

Supplementary Materials

Figure S1. Alignment of sequences corresponding to potential binders of 1C10 CDRs. **Ratio*** indicates the ratio of Abs_{450nm} obtained by testing in ELISA the clones against the target 1C10 and the depletion 2B7 constructs, respectively. ELISA was performed in triplicates. Unique sequences (27) with a ratio value > 5 were aligned and conserved sequences grouped into clusters. Amino acids corresponding to CDR3 have been highlighted. The best representatives of each cluster, according to the ELISA results, were selected for the successive step together with the unique sequences, for a total of 20 individual clones, highlighted in red.

CLUSTER 1

		Ratio*
D6	MADVQLQASGGGLVQAGGSLRLSCAASGSTFSIDWFGWYRQAPRKQRESVATVSTTGD T (26,6)	
B8	MADVQLQASGGGLVQAGESLRLSCTASESTFSFHPFGWYRQAPGKPRELVATVSRYGDTY (15,4)	
E7	MAEVQLQASGGGLVQAGESLRLSCTASESTFSFHPFGWYRQAPGKPRE*VATVSKYGDTY (16,0)	
A7	MAEVQLQASGGGLVQAGGSLRLSCVSSGSTFSVHHFGWYRQAPGKQRQTVATVTTFGDTY (28,1)	
B5	MAEVQLQASGGGLVQAGGSLRLSCASSGSTFSVHAFGWYRQAPGKQRETVATVTKTGDTY (52,9)	

D6 YADSVNGRFTVSTDNNERSVYLRMDTLHPDDSAVYDCSAGAGRYTDFWGQGTLITVS
B8 YADSVVGRFTISRDDSMSTVYLQMNSLKPEDSAVYYCYAGVAMYLDAWGQGTQVTVS
E7 YADSVVGRFTVSRRDSRSTVDLQMNSLKPEDSAVYYCYAGVAMYLDAWGQGTQVTVS
A7 YADSVKGRFTVSRRDNKSAVYLQMDNLQPEDTAVYYCYIGAACMYDDYCRQRTVITVS
B5 YADSVKGRFTISRDDNKNTVYLQMNNLQPEDTAVYYCYTGAAMYDDYCGQSNLIIVS

CLUSTER 2

B1	MADVQLQASGGGLVHAGGSLRLSCAASGHTLTNAALAWFRQAPGKEREVARITSNRGTT (38,3)	
E6	MADVQLQASGGGLVQAGGSLRLSCAASGRTLTNAALAWFRQAPGKEREVARITSNRGTT	(10,6)
D11	MAEVQLQASGGGLVQAGGSLRLSCAASGRTLTNAALAWFRQAPGKEREVARITSNRGTT (10,6)	
H7	MAEVQLQASGGGLVQAGGSLRLSCAASGRTLTNAALAWFRQAPGKEREVARITSNRGTT	(9,8)

B1 FYAYSVKDRFTISRDFAKNMVYLLMNSLKfedTAVYYCAAARSLNYDSSDYIFWGQGTQ
E6 FYADSVKDRFTISRDFAKNMVYLMQNSLKFEDTAVYYCAAARSLNYDSSDYIFWGQGT
D11 FYADSVKDRFTISRDFAKNMVYLMQNSLKFEDTAVYYCAAARSLNYDSSDYIFWGQGT
H7 FYADSVKDRFTISRDFAKNMVYLMQNSLKFEDTAVYYCAAARSLNYDSSDYIFWGQGTQ

B1 VTVS
E6 VTVS
D11 VTVS
H7 VTVS

CLUSTER 3

D12 MAEVQLQASGGGLVQTGGSLRLSCAASRRTFSASSLAWFRQAPGKEREVVAATSWTEATY
(18,8)
C3 MAEVQLQASGGGLVQTGGSLRLSCAASRRTFSASSLAWFRQAPGKEREVVAATHWTDATN
(14,6)
F1 MAEVQLQASGGGLVQTGGSLRLSCAASRRTFSASSLAWFRQAPGKEREVVAATHWTDATN
(7,9)
H3 MADVQLQASGGGLVQTGGSLRLSCAASRRTFSASSLAWFRQAPGKEREVVAATHWTDATN
(6,2)

D12 YANSAKGRFTISRDNAKSTVNLQMNSLQPEDTAVYYCAARQTGPYNLPASWNHWGQGTLV
C3 YANSAKGRFTISRDNAKSTVYLQMNSLQPEDTAVYYCAARQVGPYLPASWNHWGQGTQV
F1 YANSAKGRFTISRDNAKSTVYLQMNSLQPEDTAVYYCAARQVGPYLPASWNHWGQGTQV
H3 YANSAKGRFTISRDNAKSTVYLQMNSLQPEDTAVYYCAARQVGPYLPASWNHWGQGTQV

D12 TVS
C3 TVS
F1 TVS
H3 TVS

CLUSTER 4

C7	MAEVQLQASGGGLVQPGGSLRLSCSGNILSVNIMGWYRQAPGNQRELVAVITSDSTIN (19,1)	
B7	MAEVQLQASGGGLVQPGGSLRLSCSASGNILSINIMGWYRQAPGNQRELVAVITRDGTIN	(7,0)
F10	MADVQLQASGGGLVQPGGSLRLSCSASGNILSINIMGWYRQAPGNQRELVAVITRDGTIN	(6,7)
C7	YADSVKGRFTISKDSDMRTVYLQMDTLEPEDTGYYCYARPWAQTCVWGQRTHIVS	
B7	YADSVKGRFTISKDGDMRTVYLQMDTLEPEDTAVYYCFARPWAQTGVWGQGTLVTVS	
F10	YADSVKGRFTISKDGDMRTVYLQMDTLEPEDTAVYYCFARPWAQTGVWGQGTQVTVS	

UNIQUE SEQUENCES

D9	MADVQLQASGGGLVQTGGSLRLSCAASGRTFSISSLGWFRQAPGKEREFAATSWTD-AT (31,0)
B3	MADVQLQASGGGLVQAGGSLRLSCAASGSIFSDYVMGWYRQAPGNQRELVATITADG-WM (60,4)
A5	MADVQLQASGGGLVQPGGSLRLSCTVSGTIFTANDMGWYRQAPGKQRQAVALITTT-DT (125)
G5	MADVQLQASGGGLVQPGTSRLSCAASGFTFSSVGMNWARQAPGKGLEWISHILDDGTST (14,2)
G4	MADVQLQASGGGLVHAGDSLRLSCVTSDNSFSDHAMGWYRQAPGKERQAVATITTG-GST (17,7)
G6	MAEVQLQASGGGLVQAGGSLRLSCAVSGR---YAMGWFRQVPKGKENEFAVSISSNGRS (17,3)
D10	MAEVQLQASGGGSVQAGGSLRLSCLYSGFSLDDYTIAWFRQAPGKEREVGSCISAGERST (29,1)
B10	MAEVQLQASGGGLVQAGGSLRLSCAASGNIFSNNVMGWYRQAPGKQRELVAASISSLGD-P (84,7)
C1	MADVQLQASGGGLVQAGGSLRLSCAASGRTSSNNHMGWFRQAPGKEREFAAISWSGNRT (91,1)
A11	MAEVQLQASGGGLVQAGGSLRLSCAASGR---YTIGWFRQAPEKEREFAAIRGTRST (28,8)
G10	MAEVQLQASGGGLVQAGGSLRLSCAASGRTFSSYVTGWFRQAPGKEREFAAIRSNDGST (14,0)

D9 YYADSAKGRFTISRDDDMSTVNLMQNSLQPEDTAVYYCSARPTG-----HYDL
B3 KYADSVKGRFSISTDDDMNTVSLQMNSLKLEDTAVYYCYARY-----SPAIYGDTY
A5 TYANSVKQFTISRDN SKNTVYLLMISLKPDDTGYYCKILP-----VGDDY
G5 TYADSVKGRFITS RDNAKNMLYLMQNSLKPEDTALYYCAWGN-----I
G4 MYAGSVKGRFTISRDN GRNTLYLQMNNLKPNDTAVYYCNFLR-----VGVHY
G6 WYSDSAKGRFTISRDN NTAYLQMNNLEPEDTAVYFCVADRP GPSGLGG-GVIKYRYDN
D10 RYRDSVKGRFTISSDN AAKTVYLD MNSLKPEDTAVYYCGAH RQGYGCY---SRLTYGMDY
B10 NLVDSVKDRFAVSRDN AAKTVYLMQNSLKPEDTAVYFCFYRR-----WGTTNAD
C1 YYSDSVKGRFIISRDNAKNTVYLLMNSLKPDDTAVYYCAAHS SIAALERP-SRTVDEYDY
A11 HYADSVKERFIISRDNIK NMVYLMQNSLKPEDTAVYVCAATAPGRILS---RVRDDDYEY
G10 YYTDSVKGRFTISR DNAAKNTVYLMQNSLKPEDTAVYYCAADSRARTYYSGSYRLPTLYDY

D9 WGPWTH****
B3 WSQGTVVSVS
A5 CDPANHVSFS
G5 RGQGTLVTVS
G4 WGQGTLVTVS

G6 **R**GQGTLVTVS
D10 **W**GKGTQVTVS
B10 **W**GQETQVTVS
C1 **C**GQGTLVTDS
A11 **W**GQGTQVTVS
G10 **W**GQGTQVTVS

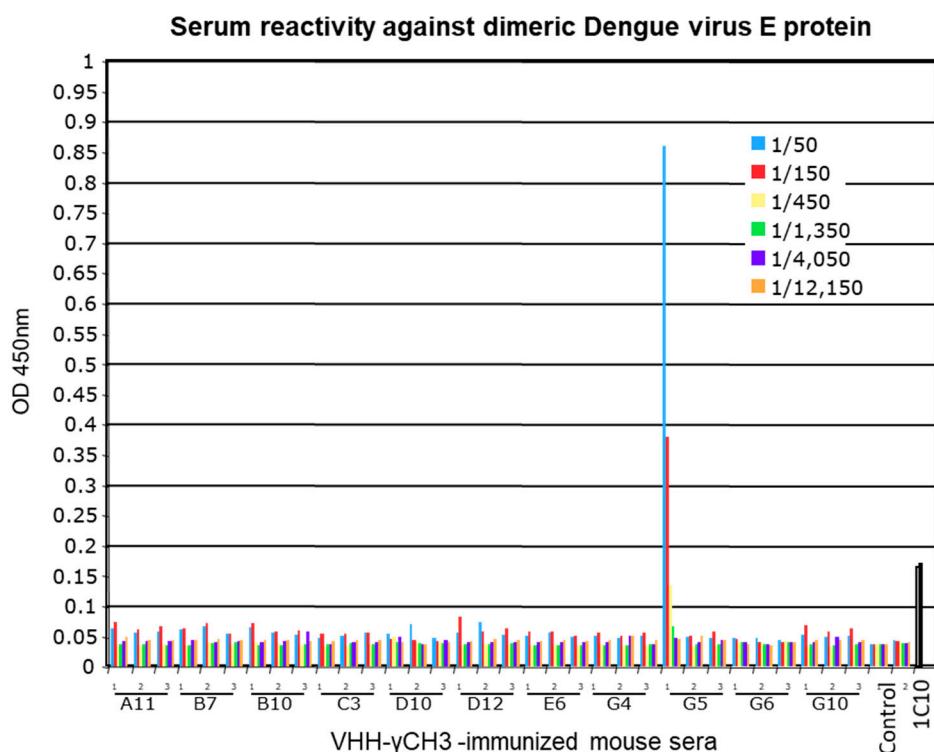


Figure S2. ELISA to test serum reactivity against Dengue CVD-Envelope. No sera showed specific activity. The only apparently positive signal detected in the experiments resulted being not specific.

Table S1. Mouse immune response towards nanobody structural components measured by ELISA. Each of the different antigens (anti-idiotypic nanobodies) was used to immunize tree mice and their sera were tested against the specific nanobody used for their immunization (specific antigen) as well as against two irrelevant nanobodies used as controls (negative controls). The rationale is that cross-reacting sera positive with any nanobody (red) recognize conserved epitope, probably belonging to the nanobody framework; non-reactive/weak-reactive sera are apparently poorly antigenic (blue); sera specific only for the nanobody used as antigen (green) should recognize the variable nanobody regions (CDRs).

Mice	Nanobodies		
	Specific antigen	Negative control 1	Negative control 2
A11.1	0.97	0.16	0.21
A11.2	0.98	0.19	0.11
A11.3	0.96	0.13	0.14
B7.1	0.71	0.68	0.56
B7.2	0.79	0.82	0.89
B7.3	0.88	0.80	0.83
B10.1	0.36	0.31	0.30
B10.2	0.24	0.20	0.18
B10.3	0.30	0.31	0.26
C3.1	0.94	0.18	0.12
C3.2	0.80	0.14	0.10
C3.3	0.82	0.19	0.25
D10.1	0.95	0.16	0.20
D10.2	0.95	0.18	0.16
D10.3	0.88	0.29	0.27
D12.1	0.74	0.26	0.21
D12.2	0.91	0.11	0.19
D12.3	0.94	0.67	0.15
E6.1	0.93	0.89	0.93
E6.2	0.92	0.92	0.84
E6.3	0.89	0.91	0.86
G4.1	0.88	0.17	0.18
G4.2	0.88	0.18	0.21
G4.3	0.82	0.21	0.12
G5.1	0.44	0.27	0.16
G5.2	0.40	0.32	0.27
G5.3	0.26	0.24	0.22
G8.1	0.69	0.68	0.70
G8.2	0.68	0.64	0.72
G8.3	0.75	0.64	0.75
G10.1	0.82	0.84	0.78
G10.2	0.85	0.86	0.36
G10.3	0.82	0.86	0.88

Summarizing, there are three kinds of serum reactivity towards the antigens: cross-reactive response (positive for any nanobody); no response (negative for all nanobodies); specific for the nanobody used for immunization (positive for the nanobody variable region corresponding to the CDRs).

Cross-reactive	pos	pos	pos
anti-CDRs	pos	neg	neg
no response	neg	neg	neg

Table S2. Mouse serum reactivity against the human γ -CH3 domain fused to the nanobodies used for mouse immunization. ELISA results were obtained by reacting mouse sera with immobilized target nanobodies; pre-immune serum and PBS buffer were used as the negative controls. Three mice were analyzed for each antigen.

Nanobody clones	Serum reactivity		
	Mouse 1	Mouse 2	Mouse 3
A11	0,919	0,94	0,927
B7	0,895	0,873	0,84
B10	0,926	1,025	0,788
C3	0,919	0,938	1,032
D10	0,878	0,874	0,841
D12	0,988	1,03	0,891
E6	0,973	0,96	0,982
G4	0,935	1,065	1,002
G5	0,889	0,784	0,953
G6	0,861	1,056	1,092
G10	0,802	0,894	0,98
Pre-Immune serum	0,038	0,034	0,033
PBS	0,039	0,038	0,038