

AWARD NUMBER: W81XWH-18-1-0255

TITLE: Tick Bites in Transmission of the Alpha Gal Allergen Eliciting Red Meat Allergy

PRINCIPAL INVESTIGATOR: Yoonseong Park

CONTRACTING ORGANIZATION: Kansas State University

REPORT DATE: JANUARY 2022

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE JANUARY 2022		2. REPORT TYPE Final Report		3. DATES COVERED 30SEPT2018 - 29SEPT2021	
4. TITLE AND SUBTITLE Tick Bites in Transmission of the Alpha Gal Allergen Eliciting Red Meat Allergy				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-18-1-0255	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Yoonseong Park Shery Fleming E-Mail:ypark@ksu.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) KANSAS STATE UNIVERSITY PRE-AWARDS SERVICESOFFICE OF RES & SPONS PROGRAM 2 FAIRCHILD HALL MANHATTAN, KS 66506-1100				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Tick bites are thought to be the cause of red meat allergy (RMA). In humans, RMA is diagnosed by 3 – 5 hour delayed urticaria and anaphylaxis to dietary red meat, is caused by an unusual occurrence of enhanced IgE antibody production against galactose-alpha-1,3-galactose (aGal), which is a common glycan in mammalian tissue with the exceptions of old world monkey and human. <u>We hypothesize that ticks mediate the 'transmission' of the aGal allergen by the injection of tick saliva containing aGal glycans acquired from a prior intrastadial (same molting stage) blood meal on non-human mammals. The objective of this study is to identify the allergenic ticks (species, stage, gender, and prior host in the feeding) and to characterize the allergenic salivary components by using a novel mutant mouse model alpha-galactosyltransferase knockout mutant mouse (aGT-KO).</u>					
15. SUBJECT TERMS NONE LISTED					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 50	19a. NAME OF RESPONSIBLE PERSON USAMRDC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	3
2. Keywords	3
3. Accomplishments	3
4. Impact	15
5. Changes/Problems	16
6. Products	18
7. Participants & Other Collaborating Organizations	20
8. Special Reporting Requirements	21
9. Appendices	22

1. INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Tick bites are thought to be the cause of red meat allergy (RMA). In humans, RMA is diagnosed by 3 – 5 hour delayed urticaria and anaphylaxis to dietary red meat, is caused by an unusual occurrence of enhanced IgE antibody production against galactose-alpha-1,3-galactose (aGal), which is a common glycan in mammalian tissue with the exceptions of old world monkey and human. A growing number of cases of RMA have been reported in the southern and eastern U.S. (1,500-3,500 cases in Virginia and North Carolina depending on the sources of the data), but it is prevalent worldwide including Europe, Asia, and South America. Studies have found the links of specific tick species of geographical regions to occurrence of RMA: *Ixodes holocyclus* in Australia, *I. ricinus* in Europe, *Haemaphysalis longicornis* in Asia, *Amblyomma cajennense* in South America, and *A. americanum* in U.S. Numerous studies have provided strong support for tick bites as the cause of RMA, although direct evidence is yet lacking. Interestingly, however, RMA occurs in only a small subgroup of the overall human population experiencing tick bites, indicating either subpopulations of ticks are culprits of change, or of human are vulnerable for development of IgE against aGal causing RMA. We hypothesize that ticks acquire the aGal allergen from non-human blood and mediate the 'transmission' the aGal allergen to humans through salivary secretions during feeding. RMA is caused by injection of tick saliva containing aGal glycans acquired from a prior intrastadial (same molting stage) blood meal on non-human mammals. This hypothesis is further expanded to implicate males of certain species of ticks (i.e., lone star tick *Amblyomma americanum* in US) as having particularly high opportunities of transmitting the allergen to humans. Objectives of this study are to test this hypothesis by using a model system alpha-galactosyltransferase knockout mutant mouse (aGT-KO). We will also identify the allergens in tick salivary glands after feeding on different host blood. Overall, biology and phenology of the ticks causing RMA will ultimately establish guidelines for the prevention of RMA.

2. KEYWORDS: Provide a brief list of keywords (limit to 20 words).

alpha galactose, red meat allergy, IgE, lone star tick, allergy, mouse model

3. ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1. Test whether the bites of ticks with prior intrastadial blood feeding on non-human mammals causes RMA by using a model system alpha-galactosyltransferase knockout mutant mouse (aGT-KO).

Milestone(s): Develop aGT-KO mouse system as the model system for study of aGAL allergy: **100 % completed**. All mice treatments (total 132 mice) have been completed and the blood from treated mice were collected. The serum was tested for titers of IgE, aGal specific IgG, and aGal specific IgE. This study provided insightful observations, which are further discussed in the Appendix. A manuscript is now submitted to Frontiers in Immunology.

SOW aligned achievements are;

Major Task 1 Eliciting RMA in aGT-KO mouse by tick-feedings (100% completed)

A total 132 mice were treated with partially fed *Amblyomma americanum* males and females. Blood samples collected from the mice have been preserved in a deep freezer.

Major Task 2. Quantifying the allergenic activities (100% completed)

The serum samples from 132 mice have been tested for total IgE, aGal specific IgE, and aGal specific IgG. The data is presented in Appendix. The data support one of our hypothesis, partially fed tick could be stronger allergic sensitizer with higher amount of sIgE production against aGAL. However, we find that the other hypothesis of partially fed MALE tick as the causal factor of RMA is NOT strongly supported in the data.

Major Task 3. Test of ticks fed on other vertebrate bloods for aGal. This task is replaced for testing other species of ticks partially fed with bovine blood (100% completed). The data analysis suggests that different species of ticks feeding on different host produce varied levels of aGal saliva. This study led to further questions that need to be answered to understand the source of aGal as the RMA sensitizer.

Specific Aim 2. Isolate, identify, and characterize the tick salivary proteins containing aGal glycosylation.

Milestone(s): Purification and identification of aGal protein and the glycan causing RMA: **70% of original proposal, or 100% modified proposal completed**, three different tick feeding stages in males and females, respectively, were tested for the glycomics and the results were published in *Vaccines* (Appendix, Park et al., 2020, *Vaccines* 8:18). Additional samples has been prepared for immunoprecipitation of the aGal protein; 181 and 316 salivary glands from partially fed female and male ticks, respectively, with bovine blood. Unfortunately, additional analysis of glycolipidomics did not work with the samples that were sent to University of Georgia, Complex Carbohydrate Research Center. Both lipid isolation and glycoprotein isolation was not successful in this trial. However, I would like to note that the glycomics and glycoproteomics were additional goals that were et in the course of project. Therefore, the original goals are completed with the published data in *Vaccines*.

SOW aligned achievements are;

Major Task 4. Purification and identification of aGal proteins (100% completed)

Glycoproteomics study is completed. However, during the course of the work, we added another goal of glycolipidomics (in the report Y3 Q1, Plan for next report). However, analysis of glycolipid relying on the service in Complex Carbohydrate Research Center, University of Georgia did not work without clear known reason.

Major Task 5. Identification of the subtypes of aGal glycan (100% completed)

Major tasks 4 and 5 were completed and published in the journal *Vaccines* (Park et al., 2020, *Vaccines* 8:18).

Specific Aim 3. Survey field-collected ticks to identify the species, stage, gender, and host of ticks carrying aGal immunogens.

Milestone(s): **50% of original proposal, or 100% modified proposal completed**. This goal has been modified for test of different species of ticks on laboratory feedings on bovine blood. Test of three species, *A. americanum*, *Ixodes scapularis* and *Dermacentor variabilis* on aGT-KO mouse and also laboratory feeding suggests that the *A. americanum* is the major species providing the aGal and also allergic response by elevated IgE. In the course of experiment, we found that the ticks have different levels of salivary aGal production, leading to a new question for the salivary aGal levels in the different species of ticks feeding on human. We will expand and continue this goal to answer the question even after the funding duration ends. SOW aligned achievements are;

Major Task 6. Field-collection of the ticks (0% completed)

This goal was modified to the test of ticks laboratory feeding system and mouse model system. We found that *A. americanum* is the most immunogenic, but other tick species need to be further examined with larger numbers to draw a solid conclusion. The modified major task 6 is 100% completed.

Major Task 7. Molecular survey of the ticks by western blot and ELISA (100% completed)

We have tested two additional species of ticks, *Dermacentor andersoni* and *Ixodes scapularis* on aGT-KO mice to evaluate the role as the aGAL allergen elicitor. As it was discussed in the Major task 6, we found that a patterned variations in different combinations of the tick species and host species. Solid conclusion needs additional expanded investigations.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Major activities in the Specific Aim 1: To test the hypothesis of “transmission” of aGal in intrastadial multiple feedings of *Amblyomma americanum*.

Summary: The males of *A. americanum* are known to have multiple intrastadial feedings, while female ticks are generally engaged in a single feeding event on a single host for full engorgement. We speculated that this behavioral difference of male ticks could provide a greater opportunity for an intrastadial switch in hosts from a nonhuman mammal to a human. Furthermore, we found that the salivary glands of male ticks that fed on bovine blood via artificial membrane feeding contained large amounts of aGal epitopes in this study. We employed alpha-galactosyltransferase knockout mutant mice (aGT-KO) to test the differences in the responses to ticks that were partially fed on bovine blood. The results showed that the aGT-KO mouse responded to tick feeding by increasing total IgE, specific IgG and IgE levels, but with large individual variations, without sufficient levels of discrimination for feedings by partially fed ticks. Although “transmission” theory was not supported in this study, we promoted the aGT-KO mouse as the model in studies of tick biting and RMA which is contrasted to the previous studies of using mouse model (C57BL/6 control mice) showing no immune response to tick bites. In addition, the results led us to conclude that ticks feeding on different host species and immune status dynamically controls the aGal level in the tick saliva.

Results and Discussions: We measured the levels of total IgE among all treatment groups in aGT-KO mice and C57BL/6 wild-type (control) mice after treatment. All treatments in the aGT-KO mice showed higher levels of total IgE in comparison to the treatments in C57BL/6 control mice (Figure 3A). The HSA immunization, unfed male and female (UM, UF), and both fed female treatments (UM, FF, FM, FF) showed statistically significant differences when compared to the “sham” group in the ANOVA test ($p > 0.05$). The chi-square test after the 95% distribution cutoff, providing the statistics for frequencies of individuals who were different from the responses of the sham control, showed significant differences across all treatments, with a tendency of higher total IgE levels in the treatments with partially fed female (FF) ticks, with few individuals presenting a hyperreaction (Fig. 1). Highly significant differences in total IgE levels were observed between the “sham” and all tick feeding treatments.

Specific IgE levels against aGal-MSA, which is considered to be involved with the major contribution of specific IgE toward aGal, but not MSA, were significantly different in the tick feeding treatments that included any combinations of partially fed ticks when compared to the “sham” group in the chi-square test for frequencies of individuals with high levels of specific IgE. In the treatments of control mice, there were no significant differences in either the ANOVA or Chi square test for specific IgE against aGal-MSA (Fig. 1). In contrast to specific IgE against aGal-MSA, the levels of specific IgE against aGal-HSA were low in all treatments with the exception of a few individuals showing highly elevated specific IgE against aGal-HSA in the mice treated with aGal-HSA but not in the mice injected with HSA.

Specific IgG levels to aGal-HSA in the aGT-KO mice were significantly increased in both HSA and aHSA immunization (Fig. 1). Tick treatments resulted in significant levels of responses in the number of individual mice in all combinatory treatments for specific IgG against aGal-HSA, which is likely contributed by specific IgG against aGal, but not to HSA. C57BL/6 mice showed the highest levels of IgG against aGal-HSA under aGal-HSA treatment, whereas these mice showed no responses to tick feeding in terms of their specific IgG levels against aGal-HSA. The levels of specific IgG against aGal-MSA in the aGT-KO mice were generally high in the tick feeding groups (Fig. 1), while those in C57BL/6 mice were very low (Fig. 1), which suggests that specific IgG against aGal-MSA in the knockout mice is primarily against the aGal epitope.

We questioned whether partially fed male ticks with bovine blood carrying high levels of aGal epitopes in the salivary glands efficiently elevate specific IgE against aGal. In the comparison between partially fed male ticks and naïve ticks, we were unable to find higher levels of specific IgE against aGal in ticks preconditioned with bovine blood. However, feedings with both treatments efficiently elevated specific IgE against aGal in aGT-KO mice with large individual variations. It is possible that the high levels of aGal in the saliva of bovine blood-fed ticks could have rapidly declined in the course of feeding on aGT-KO mice, as discussed above, resulting in no differences in the host response between the partially fed vs. unfed naïve tick treatments. We observed a lack of or low levels of aGal epitopes in the salivary glands after the ticks were fed on mice, in contrast to the case of artificial membrane feeding on bovine blood. It is also noted that a previous study reported that males of *A. americanum* that were unfed and partially fed on sheep blood lacked aGal epitopes in the salivary glands (Crispell et al., 2019).

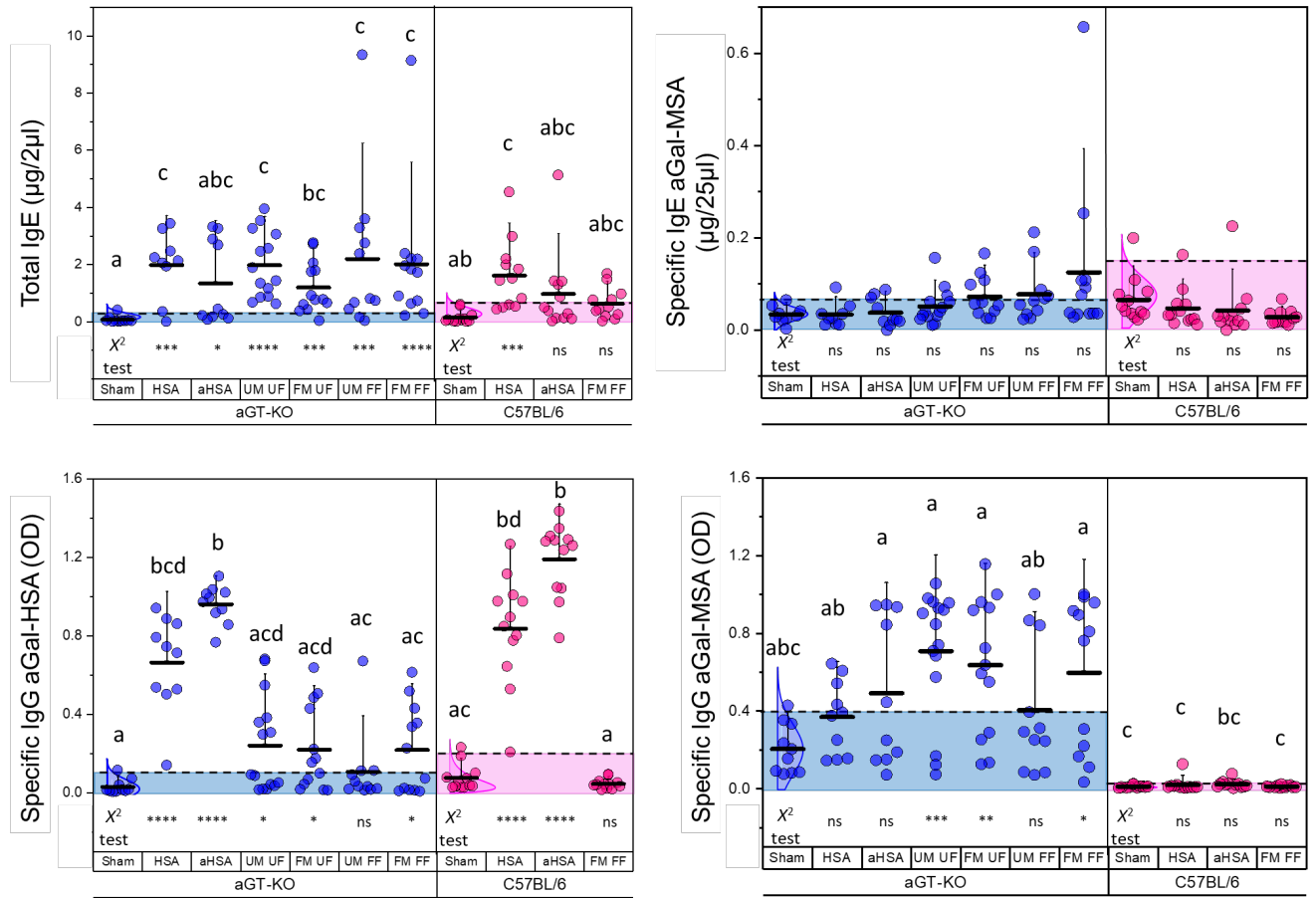


Fig. 1. Total and specific IgE to aGal and specific IgG levels to aGal-HSA and aGal-MSA. A; Total IgE levels of different treatment groups (in µg/2 µl of serum) in aGT-KO mice (blue) and C57BL/6 mice (magenta). B; Specific IgE to aGal-MSA (in µg/25 µl of serum). C; Specific IgG to aGal-HSA (OD value) and D; Specific IgG to aGal-MSA (OD value). Note that the positive control for aGal is the immunization by aHSA when specific immunoglobulins against aGal-MSA were measured. Axis labels; HSA, human serum albumin; aHSA, aGal conjugated to HSA; UM, unfed male tick; UF, unfed female tick; FM, fed male tick; FF, fed female tick. The alphabets show the significant differences at $p=0.05$ in Kruskal-Wallis ANOVA test. Lack of alphabets represents no statistical difference seen. Error bars represent the mean (lower horizontal bar) and standard deviation (upper vertical bars). Dotted lines and the shaded area show the cutoff for difference at 95% normal distribution of the control (sham control) for determining frequencies of differences, which was used for Chi-square test, and the statistics below the graph represent the Chi-square test results for significance indicated by *, $* < 0.05$, $** < 0.01$, $*** < 0.001$ and ns = not significant. Figure published in Maldonado *et al.* (In press).

Large individual variations in mouse immune responses within the same treatments could be attributed to the differences among individual mice, the differences among ticks, and the inevitably different experimental procedures for tick feedings (i.e., the length of feeding). The contributions of the differences among individual mice to the individual variations were shown by the HSA and aHSA injection responses (Fig. 1), which generally present lower levels of variation compared to the tick feeding treatments. Therefore, major causes of the differences among individuals appear to be the differences among ticks and the varied experimental procedures for tick feedings in the experimental sets. Individual differences in the protein content of the salivary glands in *A. americanum* during feeding on the same host were previously shown (Kim *et al.*, 2016a). Although this study does not support the aGal “transmission” hypothesis with the current set of data, our observations and previous reports together, as discussed above for changes in salivary contents by various factors, lead the future directions of the study to address understanding the dynamics of aGal contents in tick saliva depending on different hosts and on the immune status of the hosts. A recent report has also shown the variations in aGal levels in the tick salivary glands after feeding on different hosts (Villar *et al.*, 2021). In addition, host-specific tick salivary proteins would be further supported by the saliva containing host proteins (Kim *et al.*, 2016b, Oliveira *et al.*, 2013). Furthermore, the male specific salivary proteins (i.e. immunoglobulin binding protein, (Wang *et al.*, 1998)) may contribute to the variation in our experimental system where a pair of ticks attached on adjacent feeding sites of the mouse.

Immunoglobulins for aGal are known to be an important component as protective antigens against human pathogens (Cabezas-Cruz & de la Fuente, 2017b, Cabezas-Cruz & de la Fuente, 2017a, Tangvoranuntakul et al., 2003). Many pathogens, including those vectored by arthropods, carry aGal (Almeida et al., 1991, Avila et al., 1989, Lei et al., 2015, Moura et al., 2017, Zamze et al., 1991). A number of tick-associated bacteria have been identified as aGal producer, hence, a role for these bacteria in the sensitization of AGS has been proposed (Cabezas-Cruz et al., 2018, Sharma & Karim, 2021). Bacterial association of the AGS needs to be further studied to understand the intricate interactions among the arthropod-host-microbe. In addition, a glyco aGal-based vaccine is known to effectively protect against Chagas infection in mice (Portillo et al., 2019). Anti-aGal antibodies confer protection against malaria in mice (Yilmaz et al., 2014). Understanding the signaling pathways for triggering protective antigens instead of sensitizing the allergic response would provide ways to improve human health.

Conclusions and other achievements: The Specific Aim 1 in the proposal is 100% completed with the major tasks 1 to 3. The results showed that the aGT-KO mouse responded to tick feeding by increasing total IgE, specific IgG and IgE levels, but with large individual variations, without sufficient levels of discrimination for feedings by partially fed ticks. The data in this study do not support the “transmission” component of RMA. Positive aspects of the study includes that we confirmed that aGT-KO mice can be used as a model for RMA studies with more specific characterizations of the immune responses that were compared to the C57BL/6 control mice. Secondly, in the process of the experiment, we learned that ticks dynamically control the aGal level depending on the host species and immune status, which led us another topic of the study that is being further confirmed and expanded.

Major activities in the Specific Aim 2: Glycomics of tick salivary gland

Summary: We investigated the N-glycans in male and female lone star ticks *A. americanum* over a duration of bovine blood feeding through artificial membrane. The aGal epitopes and cross-reactive carbohydrate determinants (CCD) increases over time after the onset of blood feeding in both male and female *A. americanum*. These CCDs include xylosylation of the core mannose, 1,3-mono and 1,3- and 1,6-difucosylations of the basal GlcNAc and mono- or diantennary aGal. Combinations of both xylosylation and aGal and fucosylation and aGal were also found on the N-glycan structures. Based on the results showing high levels of aGal in the salivary glands (SGs) during blood feeding, the results suggest that ticks use aGal as a molecular mimicry to evade the immune system of nonprimate hosts, while the aGal may function as an allergy sensitizer in the atypical host human.

Results and Discussions: To explore the effect of feeding on the N-glycan profile of the lone star tick, male and female ticks were allowed to feed in an artificial membrane feeding chamber (Kim et al., 2018, Krober & Guerin, 2007). Three different time points relative to the blood feeding of the ticks were chosen for the analyses of the N-glycan profile in the SGs: before the onset of feeding, two days after, and five days after feeding attachments (0dAF, 2dAF, and 5dAF). Male ticks of this species are known to feed multiple times for short durations (Gladney & Drummond, 1970). Therefore, the 2dAF samples represent the timepoint after completion of the first feeding, and the 5dAF samples represent the timepoint in or after the second attachment in the male feeding. For females, the initial feeding is usually continued until complete engorgement. The full engorgement of *A. americanum* females took approximately 10 days of blood feeding. Therefore, the samples 5dAF are in the middle of the female feeding period and ticks are significantly bigger in their sizes.

N-glycan profiles in the lone star tick were determined for the SGs at the three timepoints described above and for the tick carcasses (CCs) at 5dAF. N-glycans were released from polypeptides by PNGase A and PNGase F and subsequently identified by Matrix-Assisted Laser Desorption/Ionization-Mass Spectrometry (MALDI-MS) and ESI-MS. Clustering analysis of the different samples based on the N-glycosylation pattern showed two main clusters based on the tissue of the samples (Fig. 2). In the analyses, same molecular weights containing mix of two different glycan were corrected by normalization factor $\frac{1}{2}$ in the percent quantity (i.e., 2244.1 and 2489.3). The samples from the male and female CCs grouped together in a cluster separated from the SG samples (Fig. 2). Within the cluster of the salivary gland samples, the samples of male and female ticks at 5dAF and 2dAF grouped based on the timepoint (Fig. 2). The N-glycan profile of the CC samples was dominated by paucimannose structures. While female CCs mainly contained non-fucosylated paucimannoses (77% of the total N-glycan profile), the majority of the paucimannose N-glycans in male CCs were monofucosylated with a high portion of fucosylation at the basal N-acetylglucosamine (GlcNAc) (61% of the total N-glycan profile) and about 17% of the total N-glycan profile was difucosylated. Except for a minor fraction (2% of the total N-glycan profile) in males, the CC samples lacked high mannose and complex glycans, while the SG samples were generally rich in high mannoses and complex glycans. The Shannon diversity H and the richness D progressively increased in the 2dAF and 5dAF SG samples in both males and females due to increases in complex N-glycan structures (Fig. 2 and 3). This increase in the diversity of the N-glycans in the SG samples is due to by the high proportion of complex N-glycans (i.e., 2244.1, 2285.1, and 2489.3).

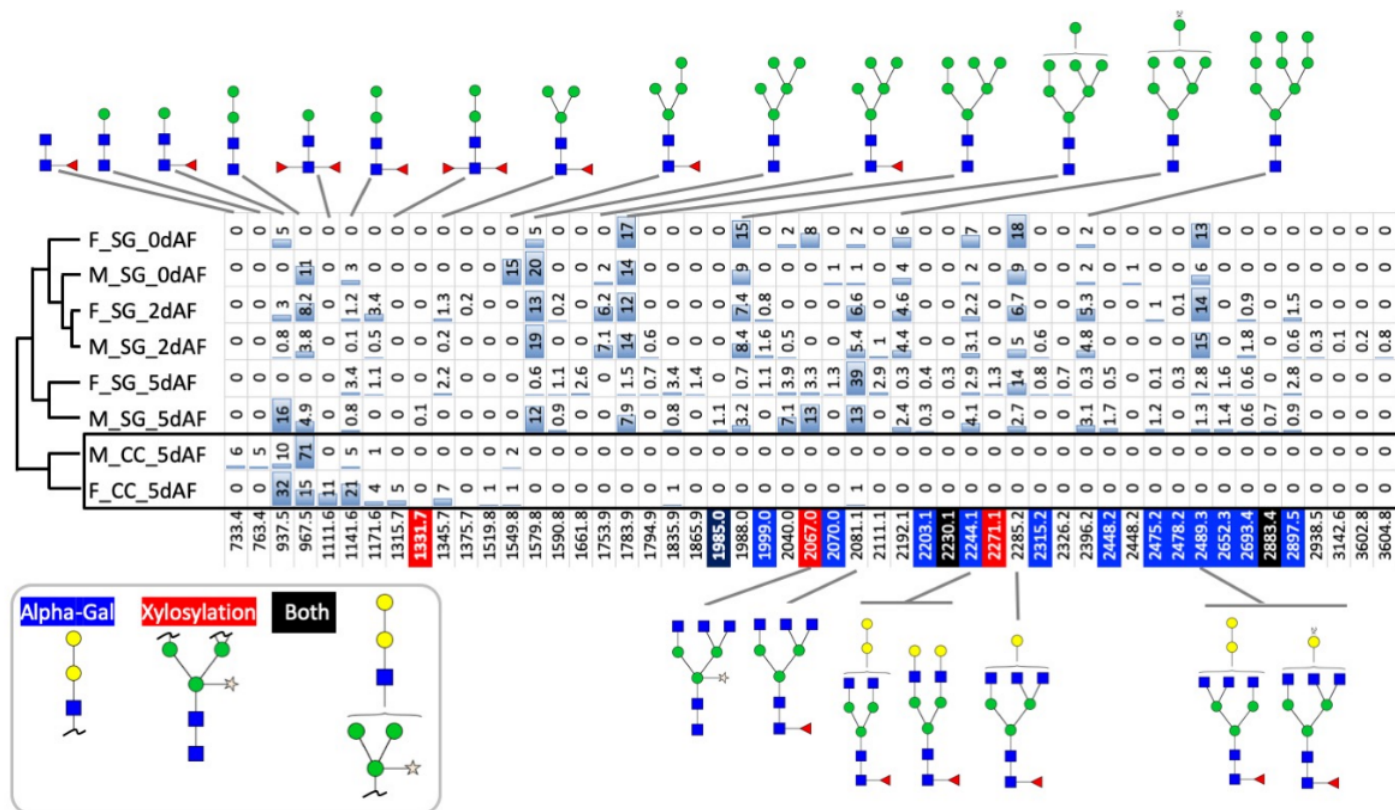


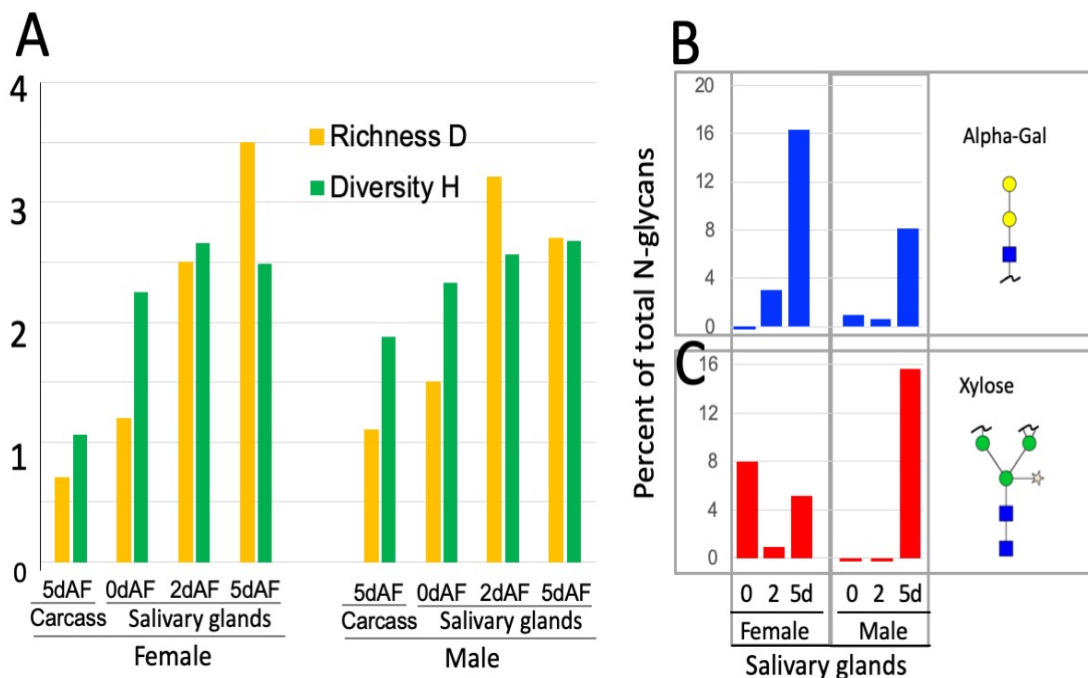
Fig. 2. Major N-glycans identified in the tick samples, carcass and salivary glands, in different feeding stages. The names of the samples indicate male/female (F or M), salivary glands/carcasses (SG or CC), and before the onset of feeding, two days after, and five days after feeding attachments (0dAF, 2dAF, and 5dAF). The number in each cell is for relative percent abundance indicated by the blue bar. ID of each glycan is shown by the molecular weight (m/z). Color codes for the ID are as follows: the ID with blue background is for the glycan containing galactose-alpha-1,3-galactose (aGal), red background is for the glycan containing xylosylation, and black background is for the glycan containing both aGal and xylosylation. In this analyses, same molecular weights containing mix of two different glycan were corrected by normalization factor $\frac{1}{2}$ in the percent quantity. See the data with the mix of more than one glycan for same ID (molecular weight) which are found in the Supplementary information 1. Structures of the glycan were shown for the glycans with more than 5% of total ion. Figure from Park et al. (2019).

The presence of N-glycans containing galactose alpha-1,3-galactose beta-1,4-N-acetylglucosamine structures, which is the most common aGal and is known as the causal factor of RMA, increased over time in the SG samples during the different feeding stages (Fig. 3). Interestingly, xylosylation at the core mannose was also commonly found in the SG samples of unfed females (8% of the total N-glycan profile) and 5dAF females (5% of the total N-glycan profile) and males (16% of the total N-glycan profile) (Fig. 3). This modification of core mannoses with xylose was mainly found in plants and some parasitic organisms (van Ree et al., 2000) and is considered an important cross-reactive carbohydrate determinant (CCD) in allergens (Altmann, 2016). Similar to insect species, paucimannose with core -1, -3 and 1, 6-fucose were commonly found (Fig. 4). In depth analysis of the monofucosylated paucimannose N-glycans by their MS decay patterns, these N-glycan structures were mainly with alpha-1,3-fucose (m/z 1011.5, 1133.6, 1141.6, 1235.6, and 1345.7).

A proportion of N-glycan structures identified in the salivary gland samples contained both a core xylose and an aGal modification or both a core mono-fucose and aGal modification (Fig. 2 and 4). However, N-glycans carrying both a core xylose and a fucose modification were not detected. Significant portions of aGal glycans were found to be di-aGal and contained the core fucose. Other trace glycans (<0.5% of the total ions) containing N-acetylneuraminic acid and N-glycolylneuraminic acid at the terminal galactose were observed in the SG samples of 2dAF females and males, respectively.

Conclusions and other achievements: The Specific Aim 2 is 70% completed with the major tasks 4 and 5. The glycomics study demonstrated that increased diversity of N-glycan in tick salivary glands over the duration of feeding is likely consistent with the changes in the repertoire of the salivary proteins. The N-glycan patterns of tick salivary glands indicates that the saliva secreted into the host may use the increasing levels of aGal as a molecular mimicry decorating the proteins to evade the host immune system as the cases of sialic acids mentioned in the introduction. Decoration of the tick salivary proteins with the host-specific glycan aGal would be an efficient way of evading the host

immune system in the case of parasitization of non-primate mammals (i.e., goat, sheep, horse, mouse, pig, and deer) that use aGal in the intrinsic system (Ogawa & Galili, 2006, Tan et al., 2010), although it may unexpectedly function as an allergy sensitizer in humans lacking aGal.



Tick N-glycans implicated in allergy as CCD

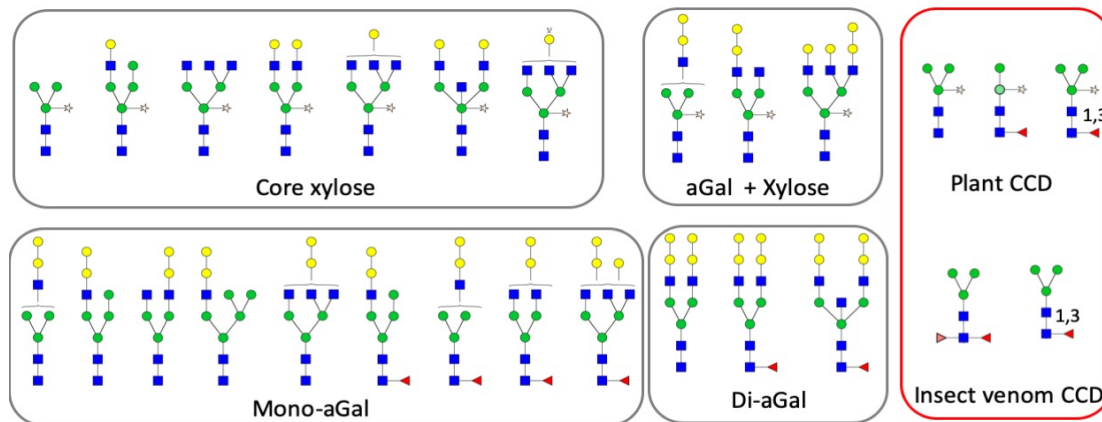


Fig. 4. N-glycans identified in the salivary glands of *Amblyomma americanum*, containing the core xylose and terminal aGal, and both. Right column shows cross-reactive carbohydrate determinants (CCDs) described for plants and insect venom. Note that these tick N-glycan are implicated in allergy by containing the CCDs.

Major activities in the Specific Aim 3: Investigations of other tick species carrying aGal immunogens.

Summary: Three species of ticks, commonly found in U.S. and highly relevant to the human health, *A. americanum*, *Ixodes scapularis* and *Dermacentor variabilis*, were tested on aGT-KO mouse to compare their immunogenic potential. Different tick species induced different levels of total IgE and specific IgG toward aGal in aGT-KO mice, indicating the saliva of different tick species have significantly different immunogenic properties.

Results and Discussions: Ticks were inoculated on the aGT-KO mouse. The experimental groups included the “sham” control group and three different species of partially fed (F) or unfed (uF) ticks. Three different species were *A. americanum* (Aa), *D. variabilis* (Dv), and *I. scapularis* (Is), which amounted to six experimental groups (uFAa, FAa, uFDv, FDv, uFIs, and FIs).

We measured total IgE levels before and after treatment with different tick species. In this trial, our tick feeding time after tick attachment was longer than that in experimental segment A (8 ± 0.4 days). The Kruskal–Wallis ANOVA and the chi square test were performed. Tick feeding efficiently induced the elevation of total IgE levels in aGT-KO mice, except the case of partially fed *I. scapularis* (Fig. 5). The total IgE of the mice infested with unfed *I. scapularis* was also relatively low compared to the total IgE induced by feedings of other species of ticks. At the levels of specific IgG against aGal-MSA, *A. americanum* treatments, both partially fed and unfed, induced (Fig. 5) high levels of the responses. Unfed *I. scapularis* also induced high levels of specific IgG in a number of individuals, while the levels were lowest in the feedings with *D. variabilis*.

Overall, *A. americanum* moderately induced total IgE but was most effective for increased specific IgG toward aGal. *D. variabilis* was the tick that induced total IgE the most efficiently, but with low levels of elevation of specific IgG toward aGal (Fig. 5). *I. scapularis* was effective in the induction of specific IgG against aGal in two individual mice. The levels of induced total IgE were correlated with the length of feeding duration to different degrees in different species (Fig. 6).

Conclusions and other achievements: The Specific Aim 3 is 50% completed (for original Aim 3, but 100% completed for modified Aim 3) with the major tasks 7, but the task 6, field collections and survey, is incomplete. Differences of the immune reactions in aGT-KO mouse were significant in the treatments of three species of tick feeding (Fig. 5). On the other hand, C57BL/6 mice infected with *A. americanum* had very low levels of total IgE elevation but no changes in specific IgG and specific IgE, which is consistent with other studies that showed low or absence of immune response to tick feedings in wild-type mice (Narasimhan et al., 2019, Wada et al., 2010). These data imply that aGal is a major player in the immune response against ticks in aGT-KO mice. Therefore, different strategies for bypassing the host immune system, the basophil-based immune system against tick feeding in the dermal layer, can be employed in different tick species (Chandrasekhar et al., 2019, Wada et al., 2010).

Incomplete in the original Aim 3 (50% of the original Aim) is for field survey of tick salivary glands for determining the aGal levels. This aim was incomplete due to Covid-19 during the project duration. We were unable to make contacts with the dairy farm owners and local veterinary clinics during Covid-19 pandemics, resulting in the failure of the sampling by collections of partially fed ticks from animals.

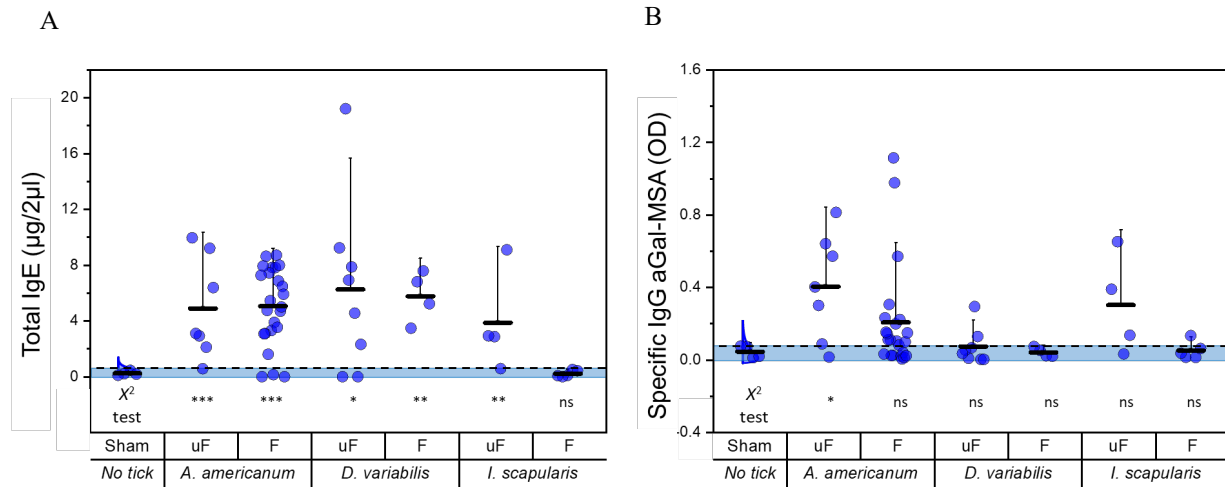


Fig. 5. Comparisons of total IgE and specific IgG to aGal-MSA before and after feeding with different ixodid tick species. A; total IgE (µg/2µl of serum) after tick infestation and B; specific IgG to aGal-MSA (OD in 2µl of serum) after tick feeding. uF; unfed, F; partially fed (on bovine blood before infestation into mice), Aa; *A. americanum*, Dv; *D. variabilis*, Is; *I. scapularis*. Kruskal-Wallis ANOVA was conducted and the lack of alphabets represents no significant differences seen with this test. Dotted lines show the cutoff for Chi square test at 0.05. Shaded areas represent the data points within the 95% distribution cutoff. The statistics below the graph represent the Chi square test (*<0.05, **<0.01, ***<0.001 and ns=not significant). Error bars represent the mean (lower horizontal bar) and standard deviation (upper horizontal bar).

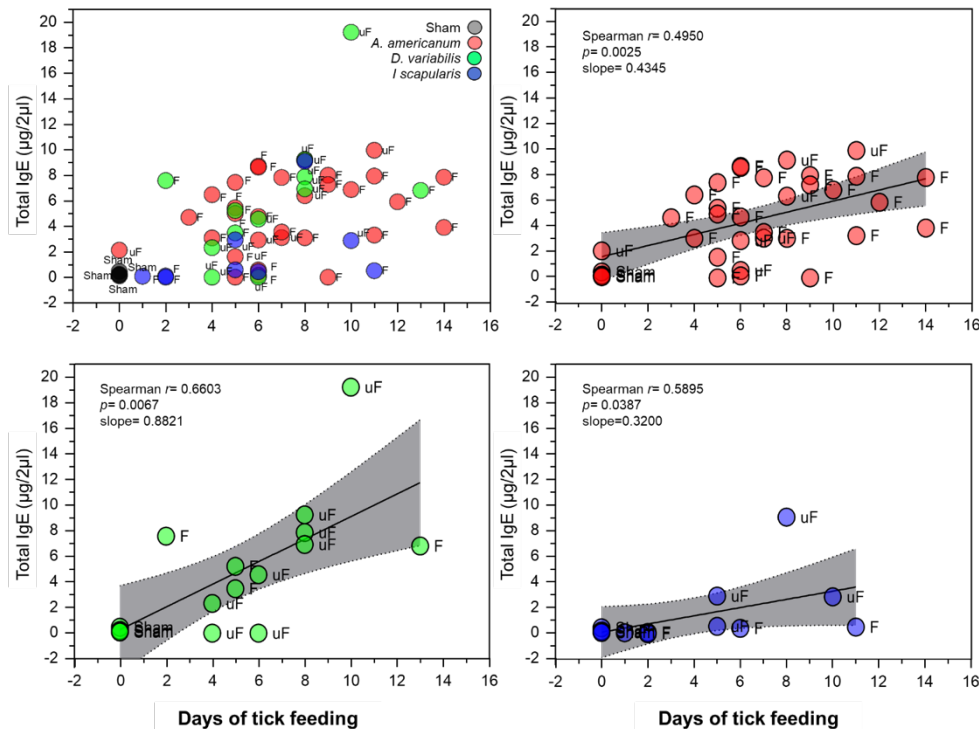


Fig. 6. Correlation of total IgE levels with days of tick feeding. A; Scatterplot showing total IgE levels (in µg/2µl of serum) in relation to days of tick feeding among the different treatments and tick species used. Gray; sham, Red; *A. americanum*, Green; *D. variabilis* and Blue; *I. scapularis*. Data labels; F; fed, uF; unfed. B; Spearman's rank correlation plot with linear regression, of *A. americanum* with days of tick feeding. C; Spearman's rank correlation plot with linear regression, of *D. variabilis* with days of feeding and D: Spearman's rank correlation plot with linear regression, of *I. scapularis* with days of feeding. Plots show positive correlation and linear regression slope. The gray shaded area represents the 95% confidence intervals.

Cited References

- Almeida, I.C., Milani, S.R., Gorin, P.A. and Travassos, L.R. (1991) Complement-mediated lysis of *Trypanosoma cruzi* trypomastigotes by human anti- α -galactosyl antibodies. *The Journal of Immunology*, **146**, 2394-2400.
- Altmann, F. (2016) Coping with cross-reactive carbohydrate determinants in allergy diagnosis. *Allergo J Int*, **25**, 98-105.
- Avila, J.L., Rojas, M. and Galili, U. (1989) Immunogenic Gal α 1–3Gal carbohydrate epitopes are present on pathogenic American *Trypanosoma* and *Leishmania*. *The Journal of Immunology*, **142**, 2828-2834.
- Cabezas-Cruz, A. and De La Fuente, J. (2017a) Immunity to α -Gal: The opportunity for malaria and tuberculosis control. *Frontiers in Immunology*, **8**.
- Cabezas-Cruz, A. and De La Fuente, J. (2017b) Immunity to α -Gal: Toward a single-antigen pan-vaccine to control major infectious diseases. *ACS Central Science*, **3**, 1140-1142.
- Cabezas-Cruz, A., Espinosa, P.J., Alberdi, P., Šimo, L., Valdés, J.J., Mateos-Hernández, L., Contreras, M., Rayo, M.V. and De La Fuente, J. (2018) Tick galactosyltransferases are involved in α -Gal synthesis and play a role during *Anaplasma phagocytophilum* infection and *Ixodes scapularis* tick vector development. *Scientific Reports*, **8**, 14224-14224.
- Chandrasekhar, J.L., Cox, K.M., Loo, W.M., Qiao, H., Tung, K.S. and Erickson, L.D. (2019) Cutaneous exposure to clinically relevant lone star ticks promotes IgE production and hypersensitivity through CD4⁺ T cell and MyD88-Dependent pathways in mice. *The Journal of Immunology*, **203**, 813 - 824.
- Crispell, G., Commins, S.P., Archer-Hartman, S.A., Choudhary, S., Dharmarajan, G., Azadi, P. and Karim, S. (2019) Discovery of α -gal-containing antigens in north american tick species believed to induce red meat allergy. *Frontiers in Immunology*, **10**.
- Gladney, W.J. and Drummond, R.O. (1970) Migration of male lone star ticks on host in relation to mating. *Journal of Economic Entomology*, **63**, 1214-&.
- Kim, D., Simo, L. and Park, Y. (2018) Molecular characterization of neuropeptide elevenin and two elevenin receptors, IsElevR1 and IsElevR2, from the blacklegged tick, *Ixodes scapularis*. *Insect Biochemistry and Molecular Biology*, **101**, 66-75.
- Kim, D., Urban, J., Boyle, D.L. and Park, Y. (2016a) Multiple functions of Na/K-ATPase in dopamine-induced salivation of the Blacklegged tick, *Ixodes scapularis*. *Scientific Reports*, **6**, 21047.
- Kim, T.K., Tirloni, L., Pinto, A.F.M., Moresco, J., Yates, J.R., Iii, Da Silva Vaz, I., Jr. and Mulenga, A. (2016b) *Ixodes scapularis* tick saliva proteins sequentially secreted every 24 h during blood feeding. *PLOS Neglected Tropical Diseases*, **10**, e0004323.
- Krober, T. and Guerin, P.M. (2007) In vitro feeding assays for hard ticks. *Trends in Parasitology*, **23**, 445-449.
- Lei, Y., Yu, H., Dong, Y., Yang, J., Ye, W., Wang, Y., Chen, W., Jia, Z., Xu, Z., Li, Z. and Zhang, F. (2015) Characterization of N-glycan structures on the surface of mature Dengue 2 virus derived from insect cells. *PLoS One*, **10**, e0132122.
- Moura, A.P.V., Santos, L.C.B., Brito, C.R.N., Valencia, E., Junqueira, C., Filho, A.a.P., Sant'anna, M.R.V., Gontijo, N.F., Bartholomeu, D.C., Fujiwara, R.T., Gazzinelli, R.T., McKay, C.S., Sanhueza, C.A., Finn, M.G. and Marques, A.F. (2017) Virus-like particle display of the α -gal carbohydrate for vaccination against *Leishmania* infection. *ACS Central Science*, **3**, 1026-1031.
- Narasimhan, S., Booth, C.J., Deponte, K., Wu, M.J., Liang, X., Mohanty, S., Kantor, F.S. and Fikrig, E. (2019) Host-specific expression of *Ixodes scapularis* salivary genes. *Ticks and Tick-borne Diseases*, **10**, 386-397.
- Ogawa, H. and Galili, U. (2006) Profiling terminal N-acetylglucosamines of glycans on mammalian cells by an immuno-enzymatic assay. *Glycoconjugate Journal*, **23**, 663-674.
- Oliveira, C.J., Anatriello, E., De Miranda-Santos, I.K., Francischetti, I.M., Sa-Nunes, A., Ferreira, B.R. and Ribeiro, J.M.C. (2013) Proteome of *Rhipicephalus sanguineus* tick saliva induced by the secretagogues pilocarpine and dopamine. *Ticks and Tick-borne Diseases*, **4**, 469-477.
- Portillo, S., Zepeda, B.G., Iniguez, E., Olivas, J.J., Karimi, N.H., Moreira, O.C., Marques, A.F., Michael, K., Maldonado, R.A. and Almeida, I.C. (2019) A prophylactic α -Gal-based glycovaccine effectively protects against murine acute Chagas disease. *npj Vaccines*, **4**, 13.
- Sharma, S.R. and Karim, S. (2021) Tick saliva and the α -gal syndrome: Finding a needle in a haystack. *Frontiers in Cellular and Infection Microbiology*, **11**, 680264-680264.
- Tan, Y.X., Gong, F., Li, S.B., Ji, S.P., Lu, Y.P., Gao, H.W., Xu, H. and Zhang, Y.P. (2010) Brief report: a new profile of terminal N-acetylglucosamines glycans on pig red blood cells and different expression of α -galactose on Sika deer red blood cells and nucleated cells. *Glycoconjugate Journal*, **27**, 427-433.
- Tangvoranuntakul, P., Gagneux, P., Diaz, S., Bardor, M., Varki, N., Varki, A. and Muchmore, E. (2003) Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid. *Proceedings of the National Academy of Sciences*, **100**, 12045-12050.
- Van Ree, R., Cabanes-Macheteau, M., Akkerdaas, J., Milazzo, J.P., Loutelier-Bourhis, C., Rayon, C., Villalba, M., Koppelman, S., Aalberse, R., Rodriguez, R., Faye, L. and Lerouge, P. (2000) beta(1,2)-xylose and alpha(1,3)-fucose residues have a strong contribution in IgE binding to plant glycoallergens. *Journal of Biological Chemistry*, **275**, 11451-11458.
- Villar, M., Pacheco, I., Mateos-Hernández, L., Cabezas-Cruz, A., Tabor, A.E., Rodríguez-Valle, M., Mulenga, A., Kocan, K.M., Blouin, E.F. and De La Fuente, J. (2021) Characterization of tick salivary gland and saliva alphagalactome reveals candidate α -gal syndrome disease biomarkers. *Expert Review of Proteomics*, null-null.
- Wada, T., Ishiwata, K., Koseki, H., Ishikura, T., Ugajin, T., Ohnuma, N., Obata, K., Ishikawa, R., Yoshikawa, S., Mukai, K., Kawano, Y., Minegishi, Y., Yokozeki, H., Watanabe, N. and Karasuyama, H. (2010) Selective ablation of basophils in mice reveals their nonredundant role in acquired immunity against ticks. *The Journal of clinical investigation*, **120**, 2867-2875.

- Wang, H., Paesen, G.C., Nuttall, P.A. and Barbour, A.G. (1998) Male ticks help their mates to feed. *Nature*, **391**, 753-754.
- Yilmaz, B., Portugal, S., Tran, Tuan m., Gozzelino, R., Ramos, S., Gomes, J., Regalado, A., Cowan, Peter j., D'apice, Anthony j.F., Chong, Anita s., Doumbo, Ogobara k., Traore, B., Crompton, Peter d., Silveira, H. and Soares, Miguel p. (2014) Gut microbiota elicits a protective immune response against malaria transmission. *Cell*, **159**, 1277-1289.
- Zamze, S.E., Ashford, D.A., Wooten, E.W., Rademacher, T.W. and Dwek, R.A. (1991) Structural characterization of the asparagine-linked oligosaccharides from Trypanosoma brucei type II and type III variant surface glycoproteins. *Journal of Biological Chemistry*, **266**, 20244-20261.
-

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Dr. Paulina Maldonado-Ruiz who was a Ph.D. student and now became postdoctoral researcher. She has been trained for tick physiology and using mice model system. She continues to utilize this training and expands her scopes in her research. Three technicians have been sequentially working on the project. All of them were trained for use of small animal mouse as the experimental system. Three undergraduate students were trained for tick collection and microdissections. In addition, all trainees expanded their scopes in red meat allergy caused by tick bites.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Two high impact publications have been the products from this research;

[Park, Y.](#), D. Kim, G. Boorgula, K. Schutter, G. Smagghe, L. Šimo, S. Archer-Hartmann, and P. Azadi (2020) Alpha-gal and cross-reactive carbohydrate determinants in the N-glycans of salivary glands in the lone star tick *Amblyomma americanum*. *Vaccines* (Impact factor 3.6), 8:18.

Maldonado-Ruiz, P., G. D. Boorgula, D. Kim, S. Fleming and [Park, Y.](#) (In press) Tick intrastadial feeding and its role on IgE production in the murine model of alpha-gal syndrome: the tick "transmission" hypothesis. *Frontiers in Immunology* (Impact factor 7.6)

What do you plan to do during the next reporting period to accomplish the goals?

If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to Report

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Impacts of the new discoveries occur by slowly infusing it into the research community and society. So far, a visible outcome of this study includes a publication of Specific Aim 2 (Park et al, 2020) in the high impact journal *Vaccines* (impact factor 3.6) which has been already 17 times cited based on the Google scholar. The second publication, combining the results of Specific Aims 1 and 3, is *in press* in the journal *Frontiers in Immunology* (impact factor 7.6). A major achievement in this study that can be further extended would be use of aGT-KO mouse in ticks and tick-borne pathogen studies, which provides a novel opportunity compared to the earlier studies using the wildtype mice which does not show the immune reactions to the tick bites. Study of the mechanisms leading the immune reactions of aGT-KO mouse against tick bite would be expandable to the studies of the roles of aGal in pathogen infection and transmission.

In addition to the impacts on the research community through the publications, the results provided additional hypotheses to be tested in the subsequent study in our research program. The most important hypothesis arose from this study is dynamic control of tick salivary aGal levels depending on the host species and host immune status. Current preliminary data indicate that the ticks biting human produce higher levels of aGal in the salivary glands than those biting other mammals. We currently look for an opportunity to verify our preliminary work and to extend the study.

We also have established collaborative networks between tick biologists and mouse immunologists. In addition to the collaborations between Park and Fleming (co-PIs), Drs. Berline Londono (vector borne disease) and Sonny Lee (Metagenomics in mouse symbionts) have been active participants in trouble shootings and designing experiments in the project and beyond.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

- 5. CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

The project started 10-01-2019 and soon after the Covid-19 pandemics became a significant impact on the proposed activities. A technician who was trained for tick inoculation on mouse, Ms. Gubavanthi Boorgula, had to leave due to her family moving to Texas, one year after the project started. Although this position was successfully replaced by other excellent researcher, Dr. Paulina Maldonado, the replacement of the main personnel caused significant delay in the proposed schedule. We requested one year no-costs extension of the project and successfully obtained the permission for extending the project to 9-30-2021 from 2020, total 2 year to 3 year. In addition to the change of 1 year no costs extension on the project, specific details of the reasons for changes and incomplete part of proposed activities are as below.

In Specific Aim 2, the original goal glycoproteomics have been incomplete while the general N-glycomics were successfully completed. The samples, tick salivary glands from partially fed ticks, were prepared from more than a total 300 ticks over a duration of almost 1 year in Park laboratory. The samples were sent for the analysis to the facility in University of Georgia Complex Carbohydrate Research Center. Unfortunately, the facility failed to identify the proteins with the aGal N-glycosylation. The reason for the failure is determined to be likely insufficiency of the aGal proteins in the samples. We concluded that an alternative approach is required for enriching aGal protein by an immunoprecipitation using affinity purified high titer antibody. Although we were unable to finish the project within the project duration, we continue this Aim by generating the affinity purified high titer anti-aGal antibody.

The Specific Aim3 has been completed for only the 50%. The incomplete original Aim 3 is caused by lacking the activities for major task 6, field collections of partially fed ticks and survey of tick salivary glands for determining the aGal levels. This aim was incomplete due to Covid-19 during the project duration. We were unable to make contacts with the dairy farm owners and local veterinary clinics during Covid-19 pandemics, resulting in the failure of the sampling by collections of partially fed ticks directly from animals.

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to Report

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume; year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

[Park, Y.](#), D. Kim, G. Boorgula, K. Schutter, G. Smagghe, L. Šimo, S. Archer-Hartmann, and P. Azadi (2020) Alpha-gal and cross-reactive carbohydrate determinants in the N-glycans of salivary glands in the lone star tick *Amblyomma americanum*. *Vaccines* (Impact factor 3.6), 8:18.

Maldonado-Ruiz, P., G. D. Boorgula, D. Kim, S. Fleming and [Park, Y.](#) (In press) Study of alpha-Gal syndrome (AGS) using a mouse model subjected to bites of the lone star tick, *Amblyomma americanum*. *Frontiers in Immunology* (Impact factor 7.6).

Books or other non-periodical, one-time publications. Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothing to Report

Other publications, conference papers and presentations. Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

Nothing to Report

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

During the study, we have improved/modified the artificial membrane tick feeding system. We have developed the system that utilizes small defined volume of feeding for a tick (100 microliter for a half day feeding), which can be a useful tool for tick-borne pathogen studies and tick physiology. Number of other laboratory members in the US have visited to my laboratory to learn this technique: Dr. Troy Anderson's lab in University of Nebraska, Dr. Daniel Swale's lab in Louisiana State University, and Dr. Andrew Li's lab in USDA-ARS in Maryland.

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name: Mary Smith
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID): 1234567
Nearest person month worked: 5

Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.

Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this award.)

Name:	Yoonseong Park
Project Role:	Professor, PI
Researcher Identifier (e.g. ORCID ID):	000-0003-1191-7335
Nearest person month worked:	0.5
Contribution to Project:	Coordination of the project, maintenance of the tick colony, tick feeding on mutant mice
Name:	Sherry Fleming
Project Role:	Professor, co-PI
Researcher Identifier (e.g. ORCID ID):	0000-0001-5385-7233
Nearest person month worked:	0.5
Contribution to Project:	Maintenance of the mouse colony, training technicians and students for IACUC using mouse in the experiments
Name:	Jennifer Rowe
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1
Contribution to Project:	Maintenance of the mouse colony
Name:	Gunavanthi Boorgula
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	6
Contribution to Project:	Maintenance of the tick colony, tick feeding, tick dissections, tick feeding on mutant mice
Name:	Paulina Maldonado-Ruiz
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	6
Contribution to Project:	Maintenance of the tick colony, tick feeding, tick dissections, tick feeding on mutant mice, plate assays for mouse samples

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner’s facilities for project activities);*
- *Collaboration (e.g., partner’s staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner’s staff use each other’s facilities, work at each other’s site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ebrap.org/eBRAP/public/index.htm> for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil/Pages/Resources.aspx>) should be updated and submitted with attachments.*

- 9. APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Two appendices are

1. Journal publication in Vaccines
2. Proof of the publication in Frontiers in Immunology



Article

Alpha-Gal and Cross-Reactive Carbohydrate Determinants in the N-Glycans of Salivary Glands in the Lone Star Tick, *Amblyomma americanum*

Yoonseong Park ^{1,*}, Donghun Kim ^{1,2}, Gunavanthi D. Boorgula ¹, Kristof De Schutter ³, Guy Smagghe ³, Ladislav Šimo ⁴, Stephanie A. Archer-Hartmann ⁵ and Parastoo Azadi ⁵

¹ Department of Entomology, Kansas State University, Manhattan, KS 66506, USA; dklome2018@knu.ac.kr (D.K.); boorgulagd@ksu.edu (G.D.B.)

² Department of Applied Biology, Kyungpook National University, Sangju 37224, Gyeongbuk, Korea

³ Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium; kristof.deschutter@ugent.be (K.D.S.); Guy.Smagghe@UGent.be (G.S.)

⁴ UMR BIPAR, INRAE, Ecole Nationale Vétérinaire d'Alfort, ANSES, Université Paris-Est, 94700 Maisons-Alfort, France; ladislav.simo@vet-alfort.fr

⁵ Complex Carbohydrate Center, University of Georgia, Athens, GA 30602, USA; sarcher@ccrc.uga.edu (S.A.A.-H.); azadi@ccrc.uga.edu (P.A.)

* Correspondence: ypark@ksu.edu

Received: 5 December 2019; Accepted: 20 December 2019; Published: 9 January 2020



Abstract: Ticks are important ectoparasites and vectors of numerous human and animal pathogens. Ticks secrete saliva that contains various bioactive materials to evade the host defense system, and often facilitates the pathogen transmission. In addition, the Lone star tick saliva is thought to be the sensitizer in red meat allergy that is characterized by an allergic reaction to glycan moieties carrying terminal galactose- α -1,3-galactose (aGal). To assess N-glycome of *Amblyomma americanum*, we examined the N-glycan structures in male and female salivary glands at three different feeding stages and in carcasses of partially fed lone star ticks. We also surveyed the genes involved in the N-glycosylation in the tick species. The aGal epitopes and cross-reactive carbohydrate determinants (CCD) increases over time after the onset of blood feeding in both male and female *A. americanum*. These CCDs include xylosylation of the core mannose, 1,3-mono and 1,3- and 1,6-difucosylations of the basal GlcNAc and mono- or diantennary aGal. Combinations of both xylosylation and aGal and fucosylation and aGal were also found on the N-glycan structures. While the enzymes required for the early steps of the N-glycosylation pathway are quite conserved, the enzymes involved in the later stages of N-glycan maturation in the Golgi apparatus are highly diverged from those of insects. Most of all, we propose that the aGal serves as a molecular mimicry of bioactive proteins during tick feedings on mammalian hosts, while it contributes as a sensitizer of allergy in atypical host human.

Keywords: alpha-gal; red meat allergy; glycosylation; xylosylation; molecular mimicry; glycan

1. Introduction

Protein glycosylation, or the attachment of (oligo)saccharides to the amino acid residues Asn (N-glycosylation) and Ser or Thr (O-glycosylation), is the most common and important post-translational modification. Protein glycosylation has important roles in protein maturation and interactions with other molecules and influences properties and activities of the protein [1]. Protein glycosylation is essential in all eukaryotic cells and is involved in multiple crucial processes in the cell. This is reflected in the conservation of glycosylation sites and glycan structures [2,3] and essential roles of the enzymes involved in glycan biosynthesis and maturation [4,5]. While the metabolic pathways for the early steps

of N-glycosylation are well conserved throughout different taxa of eukaryotes, divergence is observed at the subsequent steps generating interspecies and intraspecies specific N-glycan structures [6]. This diversity of glycans in different organisms involves evolutionary selection processes with the selection pressure on the presence/absence of functional metabolic genes, enzyme activities, and their substrate specificities. The general evolutionary pattern of N-glycan likely involved drift in neutral or near-neutral evolution based on the high variations in closely related taxa, whereas the parts of N-glycan variations were consequences of selection pressure in the arms races between pathogen and immune systems [7].

An interesting aspect of glycan function is its role in pathogen (or commensal bacteria) recognition and vector-host interactions. In the immune system, which consists of a complex network of cells and molecules that interact with each other to initiate the host defense system, many interactions involve specific carbohydrate structures and proteins that specifically recognize and bind them [8]. So are pathogens recognized through highly conserved structures, pathogen associated molecular patterns (PAMPs) [9]. Many of these PAMPs are carbohydrate structures present in the cell wall or glycan structures present on the cell surface, which are bound by the pattern recognition receptors (PRRs) of the host immune system [9]. On the other hand, the binding of pathogens to host cells in infection processes often depends on the glycan receptors on the host cell surface [10]. In the evolutionary arms race, some pathogens adopted the strategy of “molecular mimicry” and decorate their surface with host glycan structures to evade the host immune system. For example, the incorporation of sialic acid (Sia) in the gram negative bacterium *Pseudomonas aeruginosa* allows binding with the Sia-binding immunoglobulin-like lectins (siglecs) on the surface of neutrophils [11]. This interaction causes the neutrophil to recognize the bacteria as ‘self’ which dampens the neutrophil reactivity [12]. Molecular mimicry of host glycans is also known in *Trypanosoma cruzi*, which utilizes host sialic acid transferred to the terminus of mucin-like O-glycan on the surface of the parasite [13,14]. Further, molecular mimicry has been identified as one of the mechanisms by which infections can initiate or exacerbate autoimmune diseases [15].

Ticks are important hematophagous arthropods and vectors of numerous human and animal pathogens. These external parasites mainly belong to two families, the Ixodidae (hard ticks, ~700 species) and the Argasidae (soft ticks, ~200 species) [16]. During blood feeding, ticks secrete saliva that contains various bioactive materials to evade the host defense system and they can transmit various pathogens causing diseases, e.g., Lyme disease and tick-borne encephalitis [17]. Recently, glycomics of ticks have attracted attention due to certain species of ticks, including the lone star tick *Amblyomma americanum* (Ixodidae) in the U.S, were found to be causing red meat allergy (RMA). RMA in humans, which is diagnosed by a 3–5 h delayed urticaria and anaphylaxis to dietary red meat, is caused by an unusual occurrence of enhanced IgE antibody production against glycan moieties carrying terminal galactose- α -1,3-galactose (aGal). This is a common glycan in mammalian tissue with the exceptions of old world monkeys and humans [18–22].

In this study, we investigated the N-glycans in male and female lone star ticks *A. americanum* over a duration of blood feeding. Based on the results showing high levels of aGal in the salivary glands (SGs) during blood feeding, we propose that ticks use aGal as a molecular mimicry to evade the immune system of nonprimate hosts, while the aGal may function as an allergy sensitizer in the atypical host human.

2. Materials and Methods

2.1. N-Glycan Profiling

Adult ticks were purchased from Oklahoma tick rearing center. Partially engorged female ticks (4–5 days blood fed) were prepared using a modified artificial feeding system [23,24]. Dissected tissues from ~50 individual ticks were made over a duration of a month in multiple feeding chambers, and were stored at -80°C until the analyses.

The overall workflow for glycan analysis included the following steps: homogenization, tryptic digestion, N-glycan release, permethylation, and nanospray ionization–mass spectrometry (NSI-MS) including tandem mass spectrometry (MS/MS) fragmentation. All reagents were purchased from Sigma Aldrich unless otherwise mentioned. Mass spectrometric data acquisition was performed on a Thermo Scientific LTQ Orbitrap Fusion Tribrid mass spectrometer. Data analysis was performed by using Data Explorer V4.5, Xcalibur 3.0, and GlycoWorkbench 1.1.

2.2. Release of N-Linked Glycans

For the samples, ~50 pairs of dissected salivary glands or 20 carcasses, were homogenized in PBS for 30 s (Branson Sonifier), reduced for 45 min at 45 °C in a solution of 5 mM DTT (Dithiothreitol), and alkylated at room temperature in the dark in a solution of 15 mM iodoacetamide. A subsequent aliquot of DTT (5 mM) was added to quench the reaction. The reduced and alkylated samples were then digested with trypsin (porcine, Sigma) in 50 mM pH 8 Tris-HCl buffer overnight. After protease digestion, the sample was passed through a C18 Sep-Pak cartridge (Supelco), washed with 5% *v/v* acetic acid in mΩ water (Barnstead), and the glycopeptides were eluted with a blend of isopropanol (40%, 80%, and 100%) in low 5% *v/v* acetic acid before being dried by SpeedVac [25].

The dried glycopeptide eluate was digested with a combination of PNGase A (Calbiochem) and PNGase F (New England Biolabs) overnight at 37 °C to release the N-linked glycans. The digest was then passed through a C18 Sep-Pak cartridge (Supelco) with 5% *v/v* acetic acid to recover the N-glycans. The pass-through fraction containing released glycans was then dried via lyophilization.

2.3. Per-O-Methylation of N-Linked Glycans

The glycans were permethylated for analysis by ESI-MS (Electrospray ionization mass spectrometry). Briefly, the sample was dissolved in dimethyl sulfoxide (DMSO) and incubated with methyl iodide in a DMSO/NaOH mixture. The reaction was quenched with water, and permethylated O-glycans were extracted with methylene chloride.

2.4. Profiling and Fragmentation Analysis by Nanospray Ionization-Mass Spectrometry (NSI-FTMS/MS)

The sample was then dissolved in methanol, and an aliquot was taken for NSI-MS. ESI-MS and MS/MS analysis of permethylated glycans was performed on an Orbitrap Fusion mass spectrometer through an NSI probe [25]. The MSⁿ spectra collision-induced fragmentation (CID) and higher-energy collisional dissociation (HCD) of the glycans were acquired at high resolution (selected CID spectra are shown since HCD produced similar spectra). Assignment of glycan structures was performed manually, and with assistance by GlycoWorkbench software.

Full MS spectra as well as an automated “TopN” MS/MS program of the top 300 peaks were collected and fragmented with CID. These data were used to search for a Hex-Hex-HexNAc signature, both with a diagnostic fragment and expected neutral losses.

When glycoforms matching a Hex-Hex-HexNAc signature were found in some of the samples, the rest of the samples were rerun and manually fragmented at masses corresponding to each of the possible N-linked glycoforms. This was performed to confirm the presence or absence of these glycoforms even if they were in trace amounts and not detected in the full MS spectra.

2.5. Bioinformatics

Using the NGRG protein sequences from *D. melanogaster*, *T. castaneum* and humans as queries in NCBI BLAST searches, orthologous sequences were identified in the tick proteomes or genomes (taxid:6933). Identified sequences were used for back-blasts to the databases of *D. melanogaster*, *T. castaneum* and human sequences and analyzed for domain structure through Motif search (<https://www.genome.jp/tools/motif/>) to verify their orthology.

The set of putative NGRG sequences obtained in the blast searches were used for sequence alignment and phylogenetic analyses with the known *D. melanogaster*, *T. castaneum* and human

sequences. The protein sequences were aligned with MUSCLE (Multiple Sequence Comparison by Log-Expectation) using default settings in MEGA7 (Molecular Evolutionary Genetics Analysis) [26]. Neighbor-joining trees [27] were build and a phylogenetic tree is drawn with the relative scale of branch lengths computed using the Poisson correction method. Five hundred bootstrapping values are shown next to the branches [28].

3. Results

3.1. Spatial and Temporal Changes in the N-Glycosylation Patterns in the Salivary Glands of Male and Female *A. americanum*

To explore the effect of feeding on the N-glycan profile of the lone star tick, male and female ticks were allowed to feed in an artificial membrane feeding chamber [23,24]. Three different time points relative to the blood feeding of the ticks were chosen for the analyses of the N-glycan profile in the SGs: before the onset of feeding, two days after, and five days after feeding attachments (0dAF, 2dAF, and 5dAF). Male ticks of this species are known to feed multiple times for short durations [29]. Therefore, the 2dAF samples represent the timepoint after completion of the first feeding, and the 5dAF samples represent the timepoint in or after the second attachment in the male feeding. For females, the initial feeding is usually continued until complete engorgement. The full engorgement of *A. americanum* females took approximately 10 days of blood feeding. Therefore, the samples 5dAF are in the middle of the female feeding period and ticks are significantly bigger in their sizes.

N-glycan profiles in the lone star tick were determined for the SGs at the three timepoints described above and for the tick carcasses (CCs) at 5dAF. N-glycans were released from polypeptides by PNGase A and PNGase F and subsequently identified by Matrix-Assisted Laser Desorption/Ionization-Mass Spectrometry (MALDI-MS) and ESI-MS. Clustering analysis of the different samples based on the N-glycosylation pattern showed two main clusters based on the tissue of the samples (Figure 1). In the analyses, same molecular weights containing mix of two different glycan were corrected by normalization factor $\frac{1}{2}$ in the percent quantity (i.e., 2244.1 and 2489.3). The samples from the male and female CCs grouped together in a cluster separated from the SG samples (Figure 1, full list is in the Supplementary Figure S1). Within the cluster of the SG samples, the samples of male and female ticks at 5dAF and 2dAF grouped based on the timepoint (Figure 1). The N-glycan profile of the CC samples was dominated by paucimannose structures. While female CCs mainly contained non-fucosylated paucimannoses (77% of the total N-glycan profile), the majority of the paucimannose N-glycans in male CCs were monofucosylated with a high portion of fucosylation at the basal N-acetylglucosamine (GlcNAc) (61% of the total N-glycan profile) and about 17% of the total N-glycan profile was di-fucosylated (Supplementary Figure S1). Except for a minor fraction (2% of the total N-glycan profile) in males, the CC samples lacked high mannose and complex glycans, while the SG samples were generally rich in high mannoses and complex glycans. The Shannon diversity H and the richness D progressively increased in the 2dAF and 5dAF SG samples in both males and females due to increases in complex N-glycan structures (Figures 1 and 2). This increase in the diversity of the N-glycans in the SG samples is due to by the high proportion of complex N-glycans (i.e., 2244.1, 2285.1, and 2489.3 in Figure 1, Supplementary Figures S2 and S3).

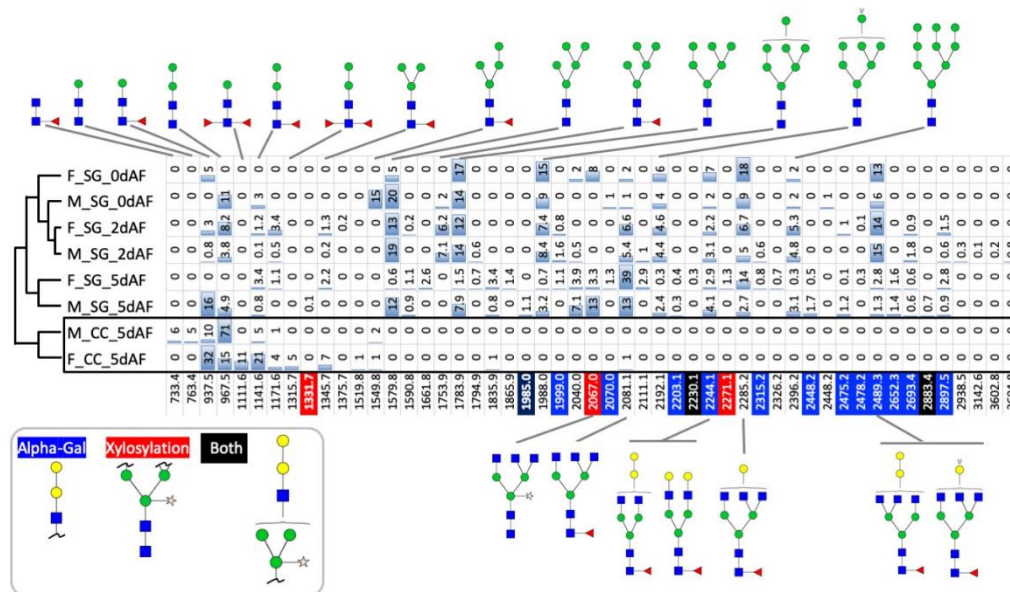


Figure 11. Major N-glycans identified in the tick samples, carcasses and salivary glands, in different feeding stages. The names of the samples indicate male/female (F or M), salivary glands/carcasses (SG or CC), and before the onset of feeding, two days after, and five days after feeding attachments (0dAF, 2dAF, and 5dAF). The number in each cell is for relative percent abundance indicated by the blue bar. ID of each glycan is shown by the molecular weight (m/z) of the glycan. The IDs are as follows: the ID with blue background is for the glycan containing galactose, alpha-1,3-galactose, beta-1,4-N-acetylglucosamine, and is for the glycan containing xylosylation, and black background is for the glycan containing both galactose and xylosylation. In this analysis, same molecular weights containing different glycan were corrected by normalization factor $\frac{1}{2}$ in the percent quantity. See the data with the 196 of data that the glycan for more than molecular weight which are followed in the Supplementary Information. Structure of the glycan shown in the figure with the shown of total glycan for detail information in a word. For details, please see Supplementary Figure S1.

In addition, the presence of N-glycans containing galactose alpha-1,3-galactose beta-1,4-N-acetylglucosamine structures, which is the most common aGal and is known as the causal factor of RMA, increased over time in the SG samples during the different feeding stages (Figure 2B). Interestingly, xylosylation at the core mannose was also commonly found in the SG samples of unfed females (8% of the total N-glycan profile) and 5dAF females (5% of the total N-glycan profile) and males (16% of the total N-glycan profile) (Figure 2B, Supplementary Figure S4). This modification of core mannoses with xylose was mainly found in plants and some parasitic organisms [30] and is considered an important cross-reactive carbohydrate determinant (CCD) in allergens [31]. Similar to insect species, paucimannose with core 1, 3 and 1, 6-fucose were commonly found (Figure 1). In-depth analysis of the monofucosylated paucimannose N-glycans by their MS decay patterns, these N-glycan structures were mainly with alpha-1,3-fucose (m/z 1011.5, 1133.6, 1141.6, 1235.6, and 1345.7, i.e., Supplementary Figure S5).

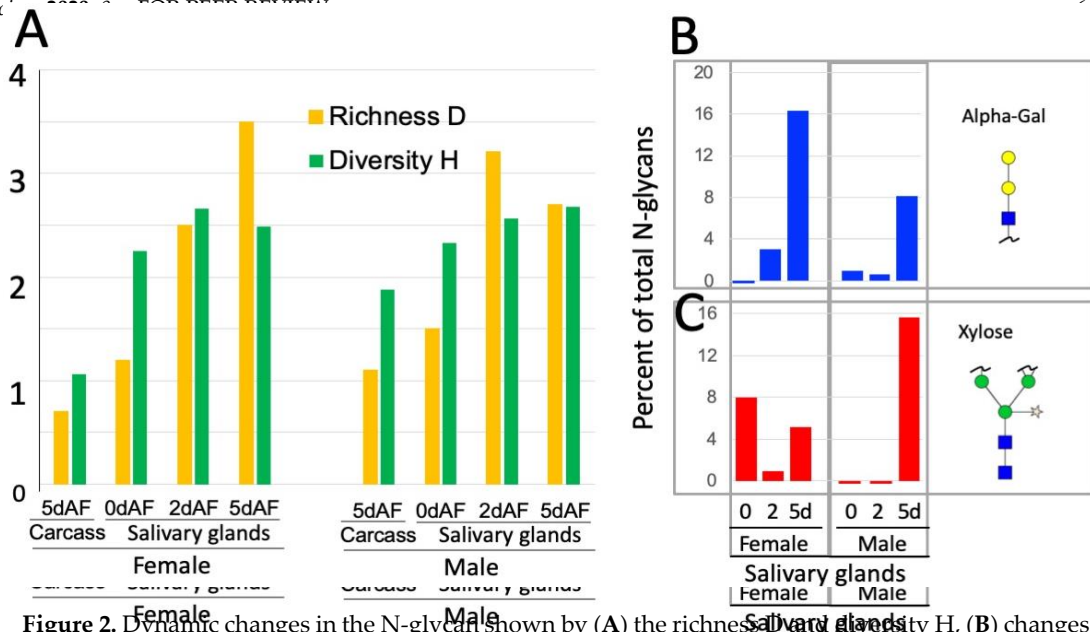


Figure 2. Dynamic changes in the N-glycan shown by (A) the richness D and diversity H, (B) changes in aGal glycans, and (C) the glycans with the core xylose during the time of feeding in the tick salivary glands.

A proportion of N-glycan structures identified in the SG samples contained both a core xylose and an aGal modification or both a core mono-fucose and aGal modification (Figure 3). However, N-glycans carrying both a core xylose and a fucose modification were not detected. Significant portions of aGal glycans were found to be di-aGal and contained the core fucose (Figure 3). Other trace glycans (<0.5% of the total ions) containing N-acetylneuraminic acid and N-glycolylneuraminic acid at the terminal galactose were observed in the SG samples of 2dAF females and males, respectively (Figure 4).

Tick N-glycans implicated in allergy as CCD

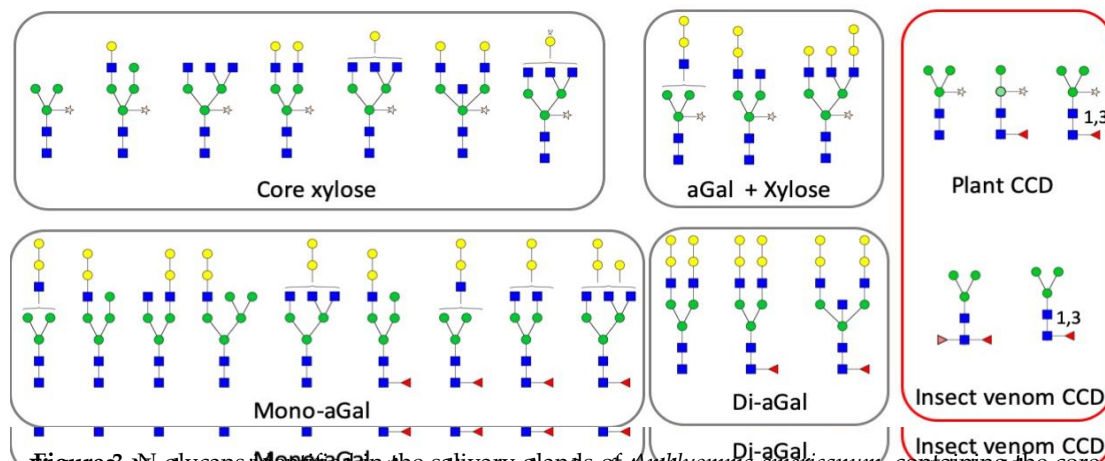


Figure 3. N-glycans identified in the salivary glands of *Amblyomma americanum*, containing the core xylose and terminal aGal, and both. Right column shows cross-reactive carbohydrate determinants (CCDs) described for plants and insect venom. Note that these tick N-glycan are implicated in allergy by containing the CCDs.

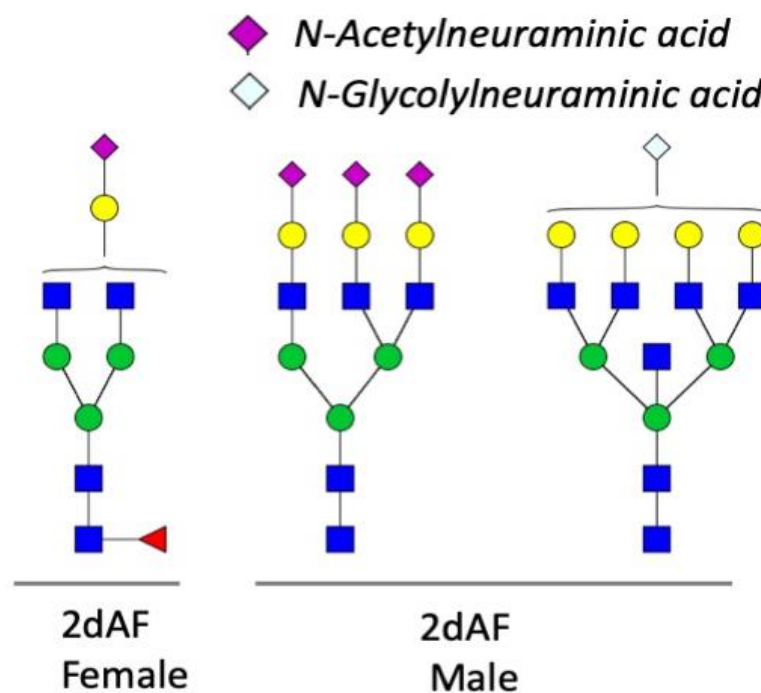


Figure 4. Trace N-glycans carrying the terminal *N-Acetylneuraminic acid* and *N-Glycolylneuraminic acid* in the salivary glands of *Amblyomma americanum*.

3.2. Genes Encoding the Enzymes for N-Glycan Processing

Protein N-glycosylation is enabled via a complex and integrated sequence of enzymatic reactions shown in the diagram in Figure 5. The putative N-glycosylation related genes (NGRGs) in the genome of ticks, encoding for the enzymatic activities of the N-glycosylation pathway, were identified through BLAST searches using the known NGRG sequences from *Drosophila melanogaster*, *Tribolium castaneum* and *Homo sapiens*. A phylogenetic analysis was performed on the identified NGRG sequences of eight tick species (*Amblyomma americanum*, *A. cajennense*, *A. meculatum*, *A. sculptum*, *A. triste*, *A. variegatum*, *Ixodes ricinus*, and *I. scapularis*), *Drosophila melanogaster*, *Tribolium castaneum* and *Homo sapiens* to analyze their evolutionary relationship. The early steps of N-glycan biosynthesis start in the endoplasmic reticulum (ER) by the transfer of the fourteen sugars N-glycan precursor from its dolichol phosphate carrier to an Asn residue of the growing peptide by the oligosaccharide transferase (OST) complex. This step is highly conserved and an ancestral character. Ticks have a highly conserved one-to-one orthology with the OST subunits STT3A and STT3B and the dolichyl-diphospho-oligosaccharide-protein glycosyltransferase subunit DAD1 (Figure 5 and Figure 6 (1)). The next step in the N-glycosylation pathway is the trimming of the terminal glucose, an essential step required for the correct folding and the quality control of the glycoproteins. This crucial role of the glucose mannosyl-oligosaccharide glucosidases GCS1 and 2, is translated in the conservation of the genes encoding these activities with insect genes (Figure 6 (2)).

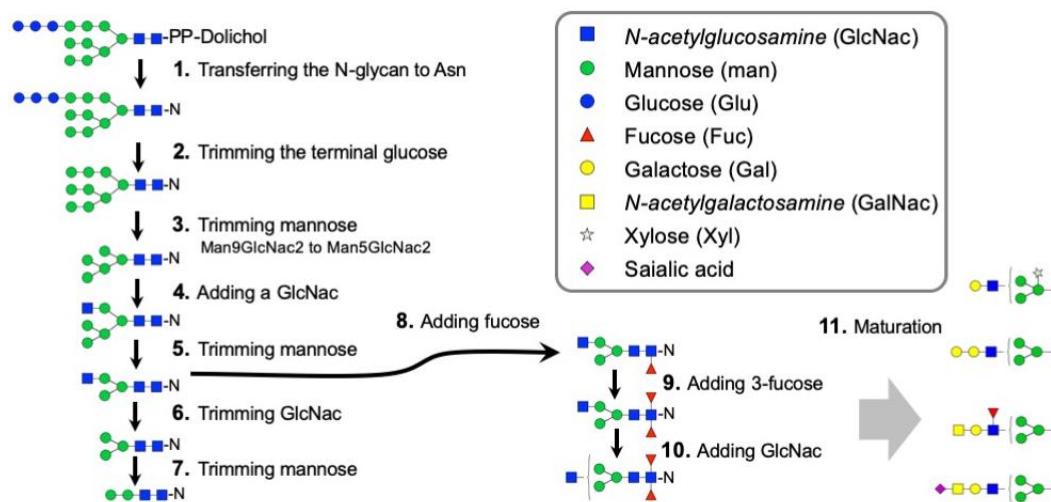


Figure 5. Generalized biochemical steps in biosynthesis of N-glycans. The numbers in each metabolic step involve the enzymes shown in Figures 6 and 7 with the matching numbers.

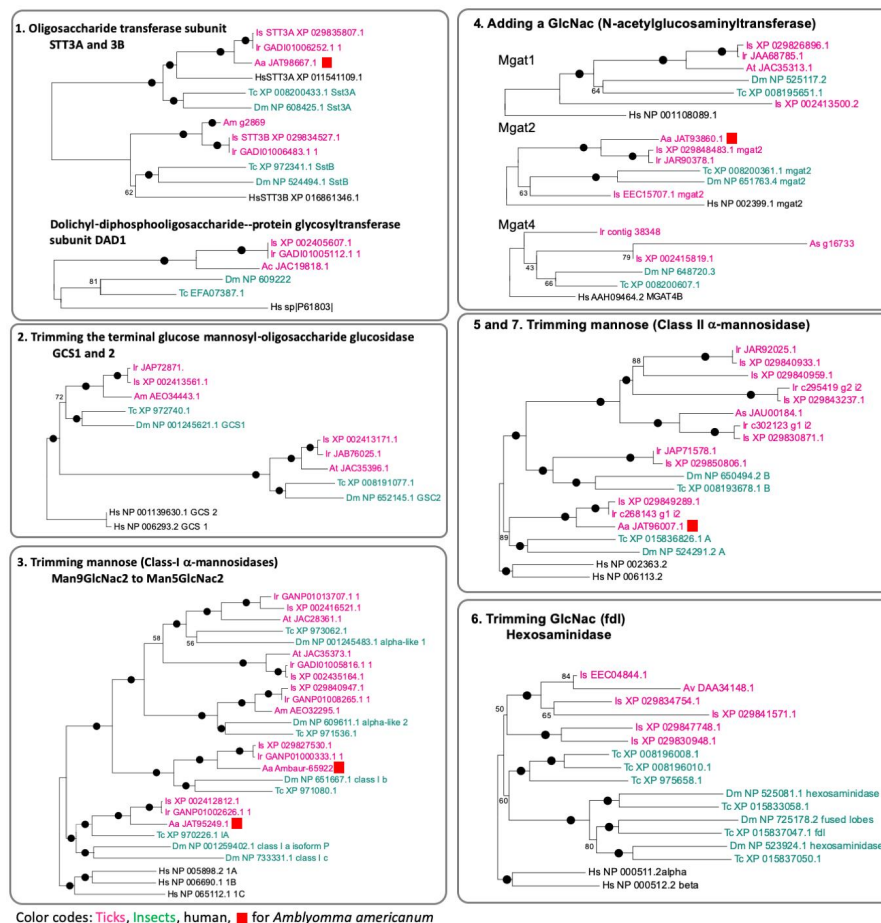


Figure 6. Phylogenetic trees for the genes involved in the N-glycan biosynthesis at the early Steps 1 to 7 in Figure 5. The numbering and the biological activities are referenced in Figure 5. Prefixes indicate the species: Aa for *Amblyomma americanum*, Ac for *A. cajennense*, Am for *A. meculatum*, As for *A. sculptum*, At for *A. triste*, Av for *A. variegatum*, Dm for *Drosophila melanogaster*, Hs for *Homo sapiens*, Ir for *Ixodes ricinus*, Is for *I. scapularis*, and Tc for *Tribolium castaneum*. Color codes are for the genes with the fonts: magenta for ticks, green for insects, and black for human. Aa (*Amblyomma americanum*) is highlighted by red square. Note that Aa sequence in the database is yet incomplete and likely caused the low levels of coverage of the subjected genes in the analyses.

The enzymes involved in the next steps of the pathway, trimming of alpha-1,2-mannoses (Step 3) and addition of a GlcNAc residue to the core alpha-1,3-mannose (Step 4) (Figure 5), appear to have a moderate degree of complexity. At least five different genes were grouped for the Class-I α -mannosidases (Man1a and b) that trim Man₉GlcNAc₂ to Man₅GlcNAc₂. Four tick genes were orthologous to those of insects, while one group was specific only in the ticks (Figure 6 (3)). The N-acetylglucosaminyltransferase, which adds a GlcNAc on trimmed mannose, could be subcategorized into three subgroups: Mgat1, alpha-1,3-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase; Mgat2, alpha-1,6-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase; and Mgat4, alpha-1,3-mannosyl-glycoprotein 4-beta-N-acetylglucosaminyltransferase. The *I. scapularis* genome contained two predicted genes for each Mgat1 and Mgat2 and one orthologous gene for Mgat4 (Figure 6 (4)).

The addition of the first GlcNAc residue is required for the further trimming of mannose in the Golgi by Class II α -mannosidases (Steps 5 and 7 in Figure 5). Ticks have two orthologues of insect Class II α -mannosidases and at least 4 additional genes in this group in the case of *I. scapularis* (Figure 6 (5) and Figure 7). Other ticks also have additional genes in this group, but because the genomes and transcriptomes of other tick species are not complete, a clear phylogenetic analysis of this group of genes could not be provided. A characteristic to the N-glycome of invertebrates is the presence of paucimannoses [32], formation of these N-glycan structures consisting of (un)modified tri- or bimanosylchitobiosyl cores requires the activity of hexosaminidases which remove the non-reducing N-acetylglucosamine (GlcNAc) (Step 6 in Figure 5). The phylogeny of hexosaminidase indicated rapid evolution in this group of genes. Five different genes in this group were found in *I. scapularis*, but as the

Vaccines 2020, 8, x FOR PEER REVIEW

10 of 17

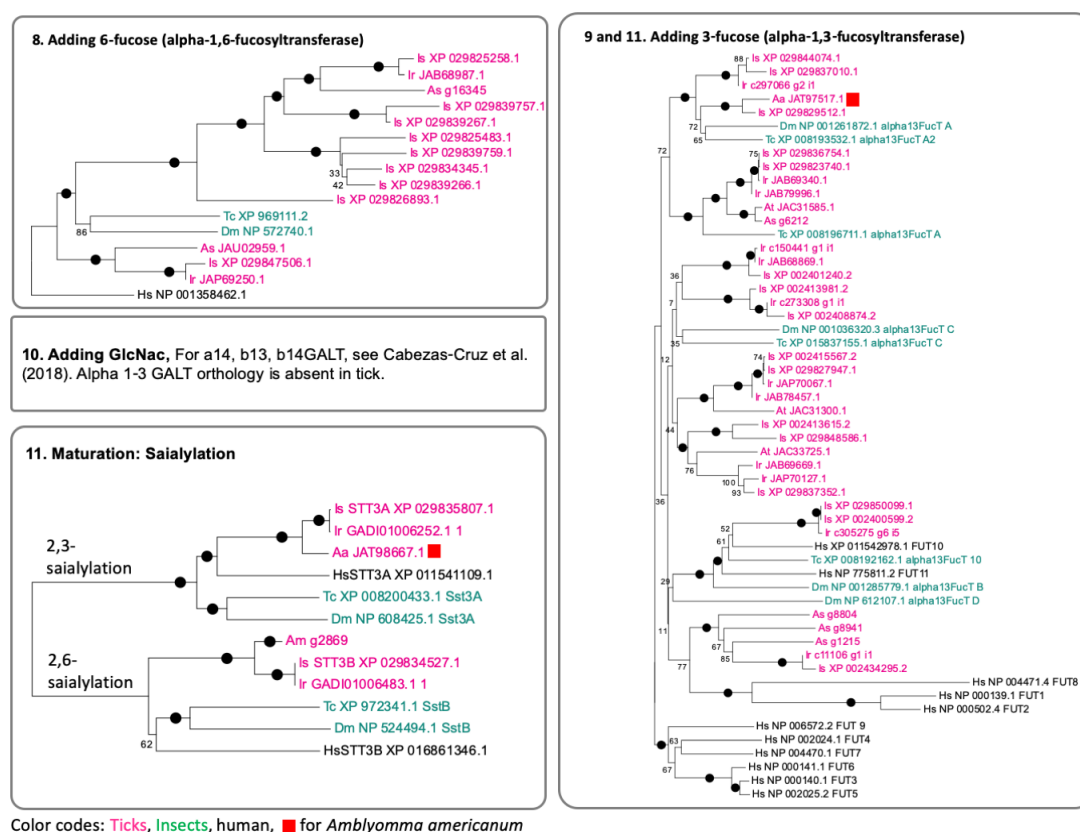


Figure 7. Phylogenetic trees for the genes involved in the N-glycan biosynthesis in the early Steps 8 to 11 in Figure 5. See figure caption for Figure 6 for the details.

More broadly, many late N-glycans showed highly modified antennae of the N-glycans (Zhu et al., 2019) (Steps 8, 9, and 11 in Figure 5). Larger (inb Steps 1, 2, 3, 4, and 11 in Figure 6 and 7). However, tick species appear to have recent gene expansions for Class II mannosidases, hexosaminidases, alpha-1,6-fucosyltransferases, alpha-1,3-fucosyltransferases, and galactosyltransferases (Steps 5, 6, 8, and 9 in Figures 6 and 7) with rapid evolution. This conclusion is yet based on the gene repertoire of *I. scapularis*, providing the most comprehensive gene sets in the genome project, while other tick species also show general patterns supporting the conclusion, but with limited numbers in the search results.

in the tick species (Figures 6 and 7. Steps 8, 9 and 11). Invertebrates can modify their N-glycans with alpha-1,3- and alpha-1,6-fucose [33]. One set of genes in *I. scapularis* was found to be orthologous to human FUT8, or alpha-1,6-fucosyltransferase. Additionally, another set of fucosyltransferase homologues, which are likely grouped with alpha-1,3-fucosyltransferase, were identified in the tick species. Large numbers of galactosyltransferases were identified, and their biochemical activities were recently studied [34]. Based on this study, the 57 genes are homologous to alpha-1,4-, beta-1,3- and beta-1,4-galactosyltransferases without alpha-1,3-galactosyltransferase orthologues in ticks. However, this study claimed that the genes in orthologous groups of beta-1,4- and alpha-1,4- galactosyltransferases showed alpha-1,3-galactosyltransferase activity for production of the terminal aGal productions. Two groups of sialyltransferases for each 2,3-sialylation and 2,6-sialylation were found in ticks with reliable orthologous relationships with insects and human sialyltransferases (Figure 7 (11)).

Overall, a number of enzymes showed high to moderate levels of conservation by having orthologous in insects and human enzymes (i.e., Steps 1, 2, 3, 4, and 11 in Figures 6 and 7). However, tick species appear to have recent gene expansions for Class II mannosidases, hexosaminidases, alpha-1,6-fucosyltransferases, alpha-1,3-fucosyltransferases, and galactosyltransferases (Steps 5, 6, 8, and 9 in Figures 6 and 7) with rapid evolution. This conclusion is yet based on the gene repertoire of *I. scapularis*, providing the most comprehensive gene sets in the genome project, while other tick species also show general patterns supporting the conclusion, but with limited numbers in the search results.

4. Discussion

4.1. Dynamic Control of the N-Glycosylation Patterns in Tick SGs

Analysis of the N-glycan profile of the carcass samples (CC) of the ticks revealed some similarity to insect glycomes. The CC N-glycome of the ticks was dominated by paucimannose N-glycans of which a significant proportion is core GlcNAc fucosylated. While the majority of the N-glycans in female CCs were non-fucosylated, in male ticks, the mono-fucosylated paucimannosid is the largest group and 17% of the total N-glycan pool is even difucosylated. This modification of the core GlcNAc with α 1,3- and α 1,6-fucoses is commonly found in insect N-glycans (reviewed in [2,35]), including phospholipase A₂ and hyaluronidase of bee venom [36], and glycosylations in *D. melanogaster* [37–39]. While in insects oligomannose and (core fucosylated) paucimannosidic N-glycans are the most common structures [37,38,40], oligomannose structures were not identified in the CC samples of the ticks. In the SGs from unfed ticks, oligomannoses are abundantly present. An interesting observation is the difference in the fucosylation levels between male and female ticks, which is associated with the increase in mono- and di-fucosylated paucimannose N-glycans in males ticks compared to female ticks. Similar pattern of difference was also observed between the N-glycome of female and male samples of the pest insect *Nilaparvata lugens* [3]. Analysis of the N-glycome of the unfed SG showed a decrease in abundance of (core fucosylated) paucimannoses, in contrast, there is a high abundance in oligomannose and complex-type N-glycans, leading to increased diversity and richness.

This increased diversity and richness of the SG N-glycans is mainly due to different numbers of terminal galactose on the core complex glycans. In addition, xylosylation in the core mannose, different branch numbers (two to four), and a small portion of sialylation at the terminal galactose (N-acetylneuraminic acid and N-glycolylneuraminic acid) were the source of the increased diversity in the SGs of later stages of tick feeding. Many complex glycans found in the tick SGs are glycans commonly found in mammals.

Tick SGs are dynamically controlled for the production of saliva [17], which has various bioactivities including modulating and eventually evading the host immune system. In addition, a large portion of the tick salivary secretion at the later stages of tick feeding was found to contain host proteins [41,42]. Therefore, the increased diversity of N-glycan in tick SGs over the duration of feeding is likely consistent with the changes in the repertoire of the SG proteins, potentially including the host proteins with N-glycan at the late feeding stage. The N-glycan patterns of tick SGs indicates that the saliva secreted

into the host may use aGal as a molecular mimicry decorating the proteins to evade the host immune system as the cases of sialic acids mentioned in the introduction. Decoration of the tick salivary proteins with the host-specific glycan aGal would be an efficient way of evading the host immune system in the case of parasitization of non-primate mammals (i.e., goat, sheep, horse, mouse, pig, and deer) that use aGal in the intrinsic system [43,44], although it may unexpectedly function as an allergy sensitizer in humans lacking aGal.

4.2. Cross-Reactive Carbohydrate Determinants (CCDs) and aGal in Tick N-glycans

Glycomics of ticks provide interesting datasets on the potential effects of ticks on the host immune system. Identification of the N-glycan structures in the tick SGs revealed several CCDs associated with severe allergic reactions [30,31,45,46]. One of these is the fucose epitopes characteristic to insects. Fucosylation of the core GlcNAc by alpha-1,3-fucose or difucosylation with alpha-1,3- and alpha-1,6-fucoses, known as the CCDs of hymenopteran venom (reviewed in [2,35]), were abundantly found in the tick SGs and CC samples. Interestingly, a study found high incidence of allergy to insect venom in aGal allergy patients [47]. Tick saliva with both allergens may be responsible for the occurrence of allergy to both insect venome and aGal allergy.

Xylose residues attached at the core mannose, a CCD found in peanut [31], was also commonly discovered in the tick SGs. In addition, a trace amount of N-glycan structures carrying the terminal N-glycolylneuraminic acid, another CCD [31], was also detected in the SGs of partially fed ticks (Figure 4). Several different CCDs are shown to interact with specific IgE (sIgE), which can mediate a hypersensitive immune response leading to allergies to certain plants and insect venoms [48]. However, clinical importance of the CCD as the causal factors of allergy is yet unclear, while correlation between sIgE activities against the specific CCD and the clinical symptoms has been shown [31,49].

4.3. aGal in the Tick SG as the Sensitizer of Red Meat Allergy

Red meat allergy (RMA) is an allergic reaction to aGal caused by a hypersensitive IgE response upon recognition of the glycan epitope. Although aGal has been well documented as an elicitor of RMA, the diagnostic test also suffers from sIgE-based assays for false-positive results [50–57]. The aGal allergy is also highly relevant to hyperreactions to xenoplasmentation [58] and to anti-cancer therapy using the monoclonal antibody cetuximab [59]. In all mammals except old world monkeys and humans, aGal is produced by an alpha-1,3-galactosyltransferase, which is mutated in humans [20,60]. A recent study proposed that RMA is elicited by glycolipids containing aGal but not by glycoproteins due to the difference in transport across the gut cell layer [61]. However, the nature of RMA sensitizers, likely originating from tick salivary secretion, needs to be further investigated.

Tick saliva is strongly supported as the factor leading to sensitization to aGal [21,53,62]. Based on our study and in previous studies [53], certain tick species contain aGal in the salivary secretion, which can be considered to be the sensitization factors for RMA. Our study found that upon feeding, the amount of aGal is progressively increased up to 16% and 8% in the SGs of *A. americanum* females and males, respectively. Some variants of aGal were di-aGal carrying aGal at both termini of biantennary glycan. In addition, N-glycan structures were identified containing more than one CCD together in a single glycan, structures with a terminal aGal and xylosylation of the core mannose or a terminal aGal and core fucosylations were commonly found in the SGs. Whether these N-glycans containing multiple CCDs can be recognized by separate sIgEs, facilitating cross-linking and aggregation of Fc-epsilon-RI (FcεRI) as an allergic reaction, is yet unknown.

Despite the presence of aGal epitopes in the N-glycome of *I. scapularis* saliva [53], it is unlikely that bites from this tick are causing RMA. *I. scapularis*, the vector for *Borrelia burgdorferi*, which causes Lyme disease, is found in a region including the northeastern United States, while RMA is mainly found in southeastern US, where *A. americanum* is the major tick. This discrepancy raises the question whether aGal in the salivary N-glycome of the tick alone is the RMA sensitization factor. In addition, as

mentioned above, the sIgE-based diagnostic assay found that the assay results included a substantial portion of false-positive results and difficulties in prediction of the allergic clinical symptoms [51,56,57].

The source of aGal in tick SGs has been recently studied. A number of galactosyltransferase homologs in ticks are involved in aGal production in tick SGs [34,54]. An alternative source of aGal could be the N-glycans on the host proteins. Tick saliva contains large numbers of host proteins [41,42], including the host immunoglobulins [63,64]. Therefore, we included male *A. americanum* in this study, which engages in multiple intrastadial feedings (our observation and [29]), unlike the females [65], which exhibit much greater fidelity for a single feeding event for a long duration on a single host. Similar to the female *A. americanum*, the male was also found to carry aGal in the SGs in this study. Males having multiple intrastadial feeding may have a higher opportunity to switch feeding hosts from nonhuman mammals to humans, while the aGal transmission or the aGal production in the SG is preactivated.

Many important pathogens including arthropod borne pathogens have been found to carry aGal including arthropod borne pathogens, such as *Trypanosoma* and *Leshmania* [66,67], *Borrelia* [68], and Dengue virus derived from mosquito cell [69]. More importantly, the fact that aGal functions as the protective antigen against Plasmodium, Trypanosoma, and Leshmania [70–72] led a basis to propose a single antigen pan-vaccine [68,73]. It would be interesting if those pathogens and the vectors commonly utilize the aGal as the molecular mimicry and the processes can be manipulated as a mean of the pathogen prevention.

5. Conclusions

The N-glycosylation patterns in the tick SGs change during tick feeding. The aGal epitopes, the presumed sensitizer of red meat allergy, increases over time after the onset of blood feeding. The aGal may serve as a molecular mimicry of bioactive proteins during tick feedings on mammalian hosts, while it functions as an allergen in non-natural host human.

N-Glycans of tick SGs proteins contain multiple CCDs: xylosylation of the core mannose, 1,3-mono and 1,3- and 1,6-difucosylations of the basal GlcNAc and mono- or diantennary aGal. Combinations of both xylosylation and aGal and fucosylation and aGal were often found in an N-glycan.

While the enzymes required for the early steps of the N-glycosylation pathway are conserved, the enzymes involved in the later stages of N-glycan maturation in the Golgi apparatus are highly diverged from those of insects.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2076-393X/8/1/18/s1>, Figure S1: N-glycan profiles in the lone star tick., Figure S2: Mass spectrometry spectra for the salivary glands of female fed for two days, Figure S3: Mass spectrometry spectra for the salivary glands of female fed for five days, Figure S4: MS spectra of a Xylose-containing glycoform found in female salivary gland after five day feeding, Figure S5: /MS spectra of a mono- and di- fucose (m/z 1345) found in female carcass after five day feeding.

Author Contributions: Conceptualization, Y.P.; methodology, Y.P., S.A.A.-H., P.A.; software, Y.P., K.D.S., L.Š.; validation, Y.P., G.S., K.D.S., L.Š., P.A.; formal analysis, Y.P., K.D.S., L.Š., S.A.A.-H., P.A.; investigation, Y.P., D.K., G.D.B.; resources, Y.P., G.S.; data curation, Y.P., S.A.A.-H., P.A.; writing—original draft preparation, Y.P., D.K., K.D.S., L.Š., S.A.A.-H.; writing—review and editing, Y.P., D.K., K.D.S., L.Š., S.A.A.-H., P.A.; visualization, Y.P., S.A.A.-H.; supervision, Y.P., G.S., P.A.; project administration, Y.P.; funding acquisition, Y.P., G.S., P.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Department of Defense TBDRP, D01 W81XWH-18-1-0255 and National Institute of Health; Grant Number: R21AI135457 to Y.P., D.K. and G.B., Special Research Fund of the Ghent University to K.D.S. and G.S., and NIH grant no. 1S10OD018530 to S.A.A.-H. and P.A. (Complex Carbohydrate Research Center).

Acknowledgments: This study is contribution no. 20-138-J from the Kansas Agricultural Experiment Station.

Conflicts of Interest: The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Helenius, A.; Aebi, M. Intracellular functions of N-linked glycans. *Science* **2001**, *291*, 2364–2369. [[CrossRef](#)] [[PubMed](#)]
2. Vandenborre, G.; Smagghe, G.; Ghesquiere, B.; Menschaert, G.; Nagender Rao, R.; Gevaert, K.; Van Damme, E.J. Diversity in protein glycosylation among insect species. *PLoS ONE* **2011**, *6*, e16682. [[CrossRef](#)] [[PubMed](#)]
3. Scheys, F.; De Schutter, K.; Shen, Y.; Yu, N.; Smargiasso, N.; De Pauw, E.; Van Damme, E.J.M.; Smagghe, G. The N-glycome of the hemipteran pest insect *Nilaparvata lugens* reveals unexpected sex differences. *Insect Biochem. Mol. Biol.* **2019**, *107*, 39–45. [[CrossRef](#)] [[PubMed](#)]
4. Walski, T.; Van Damme, E.J.M.; Smargiasso, N.; Christiaens, O.; De Pauw, E.; Smagghe, G. Protein N-glycosylation and N-glycan trimming are required for postembryonic development of the pest beetle *Tribolium castaneum*. *Sci. Rep.* **2016**, *6*, 35151. [[CrossRef](#)]
5. Li, W.; De Schutter, K.; Van Damme, E.J.M.; Smagghe, G. Synthesis and biological roles of O-glycans in insects. *Glycoconj. J.* **2019**. [[CrossRef](#)]
6. De Pourcq, K.; De Schutter, K.; Callewaert, N. Engineering of glycosylation in yeast and other fungi: Current state and perspectives. *Appl. Microbiol. Biotechnol.* **2010**, *87*, 1617–1631. [[CrossRef](#)]
7. Gagneux, P.; Aebi, M.; Varki, A. Evolution of glycan diversity. In *Essentials of Glycobiology*, 3rd ed.; Varki, A.C.R., Esko, J.D., Eds.; Cold Spring Harbor Laboratory Press: New York, NY, USA, 2017. [[CrossRef](#)]
8. De Schutter, K.; Van Damme, E.J.M. Protein-carbohydrate interactions as part of plant defense and animal immunity. *Molecules* **2015**, *20*, 9029–9053. [[CrossRef](#)]
9. Delaloye, J.; Calandra, T. Host innate immune responses to microbial pathogens. *Curr. Vasc. Pharmacol.* **2013**, *11*, 123–132.
10. Cummings, R.D.; McEver, R.P. C-Type Lectins. In *Essentials of Glycobiology*, 3rd ed.; Varki, A., Cummings, R.D., Esko, J.D., Stanley, P., Hart, G.W., Aebi, M., Kinoshita, A.G.D.T., Packer, N.H., Prestegard, J.H., Eds.; Cold Spring Harbor: New York, NY, USA, 2017.
11. Khatua, B.; Ghoshal, A.; Bhattacharya, K.; Mandal, C.; Saha, B.; Crocker, P.R.; Mandal, C. Sialic acids acquired by *Pseudomonas aeruginosa* are involved in reduced complement deposition and siglec mediated host-cell recognition. *FEBS Lett.* **2010**, *584*, 555–561. [[CrossRef](#)]
12. Carlin, A.F.; Uchiyama, S.; Chang, Y.C.; Lewis, A.L.; Nizet, V.; Varki, A. Molecular mimicry of host sialylated glycans allows a bacterial pathogen to engage neutrophil Siglec-9 and dampen the innate immune response. *Blood* **2009**, *113*, 3333–3336. [[CrossRef](#)]
13. Freire-De-Lima, L.; Oliveira, I.A.; Neves, J.L.; Penha, L.L.; Alisson-Silva, F.; Dias, W.B.; Todeschini, A.R. Sialic acid: A sweet swing between mammalian host and *Trypanosoma cruzi*. *Front. Immunol.* **2012**, *3*, 356. [[CrossRef](#)] [[PubMed](#)]
14. Previato, J.O.; Andrade, A.F.B.; Pessolani, M.C.V.; Mendoncapreviato, L. Incorporation of sialic-acid into *Trypanosoma cruzi* macromolecules—A proposal for a new metabolic route. *Mol. Biochem. Parasit.* **1985**, *16*, 85–96. [[CrossRef](#)]
15. Cusick, M.F.; Libbey, J.E.; Fujinami, R.S. Molecular mimicry as a mechanism of autoimmune disease. *Clin. Rev. Allergy Immun.* **2012**, *42*, 102–111. [[CrossRef](#)] [[PubMed](#)]
16. Guglielmone, A.A.; Robbins, R.G.; Apanaskevich, D.A.; Petney, T.N.; Estrada-Pena, A.; Horak, I.G.; Shao, R.F.; Barker, S.C. The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida) of the world: A list of valid species names. *Zootaxa* **2010**, *2528*, 1–28. [[CrossRef](#)]
17. Simo, L.; Kazimirova, M.; Richardson, J.; Bonnet, S.I. The essential role of tick salivary glands and saliva in tick feeding and pathogen transmission. *Front. Cell. Infect. Microbiol.* **2017**, *7*, 281. [[CrossRef](#)]
18. Chen, Z.C.; Radic, M.Z.; Galili, U. Genes coding evolutionary novel anti-carbohydrate antibodies: Studies on anti-Gal production in alpha 1,3galactosyltransferase knock out mice. *Mol. Immunol.* **2000**, *37*, 455–466. [[CrossRef](#)]
19. Commings, S.P.; Jerath, M.R.; Cox, K.; Erickson, L.D.; Platts-Mills, T. Delayed anaphylaxis to alpha-gal, an oligosaccharide in mammalian meat. *Allergol. Int.* **2016**, *65*, 16–20. [[CrossRef](#)]
20. Galili, U. Evolution of alpha 1, 3 galactosyltransferase and of the alpha-Gal epitope. *Subcell. Biochem.* **1999**, *32*, 1–23.

21. Commins, S.P.; Platts-Mills, T.A. Tick bites and red meat allergy. *Curr. Opin. Allergy Clin. Immunol.* **2013**, *13*, 354–359. [[CrossRef](#)]
22. Hamsten, C.; Tran, T.A.; Starkhammar, M.; Brauner, A.; Commins, S.P.; Platts-Mills, T.A.; van Hage, M. Red meat allergy in Sweden: Association with tick sensitization and B-negative blood groups. *J. Allergy. Clin. Immunol.* **2013**, *132*, 1431–1434. [[CrossRef](#)]
23. Kim, D.; Simo, L.; Park, Y. Molecular characterization of neuropeptide elevenin and two elevenin receptors, IsElevRI and IsElevR2, from the blacklegged tick, *Ixodes scapularis*. *Insect Biochem. Mol. Biol.* **2018**, *101*, 66–75. [[CrossRef](#)] [[PubMed](#)]
24. Krober, T.; Guerin, P.M. In vitro feeding assays for hard ticks. *Trends Parasitol.* **2007**, *23*, 445–449. [[CrossRef](#)] [[PubMed](#)]
25. Shajahan, A.; Heiss, C.; Ishihara, M.; Azadi, P. Glycomic and glycoproteomic analysis of glycoproteins—a tutorial. *Anal. Bioanal. Chem.* **2017**, *409*, 4483–4505. [[CrossRef](#)] [[PubMed](#)]
26. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. [[CrossRef](#)]
27. Saitou, N.; Nei, M. The neighbor-joining method—A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **1987**, *4*, 406–425.
28. Felsenstein, J. Confidence-limits on phylogenies—An approach using the bootstrap. *Evolution* **1985**, *39*, 783–791. [[CrossRef](#)]
29. Gladney, W.J.; Drummond, R.O. Migration of male lone star ticks on host in relation to mating. *J. Econ. Entomol.* **1970**, *63*, 1214. [[CrossRef](#)]
30. van Ree, R.; Cabanes-Macheteau, M.; Akkerdaas, J.; Milazzo, J.P.; Loutelier-Bourhis, C.; Rayon, C.; Villalba, M.; Koppelman, S.; Aalberse, R.; Rodriguez, R.; et al. Beta (1, 2)-xylose and alpha (1, 3)-fucose residues have a strong contribution in IgE binding to plant glycoallergens. *J. Biol. Chem.* **2000**, *275*, 11451–11458. [[CrossRef](#)]
31. Altmann, F. Coping with cross-reactive carbohydrate determinants in allergy diagnosis. *Allergy J. Int.* **2016**, *25*, 98–105. [[CrossRef](#)]
32. Dragosits, M.; Yan, S.; Razzazi-Fazeli, E.; Wilson, I.B.H.; Rendic, D. Enzymatic properties and subtle differences in the substrate specificity of phylogenetically distinct invertebrate N-glycan processing hexosaminidases. *Glycobiology* **2015**, *25*, 448–464. [[CrossRef](#)]
33. Zhu, F.F.; Li, D.; Chen, K.P. Structures and functions of invertebrate glycosylation. *Open Biol.* **2019**, *9*, 180232. [[CrossRef](#)] [[PubMed](#)]
34. Cabezas-Cruz, A.; Espinosa, P.J.; Alberdi, P.; Simo, L.; Valdes, J.J.; Mateos-Hernandez, L.; Contreras, M.; Rayo, M.V.; de la Fuente, J. Tick galactosyltransferases are involved in alpha-Gal synthesis and play a role during *Anaplasma phagocytophilum* infection and *Ixodes scapularis* tick vector development. *Sci. Rep.* **2018**, *8*, 14224. [[CrossRef](#)] [[PubMed](#)]
35. Rendic, D.; Wilson, I.B.H.; Paschinger, K. The glycosylation capacity of insect cells. *Croat. Chem. Acta* **2008**, *81*, 7–21.
36. Kolarich, D.; Leonard, R.; Hemmer, W.; Altmann, F. The N-glycans of yellow jacket venom hyaluronidases and the protein sequence of its major isoform in *Vespula vulgaris*. *FEBS J.* **2005**, *272*, 5182–5190. [[CrossRef](#)]
37. Aoki, K.; Tiemeyer, M. The glycomics of glycan glucuronylation in *Drosophila melanogaster*. *Method Enzymol.* **2010**, *480*, 297–321. [[CrossRef](#)]
38. Hagen, K.G.T.; Zhang, L.P.; Tian, E.; Zhang, Y. Glycobiology on the fly: Developmental and mechanistic insights from *Drosophila*. *Glycobiology* **2009**, *19*, 102–111. [[CrossRef](#)]
39. Katoh, T.; Tiemeyer, M. The N's and O's of *Drosophila* glycoprotein glycobiology. *Glycoconj. J.* **2013**, *30*, 57–66. [[CrossRef](#)]
40. Kurz, S.; Aoki, K.; Jin, C.; Karlsson, N.G.; Tiemeyer, M.; Wilson, I.B.; Paschinger, K. Targeted release and fractionation reveal glucuronylated and sulphated N- and O-glycans in larvae of dipteran insects. *J. Proteom.* **2015**, *126*, 172–188. [[CrossRef](#)]
41. Kim, T.K.; Tirloni, L.; Pinto, A.F.M.; Moresco, J.; Yates, J.R.; Vaz, I.D.S.; Mulenga, A. *Ixodes scapularis* tick saliva proteins sequentially secreted every 24 h during blood feeding. *PLoS. Negl. Trop. Dis.* **2016**, *10*, e0004323. [[CrossRef](#)]
42. Oliveira, C.J.; Anatriello, E.; de Miranda-Santos, I.K.; Francischetti, I.M.; Sa-Nunes, A.; Ferreira, B.R.; Ribeiro, J.M.C. Proteome of *Rhipicephalus sanguineus* tick saliva induced by the secretagogues pilocarpine and dopamine. *Ticks Tick-Borne Dis.* **2013**, *4*, 469–477. [[CrossRef](#)]

43. Ogawa, H.; Galili, U. Profiling terminal N-acetylglucosamines of glycans on mammalian cells by an immuno-enzymatic assay. *Glycoconj. J.* **2006**, *23*, 663–674. [\[CrossRef\]](#)
44. Tan, Y.X.; Gong, F.; Li, S.B.; Ji, S.P.; Lu, Y.P.; Gao, H.W.; Xu, H.; Zhang, Y.P. Brief report: A new profile of terminal N-acetylglucosamines glycans on pig red blood cells and different expression of alpha-galactose on Sika deer red blood cells and nucleated cells. *Glycoconj. J.* **2010**, *27*, 427–433. [\[CrossRef\]](#)
45. Homann, A.; Schramm, G.; Jappe, U. Glycans and glycan-specific IgE in clinical and molecular allergology: Sensitization, diagnostics, and clinical symptoms. *J. Allergy Clin. Immunol.* **2017**, *140*, 356–368. [\[CrossRef\]](#)
46. van Nunen, S. Tick-induced allergies: Mammalian meat allergy, tick anaphylaxis and their significance. *Asia Pac. Allergy* **2015**, *5*, 3–16. [\[CrossRef\]](#)
47. Choudhary, S.; Jerath, M.R.; Commins, S.P. Venom allergy is increased in alpha-gal allergy: Shared environmental or immunologic factors? *J. Allergy Clin. Immun.* **2018**, *141*, AB199. [\[CrossRef\]](#)
48. Yokoi, H.; Yoshitake, H.; Matsumoto, Y.; Kawada, M.; Takato, Y.; Shinagawa, K.; Sakurai, H.; Saito, K. Involvement of cross-reactive carbohydrate determinants-specific IgE in pollen allergy testing. *Asia Pac. Allergy* **2017**, *7*, 29–36. [\[CrossRef\]](#)
49. Hemmer, W.; Altmann, F.; Holzweber, F.; Gruber, C.; Wantke, F.; Wohrl, S. ImmunoCAP cellulose displays cross-reactive carbohydrate determinant (CCD) epitopes and can cause false-positive test results in patients with high anti-CCD IgE antibody levels. *J. Allergy Clin. Immun.* **2018**, *141*, 372. [\[CrossRef\]](#)
50. Van Nunen, S.A.; O'Connor, K.S.; Clarke, L.R.; Boyle, R.X.; Fernando, S.L. An association between tick bite reactions and red meat allergy in humans. *Med. J. Aust.* **2009**, *190*, 510–511. [\[CrossRef\]](#)
51. Jappe, U.; Minge, S.; Kreft, B.; Ludwig, A.; Przybilla, B.; Walker, A.; Varga, R.; Seidel, P.; Biedermann, T.; Anemuller, W.; et al. Meat allergy associated with galactosyl-(1,3)-galactose (-Gal) Closing diagnostic gaps by anti-Gal IgE immune profiling. *Allergy* **2018**, *73*, 93–105. [\[CrossRef\]](#)
52. Araujo, R.N.; Franco, P.F.; Rodrigues, H.; Santos, L.C.; McKay, C.S.; Sanhueza, C.A.; Brito, C.R.; Azevedo, M.A.; Venuto, A.P.; Cowan, P.J.; et al. Amblyomma sculptum tick saliva: Alpha-Gal identification, antibody response and possible association with red meat allergy in Brazil. *Int. J. Parasitol.* **2016**, *46*, 213–220. [\[CrossRef\]](#)
53. Crispell, G.; Commins, S.P.; Archer-Hartman, S.A.; Choudhary, S.; Dharmarajan, G.; Azadi, P.; Karim, S. Discovery of alpha-Gal-containing antigens in north American tick species believed to induce red meat allergy. *Front. Immunol.* **2019**, *10*, 1056. [\[CrossRef\]](#)
54. Cabezas-Cruz, A.; Hodzic, A.; Roman-Carrasco, P.; Mateos-Hernandez, L.; Duscher, G.G.; Sinha, D.K.; Hemmer, W.; Swoboda, I.; Estrada-Pena, A.; de la Fuente, J. Environmental and molecular drivers of the alpha-Gal syndrome. *Front. Immunol.* **2019**, *10*, 1210. [\[CrossRef\]](#)
55. Hilger, C.; Fischer, J.; Wolbing, F.; Biedermann, T. Role and mechanism of galactose-alpha-1, 3-galactose in the elicitation of delayed anaphylactic reactions to red meat. *Curr. Allergy Asthma Rep.* **2019**, *19*, 3. [\[CrossRef\]](#)
56. Li, J.; Fulton, R.B.; O'Connell, R.; Jang, H.S.; Fernando, S.L. Specific-IgE to galactose-alpha-1, 3-galactose (alpha-gal) has limited utility in diagnosing meat allergy in a tick-endemic population. *Ann. Allergy Asthma Immunol.* **2018**, *121*, 509–511. [\[CrossRef\]](#)
57. Wilson, J.M.; Schuyler, A.J.; Workman, L.; Gupta, M.; James, H.R.; Posthumus, J.; McGowan, E.C.; Commins, S.P.; Platts-Mills, T.A.E. Investigation into the alpha-Gal syndrome: Characteristics of 261 children and adults reporting red meat allergy. *J. Allergy Clin. Immunol. Pract.* **2019**, *7*, 2348–2358. [\[CrossRef\]](#)
58. Dahl, K.; Buschard, K.; Gram, D.X.; d'Apice, A.J.; Hansen, A.K. Glucose intolerance in a xenotransplantation model: Studies in alpha-gal knockout mice. *APMIS* **2006**, *114*, 805–811. [\[CrossRef\]](#)
59. Ayoub, D.; Jabs, W.; Resemann, A.; Evers, W.; Evans, C.; Main, L.; Baessmann, C.; Wagner-Rousset, E.; Suckau, D.; Beck, A. Correct primary structure assessment and extensive glyco-profiling of cetuximab by a combination of intact, middle-up, middle-down and bottom-up ESI and MALDI mass spectrometry techniques. *mAbs* **2013**, *5*, 699–710. [\[CrossRef\]](#)
60. Galili, U. The alpha-gal epitope and the anti-Gal antibody in xenotransplantation and in cancer immunotherapy. *Immunol. Cell Biol.* **2005**, *83*, 674–686. [\[CrossRef\]](#)
61. Roman-Carrasco, P.; Lieder, B.; Somoza, V.; Ponce, M.; Szepfalusi, Z.; Martin, D.; Hemmer, W.; Swoboda, I. Only alpha-Gal bound to lipids, but not to proteins, is transported across enterocytes as an IgE-reactive molecule that can induce effector cell activation. *Allergy* **2019**, *10*, 1956–1968. [\[CrossRef\]](#)

62. Commins, S.P.; James, H.R.; Kelly, L.A.; Pochan, S.L.; Workman, L.J.; Perzanowski, M.S.; Kocan, K.M.; Fahy, J.V.; Nganga, L.W.; Ronmark, E.; et al. The relevance of tick bites to the production of IgE antibodies to the mammalian oligosaccharide galactose- α -1, 3-galactose. *J. Allergy Clin. Immunol.* **2011**, *127*, 1286–1293.e6. [\[CrossRef\]](#)
63. Wang, H.; Nuttall, P.A. Excretion of host immunoglobulin in tick saliva and detection of IgG-binding proteins in tick haemolymph and salivary glands. *Parasitology* **1994**, *109*, 525–530. [\[CrossRef\]](#)
64. Wang, H.; Nuttall, P.A. Immunoglobulin-binding proteins in ticks: New target for vaccine development against a blood-feeding parasite. *Cell Mol. Life Sci.* **1999**, *56*, 286–295. [\[CrossRef\]](#)
65. Friesen, K.J.; Kaufman, W.R. Salivary gland degeneration and vitellogenesis in the ixodid tick *Amblyomma hebraeum*: Surpassing a critical weight is the prerequisite and detachment from the host is the trigger. *J. Insect Physiol.* **2009**, *55*, 936–942. [\[CrossRef\]](#)
66. Avila, J.L.; Rojas, M.; Galili, U. Immunogenic Gal- α -1-3gal carbohydrate epitopes are present on pathogenic American *Trypanosoma* and *Leishmania*. *J. Immunol.* **1989**, *142*, 2828–2834.
67. Zamze, S.E.; Ashford, D.A.; Wooten, E.W.; Rademacher, T.W.; Dwek, R.A. Structural characterization of the asparagine-linked oligosaccharides from *Trypanosoma brucei* Type-Ii and Type-Iii variant surface glycoproteins. *J. Biol. Chem.* **1991**, *266*, 20244–20261.
68. Cabezas-Cruz, A.; de la Fuente, J. Immunity to α -Gal: Toward a single-antigen pan-vaccine to control major infectious diseases. *ACS Cent. Sci.* **2017**, *3*, 1140–1142. [\[CrossRef\]](#)
69. Lei, Y.; Yu, H.; Dong, Y.; Yang, J.; Ye, W.; Wang, Y.; Chen, W.; Jia, Z.; Xu, Z.; Li, Z.; et al. Characterization of N-glycan structures on the surface of mature Dengue 2 virus derived from insect cells. *PLoS ONE* **2015**, *10*, e0132122. [\[CrossRef\]](#)
70. Yilmaz, B.; Portugal, S.; Tran, T.M.; Gozzelino, R.; Ramos, S.; Gomes, J.; Regalado, A.; Cowan, P.J.; d’Apice, A.J.F.; Chong, A.S.; et al. Gut microbiota elicits a protective immune response against Malaria transmission. *Cell* **2014**, *159*, 1277–1289. [\[CrossRef\]](#)
71. Moura, A.P.V.; Santos, L.C.B.; Brito, C.R.N.; Valencia, E.; Junqueira, C.; Filho, A.A.P.; Sant’Anna, M.R.V.; Gontijo, N.F.; Bartholomeu, D.C.; Fujiwara, R.T.; et al. Virus-like particle display of the α -Gal carbohydrate for vaccination against *Leishmania* infection. *ACS Cent. Sci.* **2017**, *3*, 1026–1031. [\[CrossRef\]](#)
72. Almeida, I.C.; Milani, S.R.; Gorin, P.A.J.; Travassos, L.R. Complement-mediated lysis of *Trypanosoma cruzi* trypomastigotes by human anti- α -galactosyl antibodies. *J. Immunol.* **1991**, *146*, 2394–2400.
73. Cabezas-Cruz, A.; de la Fuente, J. Immunity to α -Gal: The opportunity for malaria and Tuberculosis control. *Front. Immunol.* **2017**, *8*, 1733. [\[CrossRef\]](#)



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).



Appendix 2. Page Proof of the publication in Frontiers in Immunology

~~Alpha-Gal Syndrome and Lone Star Tick Bites in a Mutant Mouse Model: The Alpha-Gal “Transmission” Hypothesis by Tick Host Switch Is Not Supported~~

L. Paulina Maldonado-Ruiz¹, Gunavanthi D. Boorgula^{1*}, Donghun Kim², Sherry Fleming³ and Yoonseong Park^{1*}

¹ Department of Entomology, Kansas State University, Manhattan, KS, United States, ² Department of Entomology, Kyungpook National University, Daegu, South Korea, ³ Division of Biology, Kansas State University, Manhattan, KS, United States

OPEN ACCESS

Edited by:

Fabrizio Bruschi,
University of Pisa, Italy

Reviewed by:

Franco Harald Falcone,
University of Giessen, Germany
Wanessa Araujo Carvalho,
Brazilian Agricultural Research
Corporation (EMBRAPA), Brazil

*Correspondence:

Yoonseong Park
ypark@ksu.edu

Specialty section:

This article was submitted to
Parasite Immunology,
a section of the journal
Frontiers in Immunology

Received: 27 December 2021

Accepted: 10 February 2022

Published: xx xx 2022

Citation:

Maldonado-Ruiz LP, Boorgula GD,
Kim D, Fleming S and Park Y (2022)
Alpha-Gal Syndrome and Lone
Star Tick Bites in a Mutant
Mouse Model: The Alpha-Gal
“Transmission” Hypothesis by Tick
Host Switch Is Not Supported.
Front. Immunol. 13:844262.
doi: 10.3389/fimmu.2022.844262

Recent studies have provided strong evidence indicating that lone star tick bites are a cause of AGS (alpha-gal syndrome, also known as red meat allergy RMA) in humans. AGS is characterized by an increase in IgE antibody production against galactose-alpha-1,3-galactose (aGal), which is a common glycan found in mammalian tissue, except in Old World monkeys and humans. The main causative factor of AGS, the lone star tick (*Amblyomma americanum*), is broadly distributed throughout the east and midwest of the United States and is a vector of a wide range of human and animal pathogens. Our earlier glycomics study of the salivary glands of partially fed male and female ticks revealed relatively high levels of aGal epitopes. In this study, we found that partially fed males of *A. americanum* on bovine blood, which engage in multiple intrastadial feedings, carry a large amount of aGal in the salivary glands. In our current work, we aimed to test whether ticks mediate the transmission of the aGal sensitizer acquired from nonhuman blood to humans in the intrastadial host switch (referred to as the “transmission” hypothesis). To test this hypothesis, we used an alpha-galactosyltransferase knockout mutant mouse (aGT-KO) model system infested with ticks that were unfed or partially fed on bovine blood. Based on the levels of total IgE and specific IgG and IgE antibodies against aGal after tick feedings, aGT-KO mice significantly responded to tick feeding and injection of aGal (Gal α 1-3Gal β 1-4GlcNAc) conjugated to human serum albumin or mouse serum albumin (aGal-HSA or aGal-MSA) by increasing total IgE and aGal-specific IgE levels compared to those in C57BL/6 control mice. All of the treatments of aGT-KO mice involving the feeding of partially fed and unfed ticks functioned as sensitizers that increased the levels of specific IgE against aGal, with large individual variations. The data in this study do not support the “transmission” component of AGS, although they confirmed that aGT-KO mice can be used as a model for RMA studies.

Keywords: *Amblyomma americanum*, ticks, alpha-gal, red meat allergy, alpha-gal syndrome

Q9 INTRODUCTION

In humans, alpha-gal syndrome (AGS and also known as red meat allergy RMA) is caused by the increased production of IgE antibodies against galactose-alpha-1,3-galactose (alpha-gal or aGal) (1). This glycan is found in most mammals, with the exception of Old World monkeys and humans, since the alpha-1,3-galactosyltransferase gene ($\alpha 1,3GT$) has been inactivated in ancestral Old World primates in the course of evolution (2, 3). AGS or RMA is observed in susceptible individuals after the ingestion of red meat, with a clinical manifestation of urticaria and anaphylaxis (4). Worldwide reports have found that aGal allergic responses are directly linked to an initial sensitization by tick bites of different species: *Ixodes ricinus* in Europe, *I. holocyclus* in Australia, *Hemaphysalis longicornis* in Asia, *Amblyomma americanum* and other *Amblyomma* species in North America and South America, respectively (5–11). In the United States, only lone star tick (*A. americanum*) bites have been identified as the primary causal factor for RMA (12, 13). Cases of RMA have been reported in the southeastern United States, including the regions known for the distributions of the lone star tick (14). Recent studies have shown that the salivary glands of *A. americanum* contain N-glycosylated protein(s) with galactose alpha-1,3-galactose beta-1,4-N-acetylglucosamine (aGal) (11, 14–16). The source of the aGal sensitizer in tick saliva awaits further elucidation of the molecular identity and the signaling pathway leading to aGal sensitization.

The presence of glycosylated proteins carrying aGal in tick saliva might not be the sole determinant of aGal sensitization in AGS. For example, aGal N-glycan was also found in the salivary glands of *I. scapularis* in addition to those of *A. americanum* (17), while there are no supporting data for the bites of *I. scapularis* causing AGS. Likewise, a remaining question is that AGS occurs in only a small subgroup of the overall human population that experiences tick bites, which indicates that either the ticks under specific conditions are culprits of change or subpopulations of humans are vulnerable to the development of IgE against aGal, causing AGS. Therefore, the variations in the presence of the aGal sensitizer in tick salivary secretion and additional unidentified factors need to be further investigated in AGS.

A factor could stem from tick feeding behaviors that lead to a greater chance of feeding on humans, which are not a natural host. The males of *A. americanum* are known to have multiple intrastadial feedings, while female ticks are generally engaged in a single feeding event on a single host for full engorgement (18). We speculated that this behavioral difference of male ticks could provide a greater opportunity for an intrastadial switch in hosts from a nonhuman mammal to a human. Furthermore, we found that the salivary glands of male ticks that fed on bovine blood *via* artificial membrane feeding contained large amounts of aGal epitopes in this study. We employed alpha-galactosyltransferase knockout mutant mice (aGT-KO) to test the differences in the responses to ticks that were partially fed on bovine blood. The results showed that the aGT-KO mouse responded to tick feeding by increasing total IgE, specific IgG and IgE levels, but with large individual variations, without sufficient levels of discrimination for feedings by partially fed ticks.

METHODS

Mice

The C57BL/6 mouse strain and alpha-gal-knockout (aGT-KO; C57BL/6/CimlKvl-Tgaltm1Tea, obtained from the Scripps Research Institute, Janda Lab, La Jolla, CA) were maintained in the AAALAC-approved animal facility of the Laboratory Animal Care Service (LACS) at Kansas State University. All animal protocols were approved and compliant with the guidelines of the Institutional Animal Care and Use Committee (IACUC). The founders were tested for the genotype by using PCR for the mutation.

The study was divided into two experiments: Experiment 1) was sensitization to alpha-gal by various feeding stages of *A. americanum* tick bites, and Experiment 2) was sensitization to the feedings of different species of ticks. For Experiment 1, we used eighty-two aGT-KO mice (40 females, 72 males) and 48 C57BL/6 wild-type mice (24 females, 24 males). For Experiment 2, we used 58 aGT-KO mice (31 females, 27 males). All mice used were 8–12 weeks old at the time of the first sampling. Mice were anesthetized with isoflurane (2.5–5%) (Akorn Pharmaceuticals, IL, USA) *via* inhalation at each sampling point (blood draw) and euthanized immediately after the final blood collection by cervical dislocation.

Ticks and Tick Prefeeding

Unfed (naïve) 2- to 3-month-old adult male and female *A. americanum*, *Dermacentor variabilis*, and *Ixodes scapularis* ticks were obtained from the Oklahoma State University tick rearing facility. All ticks were kept at room temperature and >95% RH until use in experiments. For the treatments that required partially fed ticks, the ticks were prefed (4–5 days blood fed) with defibrinated bovine blood (Hemostat Laboratories, CA, USA) using a modified artificial feeding system (19) before being infested onto the mice.

Western Blotting of the Salivary Glands of *A. americanum* for Detection of aGal Epitope

Ticks were prefed on bovine blood for 4 days in an artificial membrane feeding system and used for dissections of the salivary glands. Three pairs of salivary glands were pooled for isolation of protein for electrophoresis. Unfed male and female salivary glands and dorsums were included, and 10 μ g of bovine thyroglobulin was used as a positive control, as it is known to contain alpha-gal epitope (20). Proteins were extracted using the protein extraction reagent T-perTM (Thermo Scientific, MA, USA), and an aliquot (~5–10 μ g of total protein) was treated with PNGase F or alpha-1,3 galactosylase enzymes following the manufacturer's protocol for digestion. PNGase F (New England Biolabs, MA, USA) was used for deglycosylation of all N-glycans, including the aGal epitope or alpha-1,3 galactosylase (P0731S, New England Biolabs, MA, USA), which exclusively cleaves the terminal aGal. Samples were loaded for SDS-polyacrylamide gel electrophoresis at ~5 μ g of protein per lane using a Mini-PROTEAN[®] TGX Stain-FreeTM gel (Bio-Rad, CA, USA), and

Precision Plus ProteinTM Unstained standard (10–250 kDa) was used as a protein size marker (Bio–Rad). The gel was transferred to a ready to use pre-wetted PVDF membrane (Bio–Rad, USA) using a Trans-Blot[®] TurboTM transfer System (BioRad) and blocked with 3% bovine serum albumin in phosphate-buffered saline supplemented with 0.05% Tween (PBST). Immunoblotting was conducted using an IgM mouse monoclonal antibody against the aGal epitope (M86) (Enzo Life Sciences, NY, USA) at 1:10 with overnight incubation. Goat-anti-mouse IgM conjugated to HRP was used as a secondary antibody at 1:500 (Novus Biologicals, CO, USA) for 3 hours and followed by incubation with the substrate 3,3'-Diaminobenzidine (0.05%) with 0.003% hydrogen peroxide in PBST. Gel image was analyzed by using the ImageJ software version 1.8.1_172 (21) for measuring the difference between pixel values. Lane with no immunoreactivity was used for subtraction of background. Thyroglobulin (suggested to contain ~4 molecules of aGal per protein molecule, or ~60 pmoles of aGal in 10µg) (22) was used to calculate an approximation of aGal concentration within the tick salivary glands.

Effects of Tick Feedings on aGT-KO Mice

Experiment 1: aGal Sensitization by

A. americanum Tick Bites in aGT-KO Mice

For the aGT-KO homozygous mice, we included three controls: 1) sham control: mice were shaved on the back and tick feeding chamber was placed without ticks, 2) Human serum albumin (HSA) control: mice were injected with HSA (25 µg, with no alpha-gal), and 3) aGal conjugated HSA (aHSA) control: mice were injected with 25 µg aHSA. Subcutaneous injection was performed 3 times at 7 ± 0.75 day intervals, as previous reports show that weekly immunizations for 3 to 4 weeks, resulted in increased IgE against the aGal sensitizer (8). Four other experimental groups were combinations of male (M) and female (F), partially fed (F) and unfed (U) ticks. Therefore, four treatments were the mice infested with one pair of ticks UMUF, FMUF, UMFF, and FMFF (**Table 1**). The control groups with wild-type C57BL/6 mice were the sham control and injections with HSA or aHSA and the treatment with partially fed males plus partially fed females (FMFF) (**Table 1**). Mice were prebled by retro orbital bleeding before treatment using a 1.1 mm

diameter capillary tube (100–300 µl) to quantify the pretreatment immunoglobulin levels (**Figure 1A**) and by cardiac puncture in the posttreatment using a 1 ml syringe with a 25 gauge needle (~1 ml).

Ticks were placed on mice inside a 15 mm circular chamber made from double layer of double sided mounting tape (Scotch, 3 M, MN, USA) ~4 mm thick covered with nylon mesh. The chamber was glued on the back with 3 M VetbondTM after shaving the fur and covered with 3 M TegadermTM adhesive tape. Finally, the chamber was secured with a mouse jacket (Harvard Apparatus, MA, USA). In the daily monitoring, if the ticks were not attached to mice within a week or died, the ticks were replaced with a new pair until successful tick attachment was observed. Ticks were removed between 4–8 days after attachment (**Figure 1A**) when the feeding ticks reached the rapidly engorging stage to repletion.

Experiment 2: Alpha-Gal Sensitization in Mice by Bites of Other Ixodid Tick Species

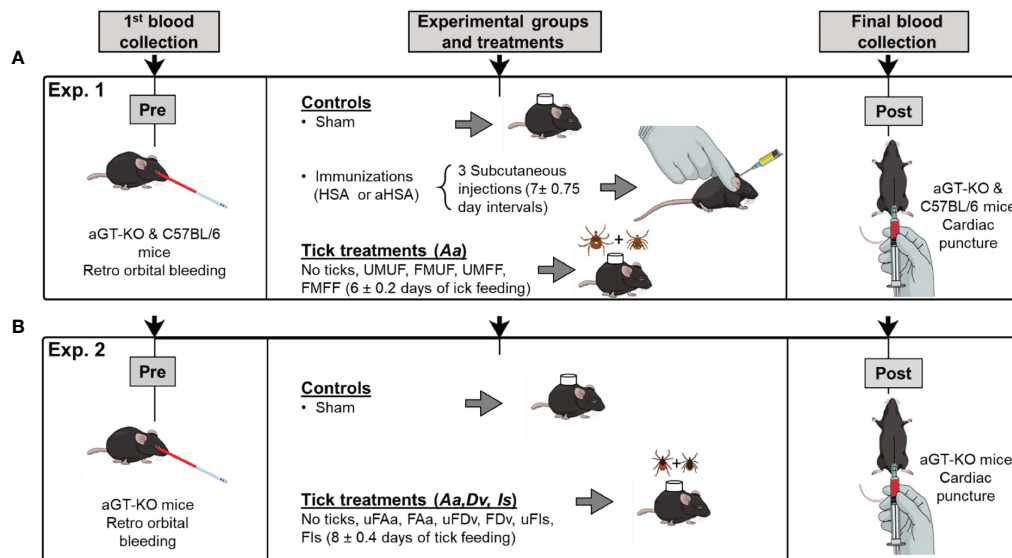
For Experiment 2, only aGT-KO mice were used, and the experimental groups included only the “sham” control group and three different species of partially fed (F) or unfed (uF) ticks. Three different species were *A. americanum* (Aa), *Dermacentor variabilis* (Dv), and *I. scapularis* (Is), which amounted to six experimental groups (uFAa, FAa, uFDv, FDv, uFIs, and FIs).

Quantification of Immunoglobulins by ELISA

For total IgE quantification, diluted mouse serum (1:50) was used in an ELISA assay. For the specific IgE quantification assay, IgG was depleted from mouse sera by using protein G resin (Genscript, NJ, USA) at a 1:1 ratio with binding buffer, following the manufacturer's instructions, and IgG-depleted sera were stored until needed for the assays. Total IgE and specific IgE against aGal conjugated to MSA or HSA were measured by sandwich enzyme-linked immunosorbent assay (ELISA) using the ELISA MAXTM kit for IgE detection (Biolegends, CA, USA) following the manufacturer's instructions. Plates were coated with mouse IgE capture antibody (Biolegends, CA, USA) or aGal-HSA or MSA (for the

TABLE 1 | Experimental groups assigned to mice and tick pre-treatments for experiment 1.

Mice	Group	Pre-treatment of ticks	Abbreviation	Male	Female
aGT-KO	Control 1	No tick feeding only Shave + chamber	Sham	5	5
	Control 2	Human serum albumin immunization	HSA	5	5
	Control 1	Human serum albumin + alpha-gal immunization	aHSA	5	5
	Exp. Group 1	Unfed Male, Unfed Female	UM UF	8	7
	Exp. Group 2	Fed Male, Unfed Female	FM UF	7	7
	Exp. Group 3	Unfed Male, Fed Female	UM FF	6	6
	Exp. Group 4	Fed Male, Fed Female	FM FF	6	6
	Control 1	No tick feeding only Shave + chamber	Sham	6	6
	Control 2	Human serum albumin immunization	HSA	6	6
	Control 3	Human serum albumin + alpha-gal immunization	aHSA	6	6
C57BL/6 mice	Exp. Group 1	Fed Male, Fed Female	FM FF	6	6



Q10

FIGURE 1 | A schematic diagram of the experimental design. **(A)** section outlines the treatments and structure for assessing RMA sensitization using pairs of *Amblyomma americanum* ticks (Experiment 1). The treatments were varied with partially fed and unfed (F,U) male and female (M,F), aGT-KO mice and C57BL/6 control mice. **(B)** experimental design for assessing comparisons between sensitization through bites of other ixodid tick species (Experiment 2), fed (F) and unfed (uF) ticks, *Amblyomma americanum* (Aa), *Dermacentor variabilis* (Dv), and *Ixodes scapularis* (Is). In all treatments involving ticks, male and female pairs of ticks were used.

detection of specific IgE against aGal) at 5 µg/ml in 100 µl/well in carbonate-bicarbonate buffer. Washing was conducted after each step with PBST (0.05% Tween) using an ELx50 Auto Strip Washer (Bio-Tek instruments, Inc. VT, USA). Blocking buffer containing 1% bovine serum albumin (BSA, Akron Biotech, FL, USA) in PBST was used for blocking. For specific IgE against aGal, mouse sera were added at a dilution ratio of 1:1 (25 µl of serum/well) in blocking buffer and at a ratio of 1:50 for total IgE (2 µl of serum/well). A mouse IgE standard (Biolegends, CA, USA) was used for IgE quantification. For color development, we incubated the plate with 3,3',5,5'-tetramethylbenzidine (TMB) (1-StepTM Ultra TMB-ELISA, Fisher Scientific, USA) for ~15 minutes. The reaction was stopped with 1 M phosphoric acid, and 450 nm absorbance was measured using a Varioskan Lux microplate reader (Thermo Fisher Scientific, MA, USA) using the SkanItTM 6.0 software.

Specific IgG against aGal-HSA or MSA was measured by ELISA using the same protocol as the procedure described above. One difference was the secondary antibody step using goat-anti-mouse IgG-HRP (1:5000) overnight at room temperature.

Chemicals and Other Compounds Used for Mice Immunizations and ELISA Assays

For immunizations we used the Freund's incomplete adjuvant (Sigma Aldrich, MO, USA) in a 1:1 ratio of saline solution containing 25 µg of human serum albumin alone (Celprogen Inc. CA, USA) or conjugated to aGal containing a 14 atom spacer and an average of 25 aGal molecules per protein molecule (Dextra Laboratories UK) for mice immunizations. For the quantification of immunoglobulins against the aGal epitope we used aGal

conjugated to HSA or MSA containing a 3 atom spacer and 33 aGal molecules per protein molecule (mouse serum albumin) (Dextra Laboratories, UK).

Statistical Analysis

For statistical comparisons between treatment groups, a Kruskal-Wallis ANOVA test (one-way ANOVA nonparametric test) was conducted with a significance level of 0.05 ($p < 0.05$). For comparisons between pre- and posttreatments, a paired sample Wilcoxon signed rank test with a significance level of 0.05 ($p < 0.05$) was used. In addition to the ANOVA test, we tested the frequencies of the different individuals at the 95% normal distribution of the "sham" group. Chi-square tests were performed for the frequencies of the different individuals from the responses in the sham group in 2 x 2 contingency tables. Data analyses were conducted in OriginPro 2020b (9.7.5.184), GraphPad Prism version 9.2.0 for Windows (GraphPad Software, San Diego, CA, USA) and R version 4.0.0 (R Foundation for statistical computing, Vienna, Austria).

RESULTS

High Levels of aGal Epitopes in the Salivary Glands of Partially Fed Male *A. americanum*

Western blots for salivary gland proteins from partially fed males and females showed high levels of reactivity to the M86 aGal monoclonal antibody, whereas the salivary glands of unfed ticks lacked reactivity (**Figure 2A**). Partially fed females revealed two

anti-aGal reactive bands of ~100 kDa and very high molecular weight (Lane 1). The salivary glands of 4-day-fed male ticks contained high levels of aGal epitopes (**Figure 2B**) with a ~25 kDa band and a smear in the range of 50-200 kDa (Lane 3 in **Figure 2B**). The band patterns were consistent in five independent trials with three different tick batches using slightly modified protocols to avoid possible artifacts, such as protein degradation or overloading. The salivary glands of unfed ticks and the dorsum of the fed ticks did not show aGal epitopes (**Figure 2C**). The immunoreactivities of the partially fed tick salivary glands were abolished with PNGase F for deglycosylation of N-glycan (Lanes 2, 4, and 6 in **Figure 2B**) and with alpha galactosylase (deglycosylation of terminal aGal, **Figure 2C**). The reactivity in bovine thyroglobulin, the positive control containing ~4 aGal per molecule (60 pmoles of aGal in 10 µg thyroglobulin), indicated that the tick salivary glands contained high levels of aGal epitope, >60 pmoles per tick.

Experiment 1: Alpha-Gal Sensitization in Mice by *A. americanum* Tick Bites

We present the pooled data for both males and females in the same treatment because the initial statistics for comparing males and females found no significant differences between sexes. We also show the data for various immunoglobulin levels in the posttreatments because we observed that all pretreatments were equally at the basal level (**Supplementary Figure S2A**) in the comparisons of total IgE and specific IgG against aGal-MSA and aGal-HSA (**Supplementary Figures S3A–C**).

A. americanum Tick Bites Increase the Levels of Total IgE and Specific IgE Against aGal

We measured the levels of total IgE among all treatment groups in aGT-KO mice and C57BL/6 wild-type (control) mice after treatment. All treatments in the aGT-KO mice showed higher levels of total IgE in comparison to the treatments in C57BL/6 control mice (**Figure 3A**). The HSA immunization, unfed male and female (UM, UF), and both fed female treatments (UM, FF, FM, FF) showed statistically significant differences when compared to the “sham” group in the ANOVA test ($p > 0.05$) (**Table S1**). The chi-square test after the 95% distribution cutoff, providing the statistics for frequencies of individuals who were different from the responses of the sham control, showed significant differences across all treatments (**Table S2**), with a tendency of higher total IgE levels in the treatments with partially fed female (FF) ticks, with few individuals presenting a hyperreaction (**Figure 3A**). Highly significant differences in total IgE levels were observed between the “sham” and all tick feeding treatments.

Specific IgE levels against aGal-MSA, which is considered to be involved with the major contribution of specific IgE toward aGal, but not MSA, were significantly different in the tick feeding treatments that included any combinations of partially fed ticks when compared to the “sham” group in the chi-square test for frequencies of individuals with high levels of specific IgE. In the treatments of control mice, there were no significant differences in either the ANOVA or Chi square test for specific IgE against aGal-MSA (**Figure 3B**). In contrast to specific IgE against aGal-

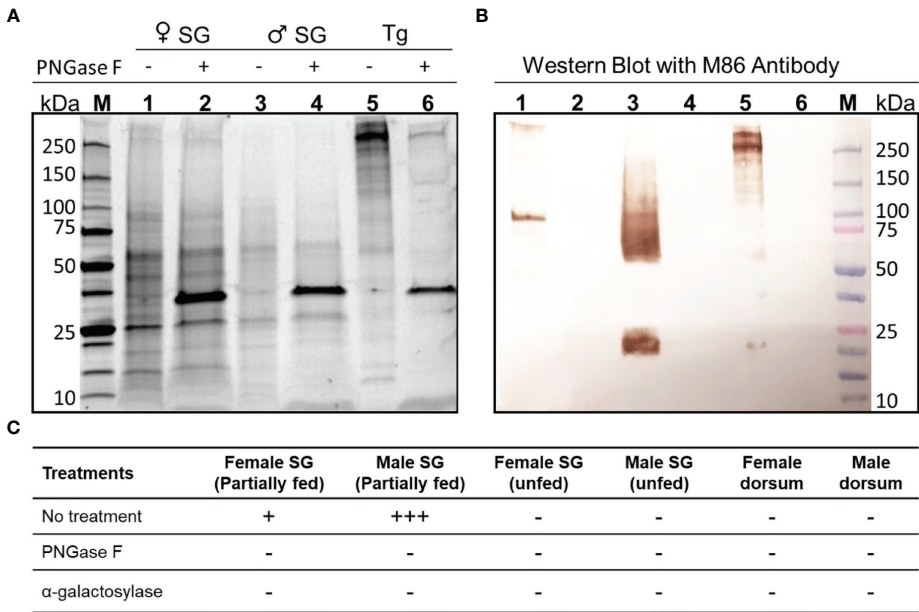


FIGURE 2 | Western blot for aGal immunoreactivity in the salivary glands of female and male *A. americanum*. **(A)** SDS-polyacrylamide gel for the female and male salivary gland (SG) proteins (1.5 pairs per lane) and bovine thyroglobulin (Tg) as the positive control. The samples were treated with PNGase F (shown by ~35kDa bands in samples 2, 4, and 6). **(B)** Western blotting with mouse monoclonal anti- aGal IgM is shown. **(C)** Summary of repeated experiments with different types of samples (column headings) with different enzymatic deglycosylations. The ‘+’ indicates the arbitrary strengths of aGal reactivity, while the ‘-’ is for lack of the reactivity.

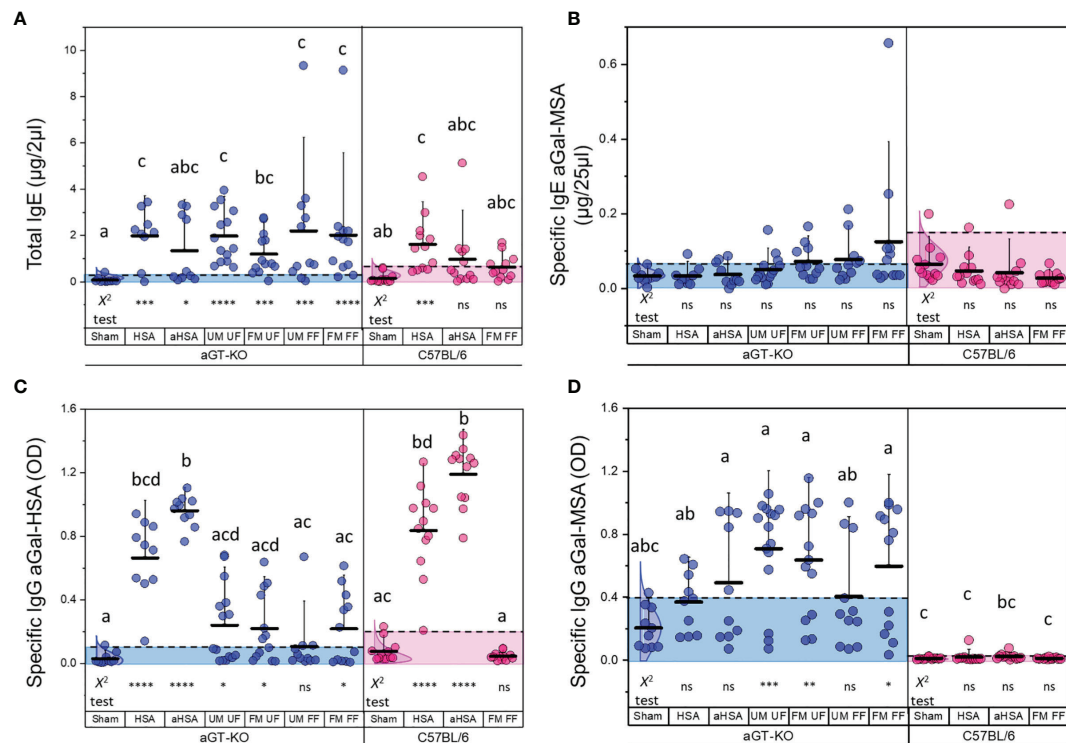


FIGURE 3 | Total and specific IgE to aGal and specific IgG levels to aGal-HSA and aGal-MSA. **(A)** Total IgE levels of different treatment groups (in µg/2 µl of serum) in aGT-KO mice (blue) and C57BL/6 mice (magenta). **(B)** Specific IgE to aGal-MSA (in µg/25 µl of serum). **(C)** Specific IgG to aGal-HSA (OD value) and **(D)** Specific IgG to aGal-MSA (OD value). Note that the positive control for aGal is the immunization with aHSA when specific immunoglobulins against aGal-MSA were measured. Axis labels; HSA, human serum albumin; aHSA, aGal conjugated to HSA; UM, unfed male tick; UF, unfed female tick; FM, fed male tick; FF, fed female tick. The alphabets show the significant differences at $p = 0.05$ in Kruskal-Wallis ANOVA test. Lack of alphabets represents no statistical difference seen. Error bars represent the mean (lower horizontal bar) and standard deviation (upper vertical bars). Dotted lines and the shaded area show the cutoff for difference at 95% normal distribution of the control (sham control) for determining frequencies of differences, which was used for Chi-square test, and the statistics below the graph represent the Chi-square test results for significance indicated by *, * < 0.05 , ** < 0.01 , *** < 0.001 and ns = not significant.

MSA, the levels of specific IgE against aGal-HSA were low in all treatments with the exception of a few individuals showing highly elevated specific IgE against aGal-HSA in the mice treated with aGal-HSA but not in the mice injected with HSA (**Supplementary Figure S1**).

Specific IgG Against aGal-HSA and to aGal-MSA

Specific IgG levels to aGal-HSA in the aGT-KO mice were significantly increased in both HSA and aHSA immunization (**Figure 3C**). Tick treatments resulted in significant levels of responses in the number of individual mice in all combinatory treatments for specific IgG against aGal-HSA, which is likely contributed by specific IgG against aGal, but not to HSA. C57BL/6 mice showed the highest levels of IgG against aGal-HSA under aGal-HSA treatment, whereas these mice showed no responses to tick feeding in terms of their specific IgG levels against aGal-HSA. The levels of specific IgG against aGal-MSA in the aGT-KO mice were generally high in the tick feeding groups (**Figure 3D**), while those in C57BL/6 mice were very low (**Figure 3D**), which suggests that specific IgG against aGal-MSA in the knockout mice is primarily against the aGal epitope.

Experiment 2: Three Different Ixodid Tick Species in aGT-KO Mice

We measured total IgE levels before and after treatment with different tick species. In this trial, our tick feeding time after tick attachment was longer than that in experimental segment A (8 ± 0.4 days). The Kruskal-Wallis ANOVA and the chi square test were performed as described above (**Table S3**). Tick feeding efficiently induced the elevation of total IgE levels in aGT-KO mice, except the case of partially fed *I. scapularis* (**Figure 4A**). The total IgE of the mice infested with unfed *I. scapularis* was also relatively low compared to the total IgE induced by feedings of other species of ticks.

At the levels of specific IgG against aGal-MSA, *A. americanum* treatments, both partially fed and unfed, induced (**Figure 4B**) high levels of the responses. Unfed *I. scapularis* also induced high levels of specific IgG in a number of individuals, while the levels were lowest in the feedings with *D. variabilis*.

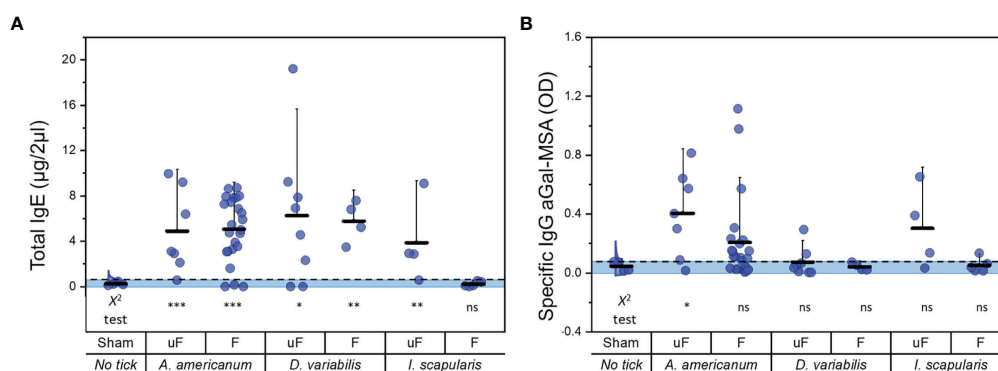


FIGURE 4 | Comparisons of total IgE and specific IgG to aGal-MSA before and after feeding with different ixodid tick species. **(A)** total IgE (µg/2µl of serum) after tick infestation and **(B)** specific IgG to aGal-MSA (OD in 2µl of serum) after tick feeding. uF; unfed, F; partially fed (on bovine blood before infestation into mice), Aa; *A. americanum*, Dv; *D. variabilis*, Is; *I. scapularis*. Kruskal-Wallis ANOVA was conducted and the lack of alphabets represents no significant differences seen with this test. Dotted lines show the cutoff for Chi square test at 0.05. Shaded areas represent the data points within the 95% distribution cutoff. The statistics below the graph represent the Chi square test (* < 0.05 , ** < 0.01 , *** < 0.001 and ns = not significant). Error bars represent the mean (lower horizontal bar) and standard deviation (upper horizontal bar).

Positive Correlation of Total IgE Levels With the Length of Tick Feeding

In our experiments, the lengths of tick feeding were determined by the approximate size of the female tick reaching the level of the rapid engorgement phase. Therefore, the lengths of tick feeding varied in each case depending on how fast the female ticks reached their size indicating rapid engorgement phase. We found that the days of tick feeding and the total IgE levels of the mice were correlated (**Figure 5A**). The positive correlation in the linear regression with the highest slope was found in *D. variabilis* regardless of being unfed or partially fed (**Figure 5B**).

DISCUSSION

Tick bites have been considered to be the causal factors of AGS (10, 12), which was initially supported by the occurrence of AGS in subjects who have experienced tick bites (13). Additional support recently provided was using aGT-KO mice that lack aGal (23) as a model for the study of AGS (24, 25). Repeated injections of crude extracts of whole bodies of ticks or of tick salivary glands (8, 24) have both been shown to elevate the levels of specific IgE against the aGal epitope in aGT-KO mice. In this study, we addressed the contribution of male and female tick bites as sensitizers in AGS and tested tick function as the “transmitters” of the aGal epitope in aGT-KO mice. Direct tick feeding in our study, compared to the injection of tick extract (24, 25), could lead to differences in the host immune response.

High levels of aGal epitopes were found in the male salivary glands of ticks fed on bovine blood. Multiple experiments, 5 independent trials, showed similar results of unusual smeared band patterns in the western blots. We conclude that the smeared immunoreactivity on the blot likely presents true heterogeneity in the tick salivary gland or with glycoproteins having highly variable glycosylation patterns. The aGal epitopes that were

abolished by PNGase F and a-galactosylase indicate that the aGal epitope is an N-glycan. The smeared band pattern in the male salivary glands can be caused by variable lengths of branches in glycosaminoglycans. N-glycosylation of highly branched mucin-like embryoglycan is an example of such a case (26, 27). Keratan sulfate I is also known to be a glycosaminoglycan with variable chain lengths anchored on asparagine (28). Large glycosaminoglycans were missed in our previous study using glycomics targeting small N-glycosylated aGal glycans (16, 17). Although the molecular identity of the aGal epitope in the male salivary glands awaits to be uncovered, the amount of aGal epitope in the male salivary gland is extremely high: $>1 \mu\text{moles}$ per tick based on the western blot compared to the standard of bovine thyroglobulin (**Figure 1**). Whether the aGal protein is directly assimilated from the host is unknown, while tick saliva is known to be rich in host proteins (16, 17, 29, 30), including the host immunoglobulins (31). *A. americanum* males are known to feed multiple times, while females rely on a single blood feeding for full engorgement (18). Together with the host-specific high levels of aGal in the saliva of male *A. americanum*, we found that *A. americanum* male feeding may play an important role depending on the prior host in the case of multiple feedings with an intrastadial host switch.

Dynamic control of the salivary components in tick feeding has been known. The factors that have been known to affect differential salivary gene expression and contents are the host species, host immune status, tick feeding stage over a week of feeding, and the presence of host pathogens (32–36). Interestingly, high levels of the aGal epitope in males fed on bovine blood were not observed in the case of male ticks fed on mice (see **Supplementary Figure S4**), which suggests that the enriched aGal epitope in the male salivary gland is host specific. The changed host blood, bovine to mouse, in our experiment may have affected the results through changes in tick salivary contents.

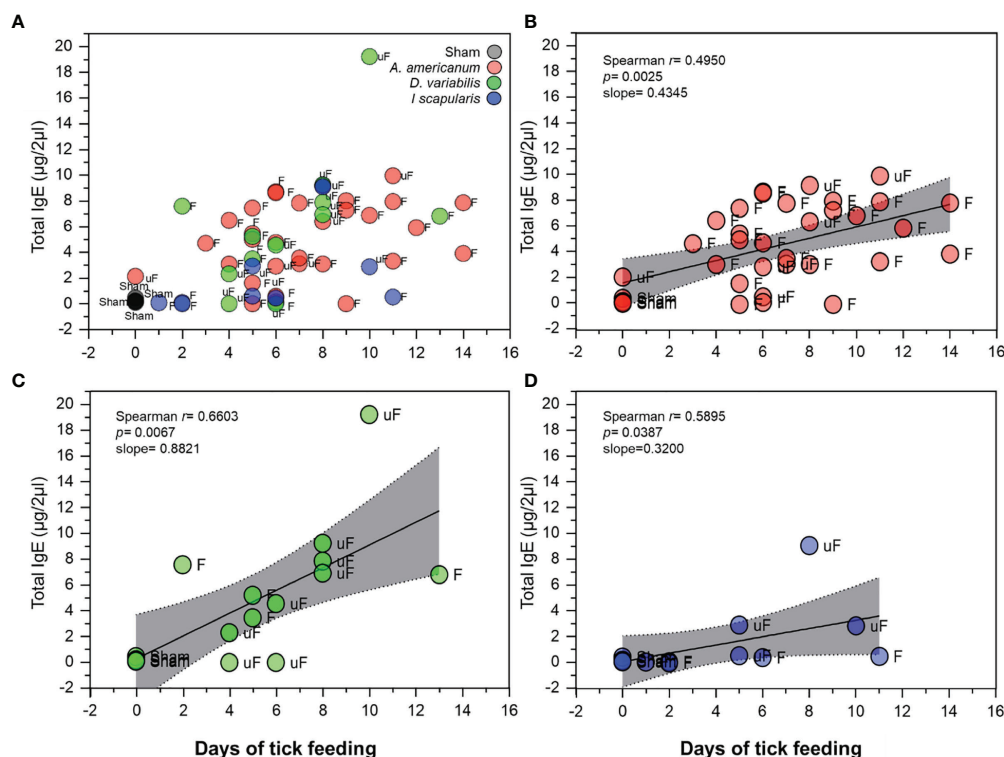


FIGURE 5 | Correlation of total IgE levels with days of tick feeding. **(A)** Scatterplot showing total IgE levels (in µg/2µl of serum) in relation to days of tick feeding among the different treatments and tick species used. Gray; sham, Red; *A. americanum*, Green; *D. variabilis* and Blue; *I. scapularis*. Data labels; F; fed, uF; unfed. **(B)** Spearman's rank correlation plot with linear regression, of *A. americanum* with days of tick feeding. **(C)** Spearman's rank correlation plot with linear regression, of *D. variabilis* with days of feeding and **(D)** Spearman's rank correlation plot with linear regression, of *I. scapularis* with days of feeding. Plots show positive correlation and linear regression slope. The gray shaded area represents the 95% confidence intervals.

Several recent reports have also shown successful elicitation of AGS in aGT-KO mice and other animal models by multiple injections of crude extract of tick salivary glands or of whole body (8, 24, 25, 37). Direct tick feeding in this study compared to immunization with crude extract of tick salivary glands likely resulted in significant differences in the host response. The location of tick feeding is intradermal compared to subcutaneous injections in most immunization protocols, which results in large differences in the immune response (38). Tick feeding likely involves long-term, slow, sequential infusion of salivary components for a week (29), while needle-mediated injections are performed in a multiple-pulse manner over a long duration (24). Most significantly, the tick salivary components change over the feeding duration, which likely depends on the host and host immune responses. Despite difficulties in the experimental approach in direct tick feeding on the host, our study demonstrated that tick feeding causes elevated total IgE, specific IgG and specific IgE toward aGal in aGT-KO mice with large individual variations but not in C57BL/6 controls.

We questioned whether partially fed male ticks with bovine blood carrying high levels of aGal epitopes in the salivary glands efficiently elevate specific IgE against aGal. In the comparison between partially fed male ticks and naïve ticks, we were unable to find higher levels of specific IgE against aGal in ticks

preconditioned with bovine blood. However, feedings with both treatments efficiently elevated specific IgE against aGal in aGT-KO mice with large individual variations. It is possible that the high levels of aGal in the saliva of bovine blood-fed ticks could have rapidly declined in the course of feeding on aGT-KO mice, as discussed above, resulting in no differences in the host response between the partially fed vs. unfed naïve tick treatments. We observed a lack of or low levels of aGal epitopes in the salivary glands after the ticks were fed on mice, in contrast to the case of artificial membrane feeding on bovine blood. It is also noted that a previous study reported that males of *A. americanum* that were unfed and partially fed on sheep blood lacked aGal epitopes in the salivary glands (17).

Large individual variations in mouse immune responses within the same treatments could be attributed to the differences among individual mice, the differences among ticks, and the inevitably different experimental procedures for tick feedings (i.e., the length of feeding). The contributions of the differences among individual mice to the individual variations were shown by the HSA and aHSA injection responses (Figure 3), which generally present lower levels of variation compared to the tick feeding treatments. Therefore, major causes of the differences among individuals appear to be the differences among ticks and the varied experimental procedures for tick

feedings in the experimental sets. Individual differences in the protein content of the salivary glands in *A. americanum* during feeding on the same host were previously shown (39). Although this study does not support the aGal “transmission” hypothesis with the current set of data, our observations and previous reports together, as discussed above for changes in salivary contents by various factors, lead the future directions of the study to address understanding the dynamics of aGal contents in tick saliva depending on different hosts and on the immune status of the hosts. A recent report has also shown the variations in aGal levels in the tick salivary glands after feeding on different hosts (11).

Different tick species induced different levels of total IgE and specific IgG toward aGal in aGT-KO mice. *A. americanum* moderately induced total IgE but was most effective for increased specific IgG toward aGal. *D. variabilis* was the tick that induced total IgE the most efficiently, but with low levels of elevation of specific IgG toward aGal (Figure 4). *I. scapularis* was effective in the induction of specific IgG against aGal in two individual mice. The levels of induced total IgE were correlated with the length of feeding duration to different degrees in different species (Figure 5). On the other hand, C57BL/6 mice infected with *A. americanum* had very low levels of total IgE elevation but no changes in specific IgG and specific IgE, which is consistent with other studies that showed low or absence of immune response to tick feedings in wild-type mice (40, 41). These data imply that aGal is a major player in the immune response against ticks in aGT-KO mice. Therefore, different strategies for bypassing the host immune system, the basophil-based immune system against tick feeding in the dermal layer, can be employed in different tick species (25, 41).

Immunoglobulins for aGal are known to be an important component as protective antigens against human pathogens (42–44). Many pathogens, including those vectored by arthropods, carry aGal (45–49). A number of tick-associated bacteria have been identified as aGal producer, hence, a role for these bacteria in the sensitization of AGS has been proposed (50, 51). Bacterial association of the AGS needs to be further studied to understand the intricate interactions among the arthropod-host-microbe. In addition, a glyco aGal-based vaccine is known to effectively protect against Chagas infection in mice (52). Anti-aGal antibodies confer protection against malaria in mice (53). Understanding the signaling pathways for triggering protective antigens instead of sensitizing the allergic response would provide ways to improve human health.

REFERENCES

1. Kaloga M, Kourouma S, Kouassi YI, Ecra EJ, Gbery IP, Allou AS, et al. Allergy to Red Meat: A Diagnosis Made by the Patient and Confirmed by an Assay for IgE Antibodies Specific for Alpha-1,3-Galactose. *Case Rep Dermatol* (2016) 8 (1):10–3. doi: 10.1159/000443631

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The mice were housed and bred in the AAALAC-approved animal facility within the Division of Biology, Laboratory Animal Care Service (LACS) at Kansas State University. Under the direction of Dr. Sherry Fleming (director of LACS), all animal protocols were approved and compliant with the guidelines of the Institutional Animal Care and Use Committee (IACUC) (IACUC #4228 and IACUC#4261).

AUTHOR CONTRIBUTIONS

LM-R: conducted experiments, sample collection, sample processing, analysis, writing of the original manuscript draft. GB: conducted experiments, sample collection, sample processing, analysis, data collection. DK: conducted tick dissections, tick feeding and processing of Western Blot and data collection. YP: study design, conducted experiments, sample collection, analysis, writing, and reviewing. All authors contributed to the article and approved the submitted version.

FUNDING

This work was conducted with the supported of the U.S. Department of Defense; DOD TBDRP, D01 W81XWH-18-1-0255.

ACKNOWLEDGMENTS

This study is contribution no. 20-138-J from the Kansas Agricultural Experiment Station.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.844262/full#supplementary-material>

2. Galili U. Evolution of Alpha 1,3galactosyltransferase and of the Alpha-Gal Epitope. *Subcell Biochem* (1999) 32:1–23. doi: 10.1007/978-1-4615-4771-6_1
3. Macher BA, Galili U. The Galalpha1,3Galbeta1,4GlcNAc-R (Alpha-Gal) Epitope: A Carbohydrate of Unique Evolution and Clinical Relevance. *Biochim Biophys Acta* (2008) 1780(2):75–88. doi: 10.1016/j.bbagen.2007.11.003

4. Stewart PH, McMullan KL, LeBlanc SB. Delayed Red Meat Allergy: Clinical Ramifications of Galactose- α -1,3-Galactose Sensitization. *Ann Allergy Asthma Immunol* (2015) 115(4):206–4. doi: 10.1016/j.anaai.2015.08.003
5. Fischer J, Lupberger E, Hebsaker J, Blumenstock G, Aichinger E, Yazdi AS, et al. Prevalence of Type I Sensitization to Alpha-Gal in Forest Service Employees and Hunters. *Allergy* (2017) 72:1540–7. doi: 10.1111/all.13156
6. Gonzalez-Quintela A, Dam Laursen AS, Vidal C, Skaaby T, Gude F, Linneberg A. IgE Antibodies to Alpha-Gal in the General Adult Population: Relationship With Tick Bites, Atopy, and Cat Ownership. *Clin Exp Allergy* (2014) 44(8):1061–8. doi: 10.1111/cea.12326
7. Hashizume H, Fujiyama T, Umayahara T, Kageyama R, Walls AF, Satoh T. Repeated Amblyomma Testudinarium Tick Bites are Associated With Increased Galactose &AgH, 1S Galactose Carbohydrate IgE Antibody Levels: A Retrospective Cohort Study in a Single Institution. *J Am Acad Dermatol* (2018) 78:113U 1141,1141.e1 1141.e3.
8. Araujo RN, Franco PF, Rodrigues H, Santos LCB, McKay CS, Sanhueza CA, et al. Amblyomma Scapularis Tick Saliva: α -Gal Identification, Antibody Response and Possible Association With Red Meat Allergy in Brazil. *Int J Parasitol* (2016) 46(3). doi: 10.1016/j.ijpara.2015.12.005
9. Hamsten C, Tran TAT, Starkhammar M, Brauner A, Commens SP, Platts-Mills TAE, et al. Red Meat Allergy in Sweden: Association With Tick Sensitization and B-Negative Blood Groups. *J Allergy Clin Immunol* (2013) 132(6):1431–4. doi: 10.1016/j.jaci.2013.07.050
10. Van Nunen SA, O'Connor KS, Fau-Clarke LR, Boyle RX, Fernando SL. An Association Between Tick Bite Reactions and Red Meat Allergy in Humans. *Med J Aust* (2009) 190(9):510–1. doi: 10.5694/j.1326-5377.2009.tb02533.x
11. Villar M, Pacheco I, Mateos-Hernández L, Cabezas-Cruz A, Tabor AE, Rodríguez-Valle M, et al. Characterization of Tick Salivary Gland and Saliva Alphagalactome Reveals Candidate Alpha-Gal Syndrome Disease Biomarkers. *Expert Rev Proteomics* (2021). doi: 10.1080/14789450.2021.2018305
12. Commens SP, Platts-Mills TA. Tick Bites and Red Meat Allergy. *Curr Opin Allergy Clin Immunol* (2013) 13(4):354–9. doi: 10.1097/ACI.0b013e3283624560
13. Commens SP, James HR, Kelly LA, Pochan SL, Workman LJ, Perzanowski MS, et al. The Relevance of Tick Bites to the Production of IgE Antibodies to the Mammalian Oligosaccharide Galactose- α -1,3-Galactose. *J Allergy Clin Immunol* (2011) 127(5):1286–93.e6. doi: 10.1016/j.jaci.2011.02.019
14. Platts-Mills TAE, Commens SP, Biedermann T, van Hage M, Levin M, Beck LA, et al. On the Cause and Consequences of IgE to Galactose- α -1,3-Galactose: A Report From the National Institute of Allergy and Infectious Diseases Workshop on Understanding IgE-Mediated Mammalian Meat Allergy. *J Allergy Clin Immunol* (2020) 145(4):1061–71. doi: 10.1016/j.jaci.2020.01.047
15. Fischer J, Riel S, Fehrenbacher B, Frank A, Schaller M, Biedermann T, et al. Spatial Distribution of Alpha-Gal in Ixodes Ricinus – A Histological Study. *Ticks Tick-borne Dis* (2020) 11(5):101506. doi: 10.1016/j.ttbdis.2020.101506
16. Park Y, Kim D, Boorgula GD, De Schutter K, Smagghe G, Šimo L, et al. Alpha-Gal and Cross-Reactive Carbohydrate Determinants in the N-Glycans of Salivary Glands in the Lone Star Tick, Amblyomma Americanum. *Vaccines* (2020) 8(1). doi: 10.3390/vaccines8010018
17. Crispell G, Commens SP, Archer-Hartman SA, Choudhary S, Dharmarajan G, Azadi P, et al. Discovery of Alpha-Gal-Containing Antigens in North American Tick Species Believed to Induce Red Meat Allergy. *Front Immunol* (2019) 10:1056. doi: 10.3389/fimmu.2019.01056
18. Gladney WJ, Drummond RO. Migration of Male Lone Star Ticks on the Host in Relation to Mating13. *J Economic Entomol* (1970) 63(4):1214–6. doi: 10.1093/jee/63.4.1214
19. Kröber T, Guerin P. The Tick Blood Meal: From a Living Animal or From a Silicone Membrane? *ALTEX* (2007) 24(1868-596X):39–41.
20. Sim DW, Lee JS, Park KH, Jeong KY, Ye Y-M, Lee J-H, et al. Accurate Assessment of Alpha-Gal Syndrome Using Cetuximab and Bovine Thyroglobulin-Specific IgE. *Mol Nutr Food Res* (2017) 61(10):1601046. doi: 10.1002/mnfr.201601046
21. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 Years of Image Analysis. *Nat Methods* (2012) 9(7):671–5. doi: 10.1038/nmeth.2089
22. Rawitch AB, Pollock HG, Yang SX. Thyroglobulin Glycosylation: Location and Nature of the N-Linked Oligosaccharide Units in Bovine Thyroglobulin. *Arch Biochem Biophys* (1993) 300(1):271–9. doi: 10.1006/abbi.1993.1038
23. Tearle RG, Tange MJ, Zannettino ZL, Katerelos M, Shinkel TA, Van Denderen BJW, et al. THE α -1,3-Galactosyltransferase Knockout Mouse: Implications for Xenotransplantation. *Transplantation* (1996) 61(1). doi: 10.1097/00007890-199601150-00004
24. Choudhary SK, Karim S, Iweala OI, Choudhary S, Crispell G, Sharma SR, et al. Tick Salivary Gland Extract Induces Alpha-Gal Syndrome in Alpha-Gal Deficient Mice. *Immun Inflammation Dis* (2021) 9(3):984–90. doi: 10.1002/iid3.457
25. Chandrasekhar JL, Cox KM, Loo WM, Qiao H, Tung KS, Erickson LD. Cutaneous Exposure to Clinically Relevant Lone Star Ticks Promotes IgE Production and Hypersensitivity Through CD4+ T Cell and MyD88-Dependent Pathways in Mice. *J Immunol* (2019) 203:813–24. doi: 10.4049/jimmunol.1801156
26. Muramatsu T. Embryoglycan: A Highly Branched Poly-N-Acetylactosamine in Pluripotent Stem Cells and Early Embryonic Cells. *Glycoconjugate J* (2017) 34(6):701–12. doi: 10.1007/s10719-016-9673-3
27. Lauder RM, Huckerby TN, Nieduszynski IA. The Structure of the Keratan Sulphate Chains Attached to Fibromodulin From Human Articular Cartilage. *Glycoconj J* (1997) 14(5):651–60.
28. Carterson B, Melrose J. Keratan Sulfate, a Complex Glycosaminoglycan With Unique Functional Capability. *Glycobiology* (2018) 28(4):182–206. doi: 10.1093/glycob/cwy003
29. Kim TK, Tirloni L, Pinto AFM, Moresco J, Yates JR, da Silva Vaz IJr., et al. Ixodes Scapularis Tick Saliva Proteins Sequentially Secreted Every 24 H During Blood Feeding. *PLoS Neglected Trop Dis* (2016) 10(1):e0004323. doi: 10.1371/journal.pntd.0004323
30. Oliveira CJ, Anatriello E, de Miranda-Santos IK, Francischetti IM, Sá-Nunes A, Ferreira BR, et al. Proteome of Rhipicephalus Sanguineus Tick Saliva Induced by the Secretagogues Pilocarpine and Dopamine. *Ticks Tick-borne Dis* (2013) 4(6):469–77. doi: 10.1016/j.ttbdis.2013.05.001
31. Wang H, Nuttall PA. Excretion of Host Immunoglobulin in Tick Saliva and Detection of IgG-Binding Proteins in Tick Haemolymph and Salivary Glands. *Parasitology* (1994) 109(4):525–30. doi: 10.1017/S0031182000080781
32. Karim S, Ribeiro JMC. An Insight Into the Sialome of the Lone Star Tick, Amblyomma Americanum, With a Glimpse on Its Time Dependent Gene Expression. *PLoS One* (2015) 10(7):e0131292. doi: 10.1371/journal.pone.0131292
33. Kim TK, Tirloni L, Pinto AFM, Diedrich JK, Moresco JJ, Yates JR, et al. Time-Resolved Proteomic Profile of Amblyomma Americanum Tick Saliva During Feeding. *PLoS Neglected Trop Dis* (2020) 14(2):e0007758. doi: 10.1371/journal.pntd.0007758
34. Perner J, Kropáčková S, Kopáček P, Ribeiro JMC. Sialome Diversity of Ticks Revealed by RNAseq of Single Tick Salivary Glands. *PLoS Neglected Trop Dis* (2018) 12(4):e0006410. doi: 10.1371/journal.pntd.0006410
35. Pham M, Underwood J, Oliva Chávez AS. Changing the Recipe: Pathogen Directed Changes in Tick Saliva Components. *Int J Environ Res Public Health* (2021) 18(4):1806. doi: 10.3390/ijerph18041806
36. Tirloni L, Kim TK, Pinto AFM, Yates JR, da Silva Vaz I, Mulenga A. Tick-Host Range Adaptation: Changes in Protein Profiles in Unfed Adult Ixodes Scapularis and Amblyomma Americanum Saliva Stimulated to Feed on Different Hosts. *Front Cell Infection Microbiol* (2017) 7(517). doi: 10.3389/fcimb.2017.00517
37. Contreras M, Pacheco I, Alberdi P, Díaz-Sánchez S, Artigas-Jerónimo S, Mateos-Hernández L, et al. Allergic Reactions and Immunity in Response to Tick Salivary Biogenic Substances and Red Meat Consumption in the Zebrafish Model. *Front Cell Infection Microbiol* (2020) 10:78–8. doi: 10.3389/fcimb.2020.00078
38. Rosenbaum P, Tchitchek N, Joly C, Rodriguez Pozo A, Stimmer L, Langlois S, et al. Vaccine Inoculation Route Modulates Early Immunity and Consequently Antigen-Specific Immune Response. *Front Immunol* (2021) 12(1362). doi: 10.3389/fimmu.2021.645210
39. Kim D, Urban J, Boyle DL, Park Y. Multiple Functions of Na/K-ATPase in Dopamine-Induced Salivation of the Blacklegged Tick, Ixodes Scapularis. *Sci Rep* (2016) 6(1):21047. doi: 10.1038/srep21047

40. Narasimhan S, Booth CJ, Deponte K, Wu MJ, Liang X, Mohanty S, et al. Host-Specific Expression of *Ixodes Scapularis* Salivary Genes. *Ticks Tick-borne Dis* (2019) 10 2:386–97. doi: 10.1016/j.ttbdis.2018.12.001
41. Wada T, Ishiwata K, Koseki H, Ishikura T, Ugajin T, Ohnuma N, et al. Selective Ablation of Basophils in Mice Reveals Their Nonredundant Role in Acquired Immunity Against Ticks. *J Clin Invest* (2010) 120(8):2867–75. doi: 10.1172/JCI42680
42. Cabezas-Cruz A, de la Fuente J. Immunity to α -Gal: Toward a Single-Antigen Pan-Vaccine to Control Major Infectious Diseases. *ACS Cent Sci* (2017) 3 (11):1140–2. doi: 10.1021/acscentsci.7b00517
43. Cabezas-Cruz A, de la Fuente J. Immunity to α -Gal: The Opportunity for Malaria and Tuberculosis Control. *Front Immunol* (2017) 8. doi: 10.3389/fimmu.2017.01733
44. Tangvoranuntakul P, Gagneux P, Diaz S, Bardor M, Varki N, Varki A, et al. Human Uptake and Incorporation of an Immunogenic Nonhuman Dietary Sialic Acid. *Proc Natl Acad Sci* (2003) 100(21):12045–50. doi: 10.1073/pnas.2131556100
45. Almeida IC, Milani SR, Gorin PA, Travassos LR. Complement-Mediated Lysis of *Trypanosoma-Cruzi* Trypomastigotes by Human Anti-Alpha-Galactosyl Antibodies. *J Immunol* (1991) 146(7):2394–400.
46. Avila JL, Rojas M, Galili U. Immunogenic Gal Alpha 1–3Gal Carbohydrate Epitopes are Present on Pathogenic American *Trypanosoma* and *Leishmania*. *J Immunol* (1989) 142(8):2828–34.
47. Lei Y, Yu H, Dong Y, Yang J, Ye W, Wang Y, et al. Characterization of N-Glycan Structures on the Surface of Mature Dengue 2 Virus Derived From Insect Cells. *PloS One* (2015) 10(7):e0132122. doi: 10.1371/journal.pone.0132122
48. Moura APV, Santos LCB, Brito CRN, Valencia E, Junqueira C, Filho AAP, et al. Virus-Like Particle Display of the α -Gal Carbohydrate for Vaccination Against *Leishmania* Infection. *ACS Cent Sci* (2017) 3(9):1026–31. doi: 10.1021/acscentsci.7b00311
49. Zamze SE, Ashford DA, Wooten EW, Rademacher TW, Dwek RA. Structural Characterization of the Asparagine-Linked Oligosaccharides From *Trypanosoma-Brucei* Type II and Type III Variant Surface Glycoproteins. *J Biol Chem* (1991) 266(30):20244–61. doi: 10.1016/S0021-9258(18)54916-4
50. Cabezas-Cruz A, Espinosa PJ, Alberdi P, Šimo L, Valdés JJ, Mateos-Hernández L, et al. Tick Galactosyltransferases are Involved in α -Gal Synthesis and Play a Role During *Anaplasma-Phagocytophilum* Infection and *Ixodes-Scapularis* Tick Vector Development. *Sci Rep* (2018) 8(1):14224–4. doi: 10.1038/s41598-018-32664-z
51. Sharma SR, Karim S. Tick Saliva and the Alpha-Gal Syndrome: Finding a Needle in a Haystack. *Front Cell Infection Microbiol* (2021) 11:680264–4. doi: 10.3389/fcimb.2021.680264
52. Portillo S, Zepeda BG, Iniguez E, Olivas JJ, Karimi NH, Moreira OC, et al. A Prophylactic α -Gal-Based Glycovaccine Effectively Protects Against Murine Acute Chagas Disease. *NPJ Vaccines* (2019) 4(1):13. doi: 10.1038/s41541-019-0107-7
53. Yilmaz B, Portugal S, Tran TM, Gozzelino R, Ramos S, Gomes J, et al. Gut Microbiota Elicits a Protective Immune Response Against Malaria Transmission. *Cell* (2014) 159(6):1277–89. doi: 10.1016/j.cell.2014.10.053

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Maldonado-Ruiz, Boorgula, Kim, Fleming and Park. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.