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Host-specific expression of *Ixodes scapularis* salivary genes

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ABSTRACT

Ixodes scapularis vectors several pathogens including *Borrelia burgdorferi*, the agent of Lyme disease. *N*ymphal and larval stages, and the pathogens transmitted by *I. scapularis* are maintained in a zoonotic cycle involving rodent reservoir hosts, predominantly *Peromyscus leucopus*. Humans are not reservoir hosts, however, accidental encounters of infected ticks with humans, results in pathogen transmission to the human host. Laboratory models of non-reservoir hosts such as guinea pigs develop a strong immune response to tick salivary proteins and reject ticks upon repeated tick infestations. Anecdotal and scientific evidence suggests that humans that get frequent tick bites might also develop resistance to ticks. *Mus musculus*, the laboratory model of natural host, does not develop resistance to *I. scapularis* upon repeated tick infestations. Addressing this dichotomy in vector-host interaction, we present data that suggest that the salivary transcriptome and proteome composition is different in mouse and guinea pig-fed *I. scapularis,* and that these differences might contribute to differences in host immune responses. These findings reveal a new insight into vector-host interactions and offer a functional paradigm to better understand the phenomenon of acquired tick-resistance.

1. Introduction

Ixodes scapularis is the tick vector of several pathogens including *Borrelia burgdorferi sensu lato*, the agent of Lyme disease (McNabb et al., 2008; Nelder et al., 2016; Steere et al., 1977). Tick-transmitted pathogens are enzootically maintained in *Peromyscus leucopus*, the white-footed mouse, the primary reservoir host for larval and nymphal stages (Radolf et al., 2012). Larvae acquire pathogens from infected mice, and infected nymphs transmit pathogens to mice during tick engorgement, and thus the life cycles of the pathogen, the reservoir host, and the vector are entwined (Barbour and Fish, 1993). When *I. scapularis* nymphs feed on non-reservoir hosts such as guinea pigs and rabbits, these animals mount an immune response and upon subsequent infestations reject the ticks within 12–24 hours of tick attachment (Allen, 1989; Wikel, 1996). This phenomenon of acquired resistance to tick feeding (Trager, 1939) is characterized by rapid recruitment of basophils to the tick bite-site (Brossard and Fivaz, 1982; Wikel and Alarcon-Chaidez, 2001), and is mediated by the concerted activation of humoral and cellular responses to salivary proteins secreted into the bite-site (Brown and Askenase, 1985; Wikel and Alarcon-Chaidez, 2001) and potentially critical for tick feeding. Understanding the molecular basis of the acquired tick-resistance would accelerate efforts to define tick salivary proteins critical for tick feeding and pave the way for the design and development of anti-tick vaccines targeting these critical salivary proteins.

Recruitment of basophils to the bite-site followed by their degranulation is invoked in effective rejection of ticks by mechanisms that are not fully understood (Brown, 1982; Brown and Askenase, 1983, 1985). In sharp contrast to non-reservoir hosts, mice, the reservoir hosts for *I. scapularis*, do not deter tick feeding upon repeated *I. scapularis* infestations (Wikel et al., 1997).

Until recently, this dichotomy in immune response was simply attributed to the lack of basophils in mice. It is now established that mice do have basophils (Sullivan et al., 2011; Wada et al., 2010), and the absence of resistance to *I. scapularis* in the murine host is clearly not due to the absence of basophils. More recently, Anderson et al (Anderson et al., 2017) have performed elegant histopathological examinations of tick bite-sites on natural (*Mus musculus* and *Peromyscus leucopus*) and non-natural hosts (*Cavia porcellus*) and demonstrate that while there is increased inflammation in the dermis of both natural and non-natural

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hosts, the architecture of the tick bite-sites are distinct. In contrast to the bite site on natural hosts, the tick bite-site in the non-natural host showed marked hyperkeratotic changes in the epidermal layer and disintegration of dermal structures (Anderson et al., 2017). These findings garner support for the immune evasion hypothesis that suggests that *I. scapularis* ticks are able to repeatedly infest permissive hosts such as white-footed mice not because of "immune incompetence" of the host, but rather, due to the ability of the tick to effectively circumvent the immune agonists of the murine host (Anderson et al., 2017).

Extending this immune evasion hypothesis further, we now show that the proteome composition of tick saliva is different when *I. scapularis* feeds on the murine (permissive host) or guinea pig (non-permissive host) and is consistent with the recent findings by Tirloni et al (Tirloni et al., 2017) and provide a new insight into the functional genome of *I. scapularis* in the context of tick-host interactions. We present evidence that suggests that guinea pig-fed salivary extracts might more readily elicit the production of IL-4 in basophils, a T_H2-defining cytokine (Pulendran and Artis, 2012). This suggests that host-specific expression of salivary proteins might provoke the host immune responses differentially and thus contribute, in part, to the differences in the immune response to tick feeding on natural and accidental hosts.

2. Materials and methods

2.1. Ethics statement

Animal care and housing essentially followed the rules described in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, USA. The protocols described below for the use of mice and guinea pigs were reviewed and approved by the Yale University Institutional Animal Care and Use Committee (YUIACUC) and the approved Protocol number is 2018-07941. All animal experiments were conducted in a Biosafety Level 2 animal facility according to the YUIACUC rules. All data generated in this work will be readily shared and available upon request.

2.2. Ticks and animals

I. scapularis adults, nymphs and larvae were obtained from a tick colony at the Connecticut Agricultural Experiment Station in New Haven CT, USA and maintained in an incubator at 23 °C and 85% relative humidity under a 14-hour light, 10-hour dark photoperiod. To obtain guinea pig fed tick salivary gland extracts, 30 pathogen free nymphs were placed on each 6-weeks old Hartley guinea pigs (Charles River, MA), and ticks fed to repletion. To obtain mice-fed salivary gland extracts 25 pathogen free nymphs were placed on head and back regions of each 4–6 weeks old C3H/HeN mice (NIH, MD) and ticks fed to repletion. Repleted nymphs were collected and dissected to remove salivary gland pairs and processed in pools of 10 pairs for RNA and 100 pairs for protein isolation.

In experiments that utilized histopathology to assess inflammation at the tick bite site in the murine host, nymphal ticks were placed on the ears/pinnae of C3H/HeN mice (5 ticks/ear) and allowed to feed for specified times. Mice were then euthanized and ears processed for histology as described below. In tick feeding experiments, 10–12 nymphs were placed on each mouse, and allowed to feed to repletion in metabolic cages approved by the Yale Animal Care and Use committee. At least 5 animals were used in each control and experimental group. Replete nymphs (repletion began approximately 72 h post tick-attachment) detached from the host were collected from the metabolic cages, and weighed on a Mettler Analytical Balance. For repeated infestations, after each round of nymphal feeding, the mice were rested for 2 weeks in routine animal cages prior to subsequent tick infestation. Repleted nymphs fed on naïve or 3-time tick infested mice were allowed to molt in groups of 20 in clean polypropylene tubes maintained in an incubator as described above, and percent molting efficiency assessed after 6–8 weeks. To assess nymphal feeding on C57BL/6 mice, nymphs were placed on C57BL/6 mice (Jackson Laboratory, ME) as described for C3H/HeN mice.

2.3. Histopathology and semiquantitative analysis of inflammation

Mice were euthanized by carbon dioxide asphyxiation, and whole ears were excised at the base of the ear, fixed in 10% neutral buffered formalin (VWR International, Batavia, IL), "bread-loafed"(Guo et al., 2009), sectioned and stained by hematoxalin and eosin (HE) by routine methods (Hladik, 1997). Semiquantitative scoring (0–5) was performed as previously described for mouse pinnae (Guo et al., 2009). Briefly, the HE-stained sections of ears were assessed at low and high power and scored for the presence and extent (severity) of the tissue changes using a semiquantitative criterion-based methodology adapted from our previous analysis of murine carditis (Montgomery et al., 2007). All foci of inflammation above background were examined at high power ($\times$40) to assess the specific nature of the inflammation characterized. The sections were evaluated for pathological changes in the epidermis, dermis, subcutis, muscle, and cartilage, including inflammation, necrosis, edema, vascular congestion, and hemorrhage. The severity scores ranged from 0 to 5, with numerical values of 0 (within normal limits, absent), 1 (minimal), 2 (mild), 3 (moderate), 4 (marked), and 5 (severe). The character of the inflammation was evaluated by light microscopy for changes due to swelling, hemorrhage, or inflammatory infiltrate, and identification of the exact type of inflammatory cell in the infiltrates was based on distinct morphological differences. Digital light microscopic images were recorded using an Axio Imager.A.1 microscope and an AxioCam MRc5 camera and AxioVision 4.7 imaging software (Zeiss). Observers were blinded to the study conditions until after the histopathologic features were assessed.

2.4. Tick RNA isolation, mouse skin RNA isolation and quantitative RT-PCR

Nymphs fed to repletion on mice or guinea pigs were dissected and salivary glands and midguts were pooled (6 pools of 10 ticks), homogenized in Trizol (Invitrogen, CA) and RNA was extracted as described by the manufacturer (Invitrogen, CA). Repleted ticks were collected at the time of repletion (when they are engorged and detach with just a gentle touch of the tick) or within a few hours of repletion (animals were monitored 2–3 times a day to ensure this) to collect the repleted and detached ticks from the pans.

Mice were euthanized as described above, ear skin obtained from naïve, and 3-time tick-infested mice, ground under liquid nitrogen, suspended in Trizol, and RNA isolated as described above for nymphs. cDNA was synthesized using the iScript RT-PCR kit (Biorad, CA), and analyzed by quantitative PCR for the expression of *mcpt4,* and *mcpt8* transcripts using primers described by Wada et al (Wada et al., 2010). Quantitative real-time PCR was performed using the iQ Syber Green Supermix (Biorad, CA) on a MJ cycler (MJ Research, CA) and data normalized to mouse *Hprt1* using primers described by Wada et al (Wada et al., 2010).

2.5. ELISA for assessment of salivary gland specific IgG and total IgE levels

96-well ELISA plates were coated overnight with 5 µg of nymphal salivary gland protein extract prepared as described earlier (Narasimhan et al., 2007) and incubated with mouse sera collected 2 weeks post tick challenge, at1:200 dilution. Bound antibody was detected with HRP-conjugated goat anti-mouse IgG and TMB substrate solution (Thermo Scientific, IL). Total IgE levels in mouse sera, collected 2 days post tick challenge, were assessed using the Mouse IgE ELISA kit (eBioscience, CA) at a serum dilution of 1:10. Sera from 5 mice from each group were examined.

2.6. Immunohistochemistry

Ears were formalin-fixed, paraffin-embedded and cut in 5-micron sections as described above. Ear sections from naïve and 3-time tick-infested mice incubated with anti-mouse MCP-8 antibody (Biolegend, CA) against basophil-specific protease Mcpt-8 (Poorafshar et al., 2000) at 4 °C overnight at a concentration of 1 μg/ml and bound antibody detected using HRP-conjugated goat anti-rat IgG at 1: 1000 dilution followed by incubation with DAB solution. The sections were then counterstained with Toluidine Blue (T-blue) as described (Narasimhan et al., 2007) to specifically co-localize Mcpt-8 staining with T-blue-stained basophils.

2.7. D protein analysis

A qualitative analysis of the *I. scapularis* salivary gland proteome of mouse-fed and guinea pig-fed nymphs was carried out by Differential 2D-Fluorescence Gel Electrophoresis (DIGE) at the W.M Keck Facility at Yale University as described earlier (Narasimhan et al., 2007). Salivary gland extracts from 100 *I. scapularis* nymphs fed on mice or guinea pigs were suspended in a cell lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 25 mM Tris, pH 8.6 at 4 °C) and protein concentration estimated by amino acid analysis at the W.M Keck Facility at Yale University. Equal amounts of protein (50 μg) from mice-fed and guinea pig-fed salivary gland extracts were then differentially labeled *in vitro* with Cy3 and Cy5 N-hydroxysuccinimidyl ester dyes as described in the Ettan DIGE manual (GE Healthcare, NJ), and isoelectric focusing was carried out in the first dimension on 24 cm Immobiline (IPG) Drystrips (GE Healthcare, NJ) using a pH 3–10 range, and a 12.5% polyacrylamide gel in the second dimension. Data were analyzed as described earlier (Wu, 2006) using the Typhoon 9410 Imager (GE Healthcare, NJ). A third dye (Cy-2) was included as an internal (pooled 25 μg of mouse-fed + 25 μg guinea pig-fed salivary gland extracts) standard to permit normalization of multiple gels and for internal normalization (Wu, 2006).

2.8. Digital gene expression analysis

RNA was prepared from 6 biological replicates of repleted salivary glands of nymphs fed on mice, and guinea pig in pools of 10 salivary gland pairs and100 ng of total RNA processed for digital gene expression on a fee-for-service basis at Nanostring Technologies, Inc, WA. The digital gene expression analysis technique was utilized using the Nanostring nCounter gene expression platform as described by Geiss et al (Geiss et al., 2008) and detailed at www.nanostring.com/applications/. 72 *I. scapularis* salivary genes encoding putative secreted salivary proteins were selected as described earlier (Narasimhan et al., 2007) and Listed in Supplementary Table 1 CodeSets were custom designed to generate a pair of target-specific 50-bp long half-site probes corresponding to a 100 bp region of each of the target genes sequence (Supplementary Table 1), of which one 50 bp long probe sequence served as a capture probe for immobilization to the nCounter cartridge and the other 50 bp probe sequence color-coded to provide the reporter barcode signal. *I scapularis* actin gene (Accession #: ISCW024111) served as the housekeeping control gene for data normalization. All probe pairs including the control actin probes were mixed and mRNA levels for all 73 genes quantified simultaneously in each of 6 biological replicates of mouse-fed and guinea pig-fed salivary gland RNA samples. Nanostring Technologies, Inc, proprietary quality control positive and negative control probes were also spiked into the samples during hybridization to ensure the performance efficiency of the experiment (Nam and Davidson, 2012; Ramadoss and Magness, 2012). Data were collected on a nCounter Digital Analyzer and expression levels presented as numbers of counts based on signal collected for each color-coded barcode specific for each target gene and is designated by its specific GenBank Accession number. The counts were normalized to *I. scapularis* actin in each biological replicate and expression levels represented as total normalized counts. Genes that provided less than 50 counts in both groups (mouse-fed and guinea pig-fed groups) were not included in the final analysis. Internal negative controls included in each sample run showed a maximum of 10 counts. The significance of the difference between the mean values of the groups for each gene was analysed using the Man-Whitney U test, at 95% confidence interval, and $P \leq 0.05$ was considered statistically significant.

2.9. ELISA and flow cytometric analysis of IL-4 production

Spleens were isolated from 4 naïve pathogen-free C3H/HeN mice and splenocytes prepared as described earlier (Borchers et al., 2002) and cells suspended in RPMI buffer at 6×10^7 cells/ml. The cells were plated into tissue-culture plates at 4×10^6 cells/ well in 2 ml of RPMI/10% FCS buffer and incubated with filter-sterilized 10 μg of mouse-fed or guinea pig-fed salivary gland extracts in triplicates and placed in a 37 °C CO_2-incubator (Panasonic Healthcare Company, IL). Four biological replicates of guinea pig-fed and mouse-fed salivary gland extracts were examined. For ELISA assessment of IL-4 production, the incubation was continued for 12 h and cell supernatant removed and IL-4 amounts in the supernatant estimated using Mouse IL-4 ELISA kit, EMIL4 (Thermo Scientific, Rockford, IL) as described by the manufacturer.

For flow cytometric analysis of IL-4 production, cells were harvested and incubated with mouse-fed and guinea pig-fed salivary gland extracts as described above. Brefeldin A (BD Biosciences, CA) was added after 2 h of incubation to maintain IL-4 intracellularly, and incubation continued for another 4 h. Control cells were incubated with filter sterilized phosphate buffered saline. After the incubation period, the cells were harvested, washed and resuspended in PBS/1% BSA and first stained for the following cell-surface markers: CD4, CD49B, FcεRI, and c-kit using fluorescent-conjugated antibodies against specific cell surface markers. Subsequently, the cells were fixed and permeabilized using the PermFix and Permwash buffers as recommended by the manufacturer (BD Biosciences, CA), and stained for intracellular IL-4 using FITC-conjugated anti-mouse IL-4 antibody. All incubations were done on ice and dark conditions. All antibodies were purchased from eBioscience, CA. The stained cells were examined on a LSRII Analytical Flow Cytometer (BD Biosciences, CA) and data analysed using FlowJo (TreeStar Inc, OR) to assess IL-4 produced by basophils defined as SSC ^{lo}, CD4$^-$, CD49B$^+$, c-kit ^{lo}, FCεRI$^+$ cells (Sullivan et al., 2011) and by CD4$^+$ T cells with results expressed as relative Mean Fluorescence Intensity. At least 3 replicate ELISA and flow cytometry experiments were conducted with similar results.

2.10. Statistical analysis

In tick feeding, molting and quantitative PCR experiments, the significance of the difference between the mean values of control and experimental groups was analysed using the non-parametric 2-tailed (Man-Whitney) test with Prism 5.0 software (GraphPad Software, CA). $P \leq 0.05$ was considered statistically significant. To assess if the rates of repletion were significantly different between control and experimental groups, two-way ANOVA (Analysis of variance)with Sidak's multiple comparison was utilized. In ELISA, and flow cytometry assessments involving more than two sample groups, the significance of difference between the groups was assessed by a non-parametric two-way ANOVA, with Tukey's multiple comparison test using the Prism 5.0 software (GraphPad Software, CA). $P \leq 0.05$ was considered statistically significant.

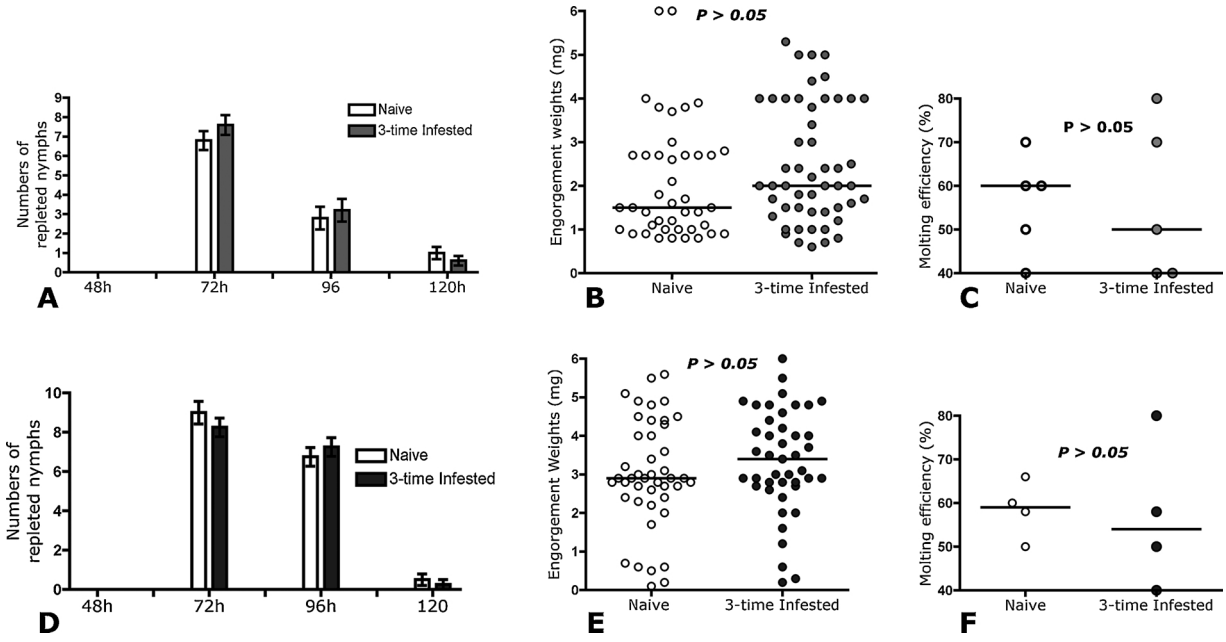

Fig. 1. Tick engorgement and rate of repletion are not impaired upon repeated tick infestations of C3H/HeN or C57BL/B6 mice. About 10 clean *I. scapularis* nymphs were allowed to engorge on each of 5 C3H/HeN female mice repeatedly infested with *I. scapularis* nymphs (3-time Infested) or naïve mice (Naïve) and scored for: **A**. Rate of repletion of nymphs; **B**. Engorgement weights of individual nymphs; and **C**. Molting efficiency. About 10 clean *I. scapularis* nymphs were allowed to engorge on each of 5 C57BL/6 female mice repeatedly infested with *I. scapularis* nymphs (3-time Infested) or naïve mice (Naïve) and scored for: **D**. Rate of repletion of nymphs; **E**. Engorgement weights of individual nymphs; and **F**. Molting efficiency. Error bars in **A** and **D** represent means ± SEM and significance of differences in repletion rates were assessed by 2-way ANOVA with Sidak's multiple comparison test. Each data point in **C** and **F** represents a pool of 20 nymphs and is collated data from 3 replicate experiments. In **B**, **C**, **E** and **F** significance of the difference between the mean values of the groups was assessed by Mann-Whitney U test and horizontal bars represent the median. A representative of 3 replicate experiments is shown.

3. Results

3.1. Repeated I. Scapularis infestations on C3H/HeN mice results in rapid recruitment of inflammatory cells to the tick-bite site

Mice (C3H/HeN) pinnae were infested with 20–25 clean *I. scapularis* nymphs three times, and then challenged with 10–15 clean *I. scapularis* nymphs to assess their feeding efficiency. Nymphs on the 3-time-infested mice fed to repletion with no significant difference in time to repletion (Fig. 1A), and in engorgement weights (Fig. 1B) when compared to nymphs that fed on naïve mice. Fed nymphs from naïve and tick-infested groups also showed comparable molting efficiency (Fig. 1C). Similar results were also obtained when C57BL/6 mice were repeatedly infested and challenged with clean *I. scapularis* nymphs (Fig. 1D-F).

Histological examination of the tick-bite sites of hematoxylin and eosin (HE)-stained sections of pinna from naïve and 3-time-infested C3H/HeN mice at 6, 12, 24, 48 and 72 h of tick feeding demonstrated that greater numbers of inflammatory cells were rapidly recruited to the tick-bite sites of mice that were 3-time tick-infested. Increased inflammation was apparent within 12 h of tick attachment at the tick-bite sites of 3-time-infested mice (Fig. 2A), and substantial by 48 and 72 h of tick feeding. Neutrophils were the predominant inflammatory cells at all time points except at 72 h (Fig. 2A) and scattered eosinophils were observed at all time points. Within 24 h of tick attachment macrophages were observed, and by 48 h macrophages were frequent and second in abundance only to neutrophils, and by 72 h macrophages were the predominant cell type (Fig. 2A). Inflammation was about 4-fold increased in the skin of the 3-time tick- infested mice compared to that in the skin of naïve mice at 12 h post-nymphal attachment (Fig. 2B). Although, the differences in the inflammation severity between the two groups decreased with time, inflammation in the skin of 3-time tick infested mice was always higher than that in the skin of naïve mice (Fig. 2B).

I. scapularis infestations on mice does not result in increased basophils and mast cells at the tick bit-site, but does increase serum IgE and IgG levels

Immunohistochemical examination of the tick bite-site using anti-Mcpt-8 antibodies to detect basophil-specific protease Mcpt-8 (Poorafshar et al., 2000) did not reveal basophils at the tick-bite site on naïve, and 3-time tick-infested mice (Fig. 3I-B, C, E, and F). Quantitative reverse transcriptase-PCR (QRT-PCR) assessment of expression of basophil-specific protease *mcpt-8* (Poorafshar et al., 2000) also did not reveal *mcpt-8* expression in the skin biopsies of the tick-bite sites of both naïve and 3-time tick-infested mice (Fig. 3-II). The expression of mast cell-specific protease *mcpt-4* was elevated in the skin biopsies of the tick-bite sites of 3-time tick-infested mice when compared to that in the biopsies of naïve mice (Fig. 3-II), however this was not statistically significant (n = 5).

ELISA assessment of salivary gland extract (SGE)-specific IgG levels, and total IgE levels in sera from animals after the first tick infestation (Naïve group) did not reveal significant differences when compared with sera obtained from animals prior to tick-infestation (Prebleed group) (Fig. 3-III and 3-IV). However, we observed significantly increased levels of (SGE)-specific IgG, and total IgE levels in 3-time tick-infested mice when compared with naïve and prebleed mice (Fig. 3-III and 3-IV).

3.2. I. Scapularis salivary genes and proteins are differentially expressed when nymphs feed on reservoir or non-reservoir host

To determine whether qualitative differences in *I. scapularis* nymphal salivary proteome might underlie the ability of *I. scapularis* nymphs to efficiently circumvent inflammation on the skin of murine host in contrast to their inability to do so on guinea pigs (Allen, 1989), we first compared the salivary proteome of *I. scapularis* nymphs fed on guinea pigs and mice by 2-Dimensional Fluorescence Difference Gel Electrophoresis (2D-DIGE) and observed marked differences in protein

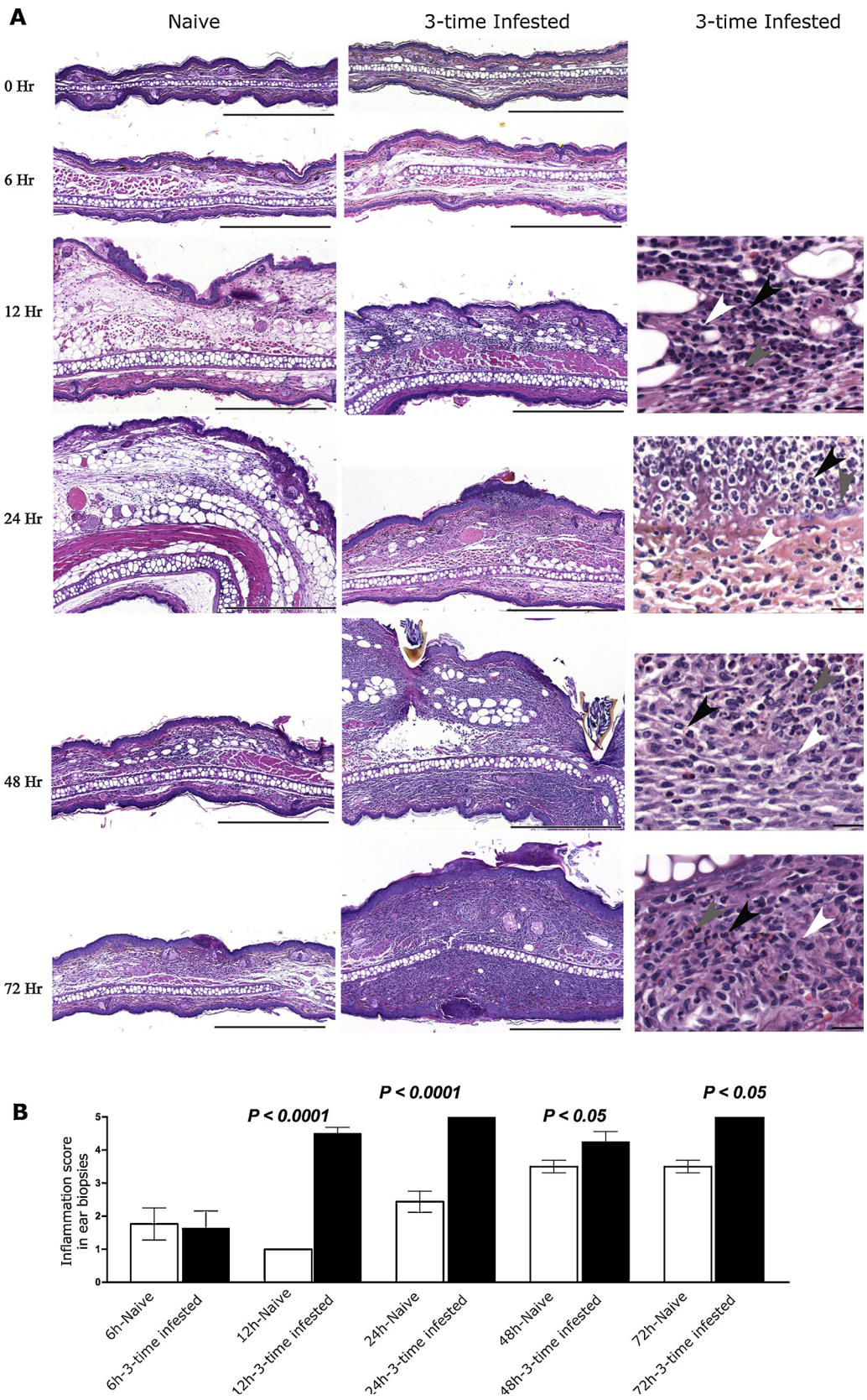

Fig. 2. Rapid recruitment of immune cells to the tick bite-site on repeatedly tick-infested mice does not impair tick engorgement. **A**. About 10 clean *I. scapularis* nymphs engorged on the ears of each of eight C3H/HeN female mice repeatedly infested with *I. scapularis* nymphs (3-time Infested) or on naïve (Naïve) mice and ears representing tick-feeding sites excised for histopathology, and representative HE-stained sections of mouse pinnae are shown at indicated time points after tick attachment. Ears excised prior to tick feeding from a representative mouse from each group (0Hr). Neutrophils (black arrowheads) predominate at 12, 24 and 48 h and macrophages (white arrowhead) predominate at 72 h (left panels); Scattered eosinophils were occasionally observed (grey arrowheads). Scale bars left and middle panels = 500 μm. right panels = 20 μm. **B**. Semi quantitative scoring shows increased Inflammation at 12, 24, 48, and 72 h on 3 time-infested mice compared to control mice. Error bars represent means ± SEM of an average of 5 sections. Mean values significantly different by two-way ANOVA with Tukey's multiple comparison test ($P < 0.05$) between the groups are indicated.

compositions (Fig. 4A). Several proteins demonstrated 2-fold or greater changes in levels between mouse-fed and guinea pig-fed salivary glands (Fig. 4B). Efforts to sequence differentially represented spots by LC–MS/MS were not successful; limited by the amounts and by the abundance of cytosolic and cytoskeletal proteins in the salivary gland extracts.

We therefore compared the expressions of a subset of salivary genes, encoding secreted salivary proteins (Supplementary Table 1), from

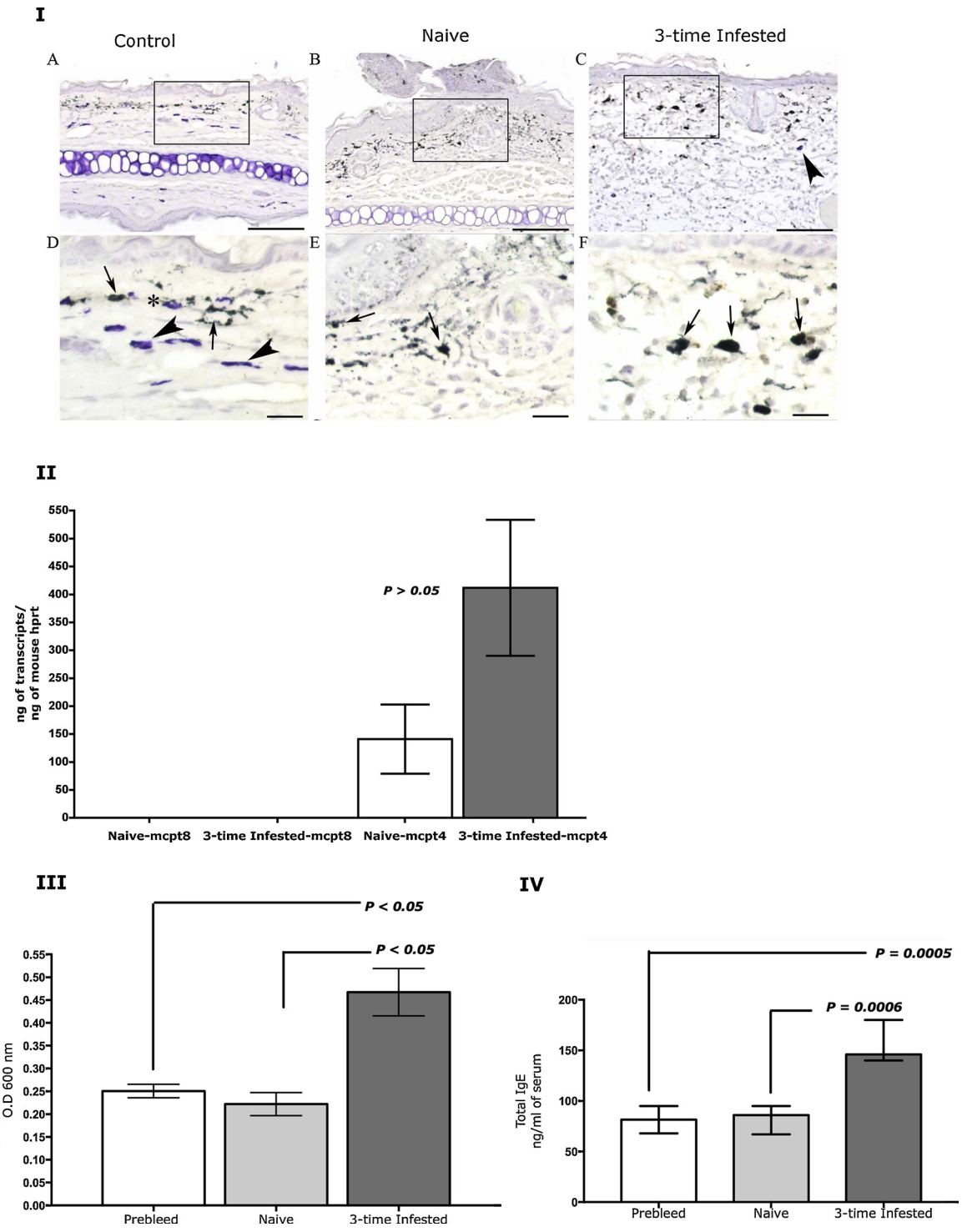

Fig. 3. Basophils not detectable at the tick bite-sites of 3-time tick infested mice. I. Immunohistochemical examination of tick-feeding sites of ears of C3H/HeN mice repeatedly infested with *I. scapularis* nymphs (3-time Infested) or naïve mice (Naïve) using mouse MCP-8 antibody followed by Toluidine (T)-Blue staining. Mcpt8$^+$ and T-Blue$^+$ basophils in Naïve and 3-Time tick infested samples not detected. T-blue positive cells in skin of mice that were not tick infested (Control) might represent tissue-resident mast cells. Arrows, T-Blue staining; Arrowheads, non-specific MCP-8 staining. Scale bars, A–C = 200 μm; D–F = 20 μm; **II**. Quantitative RT-PCR assessment of levels of *mcpt8 and mcpt4* transcripts. Error bars represent means ± SEM. n = 5. Sera from 3-time infested mice and naïve mice after challenge with *I. scapularis* nymphs assessed by ELISA for levels of: **III**. Salivary gland extract (SGE)-specific IgG; and **IV** Total IgE respectively. A representative data of 3 experiments is shown. Error bars represent means ± SEM. n = 5, and mean values significantly different by two-way ANOVA and Tukey's multiple comparison test ($P < 0.05$) indicated.

nymphs fed on mice or guinea pigs by digital gene expression analysis (Nanostring Technology, WA) (Geiss et al., 2008). Eighteen genes did not provide a robust and consistent signal (50 counts or less in both groups) and were removed from the analysis (Supplementary Table 1). Twenty one genes encoding putative protease inhibitors, histamine binding proteins and proteases were indeed differentially expressed on mice and guinea pigs (Fig. 5A and Table 1). Of the 11 putative kunitz-type protease inhibitor-encoding genes examined, 6 were upregulated

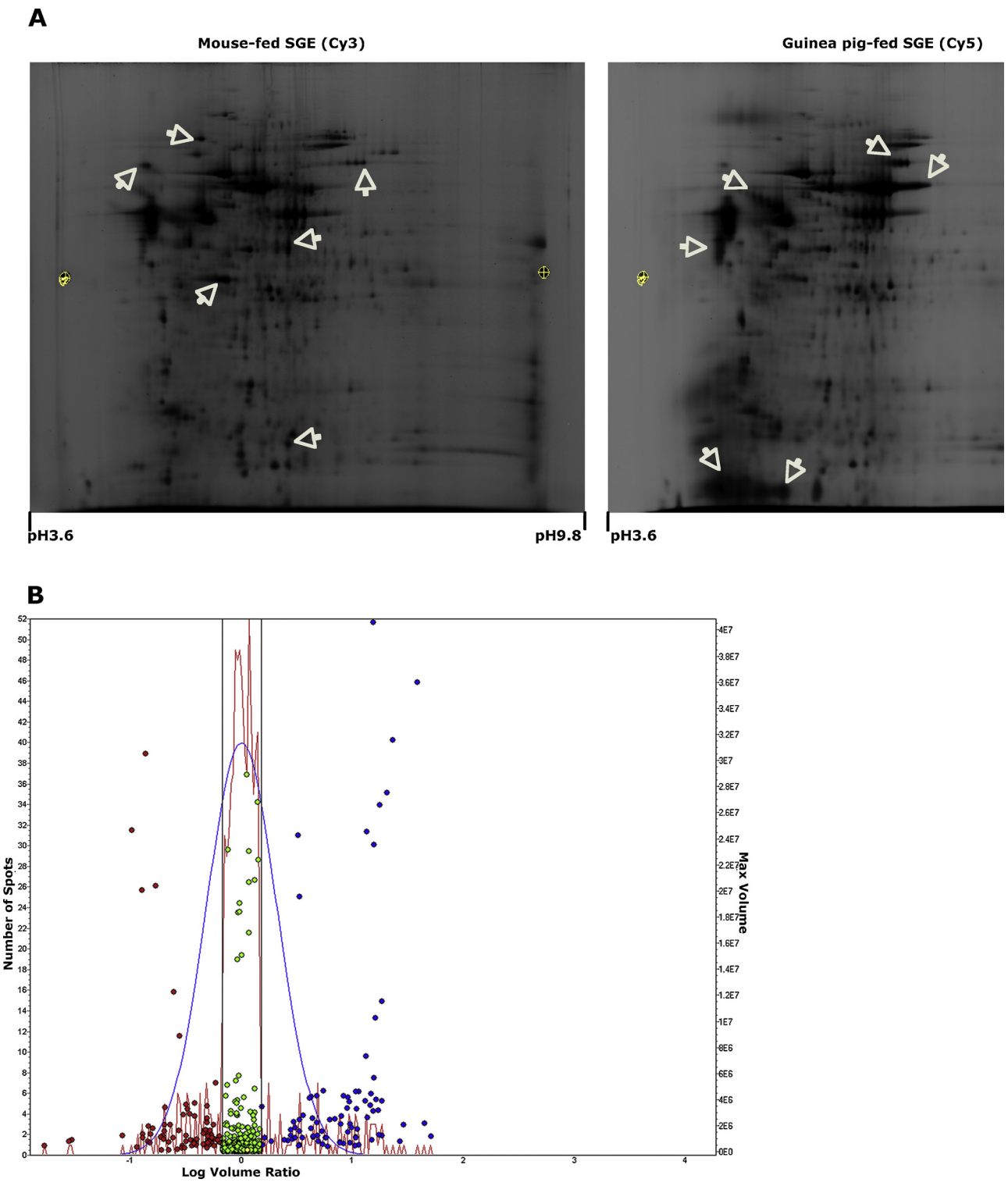

Fig. 4. Salivary proteome composition of mice fed and guinea pig fed *Ixodes scapularis* nymphs is different. **A**. Comparative analysis of mouse-fed (Cy3-labeled) and guinea pig-fed (Cy5-labeled) salivary gland protein extracts (SGE) by Differential 2D Fluorescence Gel Electrophoresis (DIGE) reveals differences (white arrows). A representative image of 2 experiments is shown. **B.** Digital assessment of spot distribution. Red curve, frequency distribution of the log volume ratios; Blue curve, normalized model frequency fitted to the spot ratios so that the modal peak is zero; Black vertical lines set at 1.5 fold difference in Cy5/Cy3 spot volume ratio. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

in the guinea pig-fed transcriptome when compared to that in mouse-fed salivary transcriptome. Of the 5 CLSP-family of protein-encoding genes 3 were increased in the guinea pig-fed transcriptome when compared to that in mouse-fed salivary transcriptome. Some of the genes encoded predicted secreted proteins that did not have a functional annotation in the database. We analyzed these secreted proteins *in silico* using the protein fold recognition software tool, Phyre2 (Kelley et al., 2015) and functional domains predicted with greater than 95% confidence assigned (Table 1).

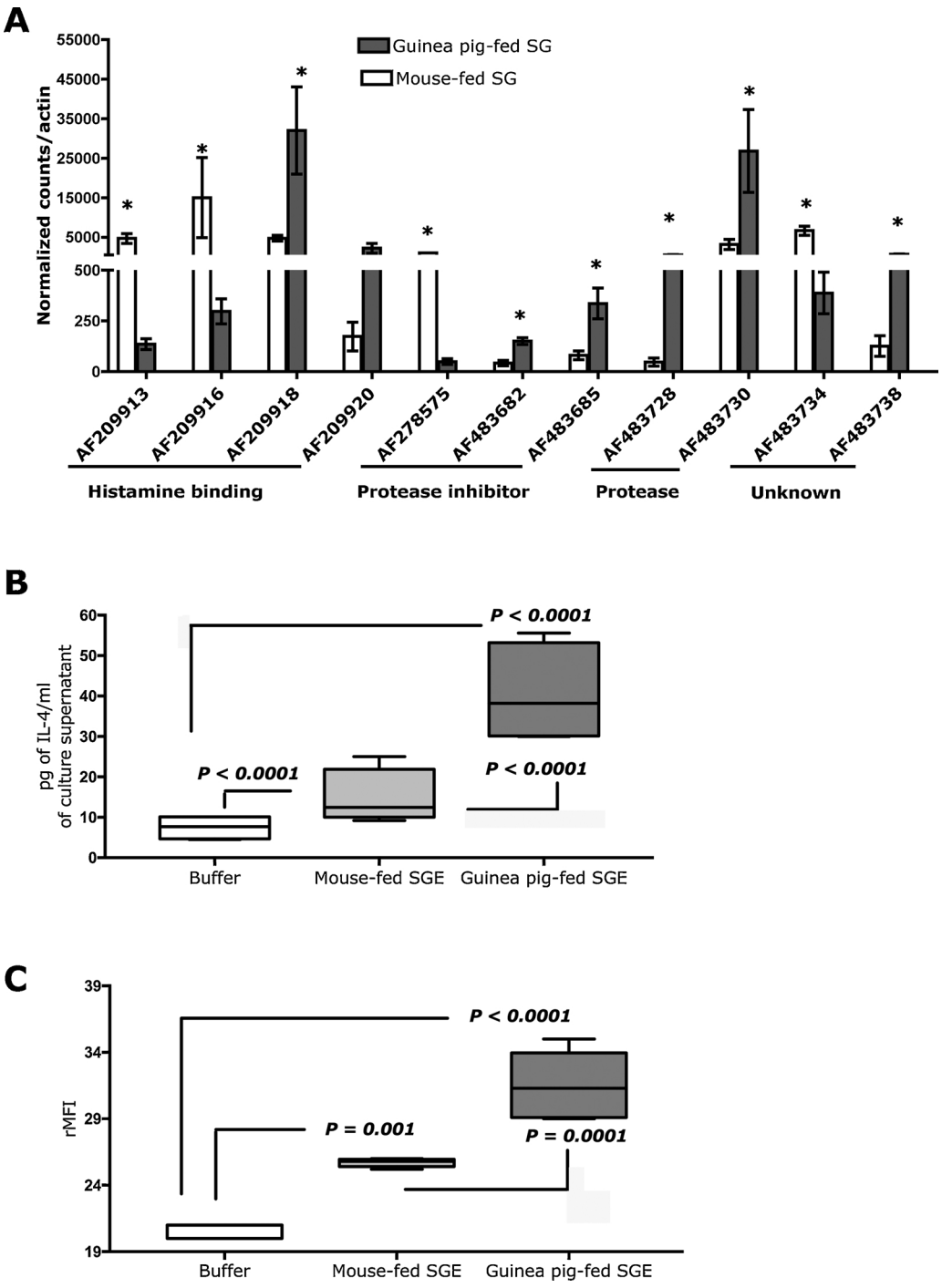

Fig. 5. Differential expression of tick salivary genes on guinea pigs and mice might modulate T_H2 responses. **A.** Normalized digital gene expression profiles in 6 biological replicates of mice-fed and guinea pig fed *I. scapularis* salivary glands reveals distinct differences in the levels of genes encoding putative histamine binding proteins, protease inhibitors, proteases and secreted proteins. Genes are designated by their specific GenBank accession numbers. Error bars represent means ± SEM, n = 6, and asterisks indicate mean values significantly different by a Mann-Whitney test ($P < 0.05$). **B.** Splenocytes of naïve mice were incubated with mouse-fed or guinea pig-fed salivary gland extracts (SGE) for 12 h and IL-4 secreted into the supernatant assessed by ELISA. **C.** Splenocytes of naïve mice were incubated with mouse-fed or guinea pig-fed salivary gland extracts (SGE) for 6 h and IL-4 levels in basophils assessed by flow cytometry. In **B** and **C** Splenocytes incubated with buffer alone served as control (Buffer). rMFI = relative Mean Fluorescence Intensity. In panels **B** and **C** error bars represent means ± SEM. n = 4, and mean values significantly different by 2-way ANOVA with Tukey's multiple comparison ($P \leq 0.05$) indicated.

3.3. Guinea pig-fed *I. Scapularis* salivary proteins induce IL-4 production in splenocytes

To determine if the differences in salivary protein profiles might influence the immune responses of the host, we tested the ability of guinea pig-fed and mice-fed salivary proteins to elicit production of IL-4, a cytokine essential for priming T_H2 immune responses (Pulendran and Artis, 2012). We isolated total splenocytes from spleens of naïve C3H/HeN mice and incubated them with equal amounts of guinea pig-fed or mice-fed salivary gland extracts for 12 h. ELISA assessment of IL-4 amounts in the culture supernatants showed significantly increased IL-4 secretion by cells incubated with guinea pig-fed salivary gland extracts when compared with cells incubated with mouse-fed salivary gland extracts (Fig. 5B).

Several studies have invoked a central role for basophils in IL-4 secretion and in the induction of T_H2 responses in the context of parasite infections, allergy, and tick rejection (Karasuyama et al., 2010, 2011). We therefore assessed if IL-4 was being secreted by activated basophils. Splenocytes from spleens of naïve C3H/HeN mice were incubated with equal amounts of guinea pig-fed or mice-fed salivary gland extracts for 6 h as described in Materials and Methods. The levels of IL-4 was then assessed by flow cytometry in total $CD4^+$ cells, and in basophils, defined by SSC^{lo} $CD4^-$, c-kit^{low}, $CD49B^+$, $Fc\varepsilon RI^+$ antigen profile (Sullivan et al., 2011). Our data suggested that guinea pig-fed salivary gland extracts preferentially increased the production of IL-4 in basophil population when compared to buffer control, and mice-fed salivary gland extracts (Fig. 5C).

Table 1
Gene expression profile of a subset of secreted salivary proteins of *Ixodes scapularis* fed on murine or guinea pig host. #CLSP, collagen like secreted protein; * mean values significantly different by Mann-Whitney U test. **a**. AF515779 annotated as a putative anticoagulant Salp9pac has been functionally characterized as an inhibitor of the lectin pathway of mammalian complement system (Schuijt et al., 2011). **b.** Homology prediction based on Phyre2 protein fold recognition analysis (Kelley et al., 2015). **c**. ADAMTS, disintegrin and metalloproteinase with thrombospondin.

GenBank Accession Number	Putative Function/conserved domain	Normalized Mean counts (Mouse-fed SG) (n = 6)	Normalized Mean counts (Guinea pig-fed SG) (n = 6)	P value
AF209911	Peroxiredoxin	1628	2227	0.633
AF209913	Histamine binding	4699	135	0.014 *
AF209915	Histamine binding	266	103	0.158
AF209916	Histamine binding	14677	464	0.0223*
AF209922	Histamine binding	17935	103649	0.121
AF483717	Histamine binding	185	8057	0.168
AF209918	Histamine binding	4770	32039	0.050*
AF209919	Histamine binding	3018	4418	0.550
AF209920	Histamine binding	220	2401	0.123
AF483718	Histamine binding	80	446	0.016*
AF483722	Histamine binding	3210	4391	0.692
AF483742	Histamine binding	81	6563	0.040*
AF483743	Histamine binding	1256	1664	0.709
AF515779[a]	Anticomplement	171047	420706	0.286
AF270496	Anticomplement	3676	13546	0.038*
AF209917	Anticomplement	3903	6274	0.345
AF278575	Protease inhibitor	1031	49	0.004*
AF483714	Protease inhibitor	1660	818	0.529
AF483724	Cystatin	517	1467	0.124
AF483727	Protease inhibitor	225	234	0.23
AF286029	Anticoagulant	25	132	0.0179*
AF483725	Anticoagulant	117	275	0.474
AF209921	Anticoagulant	27336	65355	0.354
AF483682	Kunitz-type Protease inhibitor	43	356	0.019*
AF483683	Kunitz-type Protease inhibitor	321	78	0.464
AF483685	Kunitz-type Protease inhibitor	80	315	0.038*
AF483686	Kunitz-type Protease inhibitor	433	263	0.312
AF483687	Kunitz-type Protease inhibitor	162	100	0.699
AF483690	Kunitz-type Protease inhibitor	131	176	0.379
AF483691	Kunitz-type Protease inhibitor	205	121	0.699
AF483692	Kunitz-type Protease inhibitor	16	277	0.012*
AF483693	Kunitz-type Protease inhibitor	21	294	0.042*
AF483681	Kunitz-type protease inhibitor[b]	24	244	0.0157*
AF483688	Kunitz-type protease inhibitor[b]	68	411	0.017*
AF483694	#CLSP family	81	374	0.015*
AF483704	CLSP family	1717	24413	0.029*
AF483695	CLSP family	1680	17032	0.019*
AF483696	CLSP family	725	1749	0.360
AF483698	CLSP family	968	7878	0.128
AF483715	Protease	4175	928	0.374
AF483729	Protease	84	171	0.339
AF483730	Metalloprotease	5155	26842	0.019*
AF483731	Metalloprotease	380	135	0.179
AY264367	Metalloprotease	1728	2567	0.599
AF483723	Alpha-2 macroglobulin	480	462	0.945
AF483728	Carboxypeptidase	84	591	0.0018*
AF483733	Putative 4.3 kDa secreted protein	380	522	0.359
AF483734	Putative secreted 5.3 kDa protein	8863	323	0.0015*
AF483736	Putative 7 kDa secreted protein	2427	4821	0.221
AF483737	Putative 8.4 kDa secreted protein/Potassium channel toxin[b]	2407	3679	0.409
AF483738	Putative 9.4 kDa secreted protein/ signaling function[b]	126	1254	0.047*
AF483739	Putative 8.9 kDa secreted protein/ signaling function[b]	1160	841	0.56
AF483741	Putative 18.7 kDa ADAMTS[bc]	701	602	0.903
AY234846	IgG-binding secreted protein	68	373	0.006*

4. Discussion

Repeated infestations of *I. scapularis* ticks on non-permissive vertebrate hosts (Das et al., 2001; Nazario et al., 1998), provoke a robust immune response that impairs tick feeding and promote tick rejection within 12–24 hours of attachment (Narasimhan et al., 2007). Resistance to tick feeding also effectively impairs transmission of *Borrelia burgdorferi* to the guinea pig host (Narasimhan et al., 2007; Nazario et al., 1998). Earlier works by Burke et al (Burke et al., 2005) and Krause et al (Krause et al., 2009) have suggested that humans, like other non-reservoir hosts, might also develop immune responses detrimental to tick feeding, and *B. burgdorferi* transmission. Progress towards a molecular understanding of tick-resistance has been hampered by the paucity of immunological reagents and transgenic tools for guinea pig and rabbit models of non-reservoir host. *I. scapularis* has co-evolved with its natural or reservoir host, hence *I. scapularis-P. leucopus* interactions are possibly optimized for successful tick feeding and pathogen transmission (Ribeiro, 1989). Laboratory mice strains serve as excellent models

of *I. scapularis* larval and nymphal feeding, and like the reservoir host, do not develop resistance to tick feeding upon repeated infestations (Wikel, 1996). We therefore utilized the C3H/HeN laboratory mice as a surrogate reservoir host model to begin a molecular understanding of the absence of tick resistance on the murine host. This reverse approach to unravel a mechanistic understanding of acquired tick-resistance would benefit from the availability of reagents and genetic tools to examine the murine host.

Consistent with the recent studies by Anderson et al (Anderson et al., 2017), histological examination of the tick-bite sites showed a four-fold increase in severity of inflammation at the bite-site within 12 h of tick attachment on repeatedly tick-infested mice when compared to that on naïve mice. Nevertheless, the increased inflammation had no impact on tick feeding, and subsequent development to the adult stage. These observations corroborate and extend the earlier observations by Wikel et al (Wikel et al., 1997).

Anderson et al (Anderson et al., 2017) noted distinct changes between the dermal architecture of tick bite-sites on mice and guinea pigs suggesting inherent differences in the molecular interactions between the host immune responses and the tick vector. Repeated infestations of guinea pigs by *I. scapularis* ticks promotes rapid recruitment of immune cells, predominantly basophils to the bite site, with a detrimental impact on tick feeding and consequent pathogen transmission (Narasimhan et al., 2007). Interestingly, repeated infestations of mice with cattle ticks, *Haemaphysalis longicornis,* was shown to recruit basophils to the bite site, and resultin tick rejection (Wada et al., 2010). It is worth noting that *H. longicornis* ticks normally feed on cattle, their chosen host, and upon repeated infestations of the non-natural host, mice, these ticks are rejected due to the development of acquired tick-resistance in mice and basophil recruitment, and histamine release from basophils is implicated in the rejection (Tabakawa et al., 2018). In contrast, repeated *I. scapularis* infestations of the murine host, their natural host, did not promote basophil recruitment to the bite-site as seen by HE staining of the murine skin at the tick bite-site, and by QRT-PCR assessment of basophil-specific *mcpt8*. Although expression of *mcpt4*, a mast-cell specific protease (Ugajin et al., 2009), was elevated in 3-time tick-infested mice, the increase was not statistically significant, and was consistent with the histological assessment of the tick-bite site. Repeated *I. scapularis* nymphal infestations resulted in increased salivary gland-specific IgG and total IgE levels, indicators of T_H2 responses (Pulendran and Artis, 2012), however, there was an apparent lack of basophil involvement- a potentially key player in promoting a detrimental milieu. Consistent with this, a recent study by Tabakawa et al (Tabakawa et al., 2018) suggests that basophil-derived histamine and not mast cell-derived histamine is critical for rejection of *H. longicornis* ticks on mice.

An earlier study by Carvalho et al (Carvalho et al., 2010) suggested that *Rhipicephalus microplus* salivary anti-hemostatic proteins are differentially expressed when these ticks feed on resistant and susceptible bovine hosts, and consequently alters feeding efficiency. A more recent study by Tirloni et al (Tirloni et al., 2017) has also demonstrated using unfed adults of *I. scapularis* and *Amblyomma americanum* that salivary proteomes are different when stimulated with semiochemicals of rabbits or dogs or humans. Building on these observations we hypothesized that differences in the salivary composition between reservoir host and non-reservoir host-fed *I. scapularis* might contribute, in part, to differences in tick-host interactions. Consistent with the observations by Tirloni et al (Tirloni et al., 2017), when we compared the salivary proteome of *I. scapularis* nymphs fed on guinea pigs or on mice by 2D-DIGE we observed marked differences in protein compositions with several proteins demonstrating 2-fold or greater changes in levels between mouse-fed and guinea pig-fed salivary glands. Secreted proteins would most likely be directly involved in modulating host immune responses, and differentially secreted salivary proteins are likely to modulate the immune responses to tick feeding. The salivary gland extracts contain secreted, membrane, and cytosolic proteins, and sequencing the differentially expressed 2D gel spots by LS-MS/MS was confounded by noisy data, due potentially to small amounts of proteins in individual spots.

Although, Tirloni et al (Tirloni et al., 2015) have successfully obtained saliva from engorged *H. longicorni s*nymphs, our attempts to collect saliva from engorged nymphs was not productive. Therefore, we examined the profile of a subset of 72 mRNAs encoding secreted proteins in salivary glands from nymphs fed on guinea pigs and from nymphs fed on C3H/HeN mice. Of the 72 genes assessed, 54 provided consistent results (Table 1). Our inability to obtain robust signal for 18 genes could be due to suboptimal probe design for these genes or due to the low expressions of these genes at repletion. Twenty one of the 54 genes (~ 38%) were differentially expressed. The higher proportion of differentially expressed genes in this study compared to Tirloni et al's study (Tirloni et al., 2017) that observed about 19% of genes being differentially expressed is likely due to the focus on a subset of secreted salivary proteins. Nevertheless, this observation suggests that secreted proteins are likely to be predominantly influenced by different host species and garners further support for invoking a role for differences in the salivary secreted proteome composition in the development of tick-resistance on the non-reservoir host. Expressions of several putative histamine binding protein (HBP)-encoding genes, protease inhibitor-encoding genes and proteases were differentially expressed in the guinea pig-fed and mouse-fed salivary glands. While 2 HBP encoding genes were increased in mouse-fed salivary glands, 2 HBPs were increased in guinea pig-fed extracts. HBPs are part of a diverse family of lipocalins (Paesen et al., 1999; Sangamnatdej et al., 2002) and could bind ligands other than histamines to modulate immune responses in diverse ways. Several kunitz-type protease inhibitors, and collagen-like secreted proteins (CLSP) were more highly expressed in the guinea pig-fed tick transcriptome than in the mouse-fed tick transcriptome. The tick genome encodes several paralogous genes encoding the lipocalin family of proteins, proteases and protease inhibitors (Gulia-Nuss et al., 2016) and we are yet to understand the physiological targets of the proteins encoded by all these genes. We also recognize that this analysis is limited to a subset of salivary genes-therefore we cannot fully decipher the functional consequence of these changes in gene expressions on acquired tick-resistance. Further, assessing the salivary transcripts at earlier time points of tick attachment and feeding might be more insightful.

Tirloni et al (Tirloni et al., 2017) have examined the unfed salivary transcriptome of adult female *I. scapularis* in response to dog, rabbit or human host stimuli. Given that there are stage-specific differences in the tick transcriptome (Tirloni et al., 2015), as well as differences during feeding (Chmelar et al., 2008; Kim et al., 2016; Narasimhan et al., 2007), and differences based on the different host-species on which ticks have fed -a head-to-head comparison of our data with that of Tirloni et al (Tirloni et al., 2017) cannot be done. While our study has predominantly identified histamine binding proteins, and proteases, Tirloni et al (Tirloni et al., 2017) identify a secreted lipocalin protein that also has homology with several histamine binding proteins, several hemelipoglycoproteins, cystatin, superoxide dismutase, peroxidase/oxidases and alpha-2-macroglobulins as predominantly differentially expressed secreted proteins between different host species.

Earlier work by Ribeiro has suggested that the tick salivary proteome might encode a protein repertoire geared to effectively diffuse the immune responses of its natural host, but not its accidental host (Ribeiro, 1989) and inherent differences in the immune responses of permissive and non-permissive host might direct vector-host specificity. Detailed studies focused on understanding the differential immune responses of *R. microplus*-susceptible bovine host, *Bos taurus taurus* and *R. microplus*-resistant *Bos taurus indicus* have invoked multiple factors including physical barriers such as the thickness of the skin, genetic differences including variations in IgG2 allotypes, variations in specific quantitative trait loci, changes in expressions of extracellular matrix-encoding genes and in genes involved in immune responses, increased

blood clotting time at the feeding site of susceptible host, and differences in the granulocyte milieu at the tick feeding site as potential determinants of resistance or susceptible phenotypes (reviewed by Tabor et al (Tabor et al., 2017)). An elegant study by Perner et al (Perner et al., 2018) comparing rabbit-fed and membrane-fed adult salivary transcriptome has shown that the sialome of *I. ricinus* ticks is likely influenced by the immune status of the host and these dynamic changes are critical for immune evasion. Therefore, it is likely that differences in the immune responses, due in part to inherent genetic differences between permissive and non-permissive hosts, might impact the vector transcriptome.

In this study, we suggest that the *I. scapularis* salivary proteome is influenced by the host on which it feeds and that the differential expression of salivary proteins has a functional consequence on its ability to thwart or provoke host immune responses. That guinea pig-fed salivary gland extracts induce greater IL-4 levels in splenocytes, and specifically in the basophil population when compared to mice-fed salivary gland extracts is indicative of functional differences between mouse-fed and guinea pig-fed proteomes. Extraneous contaminants were avoided during the preparation of tick salivary protein extracts, however, we do not rule out the possibility that the differential induction of IL-4 by guinea pig-fed extracts could, in part, have risen from contaminating guinea pig components in the salivary protein extracts. We also acknowledge that, while, these observations highlight the inherent differences between permissive host-fed and non-permissive host-fed salivary proteomes, a temporal comparison of the global salivary transcriptomes of mouse-fed and guinea pig-fed ticks would be essential to determine the net functional differences, and to identify pathways critically altered between mouse-fed and guinea pig-fed salivary transcriptomes. How and what signals host-specific gene expression in the tick vector remains to be determined.

Our observations corroborate recent findings (Anderson et al., 2017; Tirloni et al., 2017) and collectively provide the impetus to develop a mechanistic understanding of acquired tick-resistance leveraging on the finding that the functional salivary proteome is likely different on the natural/reservoir and non-reservoir host. An expanding understanding of this novel facet of host-specific tick gene expression is also likely to direct our search for tick antigen-based vaccines to prevent tick-transmitted diseases to humans. It is interesting to note both from our study and from Tirloni et al's (Tirloni et al., 2017) study that several genes are indeed comparably expressed on diverse hosts and likely represent the core set of functions critical for feeding. Perhaps, in the context of tick vaccine development, it is this core proteome that needs to be deciphered in detail to determine if these might be vaccine targeted to impair tick feeding and consequently thwart the transmission of tick-borne pathogens.

5. Conclusions

Tick salivary proteins injected into the mammalian host during tick feeding help subvert host immune responses and play a crucial role in tick feeding success (de la Fuente et al., 2017; Ribeiro, 1989). The ability of ticks to feed on diverse host species including natural or permissive host such as mice and on non-permissive hosts such as rabbits, guinea pigs and humans likely derives from a complex repertoire of proteins encoded by the tick genome (Gulia-Nuss et al., 2016). We provide evidence to suggest that the salivary proteome composition of ticks fed on a permissive/natural host is different from that of ticks fed on a non-permissive host. Providing a new insight into tick-host interactions this report offers a new paradigm to understand the tick functional genome in the context of tick-host interactions and to decipher a mechanistic understanding of the phenomenon of acquired tick-resistance.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2018.12.001.

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