Serum Immunoglobulin A Cross-Strain Blockade of Human Noroviruses

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Background. Human noroviruses are the leading cause of acute viral gastroenteritis, justifying vaccine development despite a limited understanding of strain immunity. After genogroup I (GI).1 norovirus infection and immunization, blockade antibody titers to multiple virus-like particles (VLPs) increase, suggesting that GI cross-protection may occur.

Methods. Immunoglobulin (Ig)A was purified from sera collected from GI.1-infected participants, and potential neutralization activity was measured using a surrogate neutralization assay based on antibody blockade of ligand binding. Human and mouse monoclonal antibodies (mAbs) were produced to multiple GI VLPs to characterize GI epitopes.

Results. Immunoglobulin A purified from day 14 post-GI.1 challenge sera blocked binding of GI.1, GI.3, and GI.4 to carbohydrate ligands. In some subjects, purified IgA preferentially blocked binding of other GI VLPs compared with GI.1, supporting observations that the immune response to GI.1 infection may be influenced by pre-exposure history. For other subjects, IgA equivalently blocked multiple GI VLPs. Only strain-specific mAbs recognized blockade epitopes, whereas strain cross-reactive mAbs recognized nonblockade epitopes.

Conclusions. These studies are the first to describe a functional role for serum IgA in norovirus immunity and the first to characterize human monoclonal antibodies to GI strains, expanding our understanding of norovirus immunobiology.

Keywords. blockade antibody; BOB assay; IgA; monoclonal antibody; neutralization; Norwalk virus; norovirus.

Noroviruses (NoVs) are a leading cause of gastroenteritis outbreaks in all age groups, contributing to an estimated 21 million illnesses per year in the United States [1]. Although genogroup II (GII).4 NoV strains predominate worldwide, a large number of diverse NoV strains cocirculate at endemic levels. Genogroup I (GI) strain infections occur most frequently in children and the elderly

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[2, 3]. Norovirus disease is usually self-limiting in healthy individuals, but it can be severe in the very young, elderly, and immunocompromised [3–5]. A NoV vaccine would benefit these vulnerable groups as well as the military, food-handlers, and support-care workers.

An understanding of the complex antigenic relationship between NoV strains will aid in vaccine development. Currently, a multivalent (GI.1/GII.4C) virus-like particle (VLP) vaccine is in phase I clinical trials [6]. Cross-strain reactive blockade Ab responses, a potential measure of strain cross-neutralization and a correlate to protective immunity in GI.1-challenged participants [7], were identified in serum samples collected during an initial reactogenicity trial of the vaccine [8]. These data support the hypothesis that some similar neutralizing antibody (Ab) epitopes might exist within both GI and GII NoV strains that could potentially provide targets for a broadly protective vaccine.

Likewise, we have previously characterized Ab responses in humans after GI.1 NoV experimental infection [9],

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and we found that GI.1 infection induced blockade Ab crossreactive within a panel of GI VLPs. In this follow-up study using the same serum samples, we extend these observations to identify serum immunoglobulin (Ig)A as a component of the cross-GI blockade Ab response, and we characterize the antigenic relationship between GI VLPs using a panel of monoclonal Abs (mAbs). These mAbs include the first reported human mAbs to GI NoV strains.

MATERIALS AND METHODS

Ethics Statement

А

GI.1 IgA Mean EC₅₀ (1/Serum Dilution)

С

10 000 -

1000

100

10

Seroresponse rate

10 000

1000

Day 0

Day 4

0/8

Day 14

7/9

Day 35

5/8

Serum samples were collected from participants infected with GI.1-1968 in an unpublished pilot study. Participants provided written informed consent, the original study was approved by the Institutional Review Board, and the guidelines for human experimentation were followed in the conduct of clinical research [9].

Human Serum Samples and Genogroup I Virus-Like Particles

The archived, deidentified serum samples collected from GI.1infected participants and GI VLPs used in these studies are described in refs. [9, 10]. Not all archival samples were available for analyses. Any missing samples are noted in the respective figure legends.

Enzyme Immunoassay, Blockade Antibody, and Blocking of **Binding Assays**

Enzyme immunoassay (EIA) and blockade Ab assays were performed as described for GI VLPs [8] with the following exceptions for the blockade assay: (1) $0.5 \,\mu\text{g/mL}$ VLP was used, and (2) the VLP-serum and VLP-porcine gastric mucin binding steps were incubated at 37°C. Blocking of binding (BOB) assay procedures [11] are the same as EIA procedures except that serial dilutions of mouse mAbs were added to GI.1-coated plates before the addition of anti-GI.1 human mAbs at a concentration equal to the EC₅₀ titer (effective concentration at 50%) [8, 11]. All sera and mAbs were assayed in duplicate in a minimum of at least 2 independent assays for each VLP.

Avidity Index Calculation

Day 0

Dav

Day 14

Day 35

В

GI.1 IgA Mean % Avidity

D

100

100

Avidity indexes were determined as described for IgG and IgA EIA as described in ref. [8], with the inclusion of a 10-minute, 7



M urea incubation step after the first wash postprimary Ab incubation [12]. The index was defined as the average ratio of optical density (OD) of Ab bound in the presence of 7 M urea compared with the average OD of Ab bound in the absence of urea at the dilution closest to the Ab EC_{50} . Samples with Ab levels below the limit of detection were assigned an index of 10 for statistical purposes. All sera and mAbs were assayed in duplicate in a minimum of at least 2 independent assays for each VLP.

Immunoglobulin A Purification

Immunoglobulin A was purified from serum samples using Peptide M/Agarose (InvivoGen) following the manufacturer's directions, except samples were diluted 6-fold in phosphatebuffered saline before filtration instead of being dialyzed. Purified IgA samples were confirmed as IgG negative by EIA.

Monoclonal Antibody Production and Purification

Mouse mAbs to GI.1 and GI.4 and human mAbs reactive to GI VLPs were isolated, produced, and purified as reported previously [13, 14].

Statistical Analysis

Statistical analyses were done using GraphPad Prism 6.02. EC_{50} values or geometric mean titers (GMTs) between days postchallenge, or VLPs were compared using the one-way analysis of variance (ANOVA) with Dunnett posttest (ANOVA), when at least 3 values were compared or a Wilcoxon test (Wilcoxon) when only 2 values were compared. A difference was considered significant if the *P* value was < .05.

RESULTS

Genogroup I .1 Infection Induces Homotypic Immunoglobulin A and Immunoglobulin G With High Titer and Increased Avidity

Genogroup I.1 infection results in significant rises in serum IgG titer to GI.1 at day 14 postchallenge [9]. To further characterize this serum response, we compared GI.1-reactive IgA and IgG titers and avidities at days 0, 4, 14, and 35. Avidity was measured as an indicator of the degree of strain-specific somatic hypermutation. Immunoglobulin A GMTs at day 0 and day 4 were below the assay limit of detection. Immunoglobulin A titers peaked at day 14 and were significantly lower at day 35 (Figure 1A) (Wilcoxon, P = .004). Immunoglobulin A avidity GMTs were corresponding low (<30%) at day 0 and day 4, peaked at day 14 (58%), and remained elevated at day 35 (44%) (Figure 1B). Immunoglobulin G GMTs were low at day 0 and 4, peaked at day 14, and remained elevated at day 35 (Figure 1C). Immunoglobulin G avidity GMTs were low at days 0 and 4 (<30%) and high at days 14 (76%) and 35 (69%) (Figure 1D). Compared with IgG, IgA titers were 3.2-fold lower at day 35 and avidities were significantly lower at days 14 (Wilcoxon, P = .01) and 35 (Wilcoxon, P = .004), indicating that the GI.1-induced IgA Ab

titers wane more quickly and are less affinity matured compared with IgG Abs.

Serum Immunoglobulin A Has Cross-Genogroup I Virus-Like Particle Blockade Activity

The ability of serum to block binding of NoV VLPs to carbohydrate ligands is used as a surrogate neutralization assay in the absence of a validated NoV cell culture system [7, 8]. We have previously shown that the day 14 sera from G1.1-infected subjects block binding of GI.1, GI.2, GI.3, and GI.4 VLPs to ligand [9]. To evaluate the role IgA plays in this broadly blocking activity, we purified IgA from day 14 sera and measured the IgA blockade Ab titer against GI VLPs. Eight participants had IgA titers high enough to facilitate IgA purification. As reported for unfractionated sera, purified IgA blocked GI.1, GI.3, and GI.4 (Figure 2). All IgA samples blocked binding of more than 1 GI VLP (Table 1). In 4 of these 8 (50%), IgA blockade potency was highest for GI.1. Of note, IgA from subject 3 blocked binding of all 3 GI VLPs similarly. Like unfractionated sera, sera depleted of IgA retained broad-GI blockade Ab function, supporting a role for other Ab isotypes, in addition to IgA, in sera blockade activity (Table 1).

Genogroup I Virus-Like Particles Share Common Antibody Epitopes

Immunoglobulin A and sera assays suggest that GI strains may share cross-reactive Ab epitopes, possibly as a result of multiple infections. Currently, little is known about GI Ab epitopes and



Figure 2. Day 14 immunoglobulin (Ig)A blocks multiple genogroup I (GI) virus-like particles (VLPS) from binding ligand. Immunoglobulin A was affinity purified from day 14 serum samples of subjects infected with GI.1 and tested for blockade potential against GI.1, GI.3, and GI.4 VLPs and mean EC_{50} titers calculated. Each color of circle represents the mean EC_{50} for a participant. The group geometric mean titer (line) and 95% confidence intervals (error bars) for each VLP are also reported. Samples with titers above the limit of detection (dashed line) were assigned the value of 32 for statistical analysis. Purified IgA was not recovered from serum from participant 1 and 7, each of which had GI.1-reactive IgA titers <200 µg/mL. *Mean EC_{50} blockade titer is significantly different from GI.1 (analysis of variance).

Subject	VLP	Serum EC ₅₀ ^{a,b}	Serum-IgA EC ₅₀ ^{a,b}	Purified IgA EC ₅₀ ^{b,c,d}
2	GI.1	308.50 (224.20–424.40)	103.40 (74.79–142.90)	7.31 (5.91–9.04)
	GI.3	94.72 (68.63–130.700)	40.56 (35.45–46.40)	32.00 (32.00–32.00)
	GI.4	2417.00 (2067.00–2827.00)	410.40 (467.90–359.90)	2.53 (2.31–2.78)
3	GI.1	1271.00 (955.00–1691.00)	257.20 (192.20–344.20)	4.46 (3.76–5.30)
	GI.3	1404.00 (1202.00–1639.00)	340.10 (302.30–382.60)	4.54 (4.11–5.03)
	GI.4	1148.00 (644.60–2043.00)	515.00 (613.60–432.10)	4.40 (4.06-4.77)
4	GI.1	430.50 (318.80–581.50)	71.20 (53.88–94.09)	5.05 (4.12-6.19)
	GI.3	193.20 (171.80–217.30)	83.62 (71.08–98.38)	10.01 (8.92–11.31)
	GI.4	575.10 (530.80–623.10)	75.49 (62.97–90.51)	8.40 (7.69–9.20)
6	GI.1	845.80 (770.60–928.50)	174.50 (147.10–207.10)	1.86 (1.69–2.05)
	GI.3	109.80 (92.68–130.20)	21.60 (16.65–28.03)	1.00 (0.90–1.11)
	GI.4	847.60 (688.60–1043.00)	381.90 (334.70–435.70)	6.20 (5.16–7.46)
7	GI.1	662.80 (502.30-874.60)	32.05 (16.01–64.15)	3.20 (2.90–3.54)
	GI.3	141.40 (122.50–163.10)	55.43 (47.71–64.40)	4.75 (4.46–5.10)
	GI.4	2313.00 (2068.00–2587.00)	315.60 (245.50–405.70)	1.16 (1.05–1.28)
8	GI.1	278.80 (220.40-352.60)	149.90 (111.70–201.20)	3.95 (3.51–4.44)
	GI.3	276.20 (225.20–338.70)	79.41 (64.31–98.06)	9.61 (7.90–11.68)
	GI.4	302.80 (190.80-480.30)	98.00 (57.19–167.90)	6.81 (5.41–8.57)
9	GI.1	342.50 (263.80-444.90)	134.00 (178.70–100.50)	5.45 (4.79–6.19)
	GI.3	270.10 (234.90-310.50)	100.10 (82.32–121.80)	12.03 (10.72–13.50)
	GI.4	593.10 (490.70–716.90)	65.85 (34.43–125.90)	8.17 (7.10–9.42)
10	GI.1	3802.00 (3097.00-4668.00)	631.40 (462.80-861.30)	0.48 (0.42-0.54)
	GI.3	5334.00 (4428.00-6424.00)	1095.00 (994.3–1206)	0.96 (0.90–1.02)
	GI.4	12095.00 (10066.00-14533.00)	5331.00 (4554.00–6241.00)	0.93 (0.79–1.09)

Table 1. Genogroup I Virus-Like Particle Blockade by Day 14 Serum Unfractionated, Serum Depleted of IgA and Purified IgA

Abbreviations: Ab, antibody; ANOVA, analysis of variance; GI, genogroup I; Ig, immunoglobulin; VLP, virus-like particle.

^a Mean blockade Ab EC₅₀ 1/serum dilution (95% confidence interval).

^b VLP mean EC₅₀ values significantly different from GI.1 mean EC₅₀ values are bolded (ANOVA).

 $^{\rm c}$ Mean blockade IgA EC_{50} $\mu g/mL$ (95% confidence interval).

^d Samples with titers above the limit of detection were assigned the value of 32 for statistical analysis.

no GI blockade epitopes have been reported. Therefore, we isolated a panel of mouse and human mAbs to directly determine whether GI strains share common epitopes or whether GI.1 infection activates multiple strain-specific Ab responses as has been reported for GII.4 strains postvaccination (Table 2) [8]. Two of 5 mouse mAbs to GI.1 were strain-specific, whereas 3 recognize multiple GI VLPs by EIA (Figure 3A). Contrary to polyclonal Ab responses post-GI.1 infection, all 5 mAbs preferentially recognize GI.1. None of the mouse GI.1 mAbs block binding of GI.1 VLP to carbohydrate ligand (Figure 3B). In comparison, all 4 mouse mAbs to GI.4 were strain-specific to GI.4 (Figure 3C) and effectively blocked binding of GI.4 to carbohydrate ligand (Figure 3D). These Abs are the first reported mAbs with GI.4 blockade activity.

Norovirus seropositivity approaches 100% in adults, and preexposure history may shape the Ab response to NoV. Therefore, we prepared GI-reactive mAbs from blood-banked human peripheral blood mononuclear cells [14] and tested for cross-GI reactive epitopes. Of the 4 human mAbs tested, 2 reacted with more than 1 GI VLP, 1 reacted with GI.1, and another reacted with GI.3 (Figure 4A). Neither of the cross-reactive mAbs blocked binding of any tested GI VLP to ligand (Figure 4B). However, the mAbs that only recognized GI.1 or GI.3 both blocked binding of the VLPs to ligand. These mAbs are the first reported human mAbs to GI strains and demonstrate the existence of both cross-GI reactive Ab epitopes and strain-specific GI blockade epitopes.

Previous studies with GII.4 NoVs have identified strainspecific epitopes on the surface of the viral particle. Using the paired human and mouse mAbs to GI.1 and an Ab binding competition assay (BOB assay) [11] allowed us to gage the relative spatial arrangement of the GI.1 blockade epitope recognized by human mAb NVB 106 to the nonblockade epitopes recognized by the mouse GI.1 mAbs. None of the mouse GI.1 mAbs were able to block binding of NVB 106 in the BOB assay, suggesting that the GI.1 blockade epitope is spatially distinct from the nonblockade epitopes (Figure 5). The GI.1E (mouse GI cross-reactive mAb) blocked binding of the human GI

Table 2. Characteristics of Anti-Genogroup I Norovirus Monoclonal Antibodies

mAb	Clone Name	Species	lsotype	Immunogen	%Avidity ^a (VLP)	Blockade
GI.1A	68.21.17	Mouse	lgG _{2a} k	GI.1	50 (Gl.1)	-
GI.1B	140.6.12	Mouse	lgG _{2a} k	GI.1	66 (GI.1)	_
					10 (GI.3)	_
GI.1C	140.42.8	Mouse	lgM	GI.1	62 (GI.1)	_
					10 (GI.3)	_
GI.1D	300.1.28	Mouse	lgG _{2a} k	GI.1	66 (GI.1)	_
					41 (GI.3)	_
					10 (G1.4)	_
GI.1E	300.4.2	Mouse	lgG _{2a} k	GI.1	66 (GI.1)	_
GI.4A	4A2.6.4	Mouse	lgG _{2b} k	GI.4	41 (GI.4)	GI.4
GI.4B	4A2.14.13	Mouse	lgG _{2b} k	GI.4	84 (GI.4)	GI.4
GI.4C	4B4.2.7	Mouse	lgG _{2a} k	GI.4	78 (GI.4)	GI.4
GI.4D	8A6.16.11	Mouse	lgG _{2b} k	GI.4	84 (GI.4)	GI.4
NVF 144	144	Human	lgG1	Natural Infection	86 (GI.3)	GI.3
NVB 106	106	Human	lgG ₁	Natural Infection	98 (GI.1)	GI.1
NVB 84	84	Human	lgG ₁	Natural Infection	86 (GI.3)	_
					72 (GI.4)	_
NVE 3	E3	Human	lgG ₁	Natural Infection	100 (GI.1)	_
					33 (GI.3)	-
					10 (GI.4)	-

Abbreviations: EIA, enzyme immunoassays; GI, genogroup I; Ig, immunoglobulin; mAb, monoclonal antibody; VLP, virus-like particle. ^a Avidity was only determined for VLPs with positive EIA results (see Figures 3 and 4).



Figure 3. Reactivity of mouse anti-genogroup I (GI) monoclonal antibodies. Mouse monoclonal antibodies against GI.1 (A and B) or GI.4 virus-like particles (VLPs) (C and D) were assayed for enzyme immunoassay (EIA) reactivity (A and C) and blockade activity (B and D) for GI.1, GI.3, and GI.4 VLPs, and mean EC_{50} titers were determined. Antibodies with EIA or blockade titer above the limit of detection (dashed line) were assigned values of 4 µg/mL or 16 µg/mL, respectively, for statistical analysis. *Mean EC_{50} significantly different from the immunizing strain VLP (analysis of variance).



Figure 4. Reactivity of human anti-genogroup I (GI) monoclonal antibodies (Abs). Human monoclonal Abs reactive with GI virus-like particles (VLPs) were assayed for enzyme immunoassay (EIA) reactivity (A) and blockade activity (B) for GI.1, GI.3, and GI.4 VLPs, and mean EC_{50} titers were determined. Antibodies with EIA or blockade titer above the limit of detection (dashed line) were assigned values of 4 µg/mL or 16 µg/mL, respectively, for statistical analysis. *Mean EC_{50} titer significantly different from GI.3 titer (analysis of variance).

cross-reactive mAb (NVE 3), indicating that the mouse mAbs generated to VLPs recognize relevant epitopes that are targeted during human infection. Pooled mouse mAbs did not block binding of NVB 106 and blocked NVE 3 less well than GI.1E alone, indicating that BOB activity may be related to epitope distance and frequency and not to steric hindrance from epitope saturation.

DISCUSSION

Noroviruses are characterized by extensive antigenic diversity among the GI and GII genotypes. Identification of type-specific and cross-genotype epitopes will provide epitope-defined diagnostic assays that will facilitate development of a broadly protective NoV vaccine and an immune therapeutic. To begin to understand the origins of cross-reactive Ab responses, we analyzed IgA and IgG serum titers and avidities in samples that present cross-reactive GI Abs at day 14 post-GI.1 infection [9]. The GI.1-specific IgG and IgA titers and avidities remained consistent at day 4 postchallenge and peaked at day 14. Compared with IgG, IgA titer and avidity waned more quickly, possibly reflecting the homing of IgA Ab-secreting cells from the peripheral blood to mucosal sites [15]. Prechallenge IgG and IgA titers with avidities above 40% were detected in 2 of the 10 (20%) participants who became infected with GI.1.



Figure 5. Genogroup (GI).1 blockade epitope is structurally distinct from GI.1 nonblockade epitopes. Mouse monoclonal antibodies (mAbs) to GI.1 nonblockade epitopes (mAbs GI.1A, B, D, E) were measured for ability to block GI.1 virus-like particle binding of human mAbs to a strain-specific blockade epitope (black circle, NVB106) or a conserved nonblockade epitope (gray circle, NVE 3) in an epitope-specific blocking-of-binding (BOB) assay. Mouse antibodies unable to block human antibody binding at titers within the limit of detection (dashed line) were assigned values of 16 μ g/mL for statistical analysis. *Mean BOB EC₅₀ titer significantly different from GI.1E for the same human antibody (Wilcoxon). Pool: 2 μ g/mL each GI.1A, B, D, and E.

Furthermore, high avidity was not predictive of blockade function among mAbs. Although the design of this study does not allow prediction of correlates of protective immunity, together, these data suggest that the location of Ab binding may be more important in determining protection from infection than Ab titer or avidity.

A functional role for serum IgG in NoV immunity has been established by demonstrating that human IgG mAbs have blockade activity [14, 16] and that IgG memory B-cell titers correlate with protection from GI.1 infection [17]. Mucosal IgA titers, as measured in saliva, have also been shown to correlate with protection from GI.1 infection [17, 18]. These studies are the first to assess the blockade activity of IgA. To distinguish IgA blockade potency from IgG, IgA was purified from the day 14 serum samples with total GI.1-reactive IgA titer greater than 200 µg/mL. All 8 IgA preparations had cross-GI blockade activity, identifying IgA as a component of the serological blockade Ab response to GI NoVs. Supporting studies with polymeric monoclonal IgA indicating that IgA is strain crossreactive [19], purified IgA did not preferentially block ligand binding of GI.1 compared with other GI VLPs. The IgA response may have been generated to cross-reactive epitopes in response to either the current GI.1 infection or an earlier GI exposure. Supporting this hypothesis, serum and serum depleted of IgA from multiple participants, and IgA from one participant blocked the 3 GI VLPs equivalently, indicating that conserved GI blockade epitopes exist or that infection with 1 strain elicits pre-exposure memory responses against other closely related GI strains. More importantly, this finding was observed using antigen-limiting conditions, designed to emphasize strain antigenic differences, as opposed to our previous analysis of these samples in which assays were optimized for sensitivity resulting in no group difference in serum IgG or blockade Ab titers between GI VLPs [8, 9]. To clearly define Ab epitopes, large panels of human mAb are needed. The data presented here suggest that clone selection processes should include IgA as well as IgG isotypes.

Mouse mAbs to GI VLPs have been described by others [20, 21], but none have identified GI blockade Ab potency or epitopes, contrary to GII.4 NoVs [14, 22]. Therefore, we developed a panel of GI mAbs to evaluate the antigenic relationship between GI VLPs. Unlike GI.1 infection in humans, GI.1 or GI.4 immunization of mice resulted in Abs that preferentially recognize the immunizing strain, again suggesting that pre-exposure history may shape the GI Ab response in humans. Six of the GI Abs appear to have type-specific blockade activity against the panel of GI VLPs tested because all recognized only 1 GI VLP. However, we cannot definitively infer from these data that blockade epitopes are strain-specific, because representatives of each of the GI genotypes were not tested. The remaining 7 mAbs recognized nonblockade epitopes. In particular, none of the 5 mouse GI.1 mAbs had blockade activity. Although atypical in our experience with NoV mAbs, it is unlikely that the lack of blockade potency is related to immunization or hybridoma production protocols because the mouse GI.1 mAbs were developed simultaneously with the mouse GI.4 mAbs and all of the GI.4 mAbs recognize a blockade epitope. Antibodies can confer protection from infection by additional means besides blocking of ligand interaction, including complement lysis, opsonization, and Ab-dependent cell cytolysis; therefore, a nonblocking phenotype does not decrease the biological significance of these Abs.

CONCLUSIONS

Most cross-reactive mouse and human GI mAbs had a higher avidity to a single GI VLP and less avidity to other GI VLPs, supporting the hypothesis that strain cross-reactive Abs are generated to sequences in a single strain that are conserved within other GI VLPs, although to varying degrees of homology. Structural studies of Ab-bound VLPs and bioinformatics and genetic tools to create VLPs with altered predicted Ab epitopes combined with a large panel of mAbs may define GI strain-specific and strain cross-reactive epitopes that can be exploited as diagnostic tools to measure epitope-specific responses and to refine the design of a broadly protective NoV vaccine. Additional human challenge studies are critical for creating mAb panels, mapping relevant epitopes, and defining correlates of protective immunity.

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Potential conflicts of interest. A. L. is the scientific founder and shareholder of Humabs BioMed SA. D. C. and M. B. are employees of Humabs Biomed, a company that develops anti-invectives human monoclonal antibodies.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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