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Supplemental Information

Immunization with AgTRIO, a Protein in *Anopheles* Saliva, Contributes to Protection against *Plasmodium* Infection in Mice

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SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Purified AgTRIO IgG reduces parasitemia in mice in a dose-dependent manner, Related to Figure 3 and Table S2. (A-F) Mice were administered a high dose of AgTRIO IgG (500 μg, AgTRIO IgG), a low dose of AgTRIO IgG (80 μg, Low TRIO IgG), or OVA IgG (500 μg). Mice were then exposed to bites from 3 *Plasmodium berghei*-infected mosquitoes. Blood was collected on day 4 (A and D), 5 (B and E), and 8 (C and F). Blood was used for RNA isolation and subsequent RT-qPCR analysis using *P. berghei 18S rRNA* with *murine β2-microglobulin* (*mouse β2m*) serving as a control (A-C) For the PCR studies, as an additional control, naïve mice were not administered IgG or exposed to *P. berghei*-infected mosquitoes (A-C). Flow cytometry analysis (D-F) The presence of *P. berghei* was determined based on the GFP signal in the blood sample. Results represent 3 combined independent experiments. Each data point represents one mouse. Error bars represent mean ± SEM. Mean values were considered significantly different using the non-parametric Mann-Whitney test when $p < 0.05$. Not significant (ns).

Figure S2. Amino acid sequence analysis of SG1 family members, as well as the D7-family member, D7r1, Related to the STAR Methods section, and Figures S3 and S4. 1. AgTRIO, 2. SG1L3, 3. SG1b, 4. D7r1. The signal peptide sequences are marked in red, and were determined using the SignalP 4.1 server.

Figure S3. AgTRIO, SG1L3, SG1b and D7r1 antisera recognize salivary proteins, Related to Figure 3, and Figures S2 and S4, and Table S2. (A-D). Rabbit antisera to AgTRIO (A), SG1L3 (B), SG1b (C), or D7r1 (D) recognize their respective proteins in salivary glands (SG). There is no reactivity with midgut proteins (MG). (E-G) Rabbit antisera to SG1L3 (E), SG1b (F) and D7r1 (G) identify their associated proteins in mosquito saliva.

Figure S4. SG1L3, SG1b and D7r1 antisera are not protective against mosquito-borne *Plasmodium* challenge, Related to Figure 3 and Figures S2 and S3, and

Table S2. (A-B) Mice were given SG1L3 (A), or SG1b (B) antisera and then challenged with *Plasmodium berghei*-infected mosquitoes. Livers were collected at 40 hours and RNA isolated. RT-qPCR was performed to determine levels of *P. berghei* RNA using *18S rRNA* and mouse *actin* (control). Each result depicts 3 independent experiments. (C-E) SG1L3 (C), SG1b (D) and D7r1 (E) antisera were administered to mice prior to exposure to *P. berghei*-infected mosquito. (C-D) On day 5, blood was collected from mice to isolate RNA and perform RT-qPCR. *P. berghei* levels were determined using *18S rRNA*, while mouse *actin* served as control. Results represent 2 independent parasitemia experiments. (E) Flow cytometry analysis. The presence of *P. berghei* was determined based on the GFP signal in the blood sample on say 5. Each data point represents one mouse. Error bars represent mean ± SEM. Mean values were considered significantly different using the non-parametric Mann-Whitney test when $p < 0.05$. Not significant (ns).

Figure S5. Antibodies raised against *Anopheles gambiae* TRIO interact with *Anopheles stephensi* TRIO, Related to Figure 3. (A-B) Salivary glands and midguts were isolated from female, clean blood-fed *A. gambiae* and *A. stephensi* mosquitoes**.** Rabbit serum to AgTRIO was used to probe against AgTRIO and AsTRIO in salivary glands (A). Midgut served as negative control (B). Actin antibody was used to detect actin. Actin levels were normalized for the loading control.

Figure S6. **AgTRIO antiserum affects immune cell populations in the skin of mice fed upon by *Plasmodium berghei*-infected *Anopheles gambiae,* Related to Figure 7.** C57Bl/6 mice were passively immunized with AgTRIO or OVA antisera and 4 infected *A. gambiae* mosquitoes were allowed to feed on the ears of each mouse. Both bitten and unbitten (naïve) ears were harvested, enzymatically digested to single cell suspension and the percentage of $CD45^+$ $Ly6G^-$ (B, C and D) or $CD45^+$ (A) cells of each population of macrophages (B), dendritic cells (C), Langerhans cells (D) and neutrophils (A) were analyzed using flow cytometry. Data shown are pooled from 3 independent experiments, with an n=16 for each group. Significance was calculated using a one-way

ANOVA with post-hoc Tukey test for multiple comparisons. Each dot represents one mouse. Results represent 3 combined independent experiments. n.s. not significant.

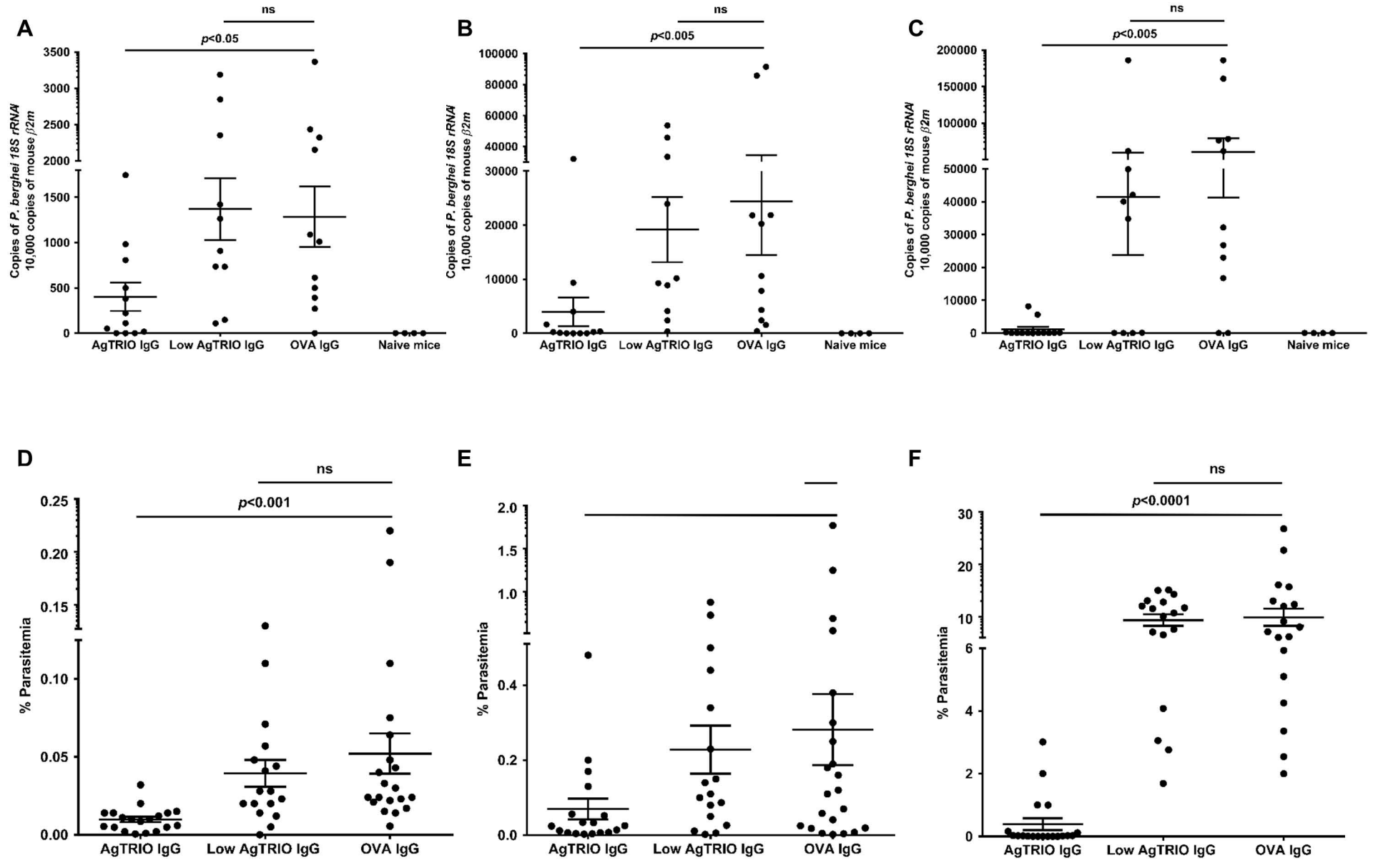

Figure S1. Purified AgTRIO IgG reduces parasitemia in mice in a dose-dependent manner

1 AgTRIO (AGAP001374-PA)
MCRGLSAVLILLVSLSAQLHVVVGEEAPKPEKEICGLKVGRLLDSVKGWLSVSQQEKCPLNKYCENKIQA
DQYNLVPLTCIRWRSLNPASPTGSLGGKDVVSKIDAAMSNFKTLFEPMKADLAKLEEEVKRQVLDAWKAL
EPLQKEVYRSTLASGRIERAVFYSFMEMGDNVKLDNYFQPANVEELLKYAWALPMHKKQRSMYDLIGQLV
QSSKSPMLQTLHAVELATVVNPELENRENLLNDQVVQLRDNLYKNSFATLVSIARHFPDHFDTLRQRLFK
LPDGSKPGADTLPNIVNFIAQLPSDELRLSSVDLLLQSLTAENGTLVQDPEYVYRLSQLAHAMPSLVDV
AHPDLQQSVDDLMAKFNTPIDGKTLQYFQNIGISPSSSVAT

2 SG1L3 (AGAP000607-PA)
MTPLIATLAACALTLSIVHSRGLPESSDKLEACGQHYGALLKASTTWNEKECNGSTKLAACVVSEHEQAY
RELKQRCQEAHDERTAKVNAIYEKLPAYLSEVSARVNVLQVSLQHDLPNLQEMVGEQRHLIEQAWQYGAQ
LQHELMLTSMESDRVQRALVLHSMLVNASLAEMVKESYQTHGADGRMVVRMLKFVRLLPGADERVAVYKQ
LAELLKSNGQDGRFPAVIFSTDVRQLEDRYKPDHAQYEGKVVERWLAELQAGTFHEVVEFARDYPEYFAR
VEEPLYETLKQQWSAEGLDRMVSFPNALPVGVQRVRALRALLETLLQHQGEQNNDVYLIRLAHETGRVEA
TVGQADAAVRQALDDVKKLFEQFKYQRGFPDYEALYKLFKGL

3 SG1b (AGAP000548-PA)
MAYRLLVSCLLLLQLVLTQADGGSFLAPSGSNYCPIPLEVLEQENGTAPADWSASCTQRRTEDHAKIEQA
VAVIKDHLEKQAAATKPIRDELRQFGGTLLPLLNSAQVKTDAAELDELRMSVLVAAIEAGRIPEAMTQFL
MLAGWNRWPQIVAQIYQNTRRDRQHIANLLEFIRIVPARDDRVAFYHELKKHMVASKDYESYLGAMFAAD
AFHVVYEADGKTPLNESDVKALYTTMLDGAGAYFQRALLTGANRYDLFLLDEHHPQLFDLLFDRIVNVSQ
ANMRKFNSWQMMGALCRVHRPMSKVLLFRKTANLLLDHFKWEKENEYYAPMLAGYFEVCLPDIRKDPATA
GLVTEVQNIFGRYKKGMNYKSISHIIGKNIHAYAG

4 D7r1 (AGAP008284-PA)
MFNKLHFVSLLACGLFVIAQANTVKKCEKKMPASLKSQLCEIRKYKLLDTPDMDKHMDCVMKALDFVRPD
GTGDYHKLIKPLNAIEKDRKHDFNLEKCGGQTQHLPVGKRANAYYKCLVESTSGEAFKKVFDTVELVKAK
KLPALSQYSSVVDKMMKKIDDKICN

Figure S2. Amino acid sequence analysis of SG1 family members, as well as the D7-family member, D7r1

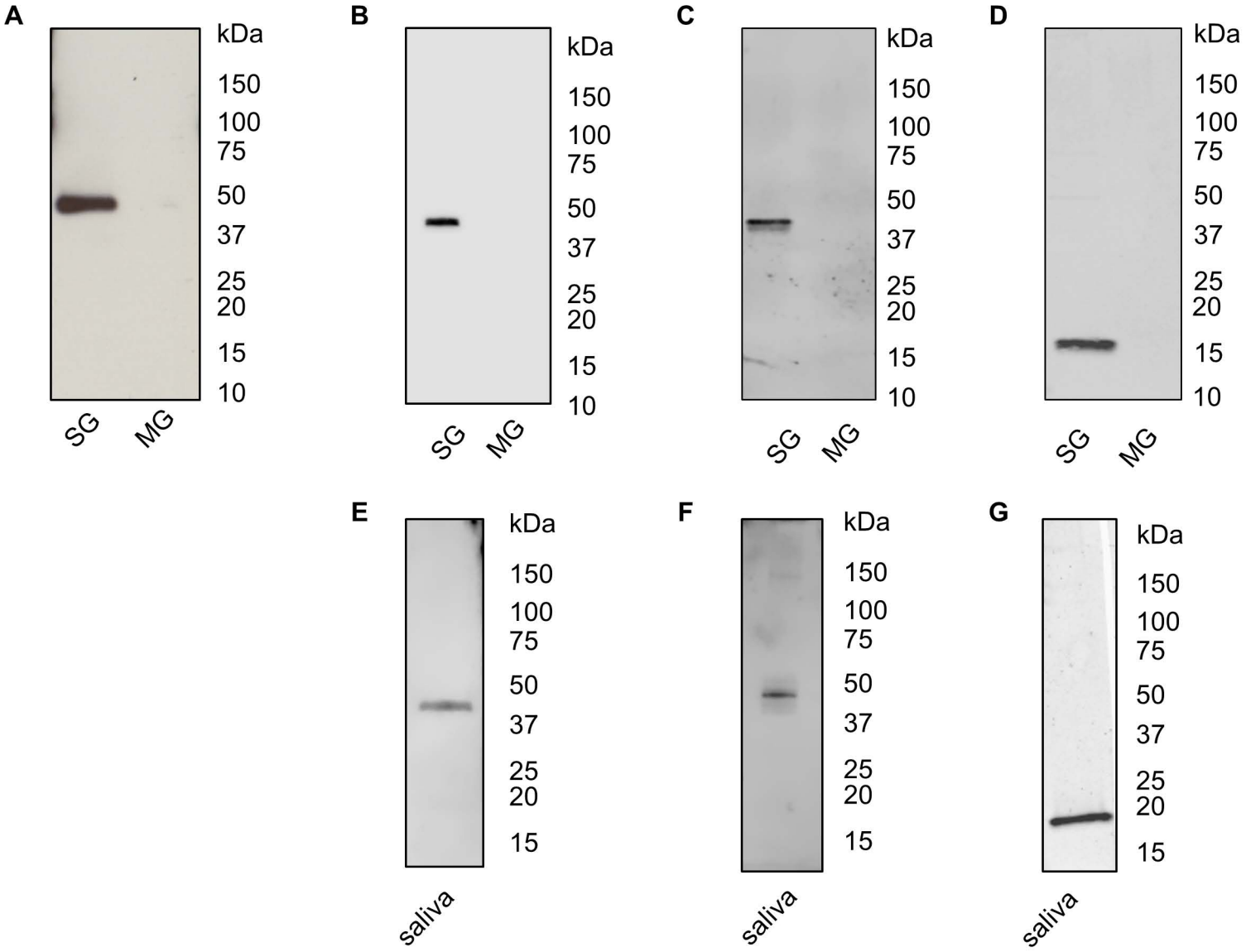

Figure S3. AgTRIO, SG1L3, SG1b and D7r1 antisera recognize salivary proteins

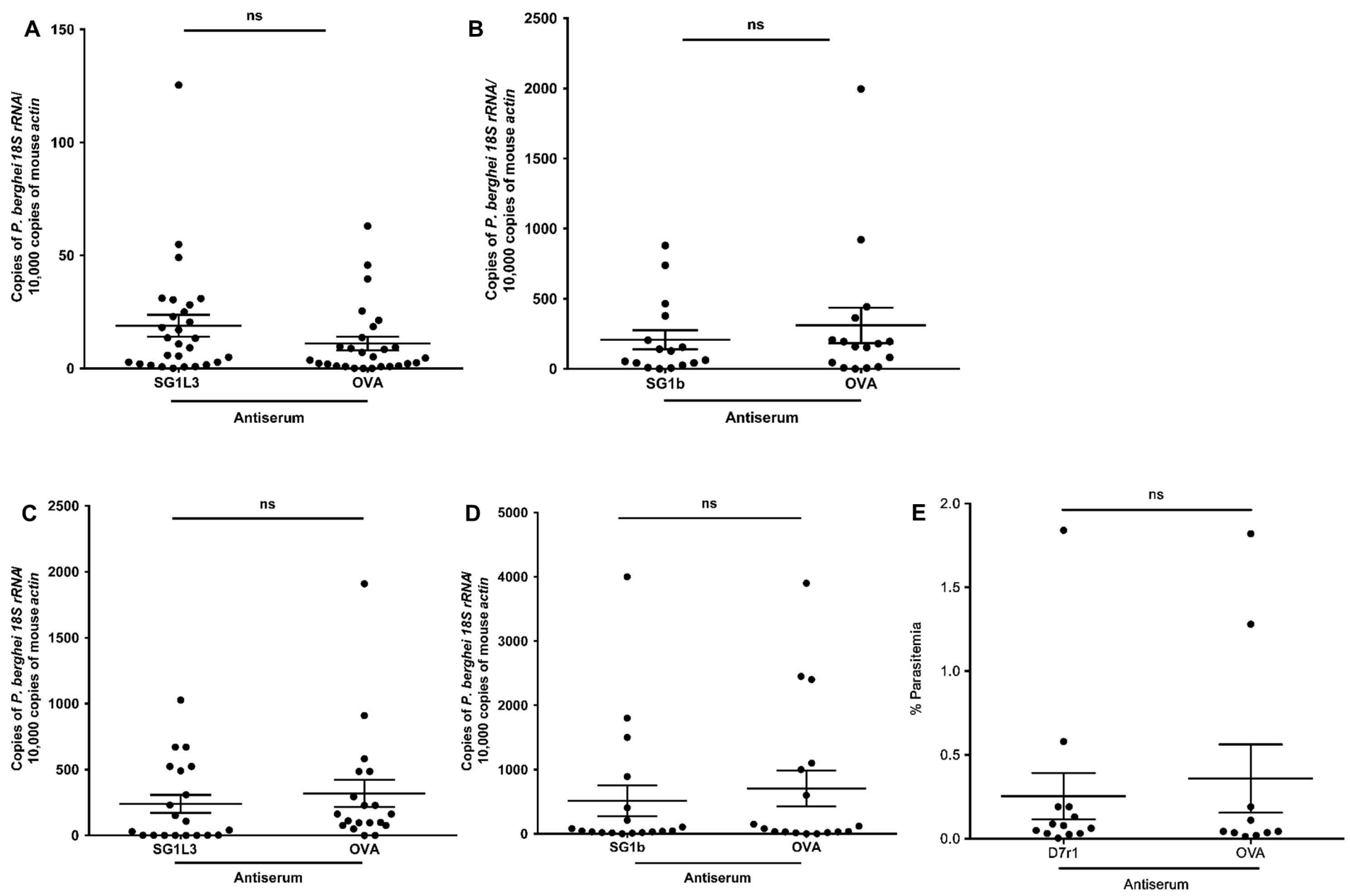

Figure S4. SG1L3, SG1b and D7r1 antisera are not protective against mosquito-borne *Plasmodium* challenge

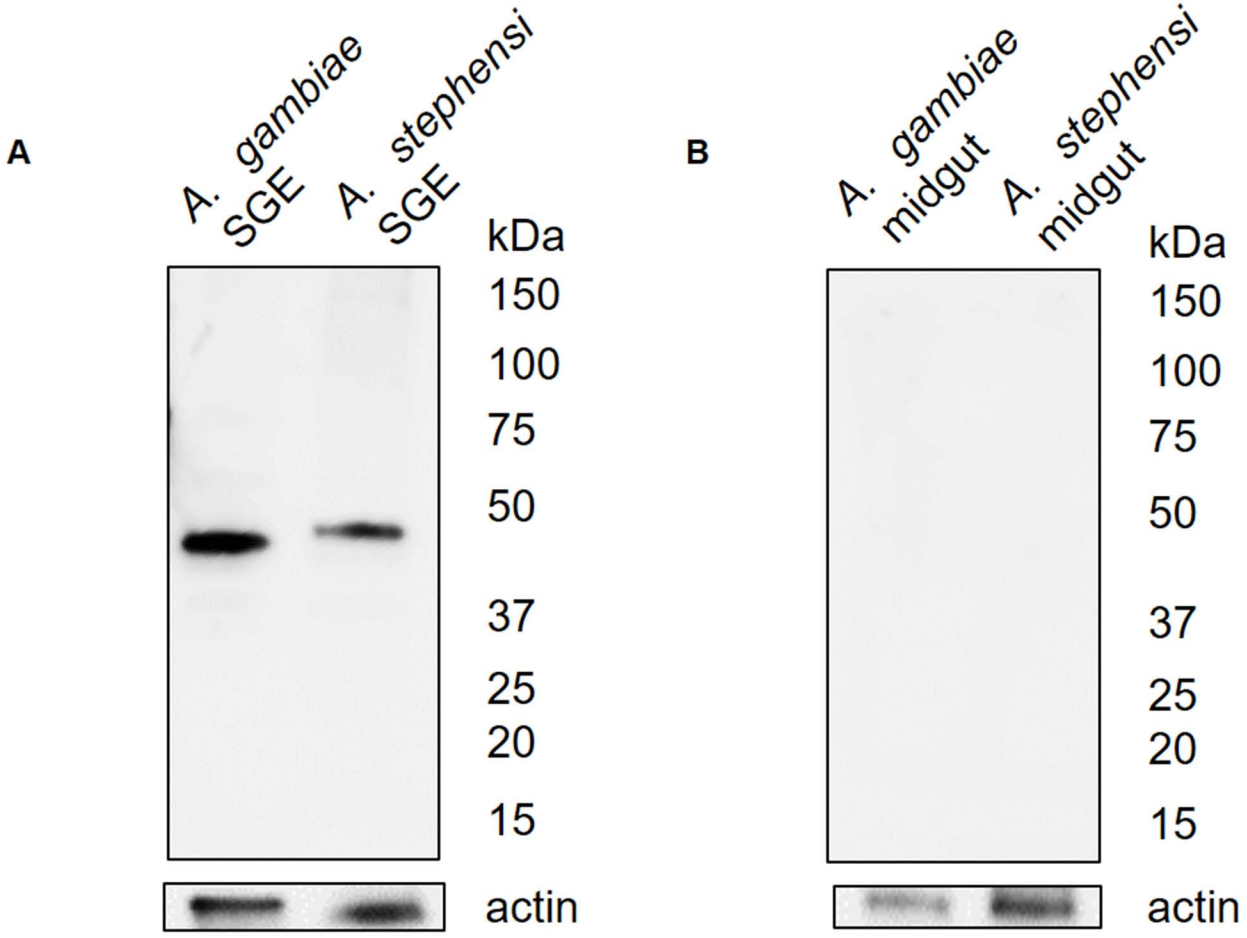

Figure S5. Antibodies raised against *Anopheles gambiae* TRIO interact with *Anopheles stephensi* TRIO

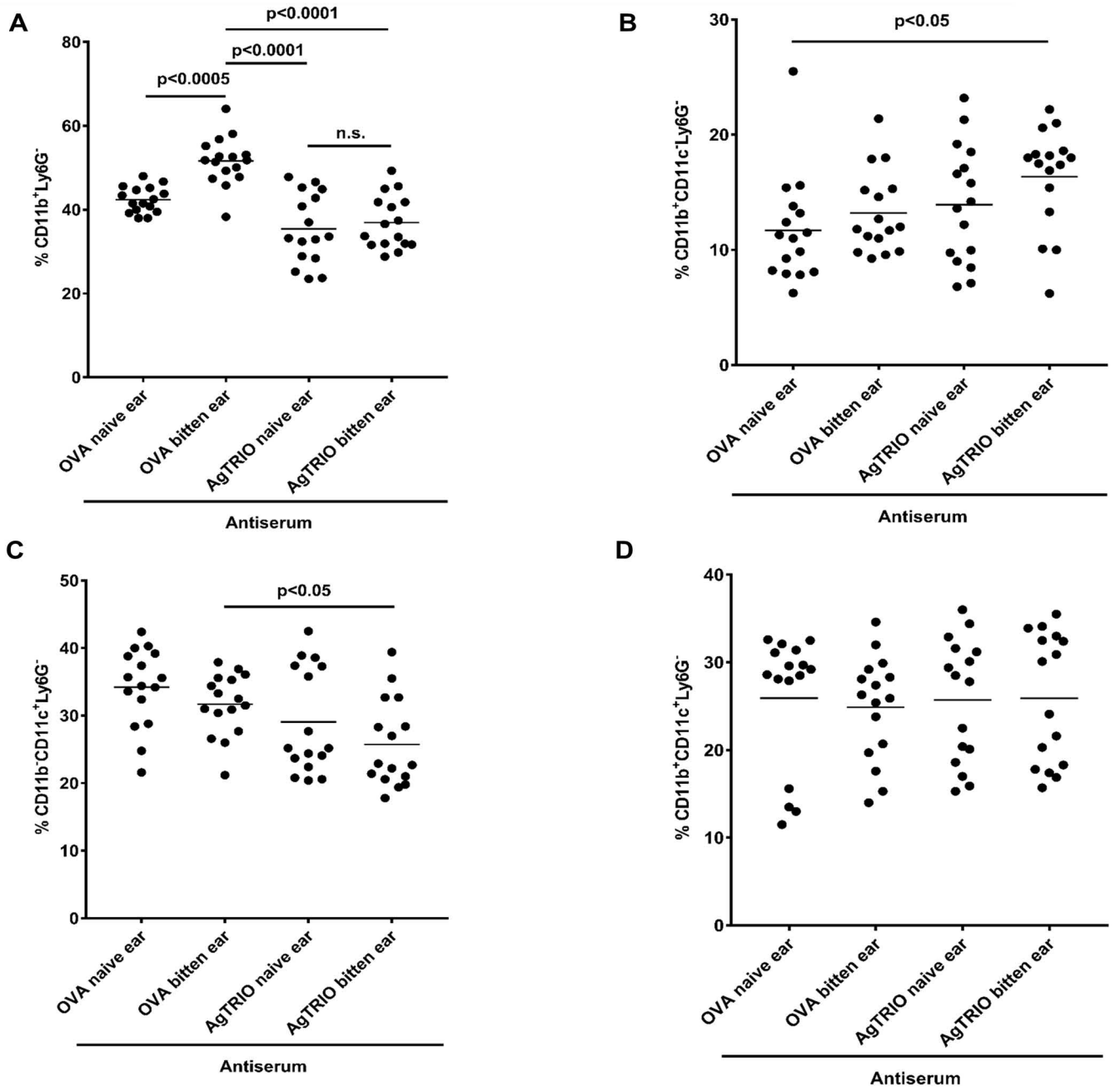

Figure S6. AgTRIO antiserum affects immune cell populations in the skin of mice fed upon by *Plasmodium berghei*-infected *Anopheles gambiae*

Table S1. *Anopheles gambiae* antigenic salivary gland proteins identified by probing an *A. gambiae* salivary gland cDNA yeast surface display library with antiserum against *A. gambiae* salivary gland extracts, Related to Figure 1. Commonly detected proteins with a putative signal sequence are listed below (AGAP001374-AGAP012401), followed by commonly detected proteins lacking a signal sequence.

Gene	Protein	Molecular weight (kDa)
AGAP001374	TRIO (Arca et al., 2005)	40
AGAP008284	D7r1: D7 short form (Arca et al., 1999)	18
AGAP008282	D7r2: D7 short form (Arca et al., 1999)	18
AGAP006504	SG2b: salivary gland protein 2-like 1 (Holt et al., 2002)	17
AGAP000610	Saglin (Ghosh et al., 2009)	49
AGAP011026	5' nucleotidase (Arca et al., 1999)	63
AGAP003251	CLIP-domain serine Protease (Gorman et al., 2000)	40

AGAP007400	Histidine acid phosphatase family member (Holt et al., 2002)	45
AGAP012401	Maltase-like protein (Zheng et al., 1995)	57
AGAP002378	Adenylosuccinate lyase (Jurecka et al., 2015)	54
AGAP010257	60S ribosomal protein L13a (Wool et al., 1995)	22
AGAP004462	40S ribosomal protein S25 (Li and Center, 1992)	17
AGAP010815	TEP1: thioester-containing protein 1 (Le et al., 2012)	132
AGAP006817	FACT complex subunit SPT16 (Adams et al., 2000)	124
AGAP008816	20S proteasome subunit alpha 5 (Groll et al., 1997)	27
AGAP010156	ATP citrate lyase (Elshourbagy et al., 1992)	118
AGAP012090	serine/threonine-protein kinase MRCK (Tan et al., 2008)	186

AGAP004164	glutathione S-transferase delta class 1 (Scian et al., 2015)	24
AGAP005660	CYP305A4: cytochrome P450 (Danielson, 2002)	60
AGAP010816	TEP3: thioester-containing protein 3 (Bou Aoun et al., 2011)	151
AGAP012089	nuclear inhibitor of protein phosphatase 1 (Van Eynde et al., 1995)	42

Table S2. Oligonucleotide primers used in the experiments, Related to Figures 2, 3, 4,6, S1, S3, and S4. The purpose of the primers is indicated, followed by the gene that was amplified. Forward primer (F) and Reverse primers (R) are indicated.

Analysis of *Plasmodium falciparum* infection of mice
P. falciparum 18S rRNA
F- CGTCGCTCCTACCGATTGAA
R- ACCTTGTTACGACTTCTCCTTCC
Human GAPDH
F- CTTGAGGCTGTTGTCATACTTC
R- GTCCACTGGCGTCTTCAC

Gene amplification for insertion into vectors for protein expression in *Escherichia coli*
AgTRIO
F- CTGCTAGGATCCGGAAGAAGCTCCCAAACCGGAGAAG
R- AAGCTAGCGGCCGCGGAGGTAGCGACCGAGGAGGAC
D7r1
F- GGATCC ATGCCAGCCTCACTC
R- GCGGCCGCGTTGCAAATCTTGTCATCG
SG1L3
F- GATCACGCGGCCGCATGACGCCATTGATCGCAACC
R- GATCTCTAGACTCAAACCCTTAAACAACTTGTA
SG1b
F- GATCGGATCCGCTTACCGACTGCTGGTGAGCT
R- GATCACGCGGCCGCCTACCCAGCATACGCGTGGAT

Quality assessment of the salivary gland cDNA library
SG1L3
F- GTGATCTTCTCGACCGATGTG
R- TGTACAGCGCTTCGTAATCC
AgTRIO
F- GGAAATGGGTGACAACGTAAAG
R- GTGACAGACGGTACACATACTC
Apyrase
F- GATCCGCTCTACAGATCCATTC
R- GTCAACCGGCCTACATACTTAC
gSG6
F- CTTGCTGTGAGCGTCAGTTA
R- GTGCTTTGCTGGCCATTTC
Lysozyme

F- GCCGTAGCCGAAGCTAAA
R- TTGAACCGTAGCCCGAATC

D7r1

F- GACACTCCCGATATGGACAAG
R- CCATCGTAGGTCCTTCCTTTAG.

Analysis of mosquito-borne *Plasmodium berghei* infection

AgTRIO

F- CGCTTCCCAACATTGTCAAC

R- TCTCAGCCGTAAGTGATTGC

Anopheles gambiae actin

F- GAAGGCTAACCGCGAGAAGATG

R- CGCCGGAGTCCAGCACGATA

P. berghei 18S rRNA

F- TGCCGAACTAAGTGTTGGATG

R- CGAATACTCGCCCCAGAAC

Mouse beta-2 microglobulin

F- CTCACACTGAATTCACCCCC

R- TCACATGTCTCGATCCCAGT

Hepatic nuclear factor 4 alpha

F- TCAAGGATGAAGAGCTTGCC

R- ACGTGTCTGATGTGATCTGC