ability (part of the data previously published in Heyndrickx et al.,[25] Visciano et al.,[26] and Borggren et al.[27]). The groups with the highest ADCC activity were further characterized for cross-reactivity between HIV-1 subtypes.

Materials and Methods

Rabbit immunizations

All rabbit experiments were performed in accordance with the Animal Experimentation Act of Denmark (permit No. 2007/561-1362) and European Convention ETS 123 (Protection of Vertebrate Animals used for experimental and other scientific purposes). Ten-week-old New Zealand White nulliparous female rabbits (Charles River Laboratories) were housed at the animal facility at Statens Serum Institut (SSI), Copenhagen, Denmark with an acclimatization period of at least 10 days. Groups of three to four rabbits were each immunized with DNA alone, protein alone, or combinations of DNA prime followed by protein boost. One group was immunized with virosomes. All groups of animals and immunogens are listed in Table 1. Neutralization and IgG titers have previously been published for 13 groups out of the 35 groups. These 13 groups include the 9 groups immunized with gp140 trimer protein, 1 group immunized with gp120 monomer protein 94UG018, and 3 groups immunized with DNA, gp150SOSIP-BX08 and SSImix140 (Table 1). ADCC data are new for all 35 immunization groups.

DNA immunizations were performed intradermally (200 μg/dose) with subsequent electroporation using the OncoVet™ system (CytoPulse Sciences/Cellectis, Romainville, France) over the injected area. Protein immunizations were performed subcutaneously (s.c.) with monomeric or trimeric Env protein (20 or 100 μg/dose) in the presence (or absence) of cationic adjuvant formulation number 1 (CAF01)[28] or cationic adjuvant formulation number 2 (CAF02) containing 100 μg/dose distearoyl-sn-glycero-3-phosphocholine (DSPC)[29] in a total volume of 400 μl. Virosomes[30] were injected s.c.

(200 μg/dose). Unless otherwise stated, immunizations were performed on weeks 0, 4, 8, and 12. DNA priming was performed thrice during the first week, a mode of "intensive" priming.[27] The protein boost was performed on week 14. Ear bleeding was performed before immunization (week 0) and 2 weeks after the last immunization (week 14 or 16), and all sera were stored at −20°C.

ADCC assay

The ADCC-GranToxiLux assay (OncoImmunin, Gaithersburg, MD) was performed as described by Pollara et al.[31] In brief, the target cells, CEM.NKR$_{CCR5}$ cells, were coated with HIV-1 envelope protein and labeled with TFL4 and NFL1. Recombinant HIV-1 representing the envelopes of subtype A, gp140 UG37 (Polymun Scientific, Klosterneuburg, Austria); subtype B, gp120 BaL (Immune Technology, New York, NY); subtype C, gp120 ZA1197MB (Immune Technology); and subtype CRF01_AE, gp120 CM243 (Protein Sciences, Meriden, CT) were used. The protein concentration used for coating (0.01 mg/ml) was decided by competing the binding of FITC-conjugated anti-CD4 antibodies (clone SK3; BD Biosciences), as described.[31]

Human peripheral blood mononuclear cells (PBMCs) were used as the source of NK cells, that is, effector cells. The PBMCs were obtained by density-gradient centrifugation of buffy coats from the Danish Blood Bank and cryopreserved until use. Before use, the PBMCs were thawed and rested overnight in R10 (RPMI; Gibco, Life Technologies, Naerum, Denmark) supplied with 10% fetal bovine serum (Gibco) and 1% penicillin–streptomycin (Gibco) at 37°C and 5% CO_2. The effector (E) and target (T) cells were tested at an E:T ratio of 30:1. Rabbit sera were tested in fivefold dilutions starting at 1:10. The positive control HIV IgG immunoglobulin (NIH AIDS Research and Reagent Program) and negative control (in-house pool of HIV-negative sera) were tested in fivefold dilutions starting at 1:250. A control without antibody was also included to confirm that there was no antibody-independent activity of NK cells against the target cells. All sera were heat inactivated (1 h at 56°C).

The cells were acquired using a BD LSRII and analyzed using FlowJo (Tree Star version 8.8.7). The threshold for a positive GzB response was >6.2% after background subtraction. These criteria were established using preimmunization rabbit samples (n = 348) and defined as the above mean plus three standard deviations. ADCC for each animal was defined as the highest percentage of granzyme B activity of the effector cells after background subtraction (i.e., preimmunization sera tested at the same dilution). For comparison, the granzyme B activity was also calculated as the area under the curve (AUC) and as the highest dilution factor above the threshold.

Anti-HIV gp120 enzyme-linked immunosorbent assay

Anti-gp120-specific IgG titers were determined by enzyme-linked immunosorbent assay (ELISA), as previously described.[27]

Neutralization assay

Neutralization of purified IgG was assayed in pseudovirus neutralization assays using TZMbl cells conducted as described previously.[25]

TABLE 1. GROUPS (*N*=35) OF ANIMALS AND IMMUNOGENS, ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY, NEUTRALIZATION, AND IGG TITER

Immunogen	Patient information[a]	Immunogen subtype	References	Dose (mg)	Administration	Adjuvant	ADCC[b]	Neutralization[c]	IgG titer[d]
DNA									
Mix (clade A, B, C) gp160	Mix of clade A, B, and C Env	B	48	200 int	i.d.+e.p.	—	— — — —	ND ND ND ND	ND ND ND ND
gp150 SOSIP-BX08	Primary, CCR5-tropic virus isolated early after infection	B	27,37,49	200	i.d. e.p. 4 weeks	—	10.4	ND	27,187 251,043 175,112
gp150 SOSIP-BX08	Primary, CCR5-tropic virus isolated early after infection	B	27,37,49	200 int	i.d.+e.p. 4 weeks	—	— ND	ND ND	161,154 —
gp150 SOSIP-BX08	Primary, CCR5-tropic virus isolated early after infection	B	27,37,49	200 int	i.d.+e.p. 2 weeks	—	7.0 ND	ND ND	172,550 312,044 409,860 286,468
SSImix gp140	Mix of three plasmids encoding gp140 from BX08 and two Danish patients, ctl21 and ctl27, displaying neutralizing activity	B	27	200 int	i.d.+e.p.	—	— — — —	ND ND ND ND	ND ND ND ND
SSImix gp140	Mix of three plasmids encoding gp140 from BX08 and two Danish patients, ctl21 and ctl27, displaying neutralizing activity	B	27	200 int	i.d.+e.p.	APOP-cells	— ND ND ND	179 250 250 ND	48,741 82,057 50,173 —
SSImix gp140	Mix of three plasmids encoding gp140 from BX08 and two Danish patients, ctl21 and ctl27, displaying neutralizing activity	B	27	200 int	i.d.+e.p.	—	ND — ND ND	85 31 114 31	— 654,505 93,715 271,282
SSImix gp140	Mix of three plasmids encoding gp140 from BX08 and two Danish patients, ctl21 and ctl27, displaying neutralizing activity	B	27	200 int	i.d.+e.p.	pFlic	9.1 — 26.1 —	108 250 31 250	66,834 53,292 49,794 29,614

(continued)

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TABLE 1. (CONTINUED)

Immunogen	Patient information[a]	Immunogen subtype	References	Dose (mg)	Administration	Adjuvant	ADCC[b]	Neutralization[c]	IgG titer[d]
Protein									
gp120 Monomer									
IIIB	Laboratory strain, CXCR4-tropic	B	34	20	s.c.	CAF02	21.3 / 16.9 / 14.0	ND / ND / ND	772,221 / 844,247 / 806,227
Bx08	Primary, CCR5-tropic virus isolated early after infection	B	25,49	20	s.c.	CAF01	— / — / — / —	ND / ND / ND / ND	ND / ND / ND / ND
94UG018	Asymptomatic pregnant woman; Uganda	A	26	20	s.c.	CAF01	— / — / — / —	218 / 240 / 250 / 203	64,846 / 108,681 / 142,973 / 161,895
gp140 Trimer									
Bx08	Primary, CCR5-tropic virus isolated early after infection	B	25,49	20	s.c.	CAF01	— / — / ND / —	ND / ND / ND / ND	890 / 11,408 / 39,092 / 1,657
ACS19642	Long-term nonprogressor, sensitive to autologous neutralization; the Netherlands	B	25,35	100	s.c.	CAF01	— / — / — / —	234 / 94 / 224 / 31	73,558 / 317,532 / 307,374 / 328,326
ACS19554	Progressor, sensitive to autologous neutralization; the Netherlands	B	25,35	100	s.c.	CAF01	13.3 / 21.7 / 28.0 / 11.9	49 / 67 / 100 / 102	278,538 / 188,776 / 85,338 / 166,544
306-9	Delayed progressor infected at birth; Italy	B	25,50	100	s.c.	CAF01	8.5 / 15.8 / — / 24.6	250 / 31 / 250 / 31	57,248 / 130,704 / 41,785 / 233,153
136-3	Slow progressor infected at birth; Italy	B	25,33	100	s.c.	CAF01	16.1 / 17.6 / 22.1 / 11.9	250 / 250 / 221 / 75	175,099 / 223,899 / 196,754 / 162,887
ITM1_4	Long-term survivor infected at birth; Rwanda	A	25	100	s.c.	CAF01	12.3 / 16.7 / 8.5 / 21.5	31 / 35 / 36 / 31	276,305 / 54,057 / 71,645 / 208,395

(continued)

TABLE 1. (CONTINUED)

Immunogen	Immunogen subtype	Patient information[a]	References	Dose (mg)	Administration	Adjuvant	ADCC[b]	Neutralization[c]	IgG titer[d]
ITM1_4	A	Long-term survivor infected at birth; Rwanda	25	100	s.c.	—	—	250	13,087
							—	146	30,824
							—	113	24,837
							—	250	16,484
ITM1_anc	A	Predicted ancestral sequence to ITM1_4	25	100	s.c.	CAF01	14.2	250	126,204
							—	48	148,287
							15.0	55	224,523
							7.5	250	159,778
94UG018	A	Asymptomatic pregnant woman; Uganda	26	100	s.c.	CAF01	9.0	89	308,206
							—	63	226,454
							—	105	336,211
							—	63	481,614
gp140 trimer-VLP									
VLP-gp140-Bx08SOSIP	B	Primary, CCR5-tropic virus isolated early after infection	27,37,51	20	s.c	gag-VLP/CAF01	—	ND	96,589
							—	ND	40,748
							—	ND	37,331
							—	ND	40,972
VLP-gp140-Bx08	B	Primary, CCR5-tropic virus isolated early after infection	27,37,51	20	s.c.	gag-VLP/CAF01	6.6	ND	25,191
							—	ND	40,498
							—	ND	42,819
							—	ND	14,987
VLP-ACS19642	B	Long-term nonprogressor, sensitive to autologous neutralization; the Netherlands	30	20	s.c.	gag-VLP/CAF01	—	ND	44,697
							—	ND	53,662
							—	ND	46,211
							—	ND	35,676
VLP-ACS19554	B	Progressor, sensitive to autologous neutralization; the Netherlands	30	20	s.c.	gag-VLP/CAF01	—	ND	39,550
							—	ND	16,596
							—	ND	57,848
							—	ND	30,008
gp120 V1–V2 mut of US92077	B	From Dr. Mauro Malnati		20	s.c.	gag-VLP/CAF01	—	ND	176,581
							—	250	27,251
							—	250	93,731
							—	250	70,950
94UG018	A	Asymptomatic pregnant woman; Uganda	26	20	s.c.	gag-VLP/CAF01	—	250	32,435
							—	250	37,045
							—	250	105,697
							—	250	153,068

(continued)

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TABLE 1. (CONTINUED)

Immunogen	Patient information[a]	Immunogen subtype	References	Dose (mg)	Administration	Adjuvant	ADCC[b]	Neutralization[c]	IgG titer[d]
ITM1_4	Long-term survivor infected at birth; Rwanda	A	25	200	s.c.	—	— — — —	250 139 250 250	10,494 5,879 8,292 12,938
94UG018	Asymptomatic pregnant woman; Uganda	A	26	20	s.c.	CAF01	7.5 14.5 6.8 6.5	ND ND ND ND	121,961 72,595 87,792 62,523
DNA prime+protein boost									
SOSIP SSImix gp140+Bx08 gp140 trimer	SSImix, mix of three plasmids encoding gp140 from BX08 and two Danish patients, ctl21 and ctl27, displaying neutralizing activity; Bx08, primary, CCR5-tropic virus isolated early after infection	B + B	25,27,49	200/20	i.d.+e.p.+s.c.		— 6.8 10.9 —	129 61 31 31	116,137 87,842 39,187 26,567
SSImix+VLP ITM4	SSImix, mix of three plasmids encoding gp140 from BX08 and two Danish patients, ctl21 and ctl27, displaying neutralizing activity; ITM4, African patient having 4E10-like antibodies	B + A	27,52	200/100	i.d.+e.p.+s.c.	CAF01	— 10.3 7.3 10.07 —	250 250 110 246	441,353 414,591 552,938 575,601
Bx08 gp140+Bx08 gp140 trimer	Primary, CCR5-tropic virus isolated early after infection	B + B	25,27,37,49	200/100	i.d.+e.p.+s.c.	CAF01	9.5 10.9 21.8 18.6	31 31 87 31	424,069 503,154 405,813 974,583
Bx08 gp150+Bx08 gp140 trimer	Primary, CCR5-tropic virus isolated early after infection	B + B	25,27,37,49	200/100	i.d.+e.p.+s.c.	CAF01	22.3 32.3 38.6 31.8	160 31 250 31	308,679 781,114 648,940 1,039,427

(continued)

TABLE 1. (CONTINUED)

Immunogen	Patient information[a]	References	Immunogen subtype	Dose (mg)	Administration	Adjuvant	ADCC[b]		Neutralization[c]	IgG titer[d]
SSImix+ITM1_4	SSImix, mix of three plasmids encoding gp140 from BX08 and two Danish patients, ctl21 and ctl27, displaying neutralizing activity; ITM1_4, long-term survivor infected at birth; Rwanda	25,27	B+A	200/100	i.d.+e.p.+s.c.	CAF01	8.1	8.4	85	407,974
							—	—	66	269,686
							7.3	—	92	575,932
							—	—	31	1,058,083
NIBSC 40-9 gp150+trimer NIBSC 40-9	Isolated from Ugandan patient with high neutralization titers	38	A+A	200/100	i.d.+e.p.+s.c.	CAF01	17.9		31	182,132
							16.8		31	503,822
							20.9		31	265,230
							14.3		42	152,739
ITM-1_4 gp150+trimer ITM1-4	Long-term survivor infected at birth; Rwanda	25	A+A	200/100	i.d.+e.p.+s.c.	CAF01	8.3		34	556,833
							9.5		98	885,290
							5.7		31	1,507,922
							7.6		79	560,139

[a]Patient from where the immunogen was derived.

[b]ADCC for each animal, defined as the highest percentage of granzyme B activity after background subtraction (i.e., preimmunization sera tested at the same dilution). The immunogens were derived from individuals infected with HIV-1 subtypes A or subtype B, and the screening of ADCC activity was performed with a matching recombinant subtype A (UG37) and subtype B (BaL) envelope protein. Two groups were immunized with a combination of subtype B and subtype A derived immunogens, there ADCC against subtype B (BaL, *left column*), as well as subtype A (UG37, *right column*), is shown. Values below the threshold for positivity, that is, above 6.2% granzyme B, are not shown.

[c]Neutralization against SF162. IC50 IgG, µg/ml.

[d]Anti-rgp120IIIb IgG titer.

ADCC, antibody-dependent cellular cytotoxicity; CAF01, cationic adjuvant formulation 1; CAF02, cationic adjuvant formulation 2; e.p., electroporation; i.d., intradermally; int., intensive prime; ND, not done; s.c., subcutaneously; SSI, Statens Serum Institut.

Statistical analysis

The data analysis was performed using GraphPad Prism 6 software version 6.0c (GraphPad Software, Inc., La Jolla, CA). Spearman's two-tailed test was used to test for correlations.

Results

ADCC activity in rabbits immunized with HIV-1 envelope immunogens

Thirty-five groups of immunized rabbits were screened for serum ADCC activity. The HIV-1 immunogens are listed in Table 1 and include envelope DNA, protein (gp120 monomer, gp140 trimer, or gp140 expressed by virus-like particles), or a combination of DNA and protein (DNA prime followed by protein boost). The immunogens were derived from individuals infected with HIV-1 subtypes A or subtype B, and the screening of ADCC activity was performed with a matching recombinant subtype A (UG37) and subtype B (BaL) envelope protein. ADCC activity was determined 2 weeks after the last immunization, that is, week 14 or 16, and a preimmunization serum from the same animal was used as background.

We assessed the ADCC-mediating activity in rabbit sera in three different ways: the highest percentage of Granzyme B activity, the magnitude of ADCC activity (by calculating the AUC), and by calculating the last dilution with ADCC activity above the threshold of 6.2%. All three analytic methods correlated well with each other ($p < .0001$, Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/aid). Hereafter, the data are shown as the highest percentage of granzyme B activity.

In 19 out of the 35 groups, ADCC activity was detected in at least 1 animal (Table 1). In eight groups, ADCC activity was detected in all animals; in three groups, ADCC was observed in three out of four animals; in four groups, ADCC was observed in two out of four animals; and in four groups, ADCC was observed in only one animal. Although the experiments were not designed to compare the same immunogen in different forms and/or immunization regimens, gp140 trimer and DNA prime plus protein boost appeared to be the most potent inducers of ADCC-mediating antibodies, with 53% and 75% of the rabbits, respectively, having induced ADCC-mediating antibodies (Fig. 1).

Correlation among ADCC activity, neutralization capacity, and IgG binding titers

To determine if the ADCC activity in the sera from the immunized rabbits correlated with the neutralizing activity, we compared the ADCC results with the neutralization capacity against the HIV-1 subtype B strain SF162. Neutralization was measured in 22 out of the 35 groups of rabbits using purified IgG in a pseudovirus-TZMbl assay (part of the data previously published in Heyndrickx *et al.*,[25] Visciano *et al.*,[26] and Borggren *et al.*[27]). As the neutralization was performed using a subtype B strain, we correlated neutralization with ADCC in animals immunized with subtype B-derived immunogens. High ADCC activity did not necessarily result in high neutralization capacity, and vice versa. Nevertheless, when including all available data, there was a correlation between ADCC and neutralization capacity against SF162 ($p = .0332$, $r = -0.3113$) (Fig. 2A and Table 1).
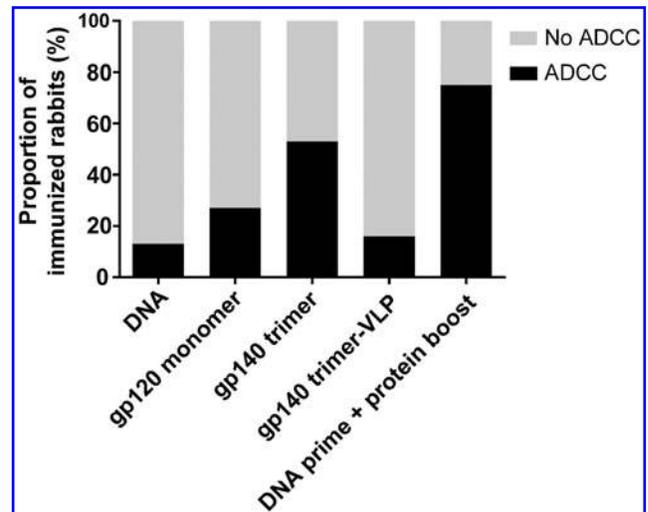

FIG. 1. Trimeric proteins with or without DNA prime were the most potent inducers of ADCC-mediating antibodies. Thirty-five groups of immunized rabbits were screened for serum ADCC activity. In this study the data are grouped according to the type of immunogen: envelope DNA, gp120 monomer, gp140 trimer, gp140 expressed by virus-like particles, or DNA prime followed by protein boost (details described in Table 1). The immunogens were derived from individuals infected with HIV-1 subtype A or subtype B, and the screening of ADCC activity was performed with a matching recombinant subtype A (UG37) and subtype B (BaL) envelope protein. ADCC activity was determined 2 weeks after the last immunization, that is, week 14 or 16, and a preimmunization serum from the same animal was used as background. The proportion of animals in which an ADCC response was detected (*black bar*) or was not detected (*gray bar*). ADCC, antibody-dependent cellular cytotoxicity.

Similarly, we correlated ADCC activity with subtype B HIV-1 envelope (IIIB)-specific IgG titers, as measured by ELISA (Fig. 2B and Table 1) (part of the data previously published in Heyndrickx *et al.*,[25] Visciano *et al.*,[26] and Borggren *et al.*[27]). We correlated ADCC activity with the ELISA IgG titers in the animals immunized with subtype B-derived immunogens. There was a significant correlation between ADCC activity and IgG titers ($p < .0001$, $r = 0.4238$). This correlation was partly driven by the fact that if there was little or no HIV-1 envelope-specific IgG present in sera, there was also no ADCC activity. Nevertheless, even when selecting only the animals with ADCC activity above the threshold for positivity (above 6.2% granzyme B), a correlation between ADCC activity and IgG titer was still observed ($p = .0136$, $r = 0.4319$).

The ADCC responses in immunized rabbits were cross-reactive between different subtypes of HIV-1

A desired feature of an effective HIV-1 vaccine is breadth, that is, the induction of an immune response that is cross-reactive between different strains and subtypes. We selected the seven groups of rabbits with the highest of ADCC response against subtype matched HIV-1 envelope and tested cross-reactivity to other HIV-1 subtypes. Four of these groups were immunized with protein, including the following: (I) trimeric
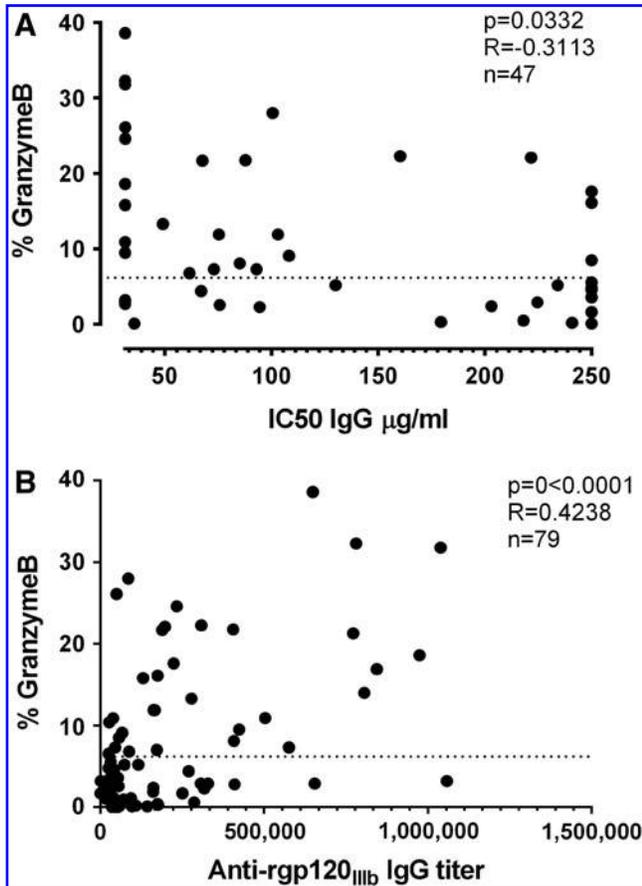
FIG. 2. ADCC activity versus neutralization and binding titers. **(A)** ADCC activity (percent granzyme B) compared with the neutralization capacity against the HIV-1 subtype B strain SF162. Neutralization was measured in 22 out of the 35 groups of rabbits using purified IgG in a pseudovirus-TZMbl assay and showed as the half maximal inhibitory concentration (IC50) $\mu g/ml$ (part of the data previously published in Heyndrickx et al.,[25] Visciano et al.,[26] and Borggren et al.[27]). **(B)** ADCC activity (percent granzyme B) compared with subtype B HIV-1 envelope (IIIB)-specific IgG titers, as measured by ELISA (part of the data previously published in Heyndrickx et al.,[25] Visciano et al.,[26] and Borggren et al.[27]). ADCC activity, neutralization, and IgG titers were determined 2 weeks after the last immunization, that is, week 14 or 16, and a preimmunization serum from the same animal was used as background. *Dotted line* denotes the threshold for ADCC activity (6.2%). ELISA, enzyme-linked immunosorbent assay.

136-3 gp140 in CAF01 adjuvant, (II) monomeric IIIB gp120 in CAF02, (III) trimeric ACS19554 gp140 in CAF01, and (IV) trimeric ITM1_4 gp140 in CAF01 adjuvant. The remaining three groups were immunized with DNA followed by a trimeric protein boost in CAF01 adjuvant: (V) Bx08 gp140 DNA followed by Bx08 gp140 trimer, (VI) Bx08 gp150 DNA followed by Bx08 gp140 trimer, and (VII) NIBSC 40-9 gp150 DNA followed by the corresponding protein (Table 1). These groups were tested for ADCC activity against HIV-1 envelopes from subtype A (UG37), B (BaL), C (ZA1197), and CRF01_AE (CM243). Two groups, monomer IIIB and trimer 136-3, displayed the broadest ADCC response, with cross-
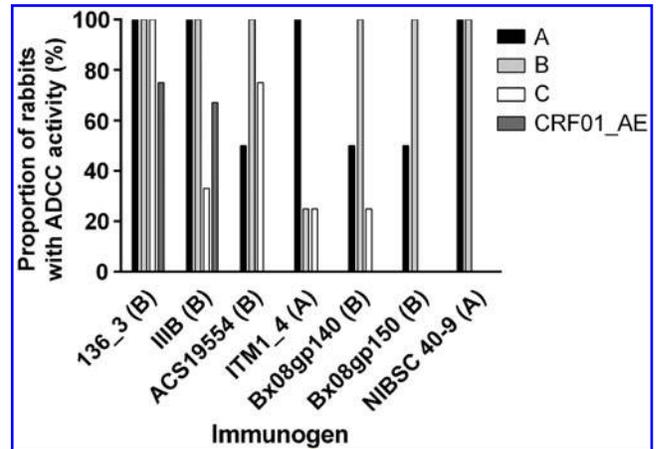
FIG. 3. ADCC activity against different subtypes. The proportion of animals in which an ADCC response was detected. ADCC activity was determined 2 weeks after the last immunization, that is, week 14 or 16, and a pre-immunization serum from the same animal was used as background. Seven groups of rabbits were immunized with trimeric 136_3 gp140, monomeric IIIB gp120, trimeric ACS19554 gp140, trimeric ITM1_4 gp140, trimeric Bx08 gp140 preceded by Bx08 gp150 DNA, trimeric Bx08 gp140 preceded by Bx08 gp140 DNA, and trimeric NIBSC 40-9 gp140 preceded by NIBSC 40-9 gp150 DNA. The sera were tested against envelopes of subtype A (UG37), subtype B (BaL), subtype C (ZA1197MB), and subtype CRF01_AE (CM243). The subtype of the immunogen is written in *parentheses*. CRF, circulating recombinant form.

reactivity between the envelopes of the four different clades tested (Fig. 3), that is, clades A, B, C, and CRF01_AE. Three groups, trimer ACS19554, trimer ITM1_4, and Bx08 gp140 DNA plus protein, induced an ADCC response that was cross-reactive among subtypes A, B, and C. The last two groups, Bx08 gp150 DNA plus protein and NIBSC 40-9 DNA plus protein, induced a narrower response against the matched subtype plus one more (Fig. 3). In all cases, the response was the most robust against the subtype corresponding to the immunogen and the lowest against subtype E.

Discussion

In this study, we identified immunogens that induced anti-HIV-1 antibodies with ADCC activity across different subtypes in a rabbit vaccination model. This is a unique study, not only identifying promising vaccine candidates but also demonstrating that it is possible to use rabbits as a small animal model when screening HIV-1 vaccine candidates for the induction of ADCC-mediating antibodies. The ADCC response was measured in a functional assay measuring the proteolytic activity of granzyme B after its delivery into target cells initiated by antibody recognition. Rabbit sera were tested, using human PBMCs as effector cells and HIV-1 envelope-coated CEM.NKR cells as target cells. It has previously been demonstrated that the results using coated target cells do not differ from when using infected target cells.[31] We confirmed that this was true also when using rabbit serum (data not shown). In this assay, the optimal dilution of rabbit sera for granzyme B activity was lower compared to what is normally observed for human sera in the same assay.[31,32] We

cannot exclude the possibility that this is due to suboptimal recognition of the rabbit IgG by the human Fc receptors. However, we compared the use of human PBMCs with rabbit PBMCs, and this did not improve the outcome but we cannot exclude that this was influenced by the mismatch of combining rabbit effector cells with human target cells (Supplementary Fig. S2).

Most of the immunogens tested in this study originated from clinical HIV-1 isolates that had elicited broadly neutralizing antibodies in HIV-1 infected individuals. However, these individuals have not been screened for ADCC-mediating antibodies.

The seven immunogens inducing the most potent ADCC antibody responses were trimeric 136-3 gp140, monomeric IIIB gp120, trimeric ACS19554 gp140, trimeric ITM1_4 gp140, Bx08 gp140 DNA prime plus protein boost, Bx08 gp150 DNA prime plus protein boost, and NIBSC 40-9 gp150 DNA prime plus protein boost. 136-3 originates from a 3-month-old child infected with subtype B HIV-1 at birth.[33] IIIB is a commonly used laboratory subtype B strain of HIV-1.[34] ACS19554 originates from a progressing HIV-1 subtype B-infected adult 47 months postinfection.[35] ITM1_4 originates from a long-term survivor, an 11-year-old child infected with subtype A HIV-1 at birth.[36] Bx08 is a clade B primary isolate from an HIV-1-infected French individual 8 months post-seroconversion.[25,27,37] NIBSC 40_9 gp150 clade A was constructed from a Ugandan patient serum scoring high in neutralization titers and breadth.[38]

These protein immunogens induced ADCC-mediating antibodies in rabbits when immunized with the adjuvant CAF01 or, in the case of IIIB, CAF02. CAF01 is a synthetic two-component liposomic adjuvant comprising the quaternary dimethyldioctadecylammonium ion (DDA) and trehalose 6,6′-dibehenate (TDB).[28] DDA facilitates antigen adsorption and presentation, while TDB potentiates the immune response through the Mincle receptor.[39] In CAF02, part of DDA was replaced with the neutral lipid of DSPC, which induces a more Th2-prone response.[29] Trimeric ITM1_4 gp140 was also tested without adjuvant, resulting in no measurable ADCC response (Table 1), demonstrating the importance of using a potent adjuvant. Moreover, ACS19554, 136-3, and ITM1_4 were all used as gp140 trimers. In fact, trimeric gp140 proteins in CAF01, with or without a preceding DNA prime, were overall the most compelling inducers of ADCC-mediating antibodies in this model.

However, the experiments were not set up in a way that enabled us to compare the same immunogen as a monomeric protein, a trimeric protein, DNA, virus-like particles, or a DNA prime followed by protein boost regimen. IIIB was immunized as monomeric gp120; however, here, it was used in combination with the adjuvant CAF02. We cannot exclude that the use of a different adjuvant enabled the effective ADCC response. Unfortunately, there was no comparison with the same immunogen using CAF01. The fact that trimeric gp140 is superior to monomeric gp120 with regard to the elicitation of binding titers, neutralizing antibodies, and ADCC-mediating antibodies has also been shown in other studies.[25,40]

Three of the most potent inducers of ADCC-mediating antibodies, Bx08 gp140, ITM1_4 gp140, and NIBSC 40-9 gp150, also induced a high level of neutralizing antibodies against the HIV-1 subtype B strain SF162, as measured in a TZMbl assay using purified IgG.[25] Moreover, we observed an overall correlation between ADCC and neutralizing capacity when including all available data points from subtype B-derived immunogens. Nevertheless, this correlation was imperfect, and there were several instances in which the neutralization and ADCC response did not correspond with each other. The V1 V2 region of gp120 have previously been shown to be important for neutralization of SF162[41]; the overall correlation we see when comparing naturalization and ADCC may therefore imply that V1 V2 specific antibodies may be important also for ADCC. There was also a general correlation between ADCC and HIV-1 envelope-specific IgG. A correlation between ADCC and neutralization has been observed in recent studies,[24,42] whereas earlier studies have shown the opposite, that is, a lack of correlation among neutralization, binding titers, and ADCC.[43–46] This could suggest an incomplete overlap in the specificities of antibodies that mediate ADCC and neutralization.

Certain IgG isotypes favor ADCC (e.g., IgG1 and IgG3). The relative distribution of IgG isotypes in sera with same IgG antibody titer may be manipulated by different adjuvants and modes of immunization, therefore influencing the ADCC measured. Because rabbits only possess one IgG isotype, this is not an issue in the rabbit model.

Antibody responses cross-reactive between different HIV-1 subtypes and strains are essential for an effective vaccine. With this in mind, we tested the seven groups displaying the highest ADCC intra-subtype responses for cross-reactivity to the three other subtypes. The responses were highest against the subtype corresponding to the immunogen. Similarly, in a recent study of a primarily subtype B-infected cohort, ADCC responses were highest against subtype B.[14,47] The monomeric IIIB gp120 and trimeric 136-3 gp140 immunogens displayed the broadest ADCC response, with cross-reactivity to all of the four different clades tested. Three others, ACS19554, ITM-4, and Bx08 gp140, induced ADCC-mediating antibodies that were cross-reactive between three different clades, as well as neutralizing antibodies.

In conclusion, we present a rabbit vaccination model suitable for screening HIV-1 vaccine candidates for the induction of ADCC-mediating antibodies, as well as neutralizing antibodies. The immunogen inducing the most potent and broadest ADCC response was trimeric 136-3 gp140. Although we were able to identify vaccine candidates concurrently inducing both types of responses, ADCC-mediating antibodies did not necessarily correlate with neutralizing antibodies, highlighting the importance of using an assay measuring the desired feature.

Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Ingrid Karlsson
Department of Virology and Special Microbial Diagnostic
Statens Serum Institut
5 Artillerivej
2300 Copenhagen
Denmark

E-mail: iks@ssi.dk

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