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Itch-induced tick removal (IITR) is a common immune defense response against ixodid ticks in guinea pigs

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Abstract

Ixodid ticks are the most pathogen-permissive group of arthropod disease vectors. Consequently, vaccine development efforts have attempted to exploit host anti-tick responses. We recently described itch-induced tick removal, an early and fast anti-tick response in guinea pigs repeatedly infested with *Ixodes scapularis*. Here, we address the question of whether itch-induced tick removal is a general host response against ixodid tick species. We show that guinea pigs acquire itch-induced tick removal to all tested ixodid tick species tested, *I. scapularis*, *I. ricinus*, *Amblyomma americanum*, *Dermacentor variabilis*, and *Rhipicephalus sanguineus*. Hallmarks of the response are shared, including itch-mediated tick removal, strong cellular infiltration of $CD3^+$ T cells and $Iba1^+$ macrophages at the bite site, and the absence of early increases in IgG/IgE titers to salivary antigens. Itch-induced tick removal to *R. sanguineus* is more intense than to other ixodid tick species, which may relate to the more aggressive feeding of this tick species. Together, our data show that itch-induced tick removal has the potential to become a pan-anti-ixodid tick vaccine strategy.

Introduction

Ixodid ticks are one of the most widely spread groups of arthropod vectors of disease, with a global range from the Arctic to Antarctica[1,2]. They have an exceptionally wide host range, parasitizing all groups of terrestrial vertebrates[3,4]. Ixodid ticks are also the most permissive group of arthropod vectors of infectious disease in the world[4–6]. They transmit a diverse array of viral, bacterial, protozoan, and nematode pathogens infective for humans and animals[2]. These pathogens are responsible for dozens of different infectious diseases, including Lyme disease, Spotted fever rickettsioses, tick-borne encephalitis, and Babesiosis[2,7,8]. Tick-borne diseases are a global source of considerable morbidity, mortality, and socio-economic damages[9,10]. However, finding treatments and vaccines against tick-borne diseases is an exceptionally complex task and exceedingly expensive.

Timely manual removal of biting ticks has been shown to prevent the transmission of several tick-borne pathogens[11], as most tick-borne pathogens require hours, if not days post the beginning of a tick bite to become transmissible[12,13]. Most ixodid ticks feed only three times on blood throughout their entire 1–2 + year-long life cycle[3]. Many tick-borne pathogens remain dormant between feeding events and use the influx of fresh blood as a trigger for reactivation[14,15]. This creates a window of opportunity to prevent host infection by rapidly disrupting tick feeding and concomitantly tick-borne pathogen transmission. Efforts to exploit this for vaccine development have focused on identifying tick-derived molecules, mostly salivary or gut proteins[16]. Molecule selection has been based on prevention of tick feeding or killing before pathogens are transmitted. This approach is inspired by the most prominently described anti-tick feeding response, acquired tick resistance (ATR)[17]. However, efforts have only been partially successful.

Using *I. scapularis* and Hartley guinea pigs (GPs), we recently described an early neuroimmunological anti-tick response that efficiently removes ticks early in tick-sensitized animals[18]. We termed this response itch-induced tick removal (IITR). IITR is rapidly acquired within 5 days post initial tick exposure. During subsequent tick exposures, IITR appears within 3 to 4 hours post tick attachment (pta) in tick-sensitized GPs. It consists of an inflammatory response to tick-derived components, presumably salivary proteins, at the tick bite site. The inflammatory response elicits a local itch by stimulation of sensory neurons. The local itch translates to targeted scratching at the tick bite site, which continues until the tick is removed. This rapid tick removal could effectively disrupt the transmission of most tick-borne pathogens. This makes IITR a great mechanism to exploit as a tick-borne pathogen transmission intervention by an immunization strategy.

Previously, we used *I. scapularis* nymphal ticks exclusively to investigate IITR. Thus, the question remained whether IITR was an *Ixodes*-specific anti-tick response, or if it was shared with other ixodid tick genera. Here, we tested if IITR is a common anti-tick response to repeated ixodid tick infestations.

Results

Guinea pigs respond to all tested ixodid tick species with increasing removal post tick exposure

In this study, we assessed IITR across three weekly exposures to four medically relevant tick species: *Amblyomma americanum*, *Dermacentor variabilis*, *Ixodes ricinus*, and *Rhipicephalus sanguineus*, by assessing tick attachment at 24, 48, and 72 hours pta. We included *I. scapularis* as our positive control.

As previously shown, GPs repeatedly exposed to *I. scapularis* nymphs removed ticks effectively (Fig. 1A). There was a statistically significant increase in tick-removal success with every subsequent tick exposure (**Supplementary Table 1A**). According to the hazard ratio, ticks were overall ~ 6.5-times and ~ 16.5-times more likely to be removed during a 2nd and 3rd tick exposure, respectively, compared to a 1st exposure (2nd : $p < 0.0001$; 3rd : $p < 0.0001$).

We then tested *I. ricinus*, the closely related European counterpart of North America's *I. scapularis*, which showed a tick removal profile in GPs similar to *I. scapularis* (Fig. 1B, **Supplementary Fig. 1A, Supplementary Table 2A**). Compared to the 1st exposure, *I. ricinus* ticks were ~ 18.6 times more likely to be removed on the 2nd exposure ($p < 0.0001$) and 43.1 times ($p < 0.0001$) more likely on the 3rd tick exposure (**Supplementary Table 1B)**. Importantly, *A. americanum*, *D. variabilis*, and *R. sanguineus* (Fig. 1C-E, **Supplementary Table 1C-E**), also induced IITR after subsequent tick exposures. Accordingly, tick-removal success increased significantly upon subsequent exposures. For *D. variabilis*, ticks were 6.7 times more likely to be removed on the 2nd tick exposure ($p < 0.0001$) and ~ 13.6 times ($p < 0.0001$) more likely on the 3rd tick exposure, respectively (**Supplementary Table 1D)**. Likewise, *R. sanguineus* ticks were 10.3 times more likely to be removed on the 2nd exposure ($p < 0.0001$) and 16.5 times more likely on the 3rd exposure ($p < 0.0001$; **Supplementary Table 1E)**.

Because *A. americanum* data did not conform to the Cox proportional hazard assumption, we employed a time transformation in the model that accommodated a change in proportional hazard over time. Our results showed significant differences among the 1st, 2^{nd,} and 3rd exposures at 24, 48, and 72 hours pta. At 72 hours pta, *A. americanum* ticks were 14 times more likely to be removed on the 2nd exposure ($p < 0.0001$) and 20.5 times more likely on the 3rd exposure ($p < 0.0001$) (**Supplementary Table 1C)**.

While there were clear similarities in IITR between tick species, we also observed nuanced differences in tick removal profiles between tick species (**Supplementary Fig. 1B-D, Supplementary Table 2B-C**). Most evidently, *R. sanguineus* caused much more rapid tick removal than *I. scapularis* during a 2nd ($p < 0.0001$) and 3rd ($p < 0.0001$) tick exposure in GPs. In contrast, *A. americanum* showed a slower tick removal during the 3rd tick exposure ($p = 0.0296$).

To assess whether IITR was a property of the outbred Hartley guinea pigs, we used the same protocol to exposed inbred Strain 13 guinea pigs three times to *I. scapularis* nymphs. These two guinea pig strains do not share a direct, common laboratory lineage. Like Hartley guinea pigs, Strain 13 animals removed nymphal ticks with increasing efficiency after repeated exposures (Fig. 1F). As with *A. americanum*, the data for Strain 13 did not conform to the proportional hazards assumption and were therefore treated in the same way. At 72 hours pta, *I. scapularis* ticks were 17.3 times more likely to be removed on the 2nd exposure from Strain 13 guinea pigs ($p < 0.0001$) and 54.3 times more likely on the 3rd exposure ($p < 0.0001$; **Supplementary Table 1F**). When directly compared, Hartley and Strain 13 guinea pigs showed no biologically relevant differences in tick removal profiles (**Supplementary Fig. 1F, Supplementary Table 2E**). Together, our data show that all tick species tested are able to induce IITR successfully and consistently in guinea pigs.

The mechanism of IITR is conserved among different ixodid tick species

To further validate IITR in other tick species, we selected *A. americanum* and *R. sanguineus* as representative ixodid tick species. Both species showed statistically significant differences in their tick removal profiles compared to *I. scapularis* (**Supplementary Fig. 1B-C, Supp. Table 2**).

First, we measured GP scratch bouts during the 1st, 2nd, and 3rd tick exposure. All three tick species caused increases in scratching behavior from the 1st tick exposure compared to subsequent tick exposures (Fig. 2A-C). However, only GPs exposed to *A. americanum* showed a statistically significant increase in scratching by the 3rd exposure (Dunn's test: $p = 0.0004$). GPs exposed to *A. americanum* also showed considerably more scratching activity during the 2nd and 3rd tick exposure than those exposed to *I. scapularis* (Dunn's test: 2nd exposure: $p = 0.0366$, 3rd exposure: $p = 0.178$) or *R. sanguineus* (Dunn's test: 3rd exposure: $p = 0.0212$).

We also graphically correlated scratch bouts and tick removal at 24 hours pta for each tick species (Fig. 2D-F). The Bayesian MANOVA-style model indicated strong evidence that each exposure round

changed the combined response for tick removal and scratch bout counts (posterior probability = 1 for all three tick species). The Count-scale follow-up model revealed in the pairwise comparison that the main effect was carried by tick removal, rather than scratch bouts, except for *A. americanum*. For *A. americanum*, we observed that on average 34.2 and 58.9 more scratch bouts were expected during the 2nd and 3rd tick exposure, respectively. With respect to tick removal, the analysis predicted on average 5.4, and 6.2, 3.49, and 4.7, 8.5 and 11.1 less ticks attached by 24 hours pta during the 2nd and 3rd exposure with *I. scapularis*, *A. americanum*, and *R. sanguineus*, respectively (**Supplementary Table 3**). This analysis confirmed that the biggest effect came from tick removal rather than scratch bouts (Fig. 2D-I), suggesting that repeated tick exposures have a significant effect on the probability of tick attachment on guinea pigs.

To test whether tick removal depended on scratching, we challenged sensitized GPs once with and once without Elizabethan collar to interfere with tick removal. Similarly to *I. scapularis*, Elizabethan collar placement also impeded removal of *A. americanum* and *R. sanguineus* nymphs (Fig. 2G-I), suggesting that scratching is the removal mechanism for these tick species. We observed a ~ 10-fold ($p < 0.0001$) and ~ 14-fold ($p < 0.0001$) reduction in tick removal success for *A. americanum* and *R. sanguineus*, respectively, resulting in statistically significant reductions (**Supplementary Table 4A-C)**.

Further, we assessed whether early tick removal was related to compromised nymphal health and feeding ability. Using the scutal index [18,19], we showed that *I. scapularis* nymphs were able to feed equally successfully on both tick-naïve and tick-sensitized GPs for the first 48 hours pta (Median scutal index: tick-naïve = 1.28, tick-sensitized 1.32, Dunn's test: adj. $p = 1$; Fig. 2J). On the other hand, for *A. americanum*, we observed a minor but statistically significant decrease in nymphal feeding success on tick-sensitized GPs compared to feeding on tick-naïve ones (Median scutal index: tick-naïve = 1.3, tick-sensitized 1.19, Dunn's test: adj. $p = 0.0062$; Fig. 2K). However, for *R. sanguineus*, we observed a large and statistically significant difference in scutal index in nymphs exposed to tick-naïve and tick-sensitized GPs (Median scutal index: tick-naïve = 1.7, tick-sensitized 1.26, Dunn's test: adj. $p < 0.0001$; Fig. 2L). Interestingly, *R. sanguineus*'s scutal index grew significantly faster than either *A. americanum* or *I. scapularis* when feeding on tick-naïve guinea pigs (Dunn's test: adj. $p < 0.0001$, $p < 0.0001$), suggesting that *R. sanguineus* nymphs fed faster than nymphs of either species. Conversely, feeding success of *R. sanguineus* nymphs on tick-sensitized GPs was comparable to *I. scapularis* (Dunn's test: adj. $p = 1$) and slightly higher compared to *A. americanum* (Dunn's test: adj. $p = 0.0204$). However, the observed differences in feeding success on tick-naïve and tick-sensitized GPs did not affect early tick removal. This was evident from the Elizabethan collar experiment, where no excessive premature tick loss was observed for either tick species when the Elizabethan collar was in place (Fig. 2A-C).

IITR does not require IgG and IgE titers to increase for its function

Our previously published work showed that repeated *I. scapularis* exposure does not increase circulating immunoglobulin G (IgG) and IgE titers for at least 4 weeks pta[18]. Here, we tracked circulating IgG and IgE

serum levels in the same group of GPs before their 1st tick exposure and over the following 10 weeks pta. During this period, GPs were repeatedly exposed to *I. scapularis* (Fig. 3A,D), *A. americanum* (Fig. 3B,E), or *R. sanguineus* (Fig. 3C,F), respectively. Pre-exposed levels served as the baseline for all subsequent measurements.

With respect to IgG, we observed no significant changes from baseline serum levels for the first 4 weeks pta regardless of tick species. Repeated exposure to *I. scapularis* showed a slow but steady increase in IgG serum levels against salivary gland homogenate (SGH) from Week 5 to 10. However, only in Week 8 and Week 10 did we observe statistically significant changes from baseline serum levels (Dunn's test: $p = 0.0119$ and $p = 0.0085$, respectively). Regardless, IgG serum levels remained low even by Week 10 (Fig. 3A). Conversely, repeated exposures to *A. americanum* induced a strong increase in IgG serum level against SGH from Week 5 to Week 6 (Fig. 3B). By Week 7, we observed statistically significant differences to baseline serum levels (Dunn's test: Week 7: $p = 0.0018$, Week 8: $p = 0.0308$, Week 9: $p = 0.0232$, Week 10: $p < 0.0001$). With *R. sanguineus*, we also observed an increase in IgG serum levels by Week 5 (Dunn's test: Week 5: $p = 0.0069$). However, no clear pattern of further significant IgG serum level increases was observed thereafter (Fig. 3C). Interestingly, IITR was observed starting Week 2 for all infesting ixodid tick species. This suggested that, in general, IITR did not rely on IgG to function.

Due to the lack of reagents for guinea pigs, IgE measurements were restricted to serum levels. We did not observe any persistent increase in IgE serum levels regardless of infesting ixodid tick species (Fig. 3D-F). Only *A. americanum* exposure caused statistically significant increases in IgE serum levels from baseline (Dunn's test: Week 1: $p = 0.0232$, Week 5: $p = 0.0069$). However, these increases were not consistent over time and did not approach positive control levels. This suggested that, in general, IITR did not rely on IgE to function.

IITR is associated with a skin cellular infiltrate at the tick bite site

We collected 3 mm skin punch biopsies from tick-naïve and tick-sensitized GPs at 48 hours pta to assess cellular infiltration at the bite site by histological staining. In this study, we also observed little to no cellular infiltration in tick-naïve GPs during the 1st tick exposure to *I. scapularis*, and strong cellular infiltration during subsequent exposures (Fig. 4). Notably, similar patterns were also observed in GPs exposed to *A. americanum* or *R. sanguineus* (Fig. 4). The cellular infiltration appeared most intense in tick-sensitized GPs exposed to *R. sanguineus*. There was also considerable damage to the epidermis and upper dermis visible in *R. sanguineus*-bitten sensitized GP skin. This correlated with the erythema and darkening of the GPs' skin around the tick bite site.

Further, we applied immunohistochemical staining to sections from the same skin biopsy blocks. We assessed T cells and macrophage infiltration using anti-CD3 and anti-Ionized calcium-binding adapter molecule 1 (Iba1) antibodies. CD3 signal was visibly increased in tick-sensitized GP skin than in tick-naïve samples, regardless of biting tick species (Fig. 5A). Interestingly, T cells in *R. sanguineus* exposed GPs were visibly organized distal to the tick hypostome in the cellular aggregate around the bite site.

Sections stained with anti-Iba1 showed a massive influx of macrophages at the tick bite site (Fig. 5B). In general, macrophages constituted the bulk of infiltrating cells. With *R. sanguineus*, the Iba1 signal was particularly pronounced proximal to the bite site and the hypostome.

Acquisition of IITR is dependent on the availability of naïve lymphocytes during the 1st tick exposure, regardless of biting tick species

We had previously shown that the acquisition of IITR was dependent on the availability of naïve lymphocytes during the 1st *I. scapularis* exposure[18]. Thus, we treated tick-naïve GPs with Fingolimod (FTY720) prior to exposure to other ixodid tick species. FTY720 is a sphingosine-1-phosphate receptor modulator, which prevents the egress of naïve lymphocytes from the lymph nodes, effectively depleting naïve lymphocytes from circulation[20]. Daily FTY720 treatment of GPs began a week prior to the 1st tick exposure and was maintained throughout the experimental tick exposures. Consistent with its established effect, we observed a statistically significant loss in circulating lymphocytes (on average > 80%) in all FTY720-treated GPs prior to the 1st tick exposure (Fig. 6A-C, **Supplementary Table 5**). Regardless of biting tick species, circulating lymphocytes remained low after the 1st tick exposure compared to PBS-treated animals.

Importantly, FTY720-treated GPs failed to establish the same degree of tick removal success during a 2nd tick exposure compared to PBS-treated GPs (Fig. 6D-F). PBS-treated GPs exposed to *I. scapularis*, *A. americanum*, and *R. sanguineus* for a second time showed a ~ 4.2-fold ($p < 0.0001$), ~ 10-fold ($p < 0.0001$), and ~ 6.7-fold ($p < 0.0001$) increase in tick removal success, respectively (**Supplementary Table 6**). FTY720-treated GPs only increased their tick removal success by ~ 1.5-fold (p 0.2977), ~ 3.4-fold (p 0.0146), and ~ 2.28-fold (p 0.0464), respectively, during the 2nd tick exposure (**Supplementary Table 6**). These findings suggest that circulating naïve lymphocytes are required for the acquisition of IITR during the 1st tick exposure regardless of biting ixodid tick species.

Further, we used laser speckle contrast imaging to assess cellular recruitment to tick bite sites at 48 hours pta in vivo. We imaged PBS- and FTY720-treated GPs after their 1st and 2nd tick exposure. Regardless of biting tick species, there was no statistically significant difference in signal between treatment groups during the 1st tick exposure (Fig. 6G-I). For the PBS-treated group, we observed a highly statistically significant increase in signal during the 2nd exposure regardless of biting ixodid tick species (Tukey's test: *I. scapularis*: $p < 0.0001$, *A. americanum*: $p < 0.0001$, *R. sanguineus*: $p = 0.0007$). This correlated with observed cellular infiltration into the tick bite site (Figs. 4 & 5). Apart from *R. sanguineus*, ticks also induced a statistically significant signal increase in FTY720-treated GPs during the 2nd exposure (Tukey's test: *I. scapularis*: $p < 0.0001$, *A. americanum*: $p < 0.0001$, *R. sanguineus*: $p = 0.4209$). However, this signal increase was significantly more moderate compared with PBS-treated GPs (Tukey's test: *I. scapularis*: $p < 0.0001$, *A. americanum*: $p < 0.0001$, *R. sanguineus*: $p = 0.0229$). Interestingly, *A. americanum* produced, in general, the strongest signal, while *R. sanguineus* produced the weakest. This is contrary to the histological observation that *R. sanguineus* biting tick-sensitized GPs attracted more immune cells into the bite site than *A. americanum*.

Discussion

Throughout this study, we have observed remarkable similarities in itch-induced tick removal (IITR) among different ixodid tick species. We previously showed that one exposure to a single *Ixodes scapularis* nymph was sufficient to induce IITR in guinea pigs (GPs), which was rapidly acquired within five days post 1st ixodid tick exposure[18]. Here, we extend these findings by showing that IITR is not restricted to *I. scapularis* but is a common response to repeated ixodid tick exposure in GPs.

Our findings further distinguish IITR from ATR at the level of adaptive immunity. Rather than directly impairing tick feeding through IgG and IgE-mediated basophilic hypersensitivity, the immune response in IITR stimulates sensory neurons in the skin[18,21]. This is evident from the absence of increases in IgG and IgE titers during the first few weeks post initial tick exposure, while IITR is already occurring. However, which immune cells produce the pruritogen(s) that stimulate the sensory neurons at the tick bite site remains unclear. What we know is that the itch signal is mediated through Trpv1-independent itch pathways[18]. This results in a rapid scratch response targeted to the local itch at the tick bite site, removing the tick rapidly.

Neither tick species used in this study elicits an immune cell response in a tick-naïve GPs, whereas tick-sensitized GPs showed a strong influx of macrophages and T cells. The Iba1$^+$ macrophage influx was most pronounced at *R. sanguineus* bite sites, where densely packed immune cells were frequently visible by 48 hours pta. The tissue damage and associated skin discoloration observed in tick-sensitized GPs exposed to *R. sanguineus* may reflect skin cavitation at the tick bite site[22]. Although retained tick hypostomescan induce granulomas[23], these structures take weeks to mature[24]. Thus, whether the structures observed here represent early stages of foreign body granuloma formation remains unclear.

The pronounced cellular recruitment may be related to the rapid removal of *R. sanguineus* and their accelerated early feeding behavior. However, their shallower anchoring in the skin and diverse sialome may also contribute to removal. Beyond their potential involvement in these aggregates, Iba1$^+$ macrophages may contribute to IITR-associated itch. In stasis dermatitis, the presence of CD68$^+$ CD31$^+$ macrophages and CD68$^+$ CD163$^+$ M2 macrophages is strongly associated with the occurrence of itch[25]. Nevertheless, the occurrence of M2 macrophages is dependent on the presence of CD3$^+$ CD4$^+$ Th2 cells, which ties in with the relevance of T cells in IITR. As of now, it remains to be seen what the exact role of macrophages is in IITR.

A role for T cells in IITR is further supported by rapid CD3$^+$ T-cell recruitment in tick-sensitized GPs and failure to acquire IITR following lymphocyte depletion before sensitization. Together with the rapid recruitment of CD3$^+$ T cells in tick-sensitized GPs, these findings support a requirement for naïve lymphocytes during the 1st tick encounter and suggest that activated T cells contribute to IITR during subsequent encounters. This may not be surprising, as CD3$^+$ CD4$^+$ T helper 2 (Th2) cells, have previously been associated with ectopic dermatitis and other pruritic skin afflictions[26] and producing pruritogens,

including IL-4, IL-13, IL-31, and Oncostatin M, which act through receptors on the peripheral Trpv1$^+$ sensory nerve endings. Interestingly, depletion of Trpv1$^+$ sensory neurons with Resiniferatoxin (RTX) in guinea pigs showed that the IITR signal acted through a Trpv1-independent pathway[18]. At this point, the precise involvement of CD3$^+$ T cells in sensory neuron stimulation remains elusive.

Although IITR was consistently observed across tick species, the efficiency and characteristics of the response also differed among tick species. *I. scapularis* and *A. americanum* show little to no statistically significant difference in feeding success on tick-naïve and tick-sensitized hosts by 48 hours pta, whereas *R. sanguineus* showed a significant reduction in feeding success on tick-sensitized GPs. Interestingly, *R. sanguineus* nymphs engorged more rapidly on tick-naïve GPs than *I. scapularis* and *A. americanum*, as reflected by their larger scutal index at 48 hours pta. This matters because these data show that, while different ixodid tick species go through the same feeding phases, the timing of these phases differs significantly between tick species. Interestingly, the attachment period for feeding in *I. scapularis* and *A. americanum* nymphs (3–5 days and 3–6 days, respectively) is estimated to be shorter than for *R. sanguineus* nymphs (4–9 days)[3,4]. Notably, despite their more rapid feeding, *R. sanguineus* nymphs were also removed much more rapidly than any other ixodid tick species tested. Possibly, accelerated feeding also accelerates IITR induction. Further studies are needed to determine how feeding rate influences IITR and whether the rapid feeding of *R. sanguineus* contributes to their more efficient removal.

Interestingly, GPs sensitized with *R. sanguineus* did not show the highest level of scratching when challenged with the same species. *A. americanum* caused significantly more scratching than any other ixodid tick species we tested. In general, increased scratching correlated with more efficient tick removal for the various tick species. However, the removal of *A. americanum* nymphs was less efficient than the removal of *R. sanguineus* nymphs. These findings suggest that it is harder for GPs to remove *A. americanum* than *R. sanguineus* or *I. scapularis*, and thus causes more persistent scratching. This could be due to *A. americanum*'s longer hypostome and the deeper anchoring in the host skin[27]. This could mean that more persistent scratching is required to dig out *A. americanum* nymphs from the host skin. Future studies will need to determine whether this association is correct.

The induction of IgG also looks different between *I. scapularis*, *A. americanum*, and *R. sanguineus*. It is of interest that no changes in IgG production were observed to either tick species for the first four weeks pta. This is consistent with the established kinetics of the humoral immune response, in which activated B cells require approximately 2–3 weeks to establish germinal center reactions in draining lymph nodes and undergo class-switch recombination, leading to increased production of IgG antibodies[28]. Despite this, IITR was readily observed in these guinea pigs. It is also of interest that none of the tick species significantly increased circulating IgE production persistently. However, most IgE is bound to immune cells, in particular mast cells and basophils, via FcεRI. Since we were unable to test for bound IgE in the GP model, the question remains whether bound IgE levels did increase. Nevertheless, the apparent independence of IITR from the humoral response contrasts with ATR, where IgG1 and IgE levels were shown to be critical[29,30].

Interestingly, IITR to repeated tick exposure is not universally observed among host species. Other studies have shown that early anti-tick responses, including early tick loss, observed in guinea pigs, do not occur in mice[22,31]. Although these studies had been conducted prior to the description of IITR, their data are consistent with our preliminary observations that standard mouse model strains do not acquire IITR. Whether this is an evolutionary adaptation, as mice are natural hosts to larvae and nymphal stages of many tick species, remains to be elucidated.

Conversely, we show for two distinct guinea pig strains of the species *C. porcellus*, Hartley and Strain 13, that IITR is readily acquired and equally effective. However, as *C. porcellus* is domesticated, it will be important to determine whether wild guinea pig species similarly acquire IITR in response to ticks in their natural habitats. We know from cattle that not all cattle species develop bovine tick resistance, which also relies heavily on grooming as a defense against ticks[32]. With the description of IITR by us, it is of interest to reevaluate bovine tick resistance to assess whether it is IITR or a distinct mechanism. Moreover, our preliminary human data suggest that humans also acquire IITR or at least some form of it. In humans, survey studies showed a correlation between the report of itch at the tick bite site and a significant reduction of Lyme disease incidence in Lyme endemic areas[33].

Overall, our data suggest that IITR is a common response to ixodid ticks in guinea pigs. At this point, we assume that tick salivary antigens are the driver for the inflammatory response that induces IITR. Given that sialomes are highly diverse among tick species[34,35], the occurrence of IITR across species suggests the involvement of shared antigens or a conserved host immune response to ixodid tick bites.

This study proposes itch as a conserved protective neuroimmune mechanism against ixodid ticks. IITR may offer an avenue for the development of a general anti-ixodid tick vaccine. The vaccine would induce sensitization and, with that, IITR during an ixodid tick bite. The itch would promote early detection of the biting ticks and their swift removal prior to the transmission of most tick-borne pathogens.

Methods

Ethics statement

All experimental animal procedures were reviewed and approved by the National Institute of Allergy and Infectious Diseases (NIAID) Animal Care and Use Committee (ACUC). The NIAID DIR Animal Care and Use Program complies with the Guide for the Care and Use of Laboratory Animals and with the NIH Office of Animal Care and Use and Animal Research Advisory Committee guidelines, which are strictly aligned with ARRIVE guidelines. Detailed NIH Animal Research Guidelines can be accessed at https://oma1.od.nih.gov/manualchapters/intramural/3040-2/. Efforts were made in the planning and execution of all animal procedures to comply with the guidelines of the National Center for Replacement, Refinement and Reduction (3Rs) of Animals in Research (https://nc3rs.org.uk/the-3rs).

Guinea pigs

3–6 weeks old outbred female albino Hartley guinea pigs, *Cavia porcellus* (Stain code: 051, https://www.criver.com/products-services/find-model/hartley-guinea-pig?region=3611), were obtained from Charles River Laboratories, Wilmington, MA, USA. 3–6 weeks old, inbred female and male Strain 13 guinea pigs were sourced internally from a breeding colony at the National Institute of Health. Guinea pigs were communally housed in groups of up to three animals under static cage conditions at the NIAID Twinbrook animal facility, Rockville, MD.

Ticks

Pathogen-free nymphal ticks of the species *Amblyomma americanum*, *Dermacentor variabilis*, *Ixodes scapularis*, and *Rhipicephalus sanguineus* were obtained from the Centralized Tick Rearing Facility, Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, OK, USA. Pathogen-free *Ixodes ricinus* nymphal ticks were kindly supplied by the Insectary of the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA. This colony was discontinued, though, at the CDC, which limited experimental use.

Nymphal ticks were commonly housed in fine mesh sealed plastic tubes or cotton wool sealed cut-off 1 ml syringes. For long-term storage, these tubes were placed in a rack in a desiccator cabinet (SP Bel-Art Secador Polystyrene Mini Gas-Purge Desiccator Cabinet; 0.3 cu. ft. from Novatech [SPB-F42075-1002]) containing water-soaked kitchen sponges in small trays (e.g., 1 ml pipette tip-box lids) for moisture. The desiccator cabinet is placed in an incubator or insect chamber set to 20°C, high humidity, and a 16 h light & 8 h dark cycle. All nymphal ticks were housed at the Laboratory for Malaria and Vector Research (LMVR), NIAID, NIH, Rockville, MD, USA.

Experimental guinea pig exposures to nymphal ticks

Experimental tick exposure of guinea pigs was recently described in detail by the authors for all tick species included in this study in Current Protocols[36]. In general, vials of nymphal ticks were removed from the cool housing incubator and double-bagged in Ziplock bags containing pieces of moistened kitchen sponges. The bagged ticks were placed in direct sunlight at room temperature for several days to break diapause, which was essential to stimulate biting. For nymphal tick exposures, GPs were anesthetized by intraperitoneal (i.p.) administration of a Ketamine (50 mg/kg) / Xylazine (5 mg/kg) mixture according to body weight. The head and neck area of GPs were shaven with electric clippers, leaving 1–2 mm stubble to allow tick burrowing into the fur. Using a fine paintbrush, nymphal ticks were transferred from vials onto the trimmed area. Except for *A. americanum*, ticks were dabbed with a fine paintbrush to stimulate burrowing and promote attachment. After 15 min, we brushed hard against the grain of the hair stubble to assess attachment. Unattached ticks were replaced with fresh ones. When GPs showed signs of recovery, they were placed in triple containment cages. ~3 hours post tick attachment (pta), GPs were anesthetized with isoflurane (Forane by Baxter, Deerfield, IL, USA) gas (4% at 2 L/min O_2 flow rate), and attached nymphal ticks were counted. If applicable, attached nymphal tick

numbers were reduced to 15 for all GPs in an experiment. GPs were also checked for nymphal ticks that had bitten off target, which were removed. In some experimental tick exposures, Elizabethan collars (e-collars; Lomir Biomedical Inc., Malone, NY, USA) were placed around the GPs' necks to prevent premature tick removal by the GPs. Tick-exposed animals were also housed individually in triple containment cages to prevent social grooming. At the end of experimental tick exposures, remaining nymphal ticks were removed with fine-tipped curved tweezers, and GPs were returned to communal housing cages.

The quantification of itch-induced tick removal efficacy was assessed as described in Current Protocols[36]. Experimentally, we counted remaining ticks on guinea pigs at 24 hours, 48 hours, and 72 hours pta during three weekly-spaced tick exposures. The tick-count data were collected as time-to-event, aka survival, data and were analyzed by the Cox proportional hazards model. The model included guinea pigs as a random factor to account for repeated measures (1st, 2nd, 3rd exposure) and having multiple ticks per guinea pig (nested data).

Tick health assessment

Assessment of tick feeding ability was recently described in detail by the authors for all tick species included in this study in Current Protocols[36]. Briefly, tick-naïve and two-times tick-sensitized GPs were exposed to ticks as described above. Elizabethan collars were applied at 3 hours pta after tick adjustments to 15 attached nymphal ticks. At 48 hours pta attached nymphal ticks were recovered, taking care not to damage the ticks on removal. Recovered nymphal ticks were immobilized on double-sided tape and imaged under a stereomicroscope (Leica M165 FC) with a Leica DFC 7000 T camera (Leica, IL, USA) using the Leica Application Suite X v.3.7.4.23463. That software was also used for measurements of the alloscutum's length and the scutum's width. These measurements were used to calculate the scutal index

$$Scutal\ index = \frac{Alloscutum\ length}{Scutum\ width}$$

The scutal index is an established indicator of tick feeding success when ticks are not allowed to feed to repletion; the larger the ratio, the greater the engorgement[19]. Further, nymphal ticks were visually assessed for intactness and movement under the stereomicroscope.

Scratch bout quantification

Behavioral video analysis of exposed GPs was recently described in detail by the authors for all tick species included in this study in Current Protocols[36]. Briefly, we employed PhenoTyper® home cages with video tracking technology and MediaRecorder 6.5 (Noldus Information Technology INC, VA, USA) to record guinea pig scratching behavior. We recorded overnight in total darkness with infrared cameras. GPs are often described as crepuscular, but studies have shown that their sleep occurs in short bursts and is evenly distributed over 24 hours[37]. As animals were recorded for 14 h, we provided custom-made absorbent cage liners (Top Layer: 85 gsm brushed/knitted polyester; Middle Layer: 250gsm Polyester

Soaker; Bottom Layer: 85 gsm brushed/knitted polyester bottom with waterproof TPU finished top) that were dyed with an infrared absorbent dye, which gave the liners a black appearance under the infrared camera. Experimentally, we exposed GPs to nymphal ticks as described above and placed them in the PhenoTyper® home cages post recovery. Black curtains were drawn around the cages to reduce stray room light and obstruct guinea pig vision of the room to avoid behavioral freezing. Video analysis was conducted by a semi-supervised deep learning pipeline, whose generation was described elsewhere[36]. Identified scratching events at tick placement sites were visually confirmed post pipeline analysis and quantified for statistical analysis.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA for IgG and IgE detection was described previously[18]. Here, we tick-exposed GPs five to six times over the course of 10 weeks, and serum was collected weekly. For IgG detection, microtiter plates were coated with 5 ug/mL of salivary gland homogenate (SGH) and incubated overnight at 4°C. Subsequently, plates were blocked with PBS/10% FBS for 1 hour at room temperature. Plates were washed six times with PBS/0.05% Tween-20 and incubated with serum diluted 1:500 in PBS/5% FBS in duplicate. After 1 hour at room temperature, plates were washed six times, and wells were filled with the secondary detection antibody (Goat anti-Guinea Pig IgG (H + L) – Invitrogen - diluted to 1:5,000 in PBS/ 5% FBS). The plates were incubated for 1 hour at room temperature. After twelve washes, the 1-Step ABTS substrate solution was added. The optical density (OD) was measured at 405 nm, using a 96-well microplate reader after 30 min.

The ELISA IgE assay was performed following the manufacturer's recommendation (MyBioSource, Inc., San Diego, CA, USA). Briefly, guinea pig sera were diluted 1:16, and spiked-in samples were used as positive control (final concentration 50 ng/mL). After 15 minutes, HRP reaction was stopped, and the OD was measured at 450 nm. IgE sera concentrations were determined using the standard curve supplied with the kit. The interpolated sample concentrations were obtained using a linear regression calculated with GraphPad Prism software.

Disruption of establishment of acquired tick removal

The use of Fingolimod (FTY720) to disrupt acquisition of IITR was previously described elsewhere[18]. Briefly, five mg of FTY720 (Sigma-Aldrich) was suspended in 3 ml of PBS (Lonza. Portsmouth, NH) under sterile conditions by pipetting the suspension up and down several times. The suspension was transferred to a 15 ml Falcon tube and cycled 3 times through 15 min heating in a water bath at 37°C, followed by vortexing for 10 min at 7,000 rpm at room temperature. Tick-naïve GPs were treated with 4 mg/kg of the FTY720 suspension starting 5–7 days prior to the 1st tick exposure. Blood was collected pre-FTY720 treatment, the day before the 1st tick exposure, and after the conclusion of the 1st tick exposure. Lymphocytes were counted in a XN-10™ Hematology Analyzer (Sysmex) according to supplier guidelines. FTY720 treatment was continued until the end of the experiment. Nymphal tick exposures were conducted as described above.

Laser Speckle

The use of laser speckle contrast imaging for the detection of inflammation/cell infiltration at the tick bite site was recently described in detail by the authors for all tick species included in this study in Current Protocols[36]. Briefly, GPs were tick-exposed as described above. 24 hours pta, GPs were anesthetized by isoflurane delivered by a SomnoFlo vaporizer (Kent Scientific, Torrington, CT, USA). To remove hair stubble around the tick bite site, Nair (Church & Dwight Co., Inc., Ewing, NJ, USA) was applied according to the supplier's instructions for 3 to 5 min (longer treatment times were detrimental to tick survival and caused chemical burns). The Nair was thoroughly washed off under running water, taking care not to submerge the GP's muzzle. The following day, GPs were anesthetized individually by i.p. administration of a Ketamine (50 mg/kg) / Xylazine (5 mg/kg) mixture according to body weight. Anesthetized GPs were placed on a PhysioSuite heating pad at 37°C (Kent Scientific, Torrington, CT, USA). Tick bite sites were visualized with a PerCam PSI HR (Perimed, NV, USA) calibrated using a LI723 CalBox PSI HR (Perimed) and the PIMsoft package v1.5 (Perimed). 1 cm^2 skin areas were recorded for 1 min at a time. Circular regions of interest (ROI; 3.47 mm^2) were drawn around each tick (excluding the tick itself, as it masked the signal) and one unbitten area per image for background signal measurements. Measurements were averaged (smoothed) over a time of interest (TOI; 20 s interval). All readings were normalized by subtracting the background value from the measurements around the tick for each respective image, producing Δ perfusion units.

Histology

Skin biopsies (ø 3 mm) of nymphal tick-bitten skin were collected by punch biopsy from CO_2-euthanized GPs. Fresh biopsies were immediately placed in 10% neutral formalin and incubated for 24 hours at 4°C for thorough tissue fixation. Fixed skin biopsies were washed two times in PBS, stored in 70% ethanol, and submitted to Histoserv, Inc. (Histoserv, Inc., MD, USA) for paraffin embedding and sectioning. Representative sections from serial sectioned biopsies were stained with hematoxylin and eosin (H&E) and provided by Histoserv. All paraffin skin sections were cut at 5 µm thickness. Samples were collected from tick-naïve and tick-sensitized GPs. Stained slides were digitized using a Motic Easyscan Pro 24 slide scanner. H&E images were processed in ImageJ for reorientation and cropping.

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tissues sections (5 µm thickness) obtained from guinea pig skin and provided by Histoserv were used to perform immunohistochemical (IHC) staining using the following antibodies: a rat monoclonal CD3 with colon CD3-12 (AbD Serotec, Catalog No: MCA1477, dilution of 1:600) and a rabbit polyclonal Iba1 (Wako, Catalog No: 019-19741, dilution of 1:800). Staining was carried out on the Bond RX (Leica Biosystems) platform according to manufacturer-supplied protocols. Briefly, 5 µm sections were deparaffinized and rehydrated. Heat-induced epitope retrieval (HIER) was performed using Epitope Retrieval Solution 1, pH 6.0, heated to 100°C for 20 minutes. The specimen was then incubated with hydrogen peroxide to quench endogenous peroxidase activity prior to applying the primary antibody. Detection with DAB chromogen was completed using the Bond Polymer Refine Detection kit (Leica Biosystems, Catalog # DS9800). Slides were finally cleared through gradient alcohol and xylene washes prior to mounting and placing coverslips. Sections were examined by a

board-certified veterinary pathologist (DAA). IHC images were processed in ImageJ for reorientation and cropping.

Statistical methodologies

Software packages

Statistical analysis of time-to-event (survival) data, two-way mixed ANOVA, and Bayesian MANOVA were executed in RStudio v.2026.04.1–526 using R v.4.6.0[38,39]. Parametric and non-parametric tests were executed in GraphPad Prism v.11.0.0. All R packages, versions, and associated references is provided in the Supplementary Methods. Moreover, all R packages employed in the data analyses are stated within the supplementary statistics report.

Statement on experimental design

We used exclusively female Hartley guinea pigs in our study, which made it easier to randomly assign guinea pigs to treatment groups. This was permissible as we had confirmed in our previous study that there is no difference compared to males. For Strain 13 guinea pigs, we used equal numbers of males and females. Animals were selected randomly into treatment groups by a researcher, taking care that treatments were mixed in communal holding cages. This avoided litter bias. The researcher who assigned animals to treatment groups did not reveal the assigned treatment until after data analysis. This ensured blinding, where possible. There was no randomization in the repeated tick exposures experiment (Fig. 1). This includes the scratch bout data (Fig. 2G-I) and correlated data (Fig. 2J-L), as these data were collected from the same guinea pigs. The Elizabethan collar experiment (Fig. 2A-C) was also not randomized, as each tick-sensitized guinea pig was tick-exposed once with and once without the collar. Guinea pigs were also not randomized for the ELISA experiment (Fig. 3), as all animals were treated the same across the experiment. Guinea pigs were randomly assigned to treatment groups in the immunosuppression experiment with FTY720.

Sample size estimations were based on data from our previous publication[18]. However, cost and use of life considerations limited the quantity of animals per experiment, underpowering the scratch bout analysis due to high data variability (Fig. 2G-I). In general, experiments were repeated twice for technical robustness. Only the serum collection for the antibody titer assessment by ELISA was done once, while all ELISA samples were run in duplicate for experimental soundness. This limited the number of animals used to the essential minimum to avoid waste of life. This approach was acceptable as we were able to compare new data to findings in our previous publication[18]. This allowed spotting experimental errors very easily. In general, 6 guinea pigs were handled per experimental replicate. Sample size calculation was repeated retrospectively to confirm that a minimum power of 80% was maintained. The scratch bout data did not conform to this after the fact. Sample *N* are stated for all experiments in the figure legends.

Experimental design decisions were made with close consideration of the 3Rs (https://nc3rs.org.uk/who-we-are/3rs). E.g., we used time-to-event data collection to monitor tick

removal. This meant that our experiments were based on 15 ticks per guinea pig (90 ticks per experiment), instead of the six guinea pigs in the experiment. This drastically reduced the required number of guinea pigs by 15-fold. The guinea pig was then integrated into a hierarchical analysis as a layer of potential data variability, while the statistical focus was on the tick.

Statement on statistical analysis

All statistical tests were two-tailed where applicable. Parametric test assumptions were considered in the selection of a parametric or non-parametric equivalent test. Questions of data independence and random sampling were considered and integrated at the experimental design phase. Data normality was assessed by the Shapiro-Wilks test, QQ plots, kurtosis, and skewness. Where small sample sizes were a concern in the normal distribution assessment, we conducted normal distribution analysis on residuals, too, for confirmation. We assessed homogeneity of variance by the Brown-Forsythe test in GraphPad Prism, or by the Levene's test in R. In the case of two-way ANOVA, assumptions of covariance and sphericity were assessed by Box'x M-test and Mauchly's test, respectively. Correlation tests were applied wherever linearity and multicollinearity between variables were a concern. In case of mild assumption violations, robust versions of the tests were applied in R. Wherever the authors had to select the p-value adjustment method manually, the False-Discovery Rate test (FDR) was selected. For comprehensibility, the results section and figure legends contain only information on the final outcomes and limit reporting p-values to < 0.05.

Preliminary analysis of time-to-event data was done by the Peto & Peto modification of the Gehan-Wilcoxon test. The full analysis was done by the Cox proportional hazards model, which supported data clustering by guinea pigs. This was essential as multiple ticks were placed on each guinea pig (at times repeatedly). The test also produced the hazard ratios stated in the results. Where necessary, the reciprocal was calculated for hazard ratios that were expressed as decimals post-calculation:

$$Reciprocal\ HR = \frac{1}{\text{Decimal HR}}$$

.

For example, the HR shown in the Supplementary Table 1C for the 72 hours' time point comparing the 2nd to the 1st tick exposure is 0.0715. The reciprocal is

$$13.99 = \frac{1}{0.0715}$$

.

In general, three versions of the Cox proportional hazards model were executed (clustered, frailty, frailty + time variable hazard) and compared by Akaike Information Criterion (AIC). The selection of the model

version also depended on whether the assumption of a steady proportional hazard was confirmed or not.

In the analysis of discrete data, statisticians generally lean toward the use of parametric regression models, like Poisson and negative binomial regression. However, when sample sizes are small, application of non-parametric tests may be more reliable. This was the case for our scratch bout data. Due to the repeated-measures structure (1st, 2nd, 3rd tick exposure), we analyzed the data using Friedman's test followed by the Dunn's test for pairwise comparison. In general, we report the pairwise comparison outcomes in the text and figure legends.

We applied a Bayesian MANOVA-style model to the output variables for tick removal and scratch bouts in a single analysis. The predictor variable was tick exposure round (1st, 2nd, 3rd). This was of interest as these two observations were derived from the same guinea pigs in the same experiment. We chose the Bayesian MANOVA-style model because the structure of our data did not naturally fit conventional MANOVA. Similarly, the Bayesian MANOVA-style model tested whether our predictor changed the combined response of scratching and tick removal. Bayesian statistics do not produce traditional p-values, but posterior probability, which are also measured in fractions between 0 and 1. They serve a similar purpose to p-values. 0.975 is equivalent to the traditional 0.05 threshold for p-values. Values ≥ 0.975 represent strong evidence for a true effect. We followed the Bayesian MANOVA-style model with a Count-scale follow-up model. The Count-scale follow-up model estimated effects in the real biological scales, which made interpretation of the pairwise comparisons biologically more relevant.

Declarations

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Additional Information

Note

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Material availability

This study does not generate new unique reagents. All data are available in the manuscript or the supplementary materials. Materials described in this manuscript are available from J.G.V. (jvalenzuela@niaid.nih.gov) on request.

Data and code availability

All data and scripts for the statistical analysis are available through GitHub (https://github.com/joedoehl/IITR-in-other-tick-species.git, DOI: 10.5281/zenodo.23675990) or upon request from the corresponding author(s).

Author contributions

Conceptualization: J.G.V., J.S.P.D., D.E.S.; Funding acquisition: J.G.V.; Investigation: J.S.P.D., L.R., R.F., M.M., C.P., T.D.S., E.I., D.A.A., D.E.S., J.G.V.; Methodology: J.S.P.D., L.R., R.F., M.M., C.P., T.D.S., E.I., D.A.A., D.E.S., J.G.V.; Project administration: J.G.V., J.S.P.D., Supervision: J.G.V., J.S.P.D., T.D.S., D.A.A.; Visualization: J.S.P.D., T.D.S, D.A.A., J.G.V., R.F., L.R., M.M.; Writing – original draft: J.S.P.D., J.G.V., D.E.S., T.D.S.; Writing – review & editing: J.S.P.D., L.R., R.F., M.M., C.P., T.D.S., E.I., D.A.A., F.O., S.K., D.E.S., J.G.V.

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Figures

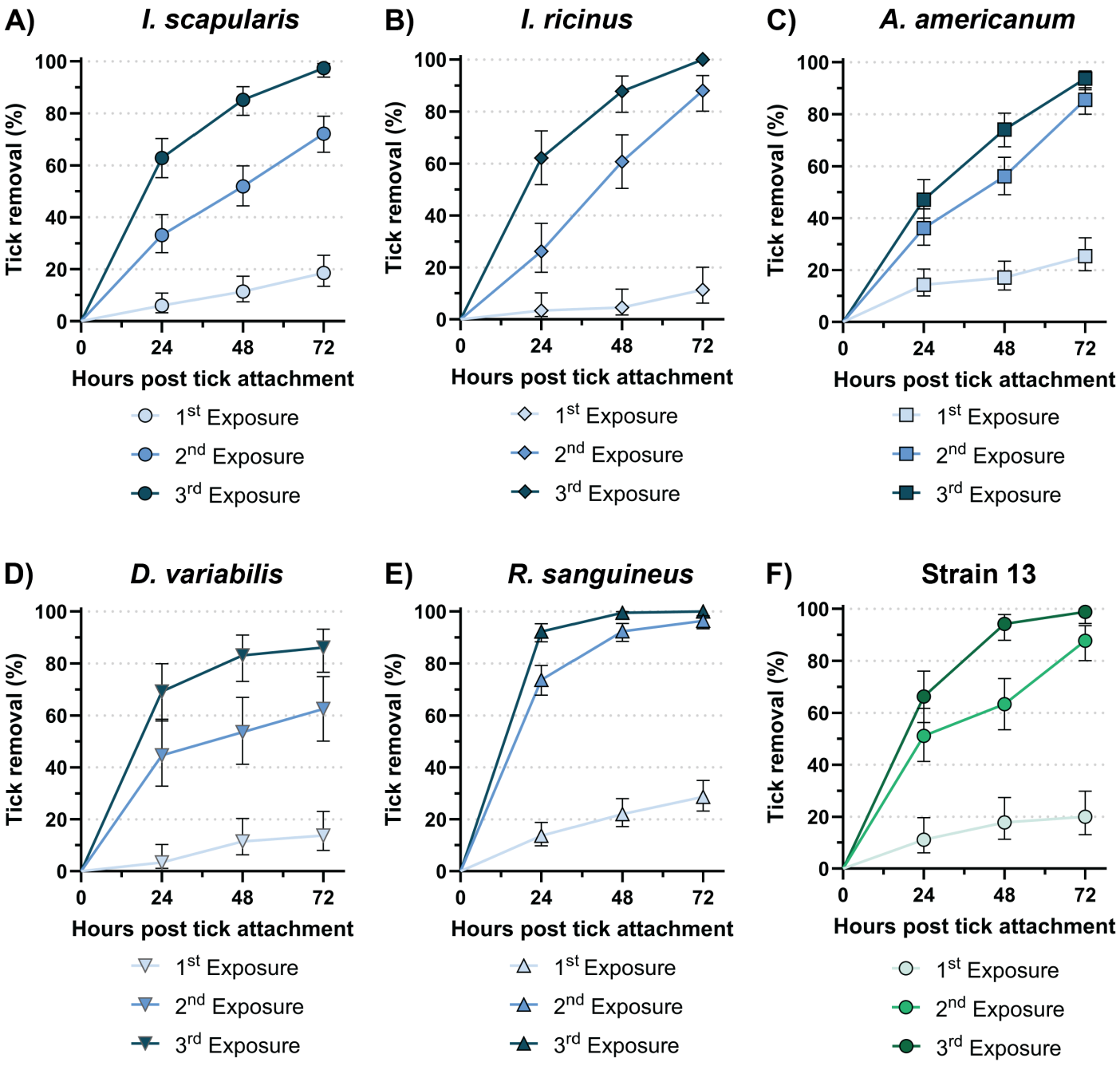


Figure 1

Repeated exposure of guinea pigs to various tick species

(A-F) Smoothed, inverted Kaplan–Meyer plots showing probability of tick removal (%) for three weekly spaced consecutive tick exposures. Animals are tick-naïve for the 1st exposure and tick-sensitized for subsequent exposures. Hartley guinea pigs exposed 3-times to (A) *I. scapularis* (*N*=11 GPs; *N*=483 ticks), (B) *I. ricinus* (*N*=6 GPs; *N*=254 ticks), (C) *A. americanum* (*N*=12 GPs; *N*=535 ticks), (D) *D. variabilis* nymphs (*N*=6 GPs; *N*=209 ticks), (E) *R. sanguineus* (*N*=16 GPs; *N*=702 ticks), and (F) Strain 13 guinea pigs exposed 3-times to *I. scapularis* nymphs (*N*=6 GPs; *N*=266 ticks). Analyzed by Cox proportional hazards model (see **Supp. Table 1**)

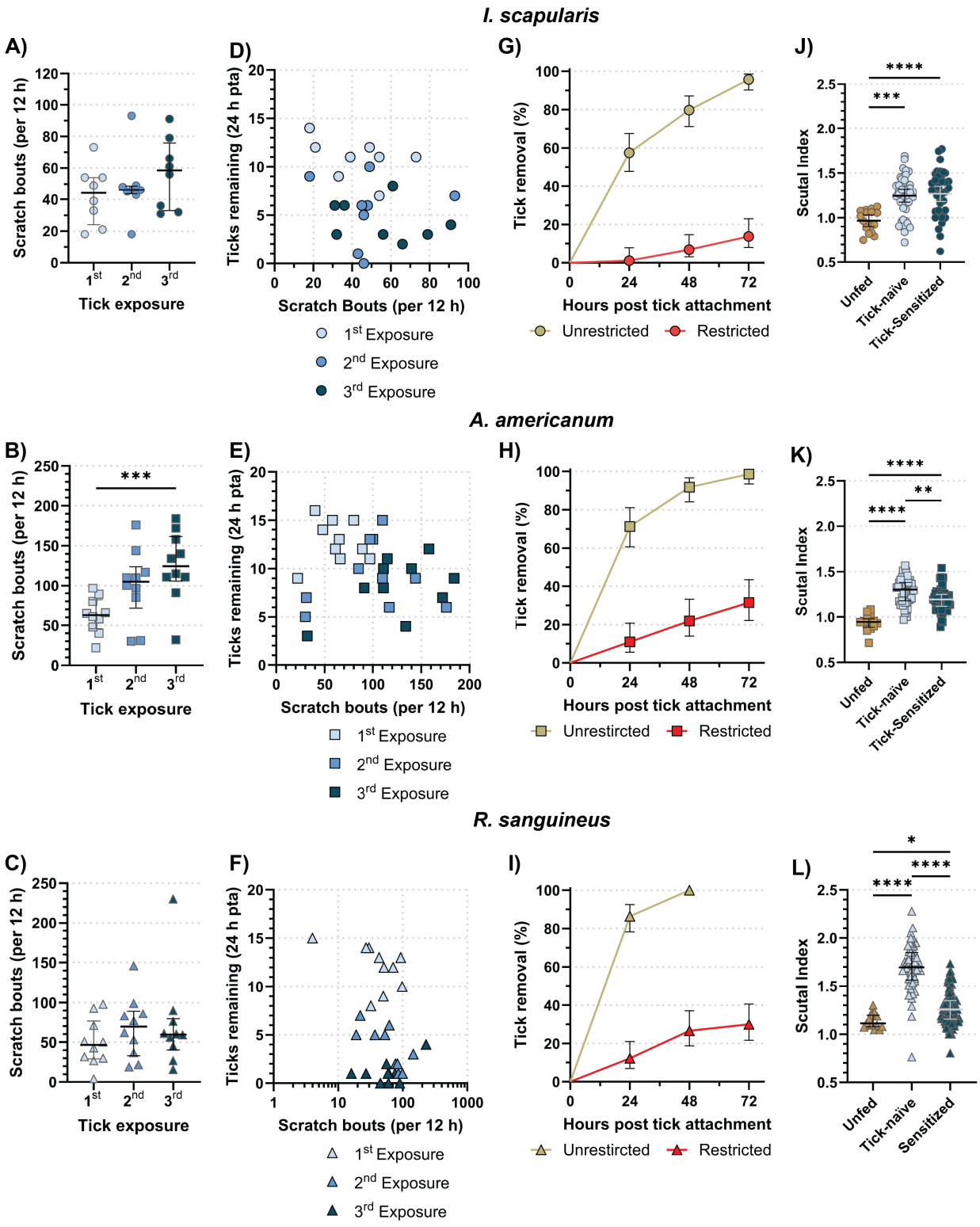


Figure 2

In-depth assessment of removal of *I. scapularis*, *A. americanum*, and *R. sanguineus*

(A-C) Scratch bouts/12 hours post tick placement in GPs (*N*=8, 10 & 10, respectively) during their 1st, 2nd, and 3rd exposure to 15 (A) *I. scapularis*, (B) *A. americanum*, (C) or *R. sanguineus* nymphs, respectively. Friedman test with Dunn's test: 1st vs. 2nd exposure: (A) p > 0.9999, (B) p = 0.076, (C) p = 0.7907; 1st vs

3rd exposure: (A) p = 0.818, (B) p = 0.076, (C) p > 0.9999; 2nd vs 3rd exposure: (A) p > 0.9999, (B) p = 0.3526, (C) p > 0.9999. Median ± IQR shown. (D-F) Scatter plot of scratch bouts/12 hours against ticks remaining at 24 hours pta in GPs (*N*=8, 10 & 10, respectively) during their 1st, 2nd, and 3rd exposure to 15 (D) *I. scapularis*, (E) *A. americanum*, (F) or *R. sanguineus* nymphs, respectively. Refer to Supp. Table 4 for information on the pairwise Count-scale follow-up model. “” > 0.05, * ≤ 0.05, ** < 0.01, *** < 0.001, **** < 0.0001. (G-I) Smoothed, inverted Kaplan–Meyer plots showing probability of tick removal (%) in restricted and unrestricted GPs (*N* = 6/group) in a 5th and 6th exposure to (G) *I. scapularis* (*N*=87 & 94 ticks, respectively), (H) *A. americanum* (*N*=73 & 73 ticks, respectively), (I) *R. sanguineus* (*N*=88 & 90 ticks, respectively). Analyzed by Cox proportional hazards model (see **Supplementary Table 3**). 95% CI is shown. (J-L) Scatter plot showing scutal index values of unfed nymphal ticks and nymphal ticks fed on tick-naïve or tick-sensitized GPs: (J) *I. scapularis* (N = 16, 42, and 38 ticks, respectively), (K) *A. americanum* (N = 15, 47, and 55 ticks, respectively), and (L) *R. sanguineus* (N = 15, 48, and 122 ticks, respectively). Kruskal–Wallis test followed by Dunn’s multiple-comparisons test; adjusted p values: unfed vs. tick-naïve: (J) p = 0.0003, (K) p < 0.0001, (L) p < 0.0001; unfed vs. tick-sensitized: (J) p < 0.0001, (K) p < 0.0001, (L) p = 0.0449; tick-naïve vs. tick-sensitized: (J) p > 0.9999, (K) p = 0.0062, (L) p < 0.0001. Confidence intervals (CIs) for the median scutal index were: (J) tick-sensitized, 1.16–1.40; (K) tick-naïve, 1.22–1.35, and tick-sensitized, 1.15–1.22; (L) tick-naïve, 1.64–1.78, and tick-sensitized, 1.19–1.28. Medians and interquartile ranges (IQRs) are shown.

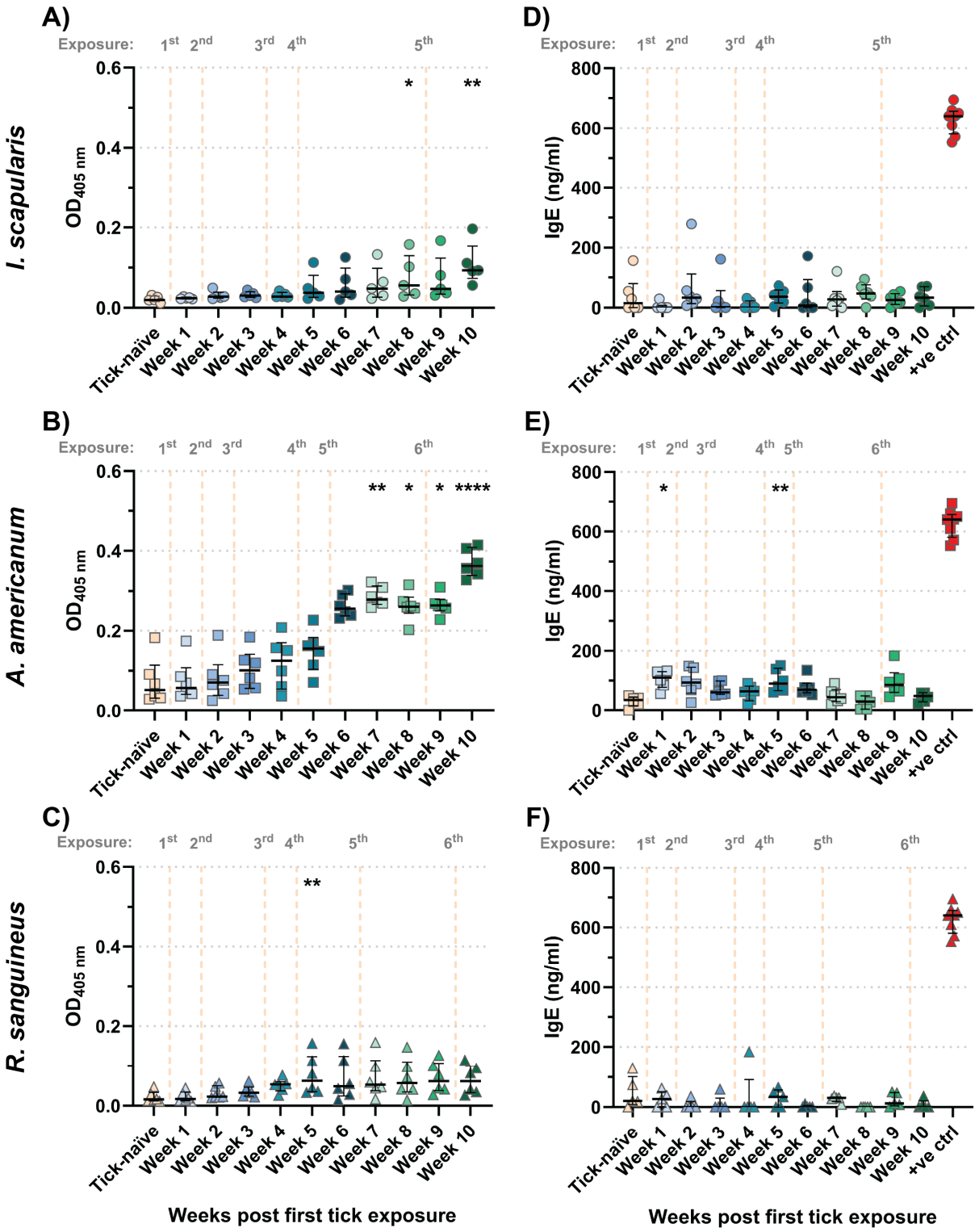


Figure 3

Weekly IgG and IgE serum levels post tick sensitization

ELISA of anti-tick saliva (A-C) IgG and (D-F) total IgE titers in GP (*N*=6 each) sera pre-exposure (tick-naïve) and weekly for 10 weeks post (A,D) *I. scapularis*, (B,E) *A. americanum* or (C,F) *R. sanguineus* nymphal tick exposure. Five to six tick exposure occurred over 10 weeks. Analyzed by Friedman test with

Dunn's test (all samples matched against Tick-naïve): IgG (A) Week 8: p = 0.0119, Week 10: p = 0.0085, (B) Week 7: p = 0.0018, Week 8: p = 0.0308, Week 9: p = 0.0232, Week 10: p < 0.0001, (C) Week 5: p = 0.0069; IgE (E) Week 1: p = 0.0232, Week 5: p = 0.0069. Median ± IQR shown. "" > 0.05, * ≤ 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

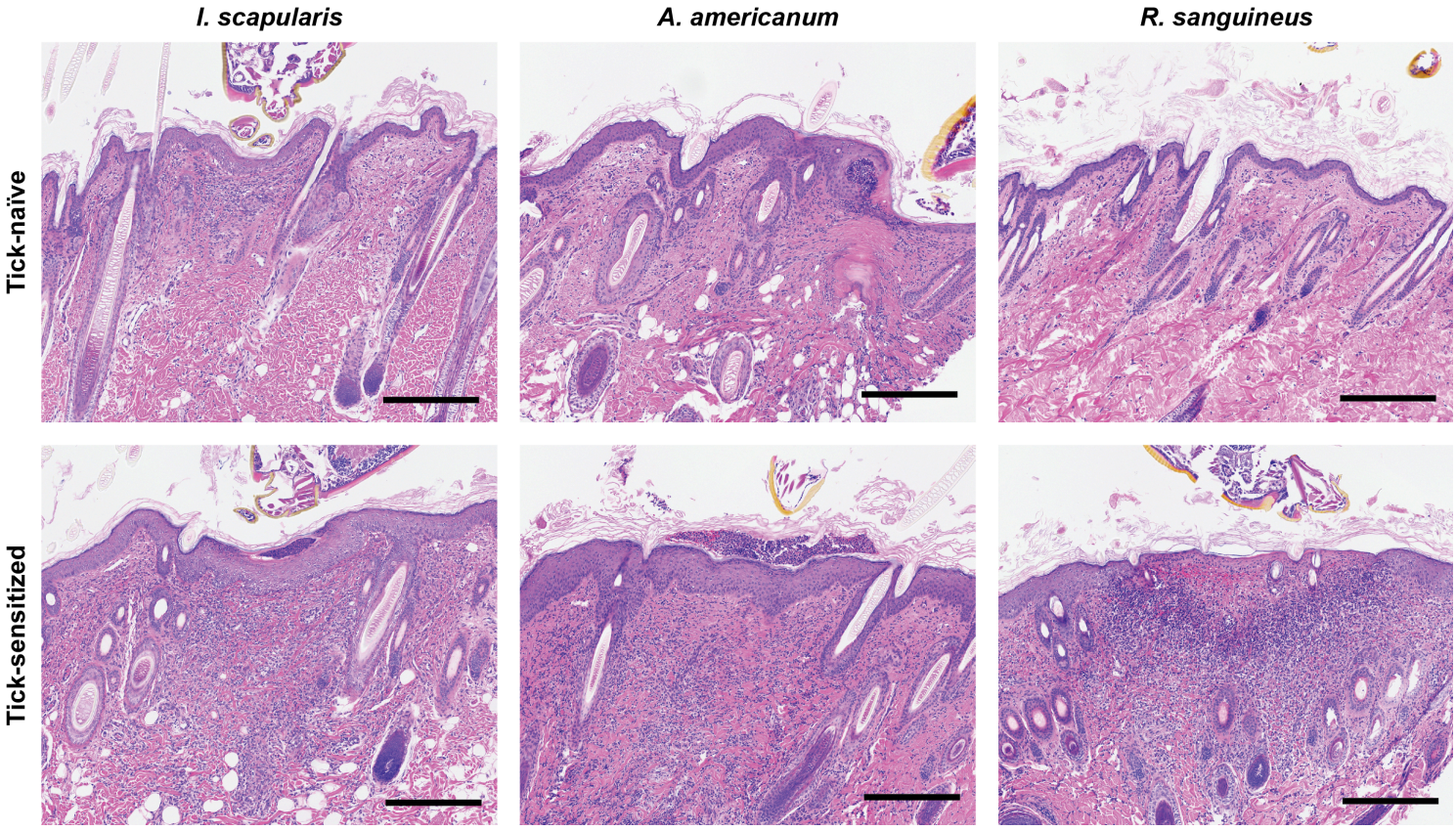


Figure 4

Hematoxylin and eosin-stained guinea pig skin sections of tick bite sites

Representative H&E skin cross-sections (>|5 µm|<) of *I. scapularis*, *A. americanum*and *R. sanguineus* nymphal tick bite sites at 48 hours pta in first-time exposed (tick-naïve) and three times weekly exposed (tick-sensitized) GPs. Black scale bar: 400 µm

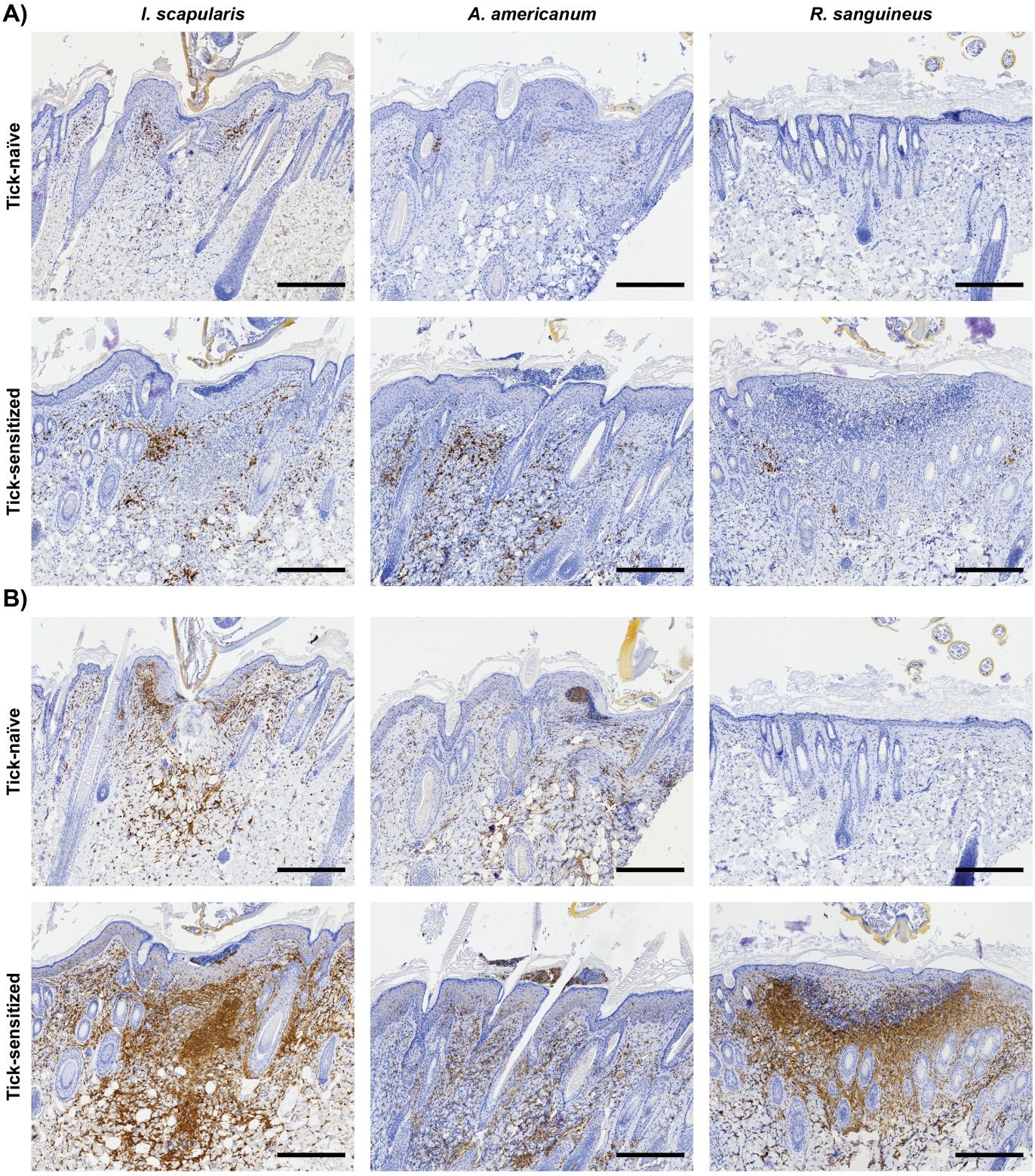


Figure 5

Immunohistochemical staining of guinea pig skin section of tick bite sites

Representative immunohistochemistry skin cross-sections (>|5 µm|<) of *I. scapularis*, *A. americanum* and *R. sanguineus* nymphal tick bite sites at 48 hours pta in first-time exposed (tick-naïve) and three

times weekly exposed (tick-sensitized) GPs. (A) Stained with anti-CD3 antibody (T cells). (B) Stained with an anti-Iba1 antibody (macrophages). Scale bare: 400 µm.

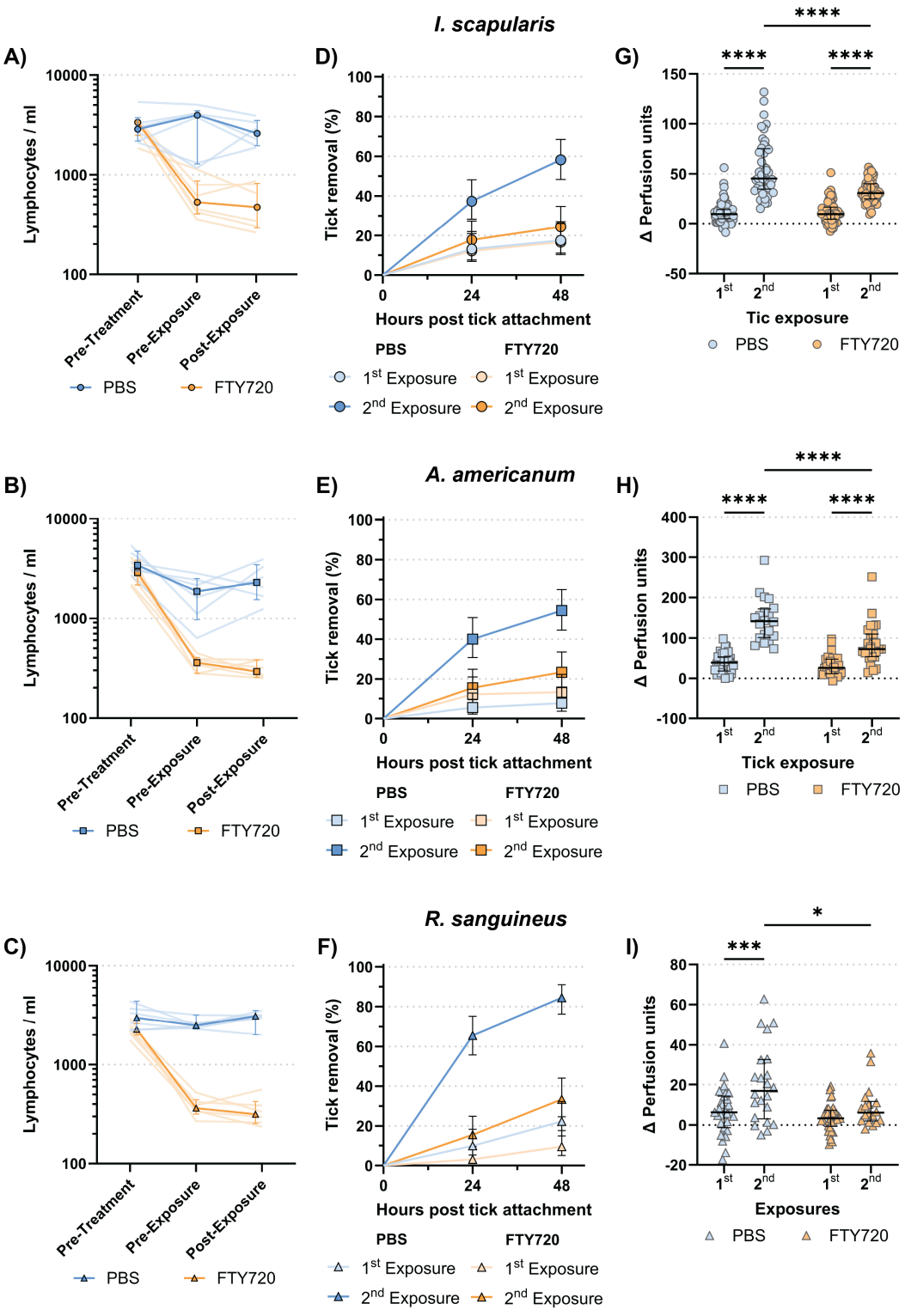


Figure 6

Loss of IITR acquisition in immunosuppressed, FTY720-treated guinea pigs

(A-C) Paired data of normalized lymphocyte counts per ml blood collect from guinea pigs (*N*=6/group) treated with either PBS or FTY720 and exposed to (A) *I. scapularis*, (B) *A. americanum*, or (C) *R. sanguineus*. Blood collections occurred before treatment (Pre-treatment; baseline), before 1st tick exposure (Pre-Exposure), and after the 1st tick exposure (Post-Exposure). Analyzed by Robust two-way mixed ANOVA with post hoc pairwise comparison: (**see Supp. Table 5**). Median + IQR shown. (D-F) Smoothed, inverted Kaplan–Meyer plots showing probability of tick removal (%) in PBS and FTY720-treated GPs (*N* = 6/group) exposed for the 1st or 2nd time to (D) *I. scapularis* (*N*=90 ticks/group), (E) *A. americanum* (*N*=90 ticks/group), or (F) *R. sanguineus* (*N*=90 ticks/group). Analyzed by Cox proportional hazards model: (see **Supp. Table 6**). 95% CI is shown. (G-I) Scatter plot: Delta Perfusion units (tick-bite site signal - image background signal) of (G) *I. scapularis* (*N*=90 ticks/group), (H) *A. americanum* (*N*=90 ticks/group), or (I) *R. sanguineus* (*N*=90 ticks/group) nymphs attached to PBS- or FTY720-treated GPs at 48 hours post tick placement. Analyzed by Robust two-way mixed ANOVA with post hoc pairwise comparison: (G) PBS (1st versus 2nd tick exposure): $p < 0.0001$, FTY720 (1st versus 2nd tick exposure): $p < 0.0001$, 2nd tick exposure (FTY720 versus PBS): $p < 0.0001$; (H) PBS (1st versus 2nd tick exposure): $p < 0.0001$, FTY720 (1st versus 2nd tick exposure): $p < 0.0001$, 2nd tick exposure (FTY720 versus PBS): $p < 0.0001$; (I) PBS (1st versus 2nd tick exposure): $p = 0.0007$, 2nd tick exposure (FTY720 versus PBS): $p = 0.229$. Median ± IQR shown. “” > 0.05, * ≤ 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalMaterialRogerioetal.pdf