APPENDIX

Arthropod exosomal glycine-rich protein as a potential vaccine candidate effectively reduces tick blood-feeding and pathogen transmission

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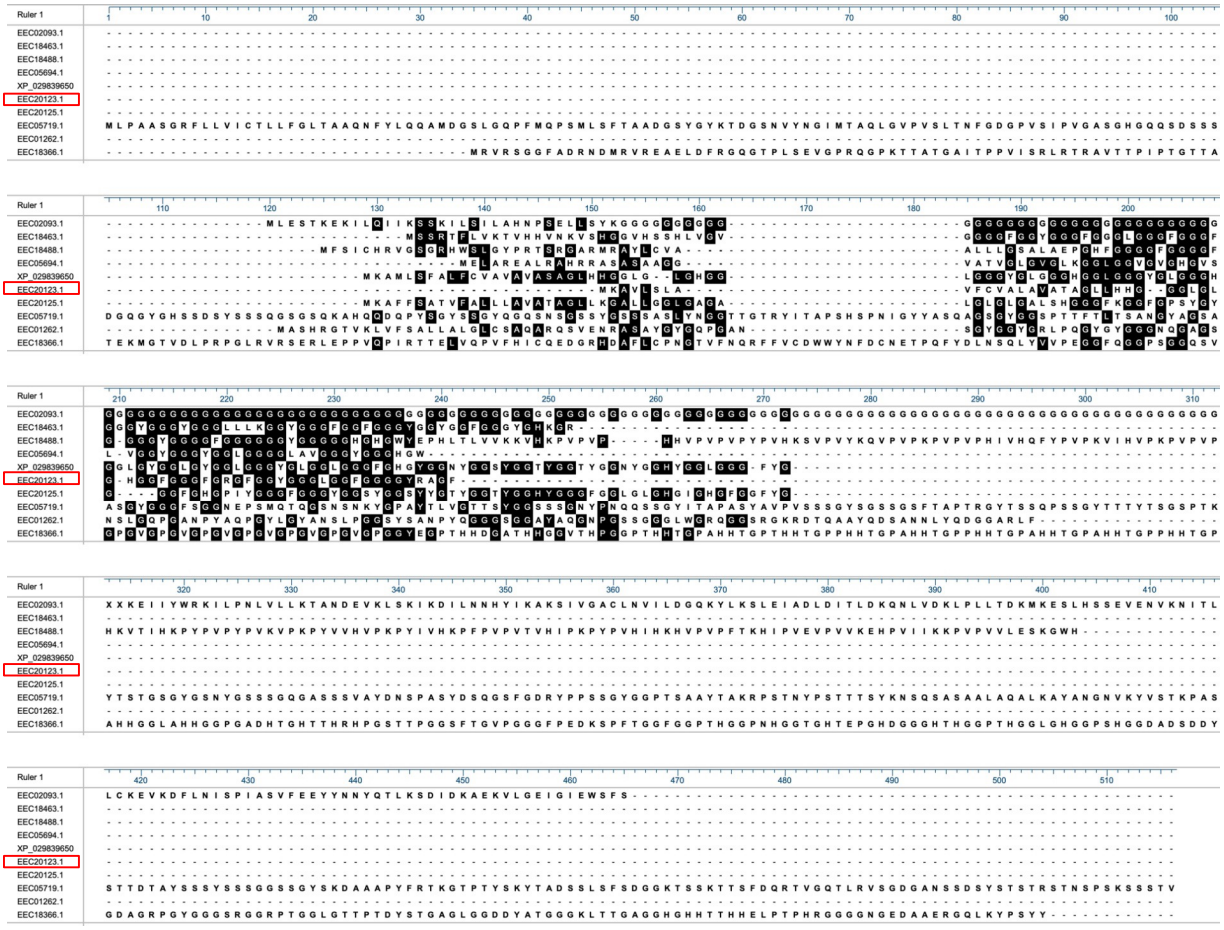
APPENDIX Information Includes

APPENDIX FIGURES AND LEGENDS

ClustalW Alignments of *Ixodes scapularis* glycine-rich family proteins



Appendix Figure S1

Appendix Figure S1. ClustalW Sequence alignment analysis of *Ixodes scapularis* GRPs. The deduced *I. scapularis* glycine-rich protein (GRP) amino acid sequence alignment using ClustalW program in DNASTAR Lasergene is presented. Black color represents the matching residues for easy identification. GenBank accession numbers of each sequence is provided at the left side and total length is shown on the top of the sequence alignment. The red boxes indicate EEC20123.1 protein.

Percentage of Identity and Phylogenetic analysis of *Ixodes scapularis* glycine-rich family proteins

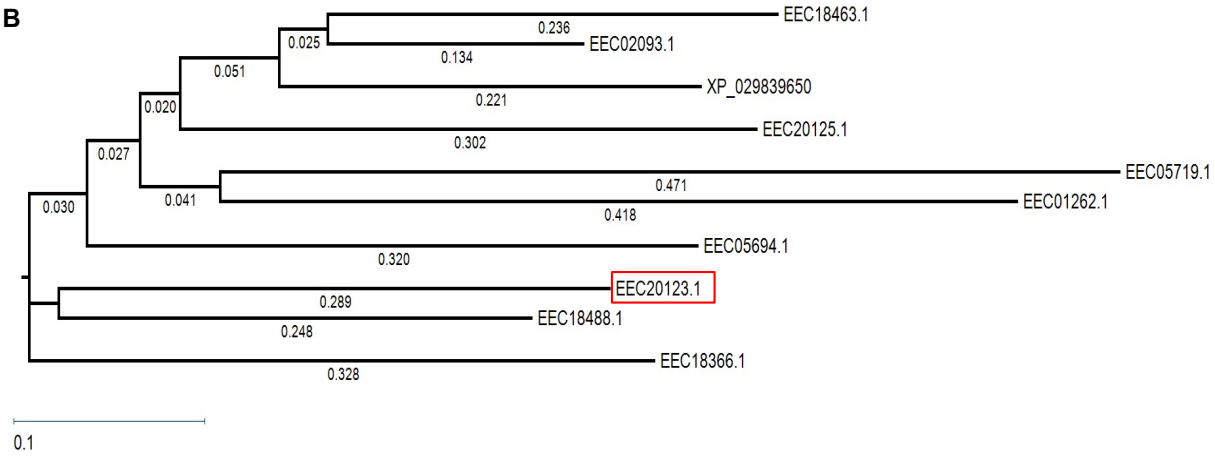
A

Percent identity

Distance Percentages

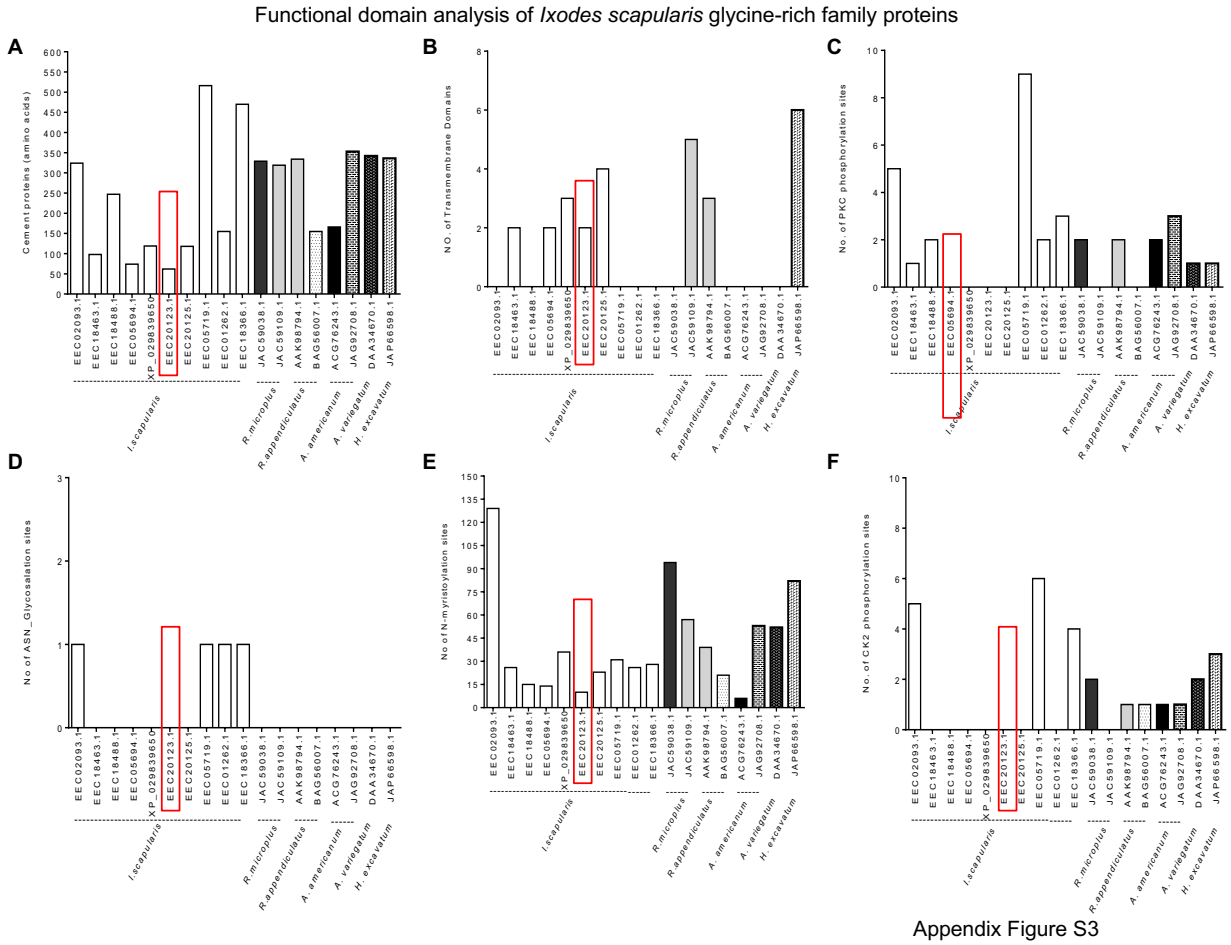
	A	B	C	D	E	F	G	H	I	J
EEC02093.1		62.96	50	48.15	62.96	42.59	44.44	22.22	27.78	35.19
EEC18463.1	0.37		35.19	27.78	50	29.63	40.74	24.07	16.67	27.78
EEC18488.1	0.5	0.65		38.89	35.19	46.3	38.89	11.11	20.37	40.74
EEC05694.1	0.52	0.72	0.61		40.74	35.19	24.07	12.96	22.22	31.48
XP_029839650	0.37	0.5	0.65	0.59		31.48	46.3	25.93	20.37	27.78
EEC20123.1	0.57	0.7	0.54	0.65	0.69		31.48	11.11	18.52	37.04
EEC20125.1	0.56	0.59	0.61	0.76	0.54	0.69		24.07	18.52	33.33
EEC05719.1	0.78	0.76	0.89	0.87	0.74	0.89	0.76		11.11	9.26
EEC01262.1	0.72	0.83	0.8	0.78	0.8	0.81	0.81	0.89		22.22
EEC18366.1	0.65	0.72	0.59	0.69	0.72	0.63	0.67	0.91	0.78	

B



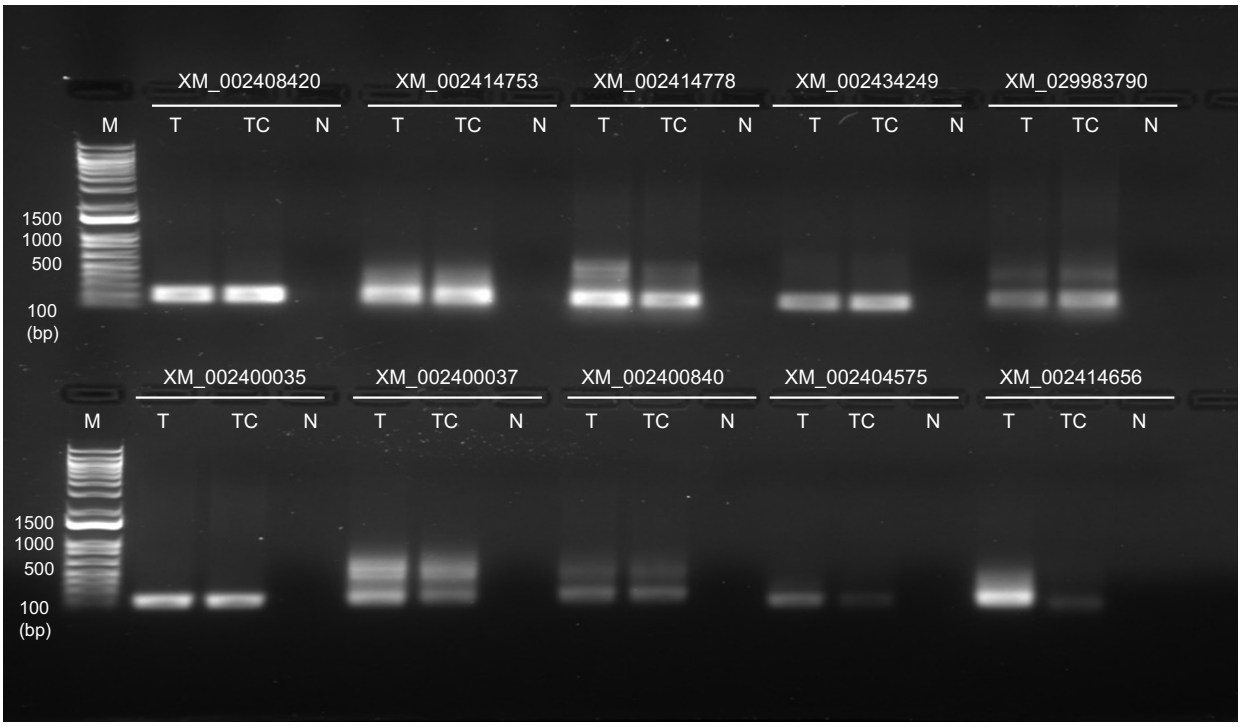
Appendix Figure S2

Appendix Figure S2. Percentage of identity/divergence and phylogenetic analysis of *Ixodes scapularis* GRPs. (A) Percent identity (horizontally presented) and distance (vertically denoted) of *I. scapularis* GRP amino acid sequence is shown. (B) Phylogenetic analysis showing *I. scapularis* GRP amino acid sequence was performed using ClustalW accurate/slow alignment method in DNASTAR Lasergene. GenBank accession numbers of each GRP molecule is provided at the left side (A) or on right side (B). The total distance in clades is shown for each GRP (B). The red boxes indicate EEC20123.1 protein.



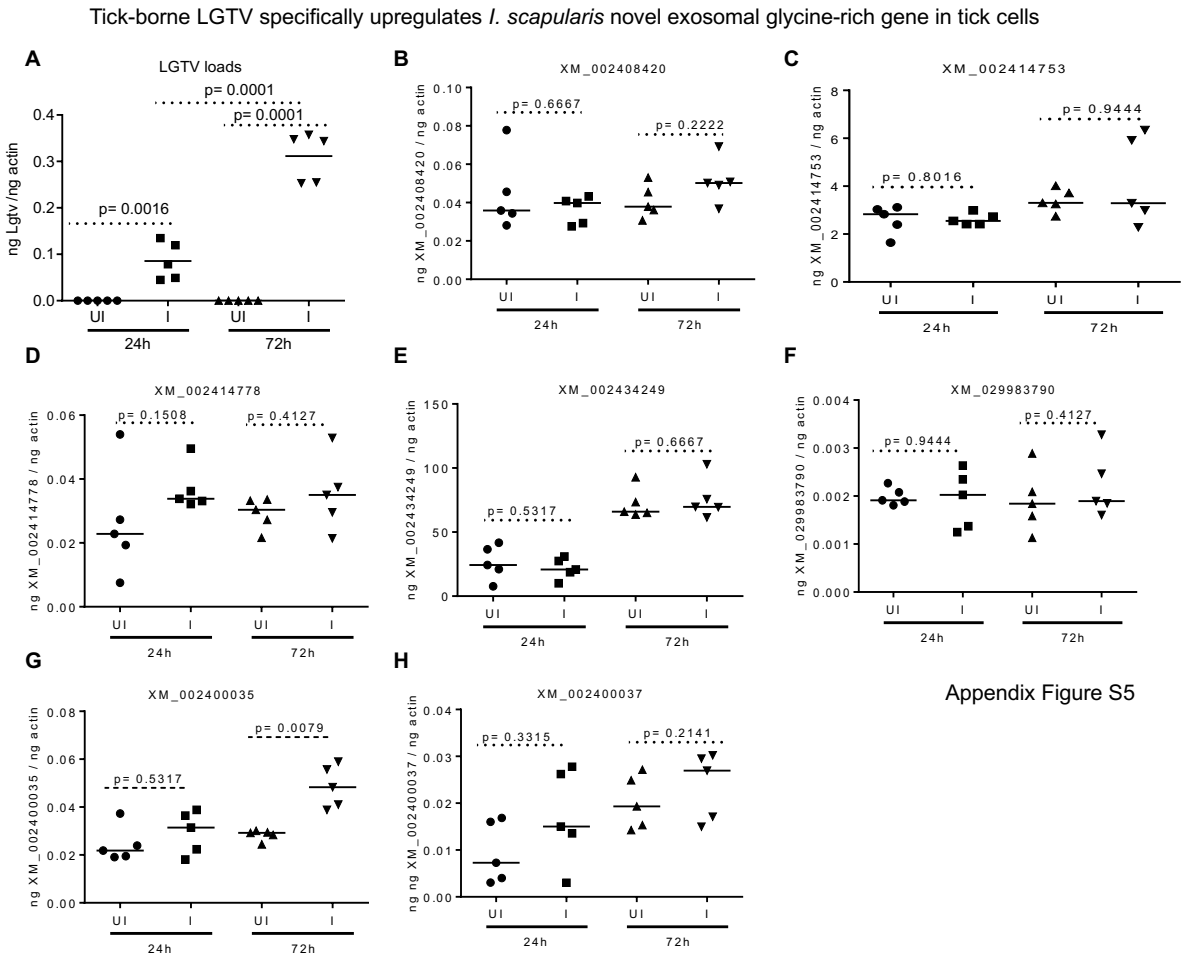
Appendix Figure S3

Appendix Figure S3. Bioinformatic and comparative analysis of GRPs from medically important ticks. Tick GRP amino acid sequences were collected from medically important vectors showing (A) total number of amino acid sequence (X-axis) with corresponding accession numbers (Y-axis), (B) Number of transmembrane domains in each molecule was analyzed using TMHMM server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/), (C) Number of PKC phosphorylation sites, (D) Number of N-glycosylation sites, (E) Number of N-myristoylation sites, and (F) Number of CK2 phosphorylation sites analyzed by PROSITE databases. The red boxes indicate EEC20123.1 protein. Organism names and GenBank accession numbers are presented at the bottom of each panel.



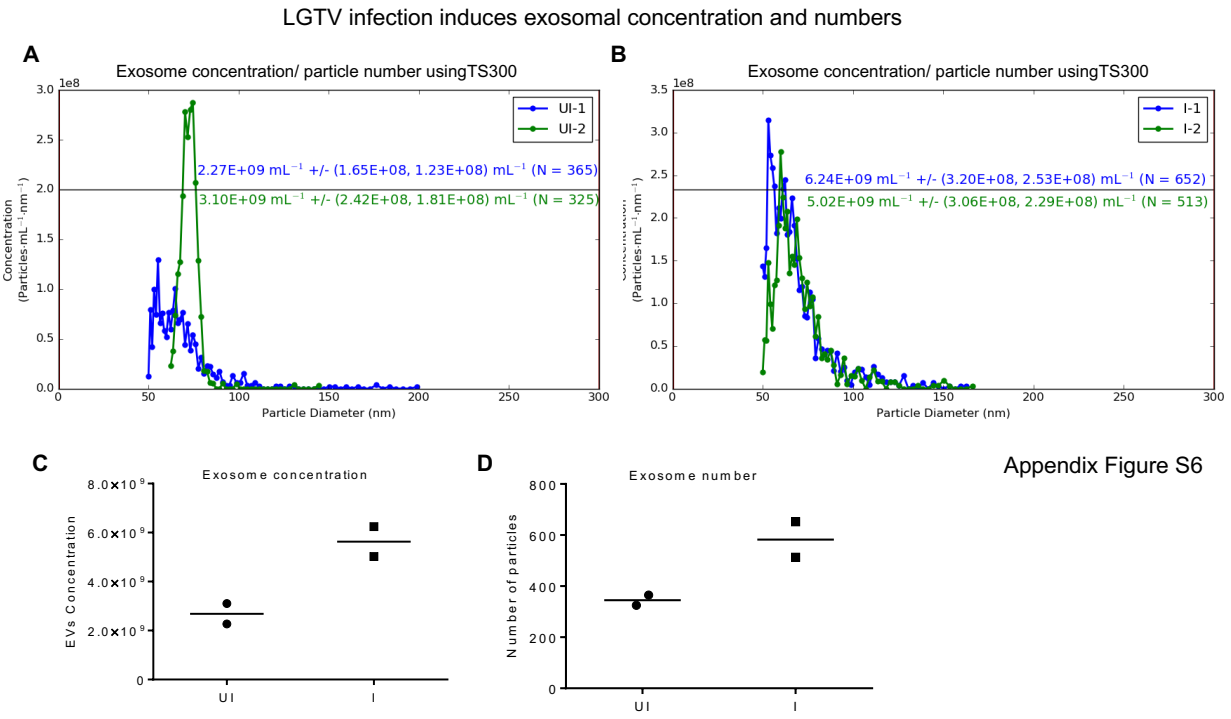
Appendix Figure S4

Appendix Figure S4. PCR Amplification of GRP genes from *Ixodes scapularis* ticks or ISE6 tick cells. Gel electrophoresis image showing amplification of *I. scapularis* GRPs in tick or tick cells. The cDNA was synthesized from total RNA isolated from tick or tick-cells and used as template for PCR amplifications. GenBank accession numbers are presented corresponding to each GRP molecule. T and TC denotes nymphal tick and tick cell cDNA, respectively. N represents no template control, corresponding to each fragment. M represents DNA marker, and bp indicates base pairs.

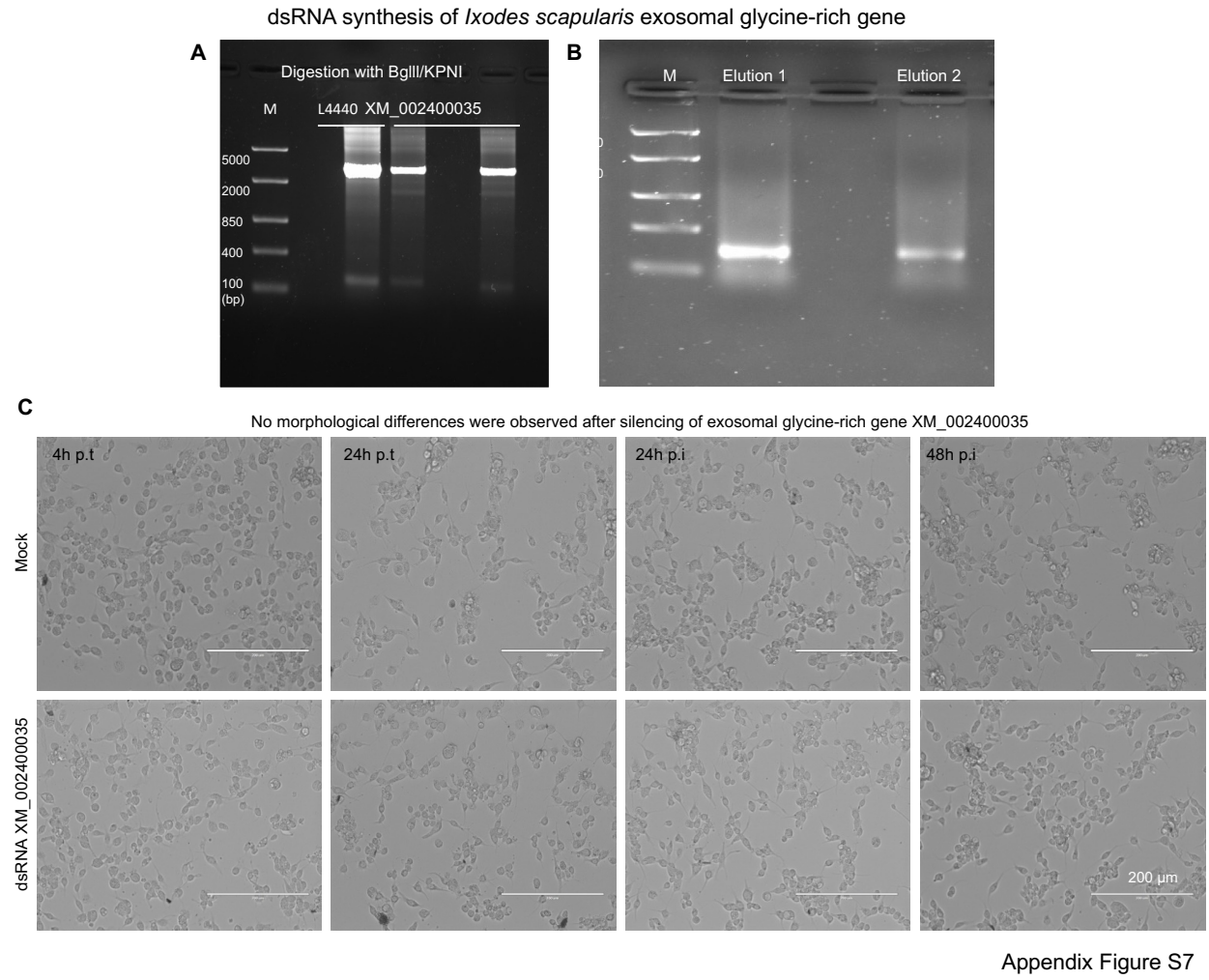


Appendix Figure S5. Analysis of *Ixodes scapularis* GRP family gene expression upon LGTV infection in ISE6 tick cells. QRT-PCR analysis showing LGTV loads (A) and expression of seven genes (B) XM_002408420, (C) XM_002414753, (D) XM_002414778, (E) XM_002434249, (F) XM_029983790, (G) XM_002400035 and (H) XM_002400037 in uninfected (UI) or LGTV-infected (I) tick cells (at 24 h and 72 h p.i., MOI 1). Each, circle, square, triangle, or inverted triangle indicates UI and I (at 24 h or 72 h p.i.) respective groups. Each circle or square or triangle/inverted triangle represents data from one independent culture well. The exact number of samples for each panel representing multiple experiments is 5 in all panels (5A-H). The viral loads and expression of genes are normalized to tick beta-actin mRNA levels. Quantities in Y-axes are variable. Statistical differences in (A) are calculated using one-

way ANOVA with Tukey's multiple comparisons test, and in (B-H) Mann-Whitney U test was used, and *p* values are shown. The $p<0.05$ are considered as statistically significant. No statistical significance is found between UI and I (in panels B-F and H).



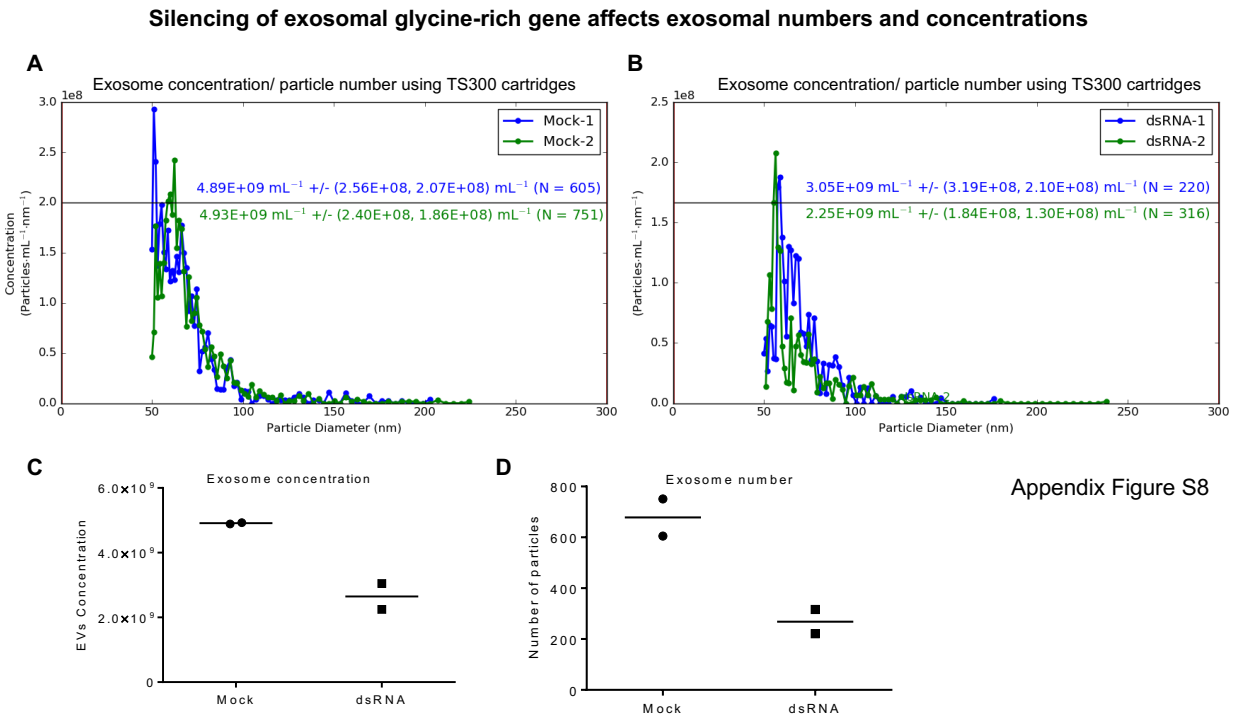
Appendix Figure S6. Tick cell-derived exosomes particle size and concentration measurement using nanoparticle analyzer. Spectradyne nCS1 nanoparticle analyzer was used to count the particle sizes using TS-300 filter/cartridge (50–300 nm particle sizes counting range) during the transit time in uninfected (UI) (A) or infected (I) (B) groups, respectively. (C) EVs concentration and number of exosome particle numbers (D) measured in tick cell-derived exosomes collected from uninfected (UI) or LGTV-infected (I) groups is shown. The exact number of samples is 2 in all panels (S6A-D). The small sample number is due to the discontinued supply of TS-300 filter/cartridges from Spectradyne, Inc. Statistical differences were not calculated as sample sizes were small. The data is shown as a part of reproducibility for data using TS-400 filter/cartridges (65–400 nm particle sizes counting range) shown in Figure 4.



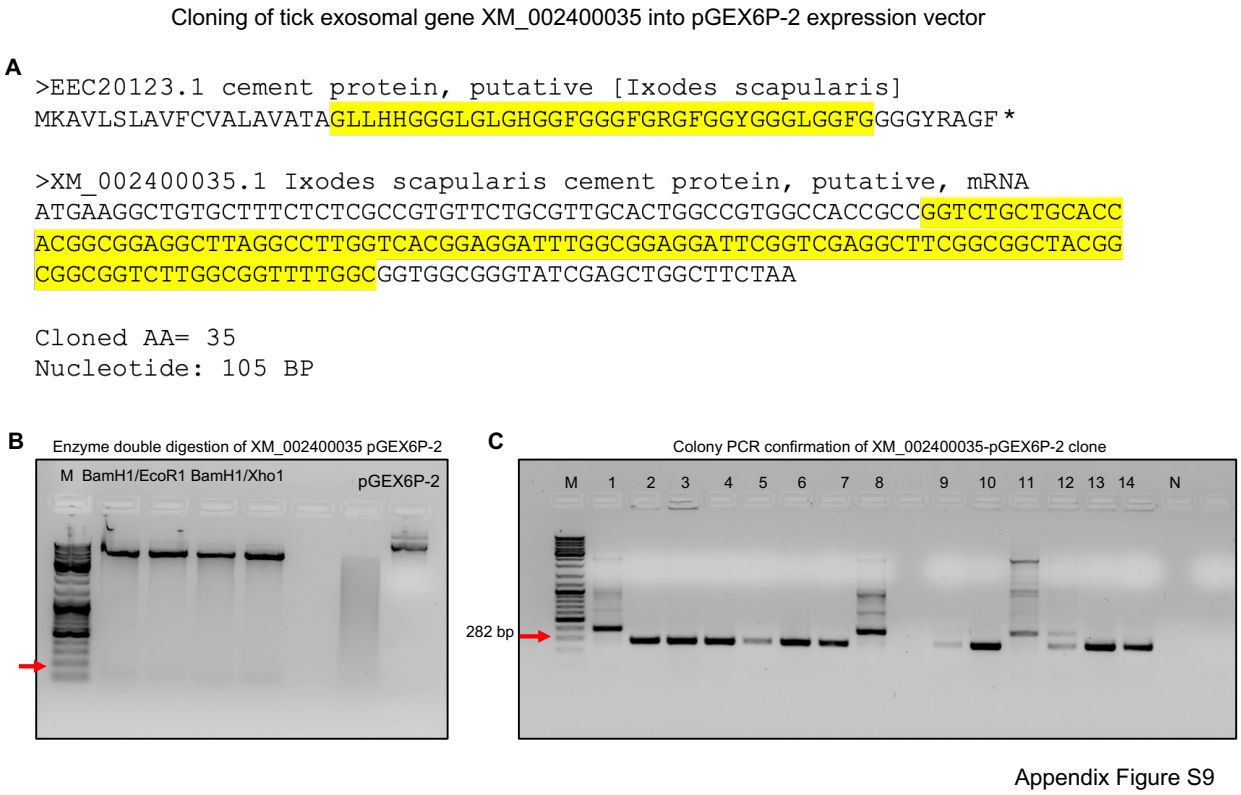
Appendix Figure S7

Appendix Figure S7. dsRNA synthesis of *Ixodes scapularis* exosomal gene XM_002400035 and morphology of tick cells after dsRNA transfections. (A) Gel electrophoresis image showing digestion of successful construct cloned in L4440 vector using *Bgl*II-*Kpn*I restriction enzyme sites. (B) The dsRNA complementary to *I. scapularis* exosomal XM_002400035 sequence was synthesized using MEGA script RNAi kit (Invitrogen, USA). Gel electrophoresis image showing elutions 1 and 2 of the synthesized dsRNA. M represents DNA marker, and bp indicates base pairs. (C) Treatment with XM_002400035-dsRNA had no cytotoxic effects or morphological changes in tick cells. ISE6 cells were treated with mock-dsRNA or XM_002400035-dsRNA to observe any cytopathic effects or morphological changes upon

XM_002400035 silencing. Representative images were captured using the EVOS auto-fluorescence System M7000 at 4 h post transfection (p.t), or 24 h p.t, and 24 h post infection (p.i.) or 48 h p.i.. Scale bar indicates 200 μm in all images.



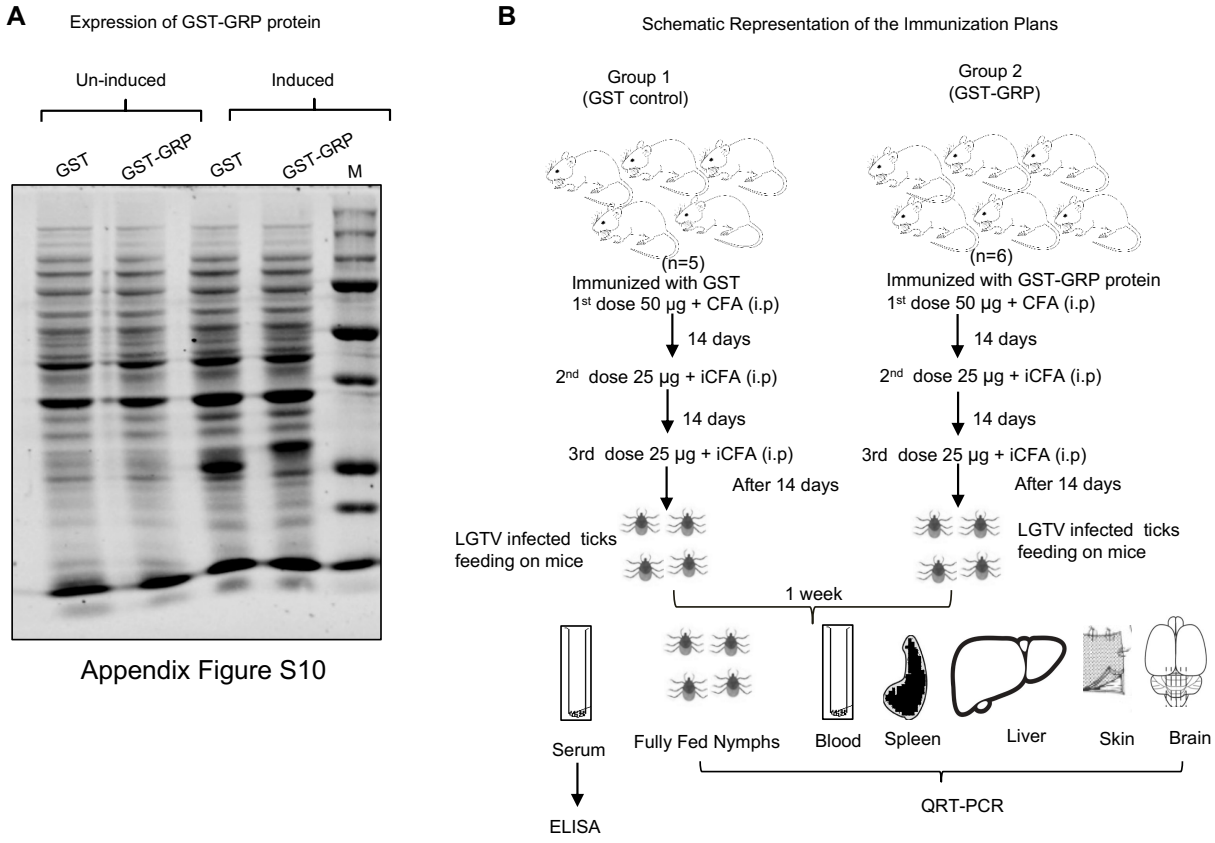
Appendix Figure S8. Mock-dsRNA or XM_002400035-dsRNA transfected tick-cell derived exosome concentration measurement using nanoparticle analyzer. Spectradyne nCS1 nanoparticle analyzer was used to count the particle sizes using TS-300 filter/cartridge (50-300 nm particle sizes counting range) during the transit time in mock-dsRNA (A) or XM_002400035-dsRNA (B) treated groups, respectively. (C) Exosome concentration and number of exosome particles (D) measured from mock-dsRNA or XM_002400035-dsRNA transfected tick cells are shown. The exact number of samples is 2 in all panels (S8A-D). The small sample number is due to discontinued supply of TS-300 filter/cartridge from Spectradyne, Inc. Statistical differences were not calculated as sample sizes were small. The data is shown as part of reproducibility using TS-400 filter/cartridge (65–400 nm particle sizes counting range) shown in Figure 6.



Appendix Figure S9

Appendix Figure S9. Cloning of *Ixodes scapularis* exosomal gene XM_002400035 in pGEX6P-2 expression vector. (A) The fragment of exosomal GRP (35 amino acids polypeptide or 105 bp PCR fragment, denoted as highlighted region) cloned into pGEX-6P-2 expression vector is shown. (B) Gel electrophoresis images showing double digestion of successful constructs cloned into pGEX6P-2 vector using BamHI-EcoRI or BamHI-XhoI restriction enzyme sites. (C) Clones from BamHI-XhoI were validated with colony PCR using F Primer from insert and reverse primer from vector. Final selected clones were validated by sequencing at EUROFINS.

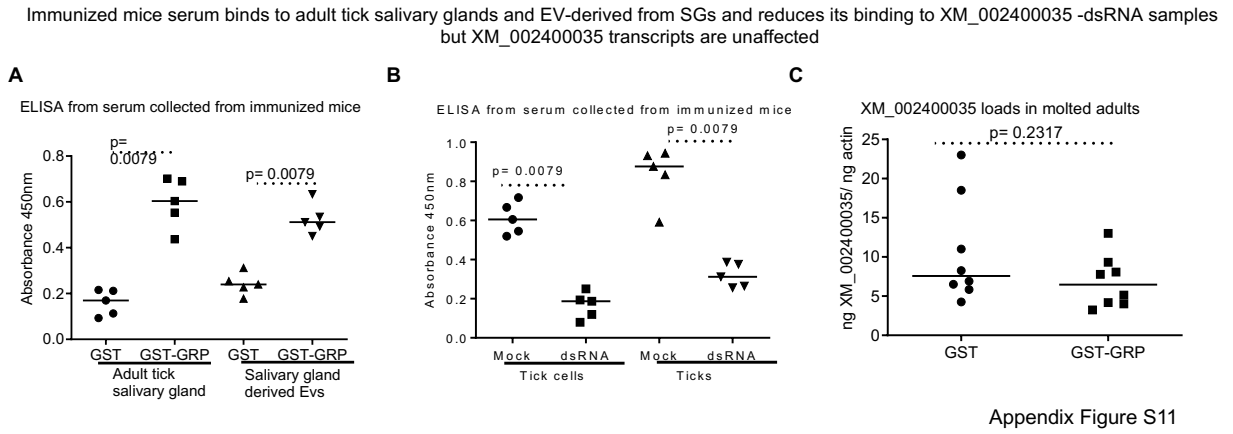
Induction of GST-GRP (glycine-rich protein) and schematic representation showing mice immunization



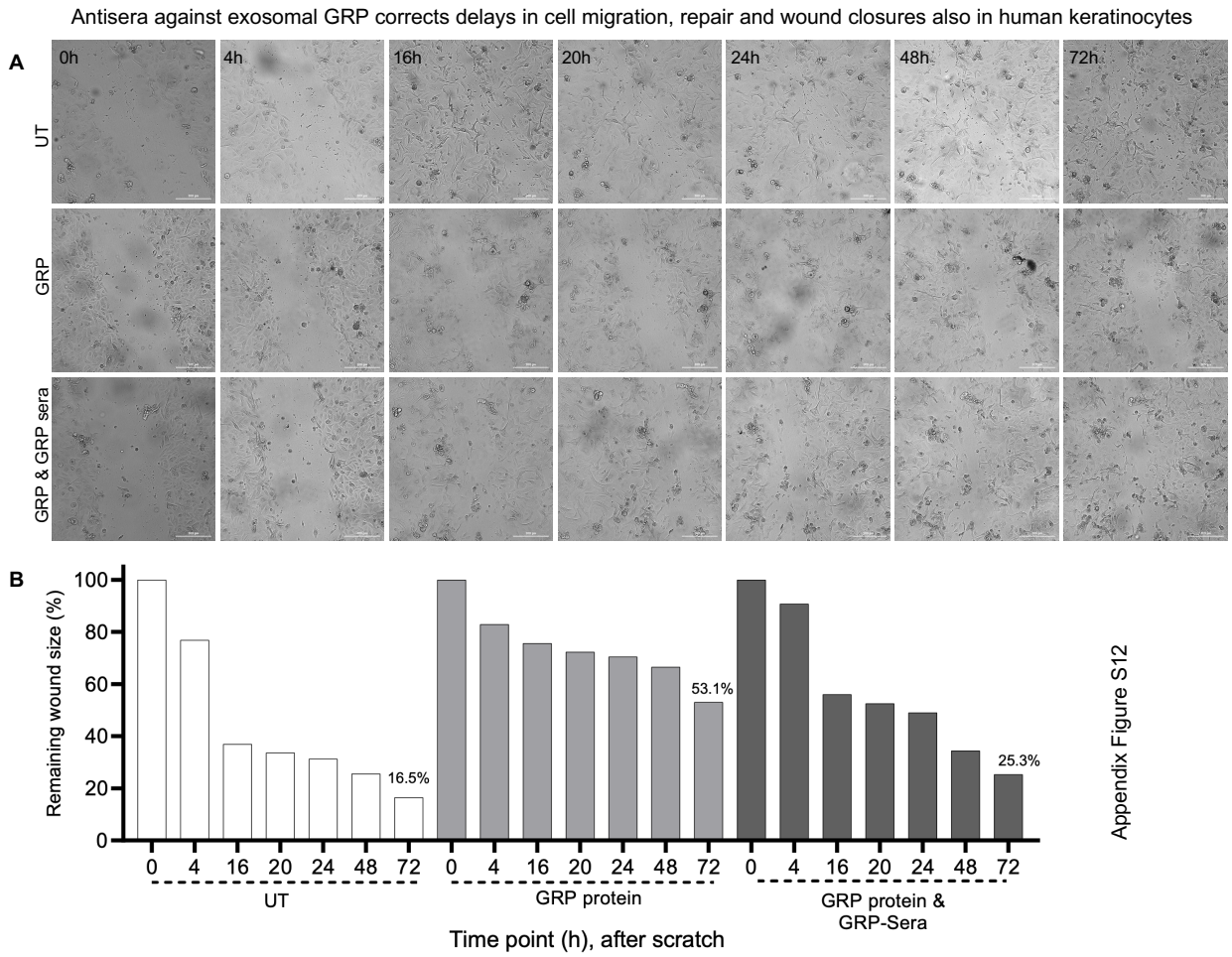
Appendix Figure S10

Appendix Figure S10. Purification of recombinant tick exosomal GRP and schematic representation of immunization in mice. (A) Coomassie stained gel image showing uninduced and induced GST-GRP or GST-alone proteins induced in *E. coli* BL-21 cell lysates. M represents the protein ladder. (B) Schematic showing C57BL/6 wild type mouse immunization with GST or GST- GRP or Freund's adjuvant at two-week of intervals. At days 0, 14, and 28, mice were actively immunized (intraperitoneal) with either GST or GST- GRP purified proteins (50 µg/mouse for first dose with Complete Freund's adjuvant and 25 µg/mouse for second and third booster dose with incomplete Freund's adjuvant). After 14 days of third booster of mice, LGTV-infected ticks were allowed to feed on these immunized mice. Ticks were allowed to fully

engorge and were collected after repletion. After 7 days of tick feeding and repletion, mice blood and tissues (brain, liver, spleen, and skin) were harvested for further analysis.

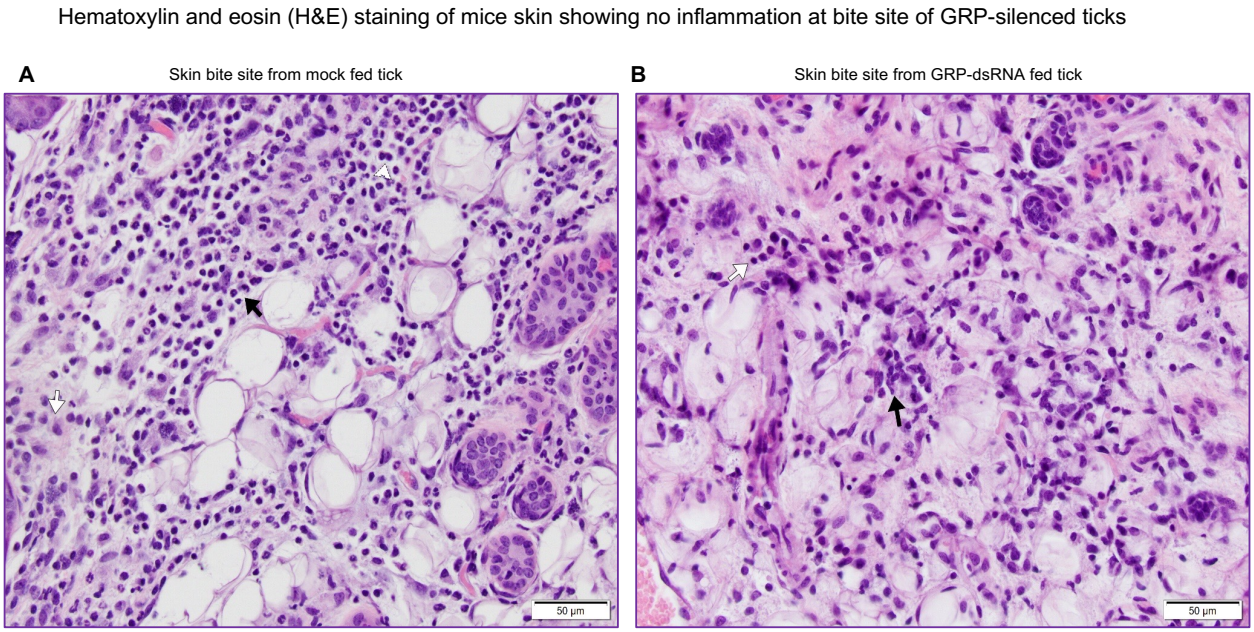


Appendix Figure S11. GST- GRP **immunized mice serum binds to endogenous protein.** (A) ELISA assay showing enhanced binding of GST- GRP protein immunized serum with endogenous lysates prepared from *I. scapularis* adult salivary glands (SGs), and EVs derived from these SGs. (B) GST- GRP protein immunized serum showing reduced binding with tick cells/fed nymphal tick lysates collected from LGTV-infected, and mock-dsRNA treated (Mock) or LGTV-infected and XM_002400035-dsRNA treated (dsRNA) groups. Each, circle, square, triangle, or inverted triangle indicates respective groups. Each circle or square or triangle/inverted triangle represents data from one replicate. The exact number of samples for each panel representing multiple experiments is 5 in both panels (S15A, B). (C) XM_002400035 transcript levels in molted adult ticks (nymphal ticks that had fed on GST or GST- GRP immunized mice and repleted were molted into adult ticks) is shown. Exact number of samples for each group representing multiple experiments (in panel C, n= 8 ticks in each group) is shown. Statistical differences are calculated using Mann-Whitney U test and p values are shown. The $p<0.05$ are considered as statistically significant.



Appendix Figure S12. Treatment with GST- GRP antisera restored wound-healing and repair process in human primary cultures of keratinocytes. (A) Scratch-based assays performed on on human keratinocytes primary cultures incubated with 2 µg/ml of GST- GRP alone (for 72 h), or GST- GRP with GST-GRP protein immunized mice sera in 1:100 dilution are shown. Phase contrast images (obtained using Cytation 7 imaging system) of human keratinocytes primary cultures were taken for selected time-points (of 0, 4, 16, 20, 24, 48 and 72 h) and using 10 x magnification. Untreated (UT) human keratinocytes primary cultures served as internal control. Scale bar indicates 1000 µm for each image per group or timepoint. (B) Measurement of remaining wound size diameters (analyzed by ImageJ software)

at different time-points (of 0, 4, 16, 20, 24, 48 and 72 h) post-treatment are shown. Wounds at 0 h is considered as 100% for all groups, including untreated (UT) control.



Appendix Figure S13

Appendix Figure S13. Novel tick exosomal GRP reduced cell infiltration/inflammation to the tick bite site. (A) Hematoxylin and eosin (H&E) staining of skin tissue of mice that allowed feeding of LGTV-infected and mock-dsRNA treated group or LGTV-infected and XM_002400035-dsRNA-treated groups is shown. In skin biopsies collected from mice fed with mock-dsRNA ticks, the panniculus contains moderate to large numbers of neutrophils (black arrow), lymphocytes (white arrowhead), plasma cells, and lower number of macrophages (white arrow/arrowhead). However, the panniculus containing moderate number of lymphocytes (white arrow) mixed with few neutrophils (black arrow) and few macrophages and plasma cells in skins samples from mice fed with LGTV-infected and XM_002400035-dsRNA-treated ticks is shown. There is mild crush artifact in this group. Both images are shown at 400x magnification. Scale bar indicates 50 µm for each image. Enlarged images from Figure 9A and B are shown again in this Supplemental Figure for better visualization.

APPENDIX TABLES

Glycine-rich family proteins combed and analyzed from *Ixodes scapularis genome*

Type	Name	Protein accession	NCBI nucleotide accession	Vector base accession	Signal peptide
1	Glycine-rich protein, putative	EEC02093.1	XM_002408420	ISCW016023-RA	no
2	Glycine-rich protein, putative	EEC18463.1	XM_002414753	ISCW014994-RA	no
3	Glycine-rich protein RIM36, putative	EEC18488.1	XM_002414778	ISCW013867-RA	yes
4	Glycine-rich protein, putative	EEC05694.1	XM_002434249	ISCW005150-RA	no
5	Glycine-rich protein 23-like	XP_029839650	XM_029983790	ISCW023479-RA	yes
6	Glycine-rich protein, putative	EEC20123.1	XM_002400035	ISCW015452-RA	yes
7	Secreted glycine rich protein, putative	EEC20125.1	XM_002400037	ISCW015455-RA	yes
8	Glycine-rich protein, putative	EEC05719.1	XM_002400840	ISCW018356-RA	yes
9	Glycine-rich protein, putative	EEC01262.1	XM_002404575	ISCW000104-RA	yes
10	Glycine rich secreted protein, putative	EEC18366.1	XM_002414656	ISCW014486-RA	no

Appendix Table S1

Appendix Table S1. Glycine-rich proteins combed from *Ixodes scapularis* genome. Ten selected GRPs proteins combed from *I. scapularis* genome are shown as types 1-10. The names have been retained from database. Protein accession numbers and NCBI nucleotide numbers are shown from GenBank. In addition, vector base accession numbers are shown for each protein. Signal peptide presence or absence was determined using SignalIP. Out of 10, 6 of the GRP molecules are shown to have N-terminal signal peptide.

Appendix Table S2. Oligonucleotides used in this study.

Forward Primer (5'-3')	Reverse Primer (5'-3')	Tick GRP Expression
GTTTGAATGTTATTTTAGATGGGCAGA	GCTTTCTTTCATTTTATCTGTTAATAGAGGT	XM_002408420
GGACATTCCTCGTGAAGACTGTG	GCCGCCAAATCCGCCA	XM_002414753
GGCACGTGGTGGGGCA	CTTCGGAGGCGGTGGCT	XM_002414778
GCGGGGTGGCGACAGT	CGACTGCCAGACCACCACCT	XM_002434249
GAAGGCTATGCTTTCTTTCGCT	CCGTGACCACCACCCAGA	XM_029983790
GCTGTGCTTTCTCTCGCCGT	CGCCGTAGCCGCCGA	XM_002400035
CCGTCTTCGCGCTTCTCCT	GCCGTAGCTGCCACCGT	XM_002400037
CCCAGAACTTCTACCTCCAGCA	CGAGACAGGACCATCACCGA	XM_002400840
GTCGCACAGAGGCACCGT	GCGCGTAGGGGTTGGCT	XM_002404575
GAGCTCGACTTCCGTGGTCA	CCGTGCCCATTTTTTCCGT	XM_002414656
Forward Primer (5'-3')	**Reverse Primer (5'-3')**	**XM_002400035 dsRNA**
TGAGATCTGCTGTGCTTTCTCTCGCCGT	CGGGTACCCGCCGTAGCCGCCGA	XM_002400035 (dsRNA synthesis)
CTTGGTCACGGAGGATTTG	TAGAAGCCAGCTCGATACC	XM_002400035 (Silencing efficiency)
TGGGATCCGGTCTGCTGCACCACGG	CGCTCGAGGCCAAAACCGCCAAGACCGCCGCCGTAGCC	XM_002400035 - pGEX6P-2 cloning
	CCTGACGGGCTTGTCTGCT	pGEX-6P2 sequencing primer
CGCCAAGGTCGTCGCCG	TTGGCTCTGGCGATGTGGC	Mouse CXCL-12 primers

Appendix Table S3: Quantification of Western blots presented in this study.

Figure	Blot	Treatment	Band intensity
Figure 5E	Tick cell lysate, NS1	UI	N/A
		I	297369
		Mock	341695
		dsRNA	119584
	Tick cell lysate, Actin	UI	36019
		I	29112
		Mock1	31164
		dsRNA	35616
Figure 5F	Tick cell EVs, NS1	UI	N/A
		I	6500
		Mock	30420
		dsRNA	7956
	Tick cell lysate, Actin	UI	44090
		I	46038
		Mock	55708
		dsRNA	48661
Figure 6E	Tick lysate, NS1	Mock	59445
		dsRNA	38025
Figure 7C	rProtein-GST	GST	712,288
	rProtein-GST-GRP	GST-GRP	947,472
	Adult-tick SG-lysate	SGs	257,472
	SGs-derived EVs	SG-EVs	140,512
	Tick cell lysates	Mock	691748
		dsRNA	282163
	Fed Nymphal ticks	Mock	154629
		dsRNA	67254

Appendix Table S4: Median values in experimental samples compared using Mann-Whitney statistical analysis.

Figure	Panel	Sample	Median values
Figure 3	3A	UI	0
		I	0.009875
	3B	UI	0
		I	4027
	3C	UI	0.2003
		I	0.4419
	3D	UI	0.0002142
		I	0.0004872
	3G	UI	1.170e+009
		I	3.240e+009
	3H	UI	594
		I	1413
Figure 4	4B	UI	0
		I	21.22
	4C	UI	1.86
		I	3.13
	4D	UI	0
		I	1.42
	4E	UI	45.4
		I	67.6
Figure 5	5A	Mock	0.0001344
		dsRNA	4.795e-005
	5B	Mock	25.68
		dsRNA	6.58
	5C	Mock	0.2786
		dsRNA	0.0320
	5D	Mock	0.817
		dsRNA	.348
	5I	Mock	3.570e+009
		dsRNA	1.310e+009
	5J	Mock	1202
		dsRNA	634
	5K	Mock	7.3
		dsRNA	1.7
	5L	Mock	18.33
		dsRNA	2.69
Figure 6	6B	Mock	5.15
		dsRNA	2.60
	6C	Mock	.29
		dsRNA	.122
	6D	Mock	0.073

		dsRNA	0.028
	6E	Mock	2.05
		dsRNA	0.0434
	6F	Mock	0.24
		dsRNA	0.58
	6G	Mock-brain	5.920e-005
		dsRNA-brain	1.630e-005
		Mock-liver	2.290e-005
		dsRNA-liver	2.800e-005
		Mock-spleen	7.970e-005
		dsRNA-spleen	2.890e-005
		Mock-Skin	9.949e-006
		dsRNA-Skin	4.480e-006
	6H	Mock	.2480
		dsRNA	.5810
Figure 7	7B	GST	.4720
		GST-GRP	.7420
	7E	GST	4.9
		GST-GRP	3.3
	7F	GST	0.006
		GST- GRP	0.005
	7G	GST	0.60
		GST- GRP	0.32
	7J	GST	3.07
		GST- GRP	1.41
	7K	GST	0.022
		GST- GRP	0.195
		GST	0.0485
		GST-v	0.0895
	7L	GST	1.3
		GST- GRP	0.33
	7M	GST-Brain	1.5
		GST- GRP -brain	0.11
		GST-Liver	0.0028
		GST- GRP -Liver	0.009
		GST-Spleen	0.10
		GST- GRP-spleen	0.01
		GST-Skin	0.82
		GST- GRP skin	0.22
Figure 8	8C	Mock	1.4
		dsRNA	0.515
	8F	GST	0.2
		GST- GRP	0.77

Supplementary Figures			
S Figure 5	S5B	UI-24	0.035
		I-24	0.039
		UI-72	0.037
		I-72	0.050
	S5C	UI-24	2.83
		I-24	2.55
		UI-72	3.3
		I-72	3.2
	S5D	UI-24	0.022
		I-24	0.033
		UI-72	0.03
		I-72	0.03
	S5E	UI-24	24.32
		I-24	20.79
		UI-72	65.9
		I-72	69.7
	S5F	UI-24	0.001
		I-24	0.002
		UI-72	0.001
		I-72	0.001
	S5G	UI-24	0.02
		I-24	0.03
		UI-72	0.02
		I-72	0.04
	S5H	UI-24	0.007
		I-24	0.015
		UI-72	0.019
		I-72	0.026
S Figure 11	S11A	GST- Tick SG	.17
		GST- GRP tick SG	.60
		GST- Tick SG-EVs	.24
		GST- GRP tick SG-EVs	.51
	S11B	Mock	0.6
		dsRNA	.18
		Mock	.87
		dsRNA	.31
	S11C	GST	7.5
		GST- GRP	6.4