Aedes aegypti AgBR1 antibodies modulate early Zika virus infection of mice

Ryuta Uraki [1], Andrew K. Hastings [1], Alejandro Marin-Lopez [1], Tomokazu Sumida [2,3], Takehiro Takahashi [3], Jonathan R. Grover [4], Akiko Iwasaki [3, 5], David Hafler [2,3], Ruth R. Montgomery [6], Erol Fikrig [1, 5*]

[1] Section of Infectious Diseases, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT, 06520, USA.

[2] Department of Neurology, Yale University School of Medicine, New Haven, CT, 06520, USA.

[3] Department of Immunobiology, Yale University School of Medicine, New Haven, CT, 06520, USA.

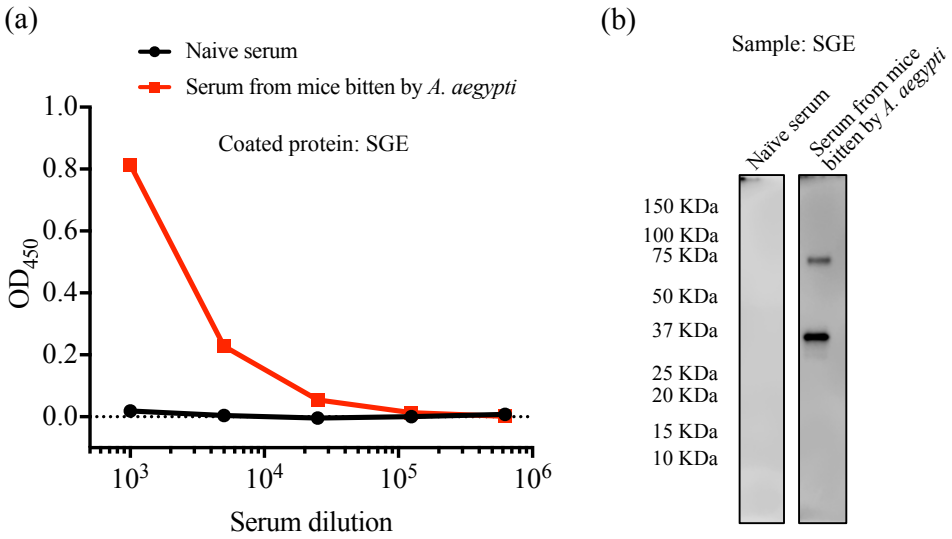
[4] Department of Microbial Pathogenesis, Yale University School of Medicine, New Haven, CT, 06520, USA.

[5] Howard Hughes Medical Institute, Chevy Chase, MD, 20815, USA.

[6] Department of Internal Medicine, Yale University School of Medicine, New Haven, CT, 06520, USA.

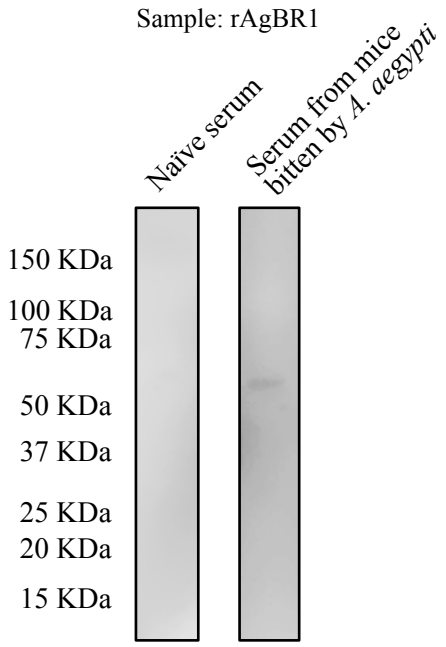
*Correspondence: Erol Fikrig, Section of Infectious Diseases, Department of Internal Medicine. Yale University School of Medicine, The Anlyan Center for Medical Research and Education, 300 Cedar Street, New Haven, CT 06520-8031. Phone: (203) 785-4140, email: erol.fikrig@yale.edu

Supplementary Information



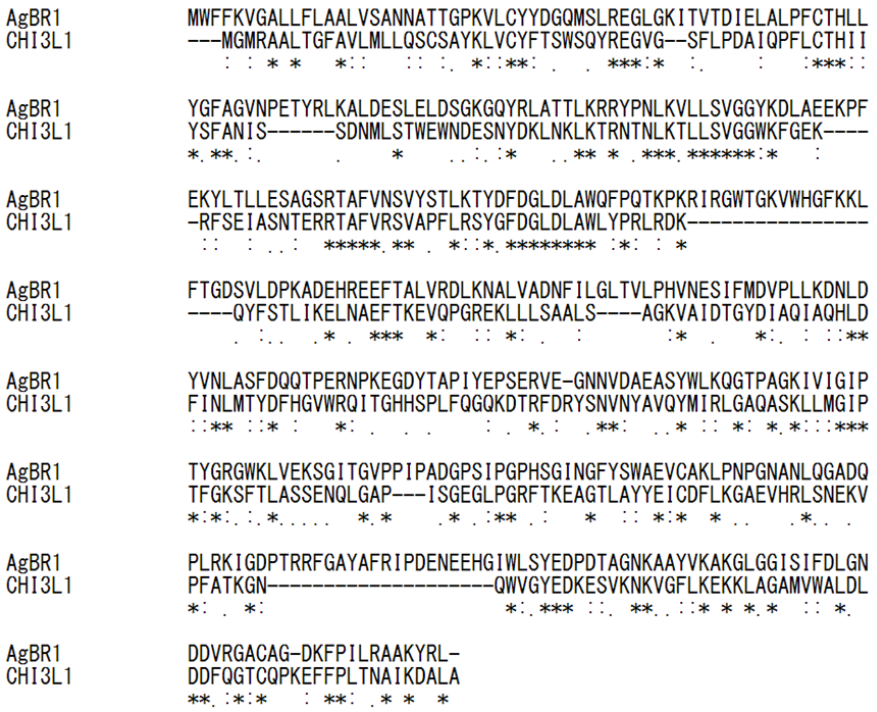
Supplementary Figure 1. Reactivity of serum from mice bitten by mosquitoes against *Aedes aegypti* salivary gland extract (SGE).

(a) ELISA and (b) Immunoblot analysis of SGE were performed by probing with naïve mouse serum and serum from mice bitten by mosquitoes. Data are representative of two independent experiments with similar results.



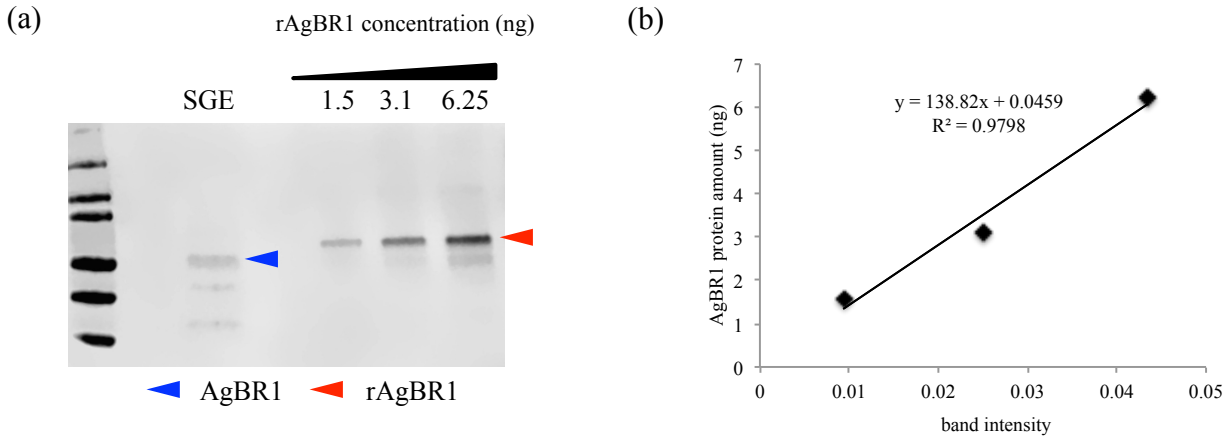
Supplementary Figure 2. Sera from mice repeatedly bitten by mosquitoes recognized recombinant AgBR1.

Immunoblot analysis of recombinant AgBR1 was performed by probing with naïve mouse sera and sera from mice bitten by mosquitoes. Data are representative of two independent experiments with similar results.



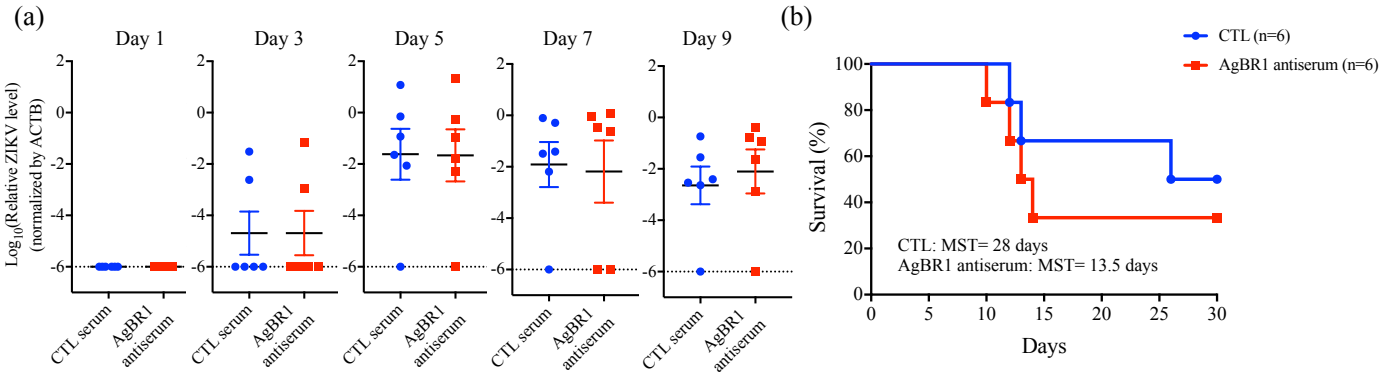
Supplementary Figure 3. Amino acid sequence similarity between AgBR1 and mouse chitinase3-like 1 protein (CHI3L1).

ClustalW alignment of AgBR1 and mouse CHI3L1. Asterisks (*) indicate an identity between two sequences; colon (:) indicates conservation between groups of strongly similar properties; period (.) indicates conservation between groups of weakly similar properties - scoring=/<0.5 in the Gonnet PAM 250 matrix.



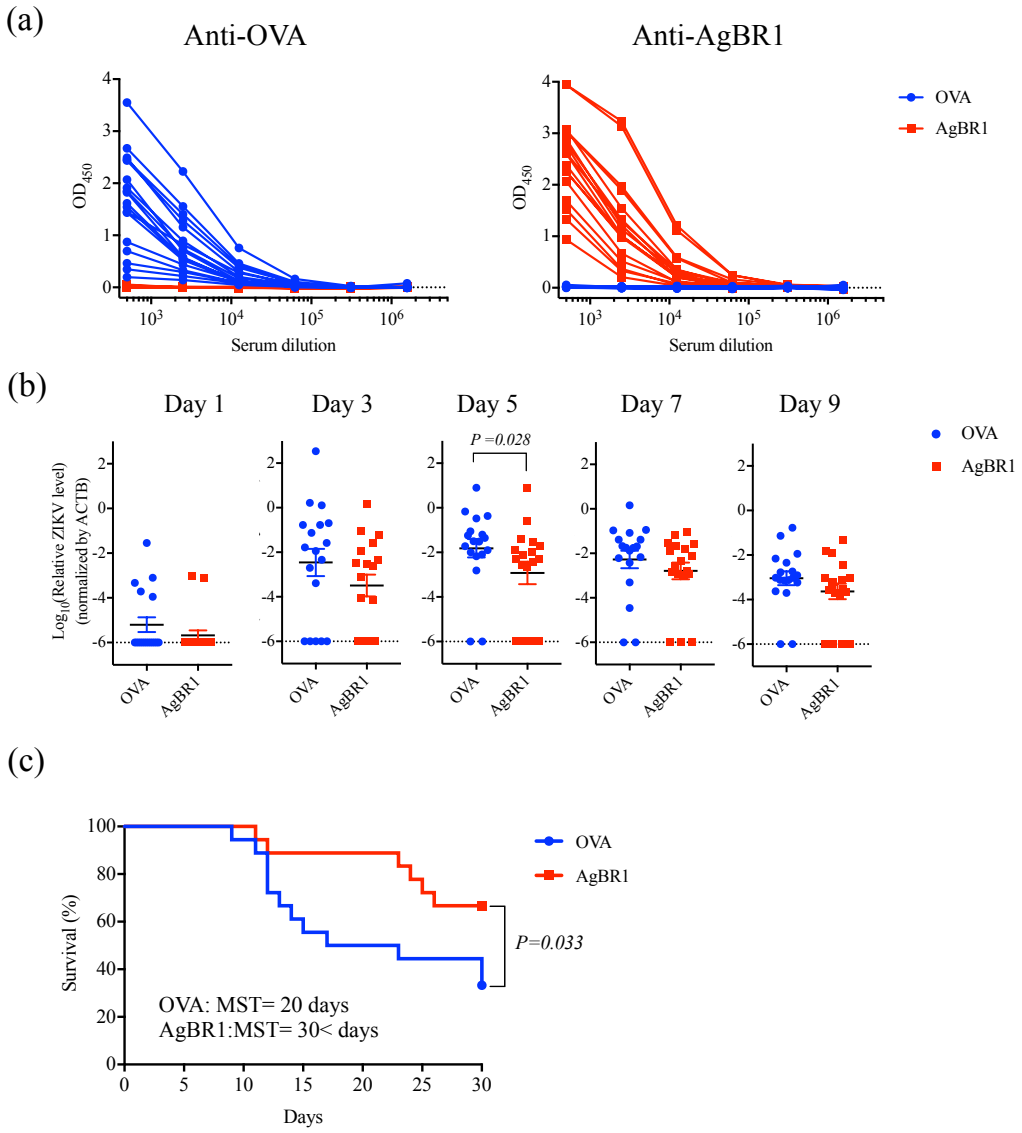
Supplementary Figure 4. AgBR1 concentration in mosquito salivary glands.

(a) Western blot of recombinant AgBR1 and *A. aegypti* salivary gland extract (SGE) at known concentrations. Blot was probed with rabbit AgBR antiserum at a 1:1000 dilution. Data are representative of two independent experiments. (b) Standard curve comparing the relative intensity of immunoblot bands for known amounts of recombinant AgBR1 and the intensity of the specific bands corresponding to native AgBR1 from SGE. Pearson correlation coefficient was calculated.



Supplementary Figure 5. AgBR1 antiserum did not protect mice from needle-injected Zika virus infection.

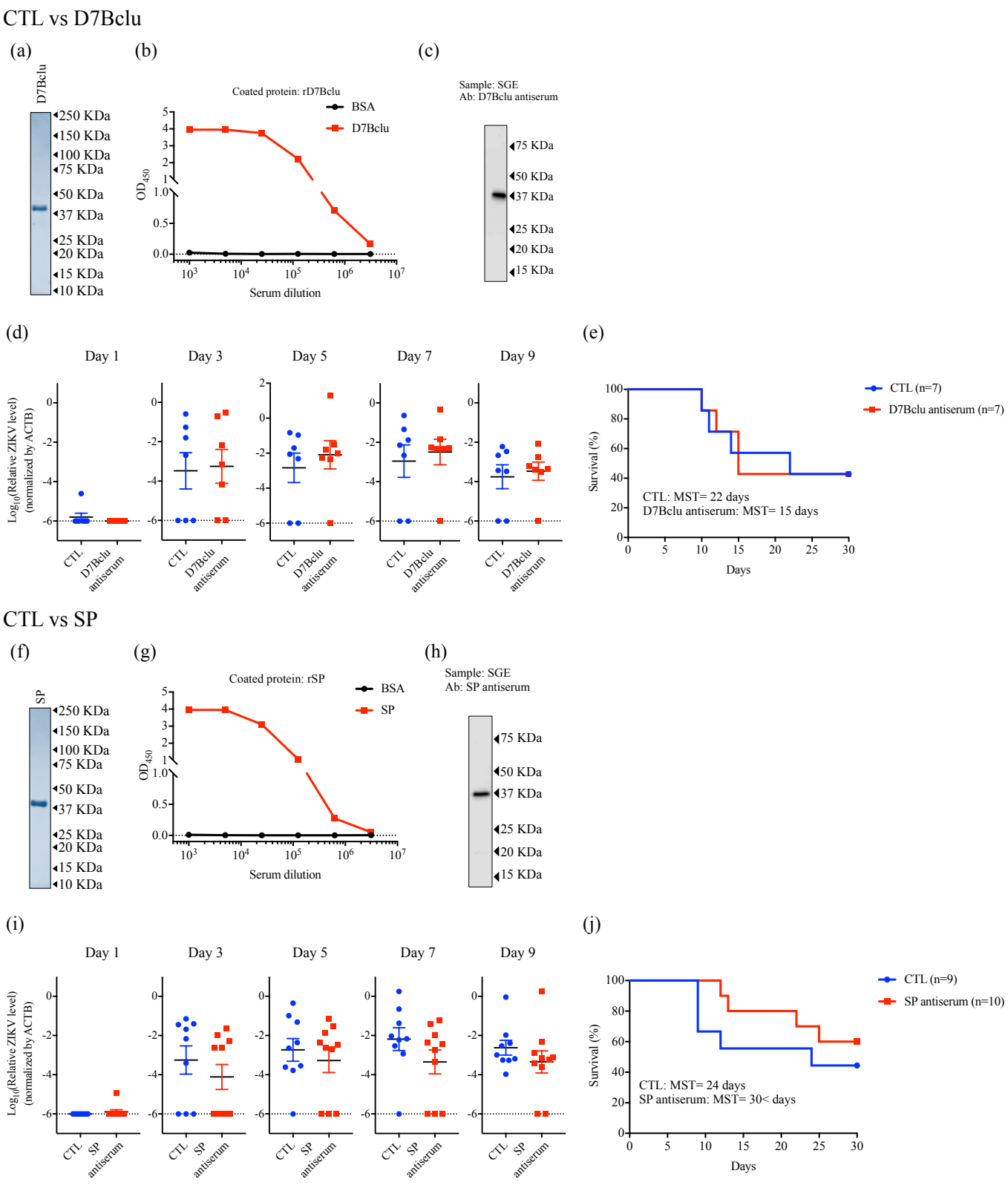
Mice were administrated with AgBR1 antiserum one day before subcutaneous 0.3 PFU of Zika virus injection. (a) Zika virus RNA levels in blood in mice treated with AgBR1 antiserum or control serum after Zika virus injection. Data are presented as mean ± s.e.m.. Each data point represents one mouse. Normalized viral RNA levels were analyzed using two-sided Wilcoxon–Mann–Whitney test. (b) Survival and median survival time (MST) were assessed using the Gehan-Wilcoxon test. (Control: n=6, AgBR1 antiserum: n=6 biologically independent samples pooled from two separate experiments.)



Supplementary Figure 6. Active immunization with AgBR1 reduces mosquito-borne Zika infection in mice.

(a) Two weeks after final immunization with AgBR1 or OVA, the sera from immunized mice were examined for specific antibodies with ELISA. Sera from AgBR1-immunized mice recognized AgBR1 (right panel), but not ovalbumin, OVA (left panel). (b) Zika virus RNA levels in immunized mice after mosquito-borne Zika virus infection. Data are presented as mean ± s.e.m.. Each data point represents one mouse. Normalized viral RNA levels were compared

using two-sided Wilcoxon–Mann–Whitney test. (c) Survival and median survival time (MST) after mosquito-feeding were assessed by a Gehan-Wilcoxon test. (Control: n=18, AgBR1 antiserum: n=18 biologically independent samples pooled from three separate experiments.)

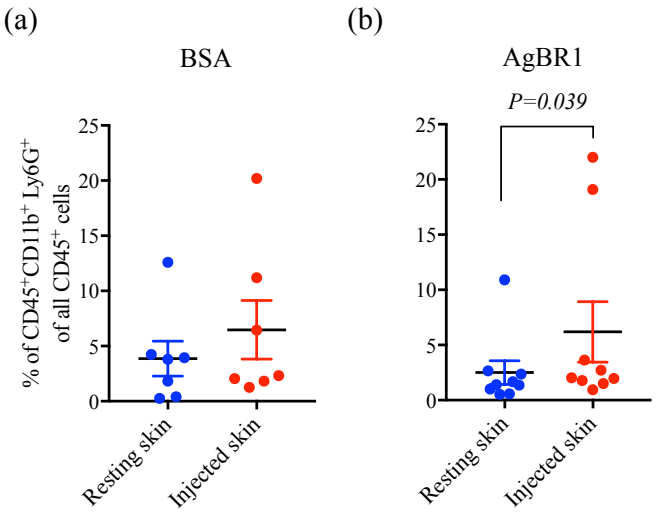


Supplementary Figure 7. Antisera against abundant proteins recognized in the yeast

display assay were not protective against mosquito-borne Zika virus infection.

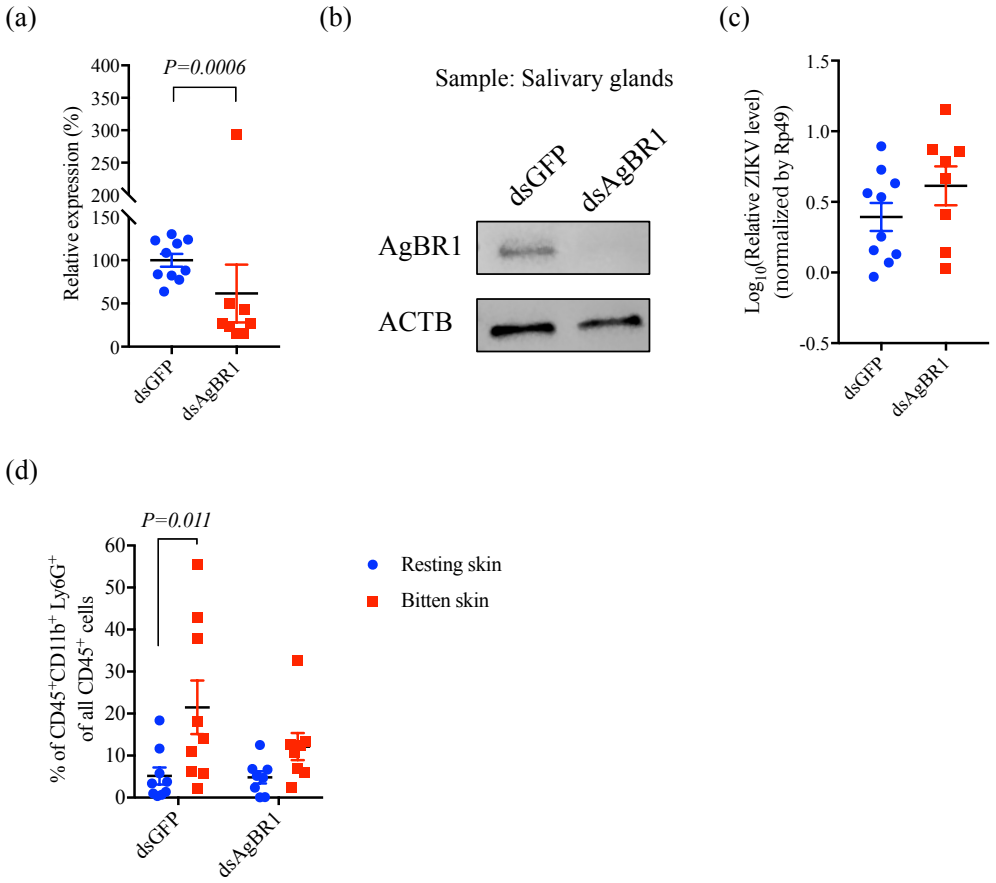
(a and f) Recombinant proteins (0.25 μg protein) were run on SDS-PAGE and stained with Coomassie Brilliant Blue. (b, c, g and h) Rabbit antisera to D7Bclu and SP recognize recombinant proteins and their respective proteins in salivary gland extract (SGE) as confirmed by ELISA (b and g) and immune blots (c and h) Data are representative of two independent experiments with similar results.

(d, e, i and j) Mice were administrated with antiserum against D7Bclu (Control: n=7, D7Bclu antiserum: n=7 biologically independent samples pooled from two separate experiments.) or SP (Control: n=9, SP antiserum: n=10 biologically independent samples pooled from three separate experiments.) one day before Zika virus-infected mosquito feeding. Immunized mice were monitored for survival for 30 days after infected mosquito-feeding. (d and i) Zika virus RNA levels in blood in mice. Data are presented as mean ± s.e.m.. Each data point represents one mouse. Normalized viral RNA levels were analyzed using two-sided Wilcoxon–Mann–Whitney test. (e and j) Survival and median survival time (MST) were assessed using the Gehan-Wilcoxon test.



Supplementary Figure 8. Direct intradermal injection of rAgBR1 into the skin recruits neutrophil.

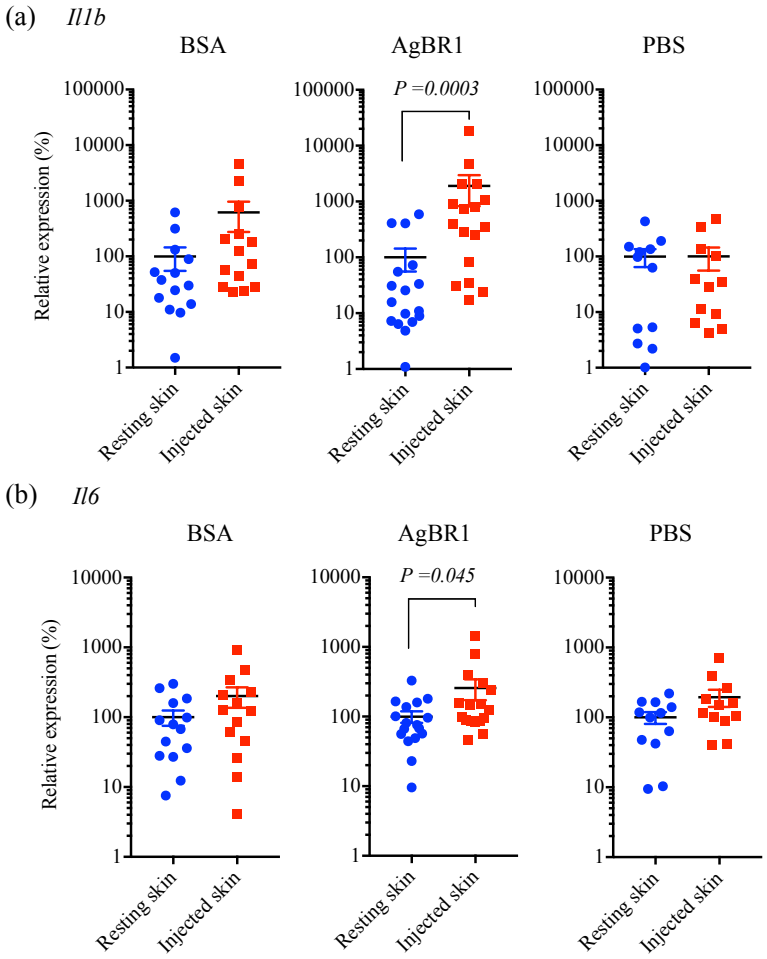
The percent of $CD45^+CD11b^+Ly6G^+$ (neutrophils) cells in $CD45^+$ leukocyte cells at 24h after intradermal BSA (a) or rAgBR1 (b) injection. Data are presented as mean ± s.e.m.. Each dot represents one mouse. Significance was calculated using two-sided Wilcoxon matched-pairs signed rank test. (BSA-resting skin: n=7, BSA-injected skin: n=7, AgBR1-resting skin: n=9, AgBR1-injected skin: n=9 biologically independent samples pooled from two separate experiments.)



Supplementary Figure 9. A partial knock-down of AgBR1 impacts neutrophil recruitment in the skin.

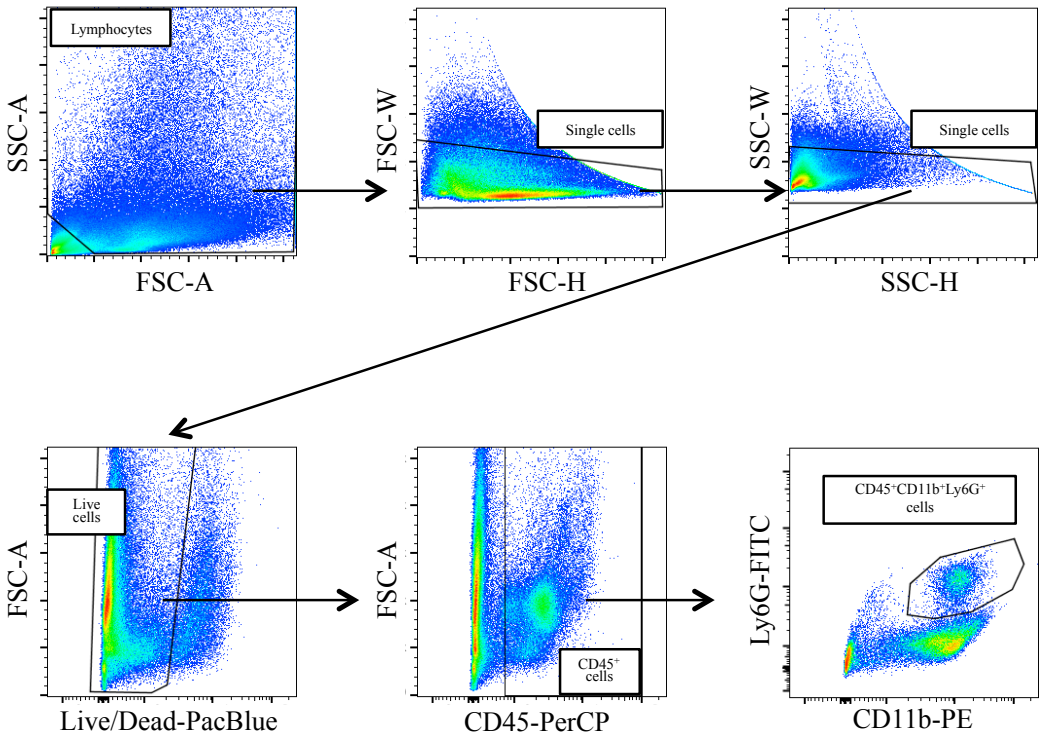
(a) QRT-PCR of salivary glands injected with either dsRNA targeting an irrelevant GFP gene or AgBR1. Data are presented as mean ± s.e.m.. Normalized AgBR1 RNA levels were compared using two-sided Wilcoxon–Mann–Whitney test. (*dsGFP*-treated mosquitoes: n=10, *dsAgBR1*-treated mosquitoes: n=8) Data are representative of three independent experiments with similar results. (b) Immunoblots of salivary glands from *dsGFP* or *dsAgBR1* injected mosquitoes. Data are representative of three independent experiments with similar results. (c) Zika virus RNA levels in the salivary glands of dsRNA treated mosquitoes at 10 days after intrathoracic Zika virus injection. Data are presented as mean ± s.e.m.. (*dsGFP*-treated

mosquitoes: n=10, *dsAgBR1*-treated mosquitoes: n=8) Data are representative of three independent experiments with similar results. (d) The percent of $CD45^+CD11b^+Ly6G^+$ (neutrophils) cells in $CD45^+$ leukocyte cells at 24h after dsRNA-treated and Zika virus-infected mosquito feeding. Each dot represents one mouse. Significance was calculated using a two-way ANOVA test for multiple comparisons. (*dsGFP* -resting skin: n=9, *dsGFP* -bitten skin: n=9, *dsAgBR1* -resting skin: n=8, *dsAgBR1* -bitten skin: n=8 biologically independent samples pooled from two separate experiments.)



Supplementary Figure 10. AgBR1 is able to induce *Il1b* and *Il6* expression in the skin.

The expression levels of *Il1b* (a) or *Il6* (b) after intradermal injection of BSA, AgBR1 or PBS without protein were examined by qRT-PCR. Expression levels were normalized to mouse *β* actin RNA levels. Data are presented as mean ± s.e.m.. Each dot represents one injected or control site. Significance was determined by two-sided Wilcoxon–Mann–Whitney test. (BSA-resting skin: n=14, BSA-injected skin: n=14, AgBR1-resting skin: n=17, AgBR1-injected skin: n=17, PBS-resting skin: n=12, PBS-injected skin: n=12 biologically independent samples pooled from two separate experiments.)



Supplementary Figure 11. Gating strategy for identifying $CD45^+CD11b^+Ly6G^+$ neutrophil cells in ear skins of mice fed by Zika virus-infected mosquitoes.

The ear skins of AG129 mice were fed by Zika virus-infected mosquitoes. Representative flow cytometry profiles are from mice harvested at 24 hours post-feeding.

Un-cropped gel or blots

Fig. 1c

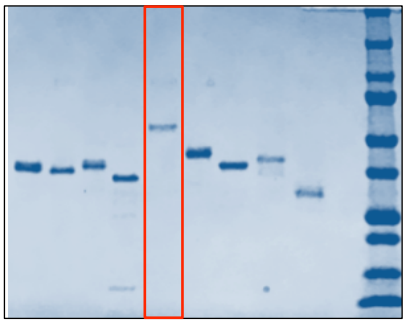


Fig. 1d

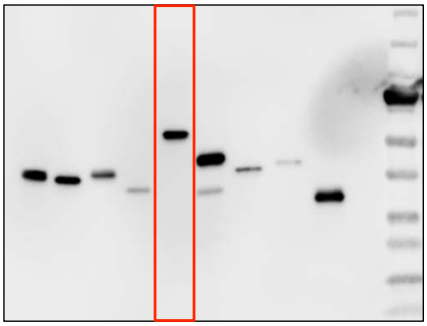
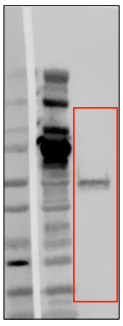
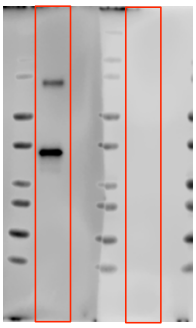


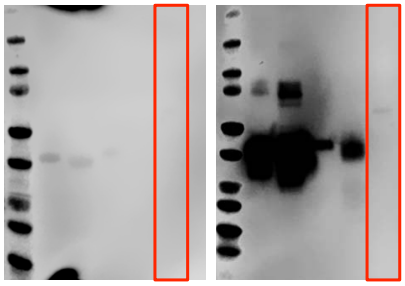
Fig. 2a



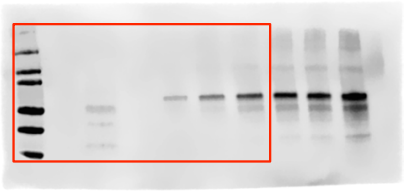
Supplementary Fig. 1



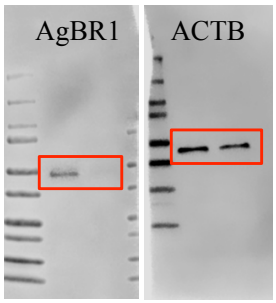
Supplementary Fig. 2



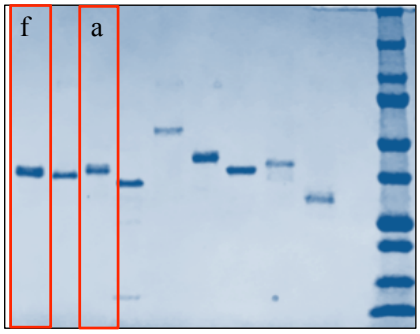
Supplementary Fig. 4



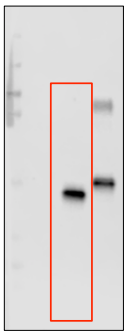
Supplementary Fig. 9



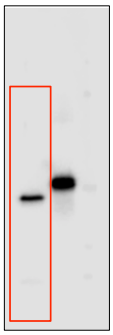
Supplementary Fig. 7



Supplementary Fig. 7c



Supplementary Fig. 7h



Supplementary Figure 12. The uncropped original gels or blots.

Supplementary Table 1. Antigenic *A. aegypti* salivary proteins identified by a Yeast Surface Display library using serum from mice bitten by *A. aegypti*.

Clone	Number of times identified in the screen	Gene Identity	Protein name	Abbreviation in this study	Signal peptide	MW (KDa) (including signal peptide)
1	19	AAEL003600	Putative 34 kDa family secreted salivary protein	SP	Yes	36
2	12	AAEL003601	Putative 34 kDa secreted protein	SSP	Yes	36
3	11	AAEL006417	Long form D7Bclu1 salivary protein	D7Bclu	Yes	39
4	7	AAEL010228	Putative 30 kDa allergen-like protein	AILP	Yes	24
5	1	AAEL001965	Bacteria-responsive protein 1	AgBR1	Yes	49

Supplementary Table 2. Endogenous AgBR1 concentration of an *A. aegypti* salivary gland.

Salivary gland extract (SGE, μg)	Relative Band Intensity	Estimated AgBR1 (ng)	Concentartion (μM)
4	0.0111	1.6	1.6-8.2

The endogenous AgBR1 concentration in an *A. aegypti* salivary gland was predicted from the calculated intensities from AgBR1 in SGE bands (Supplementary Figure 4b).

Supplementary Table 3. The histological findings scored in terms of inflammation, neutrophil infiltration, mononuclear cell infiltration and edema.

	Control	AgBR1 antiserum
Inflammation	3.6 ± 0.24	2.0 ± 0.26** (*P*= 0.0087)
Neutrophils	3.6 ± 0.24	1.7 ± 0.21**(P= 0.0022)
Mononuclear cells	2.4 ± 0.24	1.5 ± 0.22
Edema	2.6 ± 0.40	2.5 ± 0.34

The histology scores of the bite sites were compared between the AgBR1 antiserum and control group. Data are presented as mean ± s.e.m.. Statistical analysis was performed using two-sided Wilcoxon–Mann–Whitney test. n=5 (Control) or 6 (AgBR1 antiserum) biologically independent samples pooled from two separate experiments.

Supplementary Table 6. Oligonucleotide primers used in the experiments.

The purpose of the primers is indicated, followed by the gene that was amplified. Forward primer (F) and Reverse primers (R) are indicated.

1) Oligonucleotide primers for qRT-PCR

Zika virus	F: TTGGTCATGATACTGCTGATTGC R: CCTTCCACAAAGTCCCTATTGC
Mosquito Rp49	F: GCTATGACAAGCTTGCCCCCA R: TCATCAGCACCTCCAGCT
Mouse *βactin*	F: GATGACGATATCGCTGCGCTG R: GTACGACCAGAGGCATACAGG
Mouse *Tnfa*	F: TGGAACTGGCAGAAGAGGCACT R: GAGATAGCAAATCGGCTGACGG
Mouse *Il1b*	F: GCTTCAGGCAGGCAGTATCAC R: CGACAGCACGAGGCTTTTT
Mouse *Il6*	F: ATGAAGTTCCTCTCTGCAAGAGACT R: CACTAGGTTTGCCGAGTAGATCTC
Mosquito AgBR1	F: CGTCAACTTGGCTTCGTTCG R: GATGCCGGATTTCTCCACCA

2) Oligonucleotide primers for cloning into the expression vector

AgBR1	F: CTCGCTCGGGAGATCTAACAATGCCACTACCGGCCCAAAGGTCCTC R: GCCCTCTAGACTCGAGCAGCCTATACTTAGCAGCCCTCAG
SP	F: CTCGCTCGGGAGATCTCACCCAATTCCAGCCGAAGATCCCGCCAAGC R: GCCCTCTAGACTCGAGACCAAAAGCCTTCACCATGACCTTCGGATAG
D7Bclu	F: CTCGCTCGGGAGATCTGCACCTTTATGGGATGCAAAGGATCCAGAGC R: GCCCTCTAGACTCGAGGCTACACTGGATCTTGTCGATATCG

3) Oligonucleotide primers for dsRNA preparation

dsAgBR1 RNA	F: TAATACGACTCACTATAGGGGATGGACAGATGTCTCTTCGTG R: TAATACGACTCACTATAGGGCCAAATCCAATCCATCGAAA
dsGFP RNA	F: TAATACGACTCACTATAGGGGTGAGCAAGGGCGAGGAG R: TAATACGACTCACTATAGGGCATGATATAGACGTTGTGGCTGTT