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Susceptibility to Ticks and Lyme Disease Spirochetes Is Not Affected in Mice Coinfected with Nematodes

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Small rodents serve as reservoir hosts for tick-borne pathogens, such as the spirochetes causing Lyme disease. Whether natural coinfections with other macroparasites alter the success of tick feeding, antitick immunity, and the host's reservoir competence for tick-borne pathogens remains to be determined. In a parasitological survey of wild mice in Berlin, Germany, approximately 40% of *Ixodes ricinus*-infested animals simultaneously harbored a nematode of the genus *Heligmosomoides*. We therefore aimed to analyze the immunological impact of the nematode/tick coinfection as well as its effect on the tick-borne pathogen *Borrelia afzelii*. Hosts experimentally coinfected with *Heligmosomoides polygyrus* and larval/nymphal *I. ricinus* ticks developed substantially stronger systemic type 2 T helper cell (Th2) responses, on the basis of the levels of GATA-3 and interleukin-13 expression, than mice infected with a single pathogen. During repeated larval infestations, however, anti-tick Th2 reactivity and an observed partial immunity to tick feeding were unaffected by concurrent nematode infections. Importantly, the strong systemic Th2 immune response in coinfected mice did not affect susceptibility to tick-borne *B. afzelii*. An observed trend for decreased local and systemic Th1 reactivity against *B. afzelii* in coinfected mice did not result in a higher spirochete burden, nor did it facilitate bacterial dissemination or induce signs of immunopathology. Hence, this study indicates that strong systemic Th2 responses in nematode/tick-coinfected house mice do not affect the success of tick feeding and the control of the causative agent of Lyme disease.

Ticks are the most important arthropod vectors of pathogens in temperate regions of the Northern Hemisphere. In particular, ticks of the genus *Ixodes* transmit several pathogens affecting the health of humans and livestock, such as *Borrelia* spp., which cause Lyme disease (LD) or relapsing fever, and *Rickettsia* spp. of the spotted fever group, which cause diseases such as aneruptive fever and tick-borne lymphadenopathy (1). Small rodents serve as reservoir hosts for many tick-borne pathogens, and the larval stage of *Ixodes ricinus*, the main vector species in Western and Central Europe, frequently feeds on mice and voles (2). The larval tick stage efficiently acquires the pathogen when feeding on infected rodents (3). After they molt, nymphal ticks constitute the major source for infections in people (4). Two genospecies of LD spirochetes account for the majority of infections in Eurasia, *Borrelia garinii* and *Borrelia afzelii*, the latter of which mainly and efficiently perpetuates in rodents (5–8). Mice and voles in urban and rural areas are frequently infected by intestinal helminths, such as the trichostrongyloid *Heligmosomoides polygyrus* (9–11), raising the question of whether such natural coinfections may affect the feeding success of ticks and the transmission of tick-borne pathogens.

Ticks initiate type 2 T helper cell (Th2) responses during their blood meal (12). Although feeding of *I. ricinus* ticks results in protective immunity in guinea pigs and rabbits, animals nonnative to this tick's distribution, the immune responses of most autochthonous wild mouse species and laboratory mouse strains are insufficient to protect them from repeated infestations with ticks (12–16). On the other hand, feeding *Ixodes* ticks suppress Th1 responses against tick-borne LD spirochetes in mice (17), and the suppression of Th2 cytokines or the reconstitution of tumor ne-

crosis factor alpha, interleukin-2 (IL-2), and gamma interferon (IFN-γ) after tick attachment reduces infection rates in disease-susceptible laboratory mouse strains (18, 19). Infections with the enteric nematode *H. polygyrus* elicit strong local and systemic Th2 and regulatory responses (20, 21) and thereby affect the Th1-dependent control of concomitant bacterial and protozoan infections (22–28). It may be that the concurrent presence of enteric nematodes synergistically enhances Th2 and associated innate effector cell responses toward repeated tick infestations and as a result affects tick feeding success and the transmission of tick-borne pathogens. The expected strong general skewing of immune responses toward a Th2 response in mice simultaneously harboring the two macroparasites *H. polygyrus* and *I. ricinus* may restrain

the immune control of tick-borne pathogens and subsequently increase the reservoir competence of rodents.

To investigate how frequently mice simultaneously harbor enteric nematodes and ticks in nature, we captured wild rodents in Berlin, Germany, and determined the frequency of infection with *Heligmosomoides* spp. and infestation with *I. ricinus*. Using a defined experimental laboratory system, we then assessed parameters of antitick immunity and the competence of the mice as a reservoir for LD spirochetes using inbred house mice. To examine whether the expected strong Th2 immune responses associated with intestinal nematode infections interfere with, first, the experimental host's development of antitick immunity (measured by means of determination of tick-specific cytokine responses and the success of repeated tick feeding) and, second, the control of tick-borne pathogens, we established an experimental model of coinfection in inbred laboratory mice, compared susceptibility to tick feeding and to tick-borne infection with LD spirochetes, and evaluated local and systemic immune responses.

Here we show that (i) *H. polygyrus* and *I. ricinus* frequently parasitize wild *Apodemus* mice in Berlin; (ii) local anti-*I. ricinus* immune responses in laboratory house mice are not altered by a concurrent nematode infection, despite the induction of extraordinarily high systemic Th2 responses by nematode/tick coinfections; (iii) the nematode infection does not alter the feeding success of pathogen-free tick larvae and spirochete-infected nymphs or the development of partial immunity toward repeated tick infestations; and (iv) the nematode coinfection does not affect the tick-borne transmission, replication, and dissemination of *B. afzelii* spirochetes in house mice.

MATERIALS AND METHODS

Wild rodent trapping. Wild rodents were trapped at two urban study sites (Moabit, Steglitz) and two periurban study sites (Gatow, Tegel) in Berlin, Germany, in November 2010 and from April to November 2011 (license number G0210/10, Landesamt für Gesundheit und Soziales, Berlin, Germany). The sites, trapping method, and type of euthanasia used were described by J. Krücken, J. Blümke, D. Maaz, J. Demeler, S. Ramünke, D. Antolová, R. Schaper, and G. von Samson-Himmelstjerna (submitted for publication). After the animals were transferred to the laboratory, the fur was screened for ticks under a stereomicroscope. Subsequently, a complete necropsy was performed, the gastrointestinal tract was thoroughly screened for *Heligmosomoides* spp., and any *Heligmosomoides* organisms detected were stored in 80% ethanol. The remaining carcass was placed on metal grids over water in screw-top jars to allow detachment of the remaining ticks. The water and the rodent body were examined during the following week. Ticks were stored in 70% ethanol. The parasite species were determined microscopically according to the morphological literature for nematodes (29) and ticks (30–32).

Laboratory mice, parasites, and *Borrelia*. Six- to 8-week-old specific-pathogen-free (SPF) female C57BL/6JRj, BALB/cJRj, and C3H/HeNRj mice (Janvier, France) were used for the experiments. The trichostrongyloid nematode *H. polygyrus* was maintained by serial passage in C57BL/6 mice. Specific-pathogen-free larval *I. ricinus* ticks were obtained from batches of eggs from two adult females after they had fed on laboratory beagle dogs (license number H0078/10, Landesamt für Gesundheit und Soziales, Berlin, Germany). The absence of tick-borne pathogens in the tick larvae used for experimentation was surveyed by published PCRs (33, 34) for the detection of a 153-bp fragment of the *hbb* gene of *Borrelia burgdorferi sensu lato*, a 203-bp fragment of the *gltA* gene of spotted fever *Rickettsia* spp., a 602- to 639-bp fragment of the 18S rRNA gene of piroplasmida (33), and a 257-bp fragment of the 16S rRNA gene of the *Anaplasmataceae* (34) in DNA isolated (33) from subsets of 20 larvae randomly chosen from each tick batch. *Borrelia afzelii*-infected *I. ricinus*

nymphs were derived from larvae that had engorged on experimentally infected mice (license number 23-2347-A-24-1-2010, Landesamt für Umwelt, Gesundheit, und Verbraucherschutz, Potsdam, Germany).

Animal experimentation. Mice were infected with 250 *H. polygyrus* L3 larvae via oral gavage. In an initial experiment, mice of three strains (C57BL/6, BALB/c, and C3H) were infested with *I. ricinus* larvae on day 15 after *H. polygyrus* infection and dissected 20 days later. To survey the feeding success of larval ticks and the antilarval tick immune responses, C57BL/6 mice were infected with *H. polygyrus* and subsequently repeatedly infested with 50 specific-pathogen-free *I. ricinus* larvae by biweekly tick infestations on days 4, 18, and 34 postinfection (p.i.) in order to cover the early phase of local Th2 reactivity (at approximately day 6 p.i.), the phase of maximal Th2 reactivity (at about day 21 p.i.), and the chronic phase of infection with a declining magnitude of the nematode-induced Th2 reaction (at approximately day 35 p.i.). Mice exposed either to nematodes or to ticks and naive mice served as controls. To investigate the effect of *H. polygyrus* on the control of a tick-borne pathogen, nematode-infected and -free groups were infested with 4 to 6 *B. afzelii*-infected *I. ricinus* nymphs on day 16 after *H. polygyrus* infection in order to challenge the mice with *B. afzelii* at the peak of nematode-induced Th2 reactivity. Age-matched naive mice served as controls. During the period of tick infestation, all mice, including the naive controls, wore a collar to prevent removal of the ticks placed in the head region. Mice were housed individually at 80% humidity in filter-topped cages in ventilated cabinets. To optimize the recovery of detached ticks, the mice were kept on metal grids over water, and food was restricted to 3 g per day during tick feeding. Mice received water *ad libitum*. In several experiments, mice were anesthetized with isoflurane once a week for retroorbital blood collection with hematocrit capillary tubes. To assess the feeding success of the ticks, *I. ricinus* larvae or nymphs detaching from hosts were collected from the water and counted twice a day, pooled for every mouse in a screen-capped glass vial, and transferred to a desiccator with supersaturated $MgSO_4$ solution. The feeding duration was calculated as the mean recovery time of engorged ticks. Seven days after all ticks had detached, the engorgement weight of the pooled ticks was determined using a microscale, and the mean weight was calculated. The molting rates of fed larvae from the 1st and 2nd infestations or of fed nymphs were determined 3 months after infestation. All animal experiments were approved by and conducted in accordance with the guidelines of the appropriate committee (Landesamt für Gesundheit und Soziales, Berlin, Germany) under license number G0282/12.

Preparation of nematode, tick, and spirochete Ags. *H. polygyrus* antigen (Ag) was prepared as described previously (28). Antigen was acquired from specific-pathogen-free feeding *I. ricinus* larvae 48 h after attachment to C57BL/6 mice at a time of intensive saliva production. The ticks were removed from the host, washed in sterile phosphate-buffered saline (PBS), homogenized, and sonicated (3 times for 10 s each time, 60 W) in PBS on ice, and the sonicate was centrifuged at $20,000 \times g$ for 10 min at 4°C. The supernatant was sterile filtered through 0.22-μm-pore-size filters (Millipore, USA). To acquire *B. afzelii* antigen, spirochetes cultured in Barbour-Stoenner-Kelly H medium (Sigma-Aldrich, Germany) supplemented with 6% rabbit serum were washed three times by centrifugation at $10,000 \times g$ and 4°C for 30 min each time in PBS containing 5 mM $MgCl_2$ and sonicated on ice (8 times for 15 s each time), and the sonicate was centrifuged at $10,000 \times g$ at 4°C for 30 min. The protein contents of the supernatants were determined by use of a bicinchoninic acid protein assay kit (Pierce, USA). The antigens were stored at −80°C until use.

Cell culture. Splenocytes and cells were isolated from the gut-draining and head-skin-draining lymph nodes of euthanized mice by passing the organs through a 70-μm-mesh-size cell strainer (BD, USA). The cells were cultured as triplicates of 3.5×10^5 to 5×10^5 cells in 200 μl RPMI 1640 medium (PanBiotech, Germany) containing 10% fetal calf serum, 20 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cultures were stimulated with 40 μg/ml worm or tick Ag, 30 μg/ml *B. afzelii* Ag (all for 72 h), or 1 μg/ml anti-CD3 and anti-CD28 (BD, USA) for 48 h

at 37°C in a 5% CO_2 atmosphere. The supernatants were stored at −20°C for cytokine detection.

Immunohistology. Formalin-fixed tissue samples were dehydrated and embedded in paraffin. Tibiotarsal joints were decalcified before they were embedded in paraffin. The tissues in the paraffin sections (1 to 2 μm) were dewaxed, hydrated, and histochemically stained with hematoxylin and eosin (Merck) to obtain an overview, toluidine blue to observe mast cells, and a modified Sirius red staining protocol to observe eosinophil granulocytes (35). Sections were stained with toluidine blue (Sigma) for 10 min, washed with distilled water, and dehydrated by dipping them in 1% acetic acid and 100% ethanol. Sections were cleared in xylol, and coverslips were mounted with Corbit balsam (Hecht). For staining of eosinophils, sections were stained for 1 min with hematoxylin (Merck), washed with tap water, and dehydrated with 100% ethanol before staining with direct red 80 dye (Sigma) for 2 h. Sections were washed with tap water, dehydrated with 100% ethanol, and cleared in xylol before coverslips were mounted with Corbit balsam. Images were acquired using a fluorescence microscope (AxioImager Z1) equipped with a charge-coupled-device camera (AxioCam MRm) and processed with Axiovision software (Carl Zeiss AG, Germany).

ELISA. Culture supernatants were screened by enzyme-linked immunosorbent assay (ELISA) for IFN-γ, IL-13 (Ready-Set-Go! ELISA; eBioscience, USA), and IL-10 (BD, USA) according to the manufacturer's instructions.

Flow cytometry. Blood samples were placed in PBS with 0.2% bovine serum albumin (BSA) and 5 mM EDTA, and cells were stained with anti-CD4–fluorescein isothiocyanate (clone RM4-5; eBioscience, USA) and fixable viability dye eFluor 780 (eBioscience, USA) for dead cell exclusion. Red blood cells were lysed in fluorescence-activated cell sorting (FACS) lysing solution (BD, USA), before leukocytes were stained intracellularly with anti-Foxp3–eFluor 450 (clone FJK-16S; eBioscience, USA), anti-GATA-3–eFluor 660 (clone TWAJ; eBioscience, USA), and anti-T-bet–phycoerythrin (PE) (clone eBio4B10; eBioscience, USA) using a fixation/permeabilization buffer kit (eBioscience, USA). Cells from the spleen and lymph nodes were labeled with anti-CD4–peridinin chlorophyll protein–eFluor 710 (clone GK1.5; eBioscience, USA) and fixable viability dye eFluor 780 (eBioscience, USA) in PBS with 0.2% BSA. Transcription factors were stained as described above for the white blood cells. For the intracellular detection of cytokines, cells were incubated with 1 μg/ml phorbol myristate acetate (PMA) and ionomycin for 30 min and 5 μg/ml brefeldin A (Sigma, Germany) for another 2.5 h. Subsequently, they were stained with anti-IFN-γ–eFluor 450 (clone XMG1.2; eBioscience, USA), anti-IL-13–Alexa Fluor 647 (clone eBio13A; eBioscience, USA), anti-IL-10–PE (clone JES5-16E3; eBioscience, USA), and anti-IL-17–Alexa Fluor 488 (clone TC11-18H10; BD, USA). Cells were acquired using LSRII and Canto II flow cytometers (BD, USA) and analyzed using FlowJo (version 8.8.7) software (TreeStar, USA).

Spirochete quantification. For quantification of spirochetes in mouse organs in relation to tissue weight, a touchdown quantitative PCR (qPCR) assay for absolute quantification was developed, and λ phage genome copies were used as an external standard. For DNA isolation, 80 ± 1 mg of head skin and 70 ± 1 mg of heart were sampled with sterile scalpel blades, and the skinned left tibiotarsal joint (weight range, 32.6 to 39.1 mg) was obtained using clean scissors. Prior to DNA isolation with a FastDNA Spin kit with Lysing Matrix A (MP Biomedicals, France), 10^4 copies of the 48,502-bp λ phage genome (Thermo Fisher Scientific, USA) per mg head skin or heart and 10^5 copies per tibiotarsal joint were added as an external standard for tissue weight. To calculate copy numbers, the DNA concentration (0.3 μg/μl, measured spectrophotometrically) and the molecular weight of the genome were used. Samples were homogenized with a Fast-Prep 24 kit (MP Biomedicals, France) 4 times for 30 s each time at 6.0 m/s and cooled on ice between homogenization steps. Variations in the amount of DNA in the samples used in the qPCR have a major impact on the accuracy of absolute quantification, since qPCR efficiency is decreased with higher DNA concentrations. As common spectrophotometric DNA

quantification is strongly impacted by contamination with salts and proteins, a more precise fluoroscopic DNA quantification assay for fluorescence measurement was developed using LCGreen Plus 10× dye (BioChem, USA), exclusive binding on double-stranded DNA (dsDNA), and a LightCycler 480 II system (Roche Molecular Diagnostics, USA). Accurate DNA quantification over the range of 1 to 100 ng was obtained. DNA samples, standards (20, 40, 60, 80, and 100 ng λ phage DNA), and water controls were prepared in duplicate in a 10-μl assay volume for each well in 96-well plates. After vigorous vortexing of the sealed plates, samples were centrifuged and fluorescence was measured by the LightCycler 480 II system after 2 min of incubation at 25°C during a short heating from 25 to 26°C with 100 acquisitions per degree Celsius. For extrapolation of the dsDNA quantity in the samples, a calibration curve was calculated from the fluorescence values of the standards using a 4-parameter logistic regression model. Two real-time PCR assays targeting a 153-bp fragment of the *hbb* gene of LD spirochetes and a 158-bp fragment of the λ phage genome were conducted with the LightCycler 480 II system using LightCycler 480 SYBR green I Master mix (Roche Molecular Diagnostics, USA), 0.5 μM each primer, and exactly 100 ng sample DNA in a 20-μl volume. The primers used for the *hbb* fragment are published elsewhere (36), and the forward and reverse primers used for the λ phage genome were 5′-TTTTGATTTTGCTGTTTCAA-3′ and 5′-ACCTTTTCCATGA ATTGGTA-3′, respectively. All PCRs were performed in duplicate and included a negative control and standards, each with 100 ng target-free mouse DNA from the respective organ. The standards consisted of a dilution series of 10^4 to 10^1 and 3 copies of a plasmid carrying the *hbb* gene standard or λ phage DNA, respectively. The thermocycling conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 50 cycles of 95°C for 5 s, annealing for 30 s (at an initial temperature of 62°C and then a decrease in the temperature by 0.25°C/cycle to 57°C), and 72°C for 15 s. Touchdown amplification was performed to increase the binding specificity of the primers during the first cycles. After amplification, a high-resolution melting step consisting of 95°C for 5 s, 40°C for 1 min, and progressive heating from 65 to 95°C with 20 acquisitions per degree Celsius was performed to identify target fragments by melting temperature in combination with their fragment size in subsequent agarose gel electrophoresis. The numbers of *Borrelia hbb* copies extrapolated from the standard curve were multiplied by the ratio of the extrapolated λ phage DNA copy numbers in the sample and the number of copies previously added per milligram of skin/heart or per tibiotarsal joint. The detection limit was less than 3 copies, and the quantification limit was 5 to 10 copies per 100 ng DNA for both touchdown qPCR assays.

Statistics. The confidence intervals (CIs) of the prevalences were calculated as Wilson score intervals using R Statistics software and the Plot-CIs package. Negative binomial regression of tick numbers was performed using R Statistics software and the MASS package to estimate the influence of the variable *Heligmosomoides* sp. count and the categorical variables mouse species (*Apodemus flavicollis* and *Apodemus sylvaticus*) and trapping location (Gatow, Moabit, Steglitz, and Tegel) on the number of *I. ricinus* ticks on wild mice.

GraphPad Prism (version 5.01) software (San Diego, CA, USA) was used for the plotting and statistical analysis of the experimental infection data. The data were tested for statistically significant differences using an unpaired *t* test, one-way analysis of variance (ANOVA) with a Bonferroni-corrected posttest (see Fig. 3 and 5), and two-way-ANOVA (see Fig. 2) under the assumption of a normal distribution of the FACS fluorescence data, ELISA optical density data, and the majority of the experimental parasite infection data. The posttests following the two-way ANOVA for individual pairs using the Bonferroni correction were performed with the posttest calculator (GraphPad Software). The number of engorged tick nymphs and the molting rate in Fig. 7A and D, respectively, and the cell counts in Fig. S1B in the supplemental material were not normally distributed, and differences were tested using the Mann-Whitney U test. Differences with *P* values below 0.05 were considered to be significant.

TABLE 1 Numbers of wild *Apodemus* (*Sylvaemus*) mouse species trapped at the different study sites and *I. ricinus* and *H. polygyrus* infection parameters[a]

| Trapping location | No. of mice trapped | | *Ixodes ricinus* (larvae and nymphs) infection parameters | | | | *H. polygyrus* infection parameters | | | |
| | | | Prevalence | | Intensity | | Prevalence | | Intensity | |
	Apodemus flavicollis	*Apodemus sylvaticus*	% of specimens	95% CI	Mean	Range	% of specimens	95% CI	Mean	Range
Gatow	33		81.8	65.6–91.4	15.7	1–52	9.1	3.1–23.6	2.7	1–6
Tegel	22		54.5	34.7–73.1	3.8	1–9	40.9	23.3–61.3	24.1	1–200
Steglitz	27		51.9	34.0–69.3	8.9	1–59	66.7	47.8–81.4	7.6	1–52
Moabit		25	24.0	11.5–43.4	2.5	1–6	84.0	65.4–93.6	29.2	1–246
Total[b]	82	25	55.1	45.7–64.2	10.3	1–59	47.7	38.5–57.0	19.1	1–246

[a] Prevalences (in percent with 95% CIs) and intensities (mean number of parasites per infected/infested mouse and range) are shown for *H. polygyrus* and subadult live stages of *I. ricinus*.

[b] Total, the sums of the number of trapped mice per species as well as the infection parameters for all trapped mice ($n = 107$).

RESULTS

Nematode/tick coinfection is frequent in urban and periurban wild mice. A total of 107 potential murine hosts of a *Heligmosomoides* sp. representing 82 yellow-necked mice (*Apodemus flavicollis*) and 25 long-tailed wood mice (*A. sylvaticus*) were trapped alive at 2 urban sites (Moabit, Steglitz) and 2 periurban sites (Gatow, Tegel) in Berlin and examined for their parasite burden. No house mice (*Mus musculus*) were caught, as the traps were placed exclusively outside buildings. Both species frequently hosted *H. polygyrus* (47.7%; 95% CI, 38.5 to 57.0%; $n = 107$) with intensities of from 1 to 246 specimens (Table 1). In addition, 55.1% (95% CI, 45.7 to 64.2%) were infested by up to 59 subadult *I. ricinus* ticks (92.1% larvae, 7.9% nymphs). A high percentage (41.3%; 95% CI, 29.1 to 53.4%) of tick-infested mice ($n = 59$) were simultaneously infected by *H. polygyrus*. A negative binomial regression model including all 107 mice showed an important influence of the trapping location and/or the rodent species on the number of *I. ricinus* ticks on the rodents but could not distinguish between the two variables because of total colinearity (Fig. 1). *A. sylvaticus* mice were exclusively trapped in Moabit, whereas *A. flavicollis* occurred only at the three other locations. *A. flavicollis* mice from Gatow were infested with significantly more ticks than those from Steglitz ($P < 0.05$) and Tegel ($P < 0.001$) and *A. syl-*
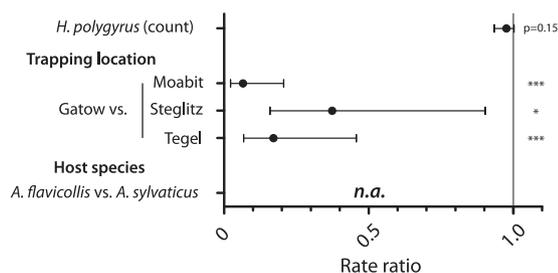

FIG 1 Incident rate ratios with 95% CIs for risk factors affecting the number of host-associated *Ixodes ricinus* ticks on wild mice. A negative binomial regression for the *I. ricinus* count (larvae and nymphs) as the dependent variable was performed using the *H. polygyrus* worm count and the categorical factors trapping location with three dummy variables and host species as independent variables. Reference categories for the trapping location and host species were Gatow and *A. flavicollis*, respectively. *, $P < 0.05$; ***, $P < 0.001$; n.a., not applicable because of total colinearity with the trapping location. Data are for 107 wild mice.

vaticus mice from Moabit ($P < 0.001$). Although the difference was not significant ($P = 0.15$), the number of *Heligmosomoides* organisms showed a negative trend versus the number of ticks, with every nematode reducing the number of ticks by 2.4% (95% CI, −6.6% [decrease] to +0.17% [increase]). Likelihood ratio tests confirmed that the negative binomial model was more appropriate than a Poisson model ($P < 0.001$) and a significant improvement over the null model without coefficients ($P < 0.001$). In order to assess a possible influence of a nematode infection on the parameters of tick infestation, host development of antitick immunity, and dissemination of tick-borne pathogens, we established an experimental nematode/tick coinfection model under defined laboratory conditions with house mice.

Nematode/tick coinfection leads to highly polarized Th2 responses in inbred mouse strains. To compare the spectrum of Th2 reactivity against ticks and nematodes depending on the host's genetic background, mice of three inbred strains with reported differences in immune reactivity (13, 37, 38) were either infested with *I. ricinus* larvae alone or infested with *I. ricinus* larvae and coinfected with the nematode *H. polygyrus* (Fig. 2A). Systemic Th2 responses in spleens were characterized by flow cytometry 20 days after *I. ricinus* infestation on the basis of cytokine (IL-13) production and expression of the lineage transcription factor of Th2 cells, GATA-3 (Fig. 2B). Coinfected mice of all strains exhibited elevated Th2 responses compared to the responses of the controls infested only with ticks. C57BL/6 mice produced more splenic IL-13-producing (IL-13^{+}) CD4^{+} cells and exhibited higher frequencies of GATA-3-expressing (GATA-3^{+}) CD4^{+} cells in response to coinfection or infestation with ticks only than BALB/c and C3H mice. Following the hypothesis that a strong Th2 response during nematode/tick coinfection might affect the feeding success of ticks and the control of tick-borne pathogens, C57BL/6 mice were chosen for subsequent experiments.

The frequencies of Th2 cells peak during acute nematode and tick coinfection. To evaluate the effects of a nematode infection on antitick immune responses, we next determined the kinetics of the systemic Th2 and regulatory T cell responses provoked by the nematode infection, repeated tick infestations, and a coinfection regimen with repeated tick feeding. We chose an infective dose of 250 *H. polygyrus* L3-stage larvae and 50 *I. ricinus* larvae to reflect the parasite burdens also detectable in wild mice (Table 1).
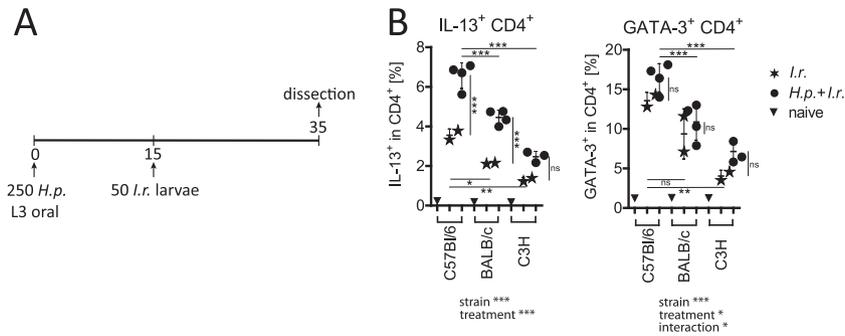
A

FIG 2 Comparison of systemic Th2 responses to tick and nematode/tick coinfections in three inbred mouse strains. (A) Experimental setup showing the time line (in days) of exposure to *H. polygyrus* (*H.p.*) and to *I. ricinus* (*I.r.*) larvae. (B) Frequencies (mean ± SD) of splenic IL-13⁺ CD4⁺ T cells in response to PMA-ionomycin stimulation (left) and GATA-3⁺ CD4⁺ cells (right) detected by flow cytometry. Values of significance determined by two-way ANOVA are shown below the graph. Values of significance determined by individual Bonferroni-corrected pairwise posttests are depicted with horizontal lines for comparisons between mouse strains (lines at the top are for mice coinfected with *H. polygyrus* and *I. ricinus*; lines at the bottom are for mice infested with *I. ricinus*) and vertical lines for comparison between treatments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. Data are for 1 to 4 mice per group.

C57BL/6 mice were either infested with specific-pathogen-free *I. ricinus* larvae three times at biweekly intervals or first infected with *H. polygyrus* and subsequently infested three times with *I. ricinus* starting on day 4 after nematode infection (Fig. 3A), a regimen chosen to cover the early, acute, and chronic phases of nematode infection reflecting expanding, maximal, and declining nematode-driven Th2 reactivity, respectively. Mice infected only with *H. polygyrus* and naive littermates served as controls. To survey the development of systemic Th2 and regulatory responses, blood was sampled once a week and GATA-3 and Foxp3 protein expression by CD4⁺ T cells was monitored (Fig. 3B). Infection with *H. polygyrus* alone led to the typical pattern of a strong systemic Th2 response during the acute phase of infection and the subsequent decline of Th cell levels to near the background levels in the chronic phase of infection (Fig. 3C), despite the presence of high worm burdens at the end of the experiment on day 41 p.i. (Fig. 3E). Tick infestations resulted in strong systemic Th2 responses of similar magnitudes after each infestation and a subsequent decline in Th2 cell frequencies (Fig. 3C). In mice coinfected with both parasites, systemic Th2 cells were substantially more numerous, roughly reflecting the sum of the Th2 cell populations detected in controls infected/infested with a single pathogen. Interestingly, the suppression of nematode-elicited systemic immune responses in the chronic phase of *H. polygyrus* infection at day 41 did not spill over to tick-induced responses, as GATA-3 cells expanded systemically in response to the third tick infestation with a magnitude similar to that in the controls infested with ticks only (Fig. 3C). Infections with *H. polygyrus* activate Foxp3-expressing regulatory T cells (Tregs), which control the developing Th2 response (39–41). The frequencies of Foxp3⁺ Tregs in the peripheral blood of mice exposed only to *H. polygyrus* or only to *I. ricinus* were not significantly altered compared to those in the peripheral blood of the naive controls (Fig. 3D). In coinfected mice, however, the dominant increase in GATA-3-expressing Th2 cells led to a reciprocal transient decline in the frequencies of Tregs detected in peripheral blood. Hence, we found that systemic Th2 responses were greatly increased in mice that were acutely infected by intestinal nematodes and concomitantly infested by ticks.

Th2 cytokine responses to ticks are not affected by concurrent nematode infection. We next analyzed whether systemic and local cytokine responses to repeated tick infestations were affected

by a concurrent nematode infection. For this purpose, mice were dissected 1 day after the last *I. ricinus* larva of the third infestation had detached, and cells from spleen and local skin-draining lymph nodes (SLNs) were harvested and stimulated for cytokine production. As expected, the frequencies of splenic CD4⁺ T cells producing IL-13 in response to unspecific PMA-ionomycin-induced stimulation were the highest in coinfected mice (Fig. 4A). In response to larval tick antigen, however, similar levels of IL-13 were detected in cultures of splenocytes from coinfected mice and control mice infested only with ticks, a pattern also reflected by anti-CD3/anti-CD28 antibody-induced stimulation (Fig. 4A and not shown). The tick-specific local IL-13 responses of cells from cervical and axillary lymph nodes draining the head and neck skin region were also equivalent (Fig. 4A). A similar pattern was detected for IL-10: the highest frequencies of IL-10⁺ CD4⁺ cells were detected after unspecific activation of spleen cells from coinfected/infested mice, and systemic and local *I. ricinus*-specific IL-10 responses were equal in both tick-infested groups (Fig. 4B). Splenocytes from coinfected mice contained the highest proportion of CD4⁺ cells responding to PMA-ionomycin with IFN-γ production; however, IFN-γ-positive (IFN-γ⁺) responses to tick antigen in spleen cells were close to the detection limit of the assay and similar in spleen and SLNs for both tick-infested groups (Fig. 4C). Because Th2-associated innate effector cells contribute to immunity against ticks (42, 43), we compared the mast cell and eosinophil numbers of tick-infested and *H. polygyrus*-infected mice by histological analyses of the skin region affected by repeated tick feeding. They did not vary between the tick-infested experimental groups (see Fig. S1 in the supplemental material). Taken together, mice coinfected with nematodes and ticks develop very strong systemic Th2 responses, while local antitick immune responses are unaltered by an infection with enteric nematodes.

Tick feeding success is not affected by concurrent *H. polygyrus* infection. In line with the unaltered tick-specific local cytokine responses in coinfected mice, the parameters of the success of tick feeding and subsequent molting were unaffected by the presence of *H. polygyrus* (Fig. 5). During successive infestations, the number of successfully engorged larval ticks and their feeding duration did not differ among the single infection and coinfection groups. Although ticks feeding on nematode-infected hosts dur-
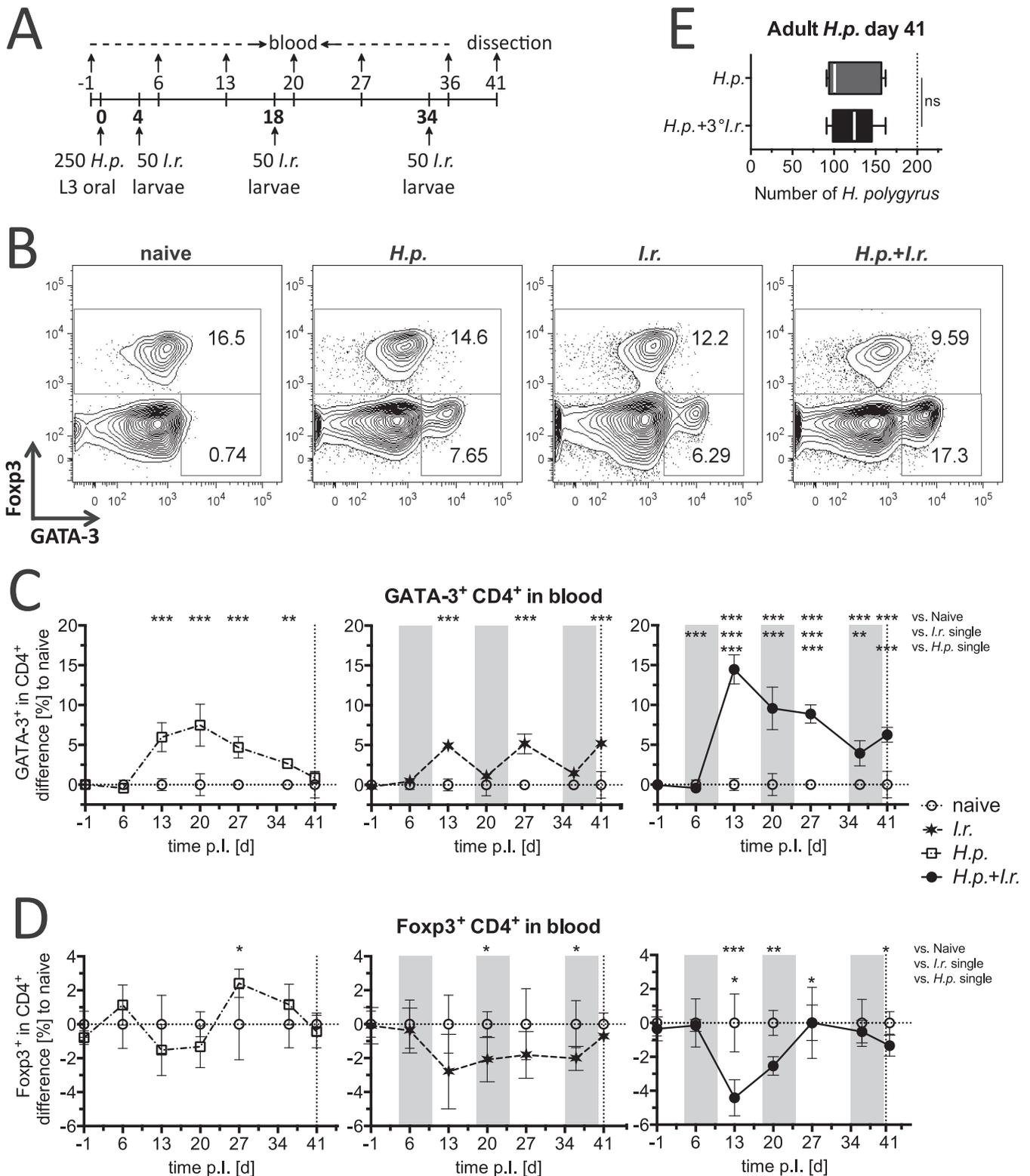
FIG 3 Kinetics of GATA-3+ CD4+ Th2 cells from C57BL/6 mice during infestation with *I. ricinus* ticks alone and during infestation with *I. ricinus* ticks and coinfection with *H. polygyrus*. (A) Experimental setup showing the time line (in days) of exposure to *H. polygyrus* (*H.p.*) and to *I. ricinus* (*I.r.*) larvae and blood collection. (B) Representative flow cytometry density plots of peripheral blood CD4+ cells stained for Foxp3 and GATA-3 on day 13. (C, D) Frequencies of GATA-3+ (C) and Foxp3+ (D) CD4+ cells (mean ± SD) in the peripheral blood of *H. polygyrus*-infected mice (left), *I. ricinus*-infested mice (middle), and *H. polygyrus*- and *I. ricinus*-coinfected mice (right) normalized to those in the peripheral blood of naive controls. Gray shading and vertical lines, period of larval tick feeding and the day of dissection, respectively. One-way ANOVAs with a Bonferroni-corrected pairwise *t* test were performed for every time point in the kinetic analysis. d, day. (E) Number of adult *H. polygyrus* worms isolated from small intestines on day 41. The box plot shows the medians and quartiles, with whiskers indicating 95% CIs. Differences were tested using an unpaired *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. Data are for 5 or 6 mice per group.
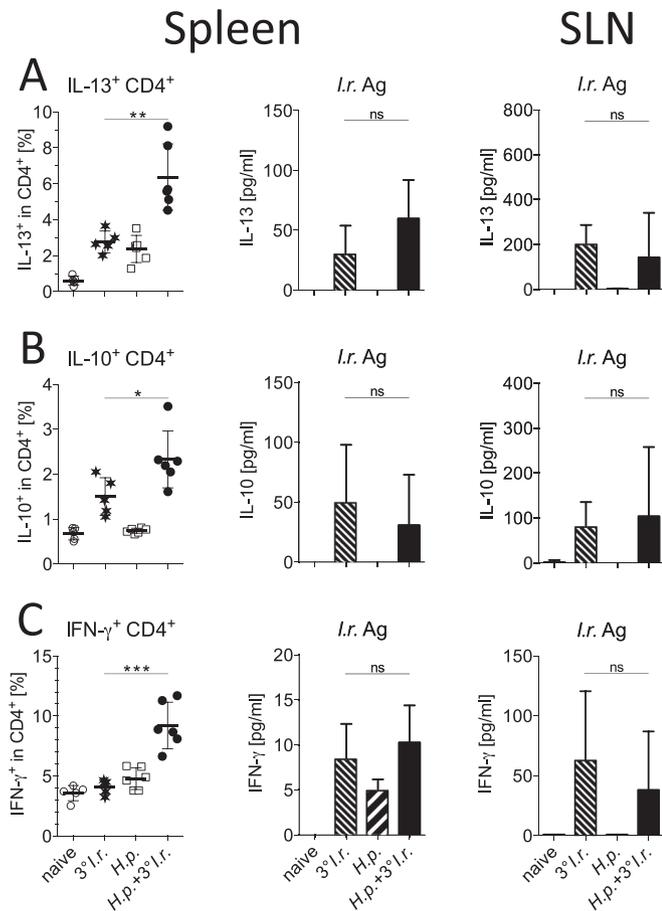
Spleen

FIG 4 Systemic and local cytokine responses of mice infected with *H. polygyrus* and *I. ricinus*. The production of IL-13 (A), IL-10 (B), and IFN-γ (C) was measured in spleen CD4$^+$ T cells by flow cytometry after intracellular cytokine staining following PMA-ionomycin stimulation (left) and in bulk culture supernatants of spleen and SLN cells by ELISA after specific stimulation with larval *I. ricinus* antigen (middle and right, respectively). Means and SDs are shown. Symbols represent the intracellular cytokine responses detected in cells from individual mice. Differences between groups were tested using an unpaired *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. Data are for 5 or 6 mice per group.

ing the 1st infestation were significantly lighter than ticks feeding on nematode-free mice, no such difference was observed during subsequent infestations. The effect of partial immunity against larval ticks after repeated feeding was also similar in both groups when the detachment rates of engorged ticks of the primary and tertiary infestations, their feeding durations, and their weights were compared (Fig. 5). The rates of molting to the nymphal stage did not differ for ticks feeding on worm-free and worm-infected hosts after the primary and secondary infestations. Ticks from the tertiary infestations, unfortunately, succumbed to fungal growth. Hence, we detected a partial protection of laboratory house mice against repeated tick feeding which was unaffected by a concurrent nematode infection.

Concurrent *H. polygyrus* infection does not significantly affect Th1 responses to tick-borne *B. afzelii* infection. Next, we examined whether increased systemic Th2 responses in mice coinfected with ticks and intestinal nematodes might inhibit Th1 responses against tick-borne spirochetes. *Borrelia afzelii*-infected *I. ricinus* nymphs were permitted to feed on mice infected with *H. polygyrus* and naive controls. As the maximum systemic Th2 response, based on peripheral blood Th2 cell frequencies, occurred in the second week of *H. polygyrus* infections (Fig. 3C), we chose day 16 after nematode infection for tick attachment (Fig. 6A). High frequencies of GATA-3$^+$ Th2 cells in the peripheral blood briefly before and during tick feeding were confirmed (Fig. 6B). Mice were dissected 19 days after *I. ricinus* infestation, when *B. afzelii* dissemination in the natural rodent hosts results in maximum infectivity for feeding tick larvae (3). Upon PMA-ionomycin-induced stimulation, spleen cells from mice coinfected with nematodes and ticks again responded with the highest frequencies of CD4$^+$ cells producing IL-13, IL-10, and IFN-γ (Fig. 6C to E). A similar response to PMA-ionomycin-induced stimulation was observed for local CD4$^+$ T cells derived from SLNs (Fig. 6C to E). To survey antispirochete responses, bulk cultures were stimulated with *B. afzelii* antigen. Surprisingly, low levels of spirochete-specific IL-13 production by splenocytes but not by SLN cells were detectable in both groups of mice infested with *B. afzelii*-infected ticks (Fig. 6C). Splenocytes from mice infested only with *Borrelia*-infected ticks produced high levels of IL-10 in response to spirochete antigen, but this response was significantly suppressed in spleen cells derived from mice coinfected with nematodes and
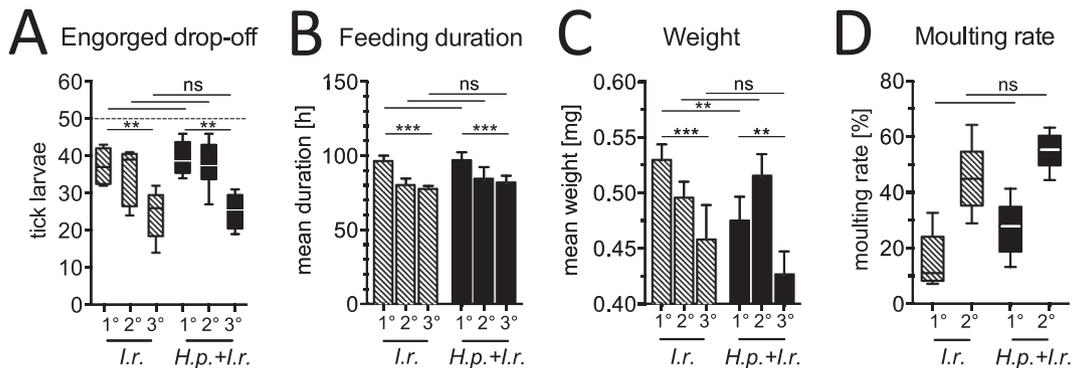

FIG 5 Feeding success of *I. ricinus* larvae on mice with and without *H. polygyrus* coinfection. (A and D) The number of engorged larvae recovered after they dropped off the mice (A) and the rates of molting to the nymph stage by engorged larvae (D) are depicted as box plots (medians and quartiles, with whiskers representing 95% CIs). (B and C) The feeding duration (B) and tick weight after detachment (C) are shown as means ± SDs. The dotted line in panel A depicts the total number of ticks applied to individual mice. Asterisks indicate the significance values obtained by Bonferroni-corrected pairwise posttests following one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. Data are for 5 or 6 mice per group.
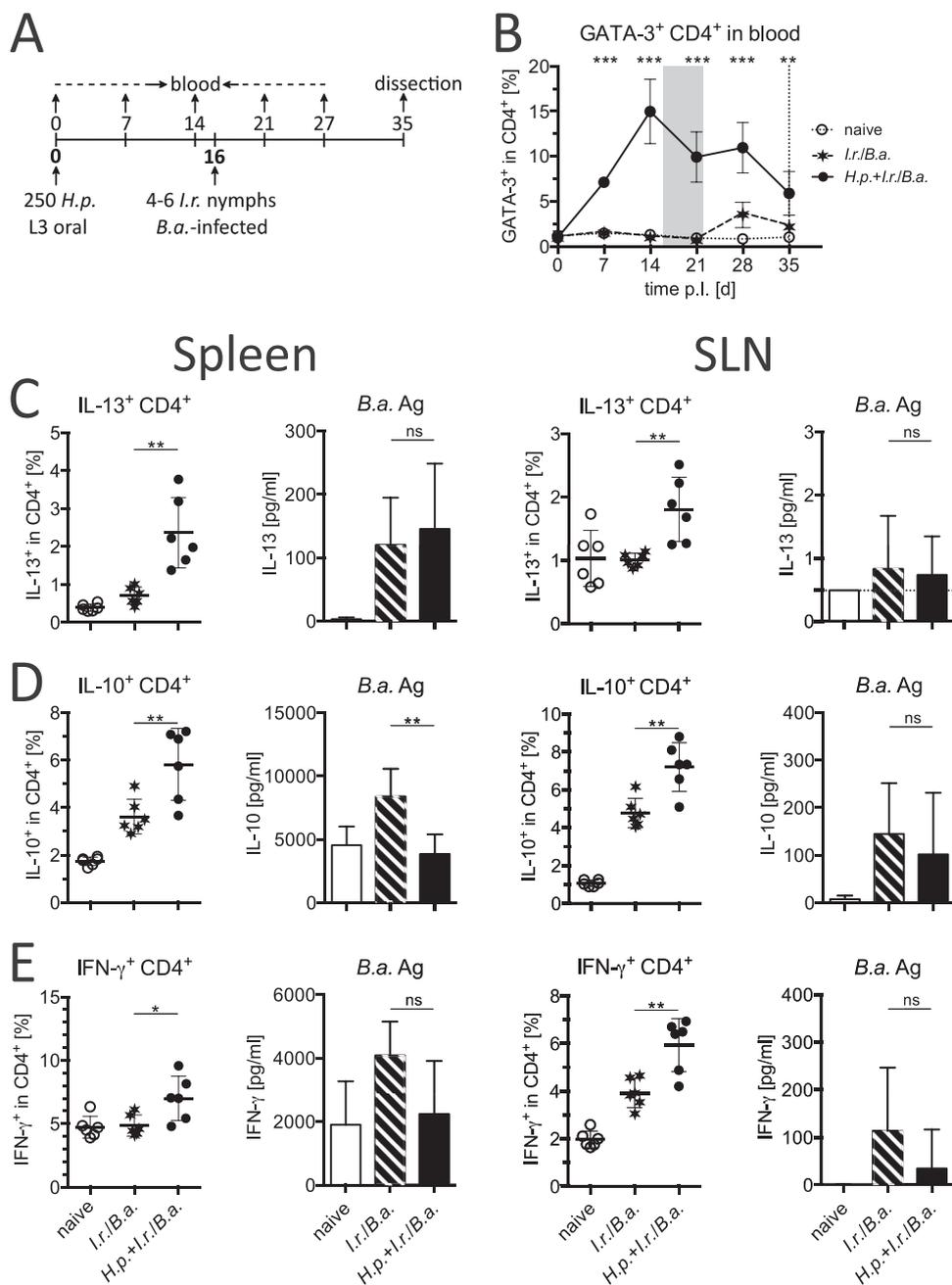
FIG 6 Systemic and local immune responses in mice coinfected with *H. polygyrus* and *I. ricinus*-transmitted *B. afzelii* (*B. a.*). (A) Experimental setup showing the time line (in days) of exposure to parasites and blood sampling. (B) Kinetics of GATA-3$^+$ CD4$^+$ cell frequencies (mean ± SD) in peripheral blood. Gray shading and the vertical line, period of nymphal tick feeding and the day of dissection, respectively. Asterisks indicate significant differences between the coinfected group and the group infested with *I. ricinus* only for every time point. (C to E) Production of IL-13 (C), IL-10 (D), and IFN-γ (E) by spleen and SLN CD4$^+$ T cells stimulated with PMA-ionomycin and bulk cultures stimulated with *B. afzelii* antigen. Means and SDs are shown. Symbols represent the intracellular cytokine responses detected in cells from individual mice. Differences between groups were analyzed using unpaired *t* tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. Data are for 6 mice per group.

ticks. In contrast, spirochete-specific IL-10 production by local SLN cells was unaffected by the presence of intestinal nematodes (Fig. 6D). Although the highest frequencies of CD4$^+$ IFN-γ$^+$ cells were detected in the spleens and SLNs of coinfected mice, the *B. afzelii* antigen-specific responses of splenocytes and SLN cells appeared to be slightly but not significantly lower in mice coinfected with nematodes and ticks, which was also reflected by anti-CD3/

anti-CD28 antibody stimulation (Fig. 6D and not shown). Thus, systemic and local *Borrelia*-specific Th1 reactivity is only mildly affected, if it is affected at all, by a concurrent intestinal nematode infection.

H. polygyrus infection does not affect the transmission or control of tick-borne B. afzelii infection. Because coinfections with intestinal nematodes reduce the control of concomitant bac-
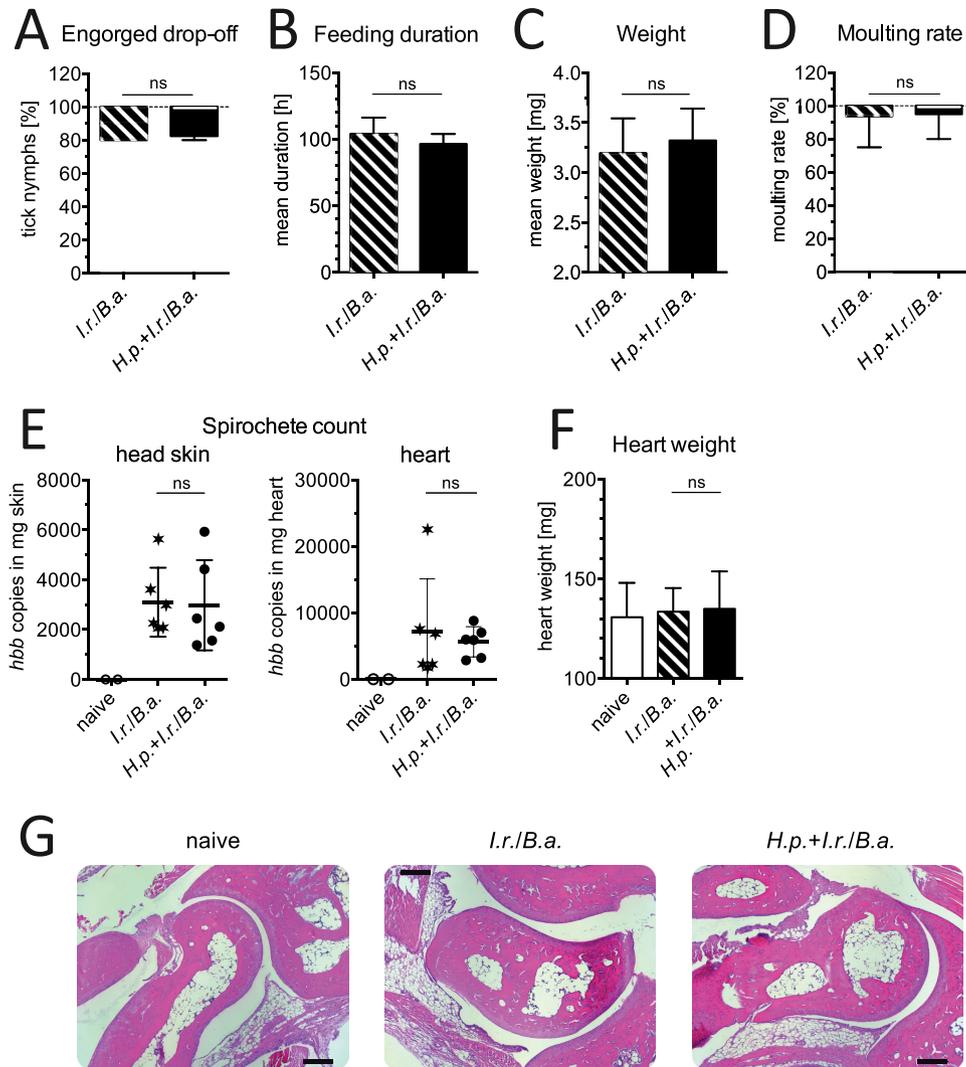
FIG 7 Feeding parameters, spirochete load in mouse tissues, and tibiotarsal joint histology after infection with *H. polygyrus* and tick-*B. afzelii* infection. (A and D) The number of engorged nymphs recovered after detachment (A) and rates of molting to adult ticks (D) are depicted as box plots (medians and quartiles, with whiskers representing 95% CIs). The dotted line in panel A depicts the total number of ticks applied to individual mice, and that in panel D depicts the total number of engorged ticks. (B and C) Feeding duration (B) and tick weight after detachment (C) are shown as means ± SDs. (E) Number (mean ± SD) of *B. afzelii* spirochetes per milligram of head skin (left) and heart (right), measured by absolute quantification of the number of copies of the *hbb* gene via touchdown qPCR. (F) Heart weight of mice (mean ± SD). (G) Representative cross sections from tibiotarsal joints stained with hematoxylin and eosin for histopathological scoring. Bar, 100 μm. Differences between groups were tested using unpaired *t* tests (B, C, E, F) or the Mann-Whitney U test (A, D). ns, not significant. Data are for 6 mice per group.

terial infections (22–24), we examined whether the transmission and dissemination of tick-borne LD spirochetes were affected in nematode-infected hosts. As was observed during primary infestation with larval *I. ricinus*, *H. polygyrus* did not affect the feeding success of *B. afzelii*-infected *I. ricinus* nymphs (Fig. 7A to D). Nearly all ticks engorged and subsequently molted successfully. We quantified the number of *B. afzelii* bacteria in the skin of the head, the heart, and tibiotarsal joint by applying a newly established touchdown real-time PCR assay quantifying spirochetes in relation to organ weight. All mice had acquired *B. afzelii* from the infected nymphal *I. ricinus* ticks. They harbored about 3,000 spirochetes per mg head skin and about 6,400 spirochetes per mg heart, while the spirochete numbers in the tibiotarsal joints were under the detection limit (Fig. 7E). Spirochete numbers were independent of the presence of *H. polygyrus* infections. All mice were

free of clinical signs of Lyme disease, such as carditis or arthritis. The weight of the hearts of infected mice was comparable to that of the hearts of naive mice (Fig. 7F), and the low numbers of spirochetes that had disseminated to the joints did not lead to signs of pathological inflammation (Fig. 7G). Furthermore, food and water intake, mobility, and body weight were unaltered in *B. afzelii*-infected mice (data not shown). Taken together, we demonstrate that coinfection with an intestinal nematode fails to facilitate the proliferation and systemic dissemination of tick-borne Lyme disease spirochetes in house mice under experimentally controlled conditions.

DISCUSSION

Small rodents serve as reservoir hosts for several tick-borne pathogens in Europe, and the *I. ricinus* tick is the vector for the agents of

Lyme disease. Numerous mice and voles inhabit urban areas and thus constitute a considerable source for tick-borne pathogens in close proximity to humans. Hosts serving as competent reservoirs for tick-borne pathogens must permit repeated tick feeding, must be susceptible to the pathogen, and must become and remain infectious for feeding ticks for prolonged periods. Acquired immunity to repeated tick infestations, observed in some artificial hosts, concomitantly reduces the susceptibility to tick-borne pathogens and thus curtails the pathogens' transmission to a new vector (44–46). Currently, it is not known whether and to what extent coinfections affect the susceptibility of rodents to tick feeding and the competence of rodents as a reservoir for tick-borne pathogens. As nematode coinfections are highly prevalent in wild mice and the worm burdens can reach high levels (9–11), they might influence the success of pathogen transmission by repeated tick feeding and the immune control of tick-borne pathogens, such as *B. afzelii*. Thus, coinfections of rodents with macroparasites may increase the efficiency of transmission of tick-borne pathogens and thereby affect public health. In this study, we present epidemiological data derived from wild mice showing that coinfections with ticks and nematodes are very frequent and combine them with immunological data and parameters related to the competence of the rodent as a reservoir for LD spirochetes obtained from a defined experimental laboratory system. This system was chosen by consideration of the fact that factors likely to affect the surveyed parameters, such as preexposure to tick infestations and LD spirochetes, the time since spirochete infection, and the number of bites by infected ticks, were unknown for rodents trapped in the wild.

We analyzed wild mice captured in Berlin for the occurrence of a *Heligmosomoides* sp. and *I. ricinus* and found that both are frequent parasites in wild mice from urban and periurban sites in Berlin; i.e., 41.3% of tick-infested *Apodemus* mice were coinfected by the nematode. This is in line with the findings of a previous Italian field study reporting that 68% of tick-infested yellow-necked mice were coinfected with *H. polygyrus* (47). A regression analysis revealed that rodent species and the trapping location mainly affect the number of ticks on the rodents. Only a trend for a negative influence of the number of *Heligmosomoides* organisms on the tick count was observed, although it did not reach statistical significance. Ferrari et al. (47) reported that besides the trapping location and different host factors, *H. polygyrus* had a negative influence on the number of *I. ricinus* ticks infesting wild *A. flavicollis* mice. These statistical results suggest that the nematode infection may influence the tick infestation, but validation by use of an experimental infection under defined laboratory conditions was lacking. Hence, we examined experimentally whether concurrent nematode infections influence the development of (i) antitick immunity and (ii) the control of the tick-borne pathogen *B. afzelii*.

Antitick immunity is associated with the development of Th2 immune responses and is mainly accomplished by basophils, mast cells, and eosinophils, which are Th2-associated innate effector cells (13, 42, 48). In our experimental model, C57BL/6 mice acquired partial immunity against repeated tick infestations, detected as a significantly reduced number of larval ticks and a significantly reduced engorgement weight of the larval ticks after the third infestation. A reduction in feeding time during a third tick infestation, as previously documented in BALB/c mice (16), was also evident in our study but may not reliably reflect the immune status of the infested host (49). Our observations during repeated larval infestations are in contrast to those in a study in which several laboratory inbred mouse strains, including C57BL/6 mice, did not develop protective immunity in response to repeated feeding of nymphal *I. ricinus* ticks (13). It is known that the pattern of tick antigenic molecules recognized by the tick hosts is dependent on the tick life stage (50); therefore, it is possible that the larval *Ixodes* stage is more immunogenic or more susceptible to discrete immune defense mechanisms than the nymphal stage.

In our experimental coinfections of laboratory mice with nematodes and ticks, the systemic Th2 immune responses against the distinct macroparasites resulted in the generation of extraordinarily high levels of Th2 cells in the peripheral blood and spleens, and these peaked during the second tick infestation and simultaneous acute nematode infection. This, however, did not affect the anti-tick Th2 responses measured locally in skin-draining lymph nodes after repeated infestations with larval ticks. Consequently, the success of larval tick feeding was unaffected by prepatent, acute, and chronic nematode coinfection, and partial immunity to tick feeding developed independently of the concurrent nematode infection. In addition, infestation with infected nymphal ticks was unaffected. Taken together, we demonstrate that a concurrent *H. polygyrus* infection does not reduce the susceptibility of mice to tick feeding.

A previous study revealed a negative correlation between *H. polygyrus* infection (the number of eggs per gram in feces) and tick numbers on wild yellow-necked mice and showed that tick infestation was increased when mice were dewormed, released, and recaptured compared to the level of infestation for the nontreated controls (47). Competition for space and food could not explain that correlation between these two macroparasites, and the influence of factors such as sex and host sex-related behavior, age, breeding condition, and habitat was at least partly ruled out, as the sites where the treated and control mice were trapped were in close proximity and similar parasite abundances were observed on the mice *a priori* (47). The intensity of grooming behavior leading to reduced tick infestation and increased ingestion of nematode larvae from the fur could also explain the negative correlation but not the increased level of tick infestation after deworming in the study of Ferrari et al. (47). Our findings suggest that the possibility of an immunologic basis for this negative correlation or the release of toxic products by one parasite affecting the other, which was suggested by the authors of the previous study (47), can also be excluded.

Several reasons may account for the differences observed between the mice obtained from the wild and those raised in the laboratory. First, the antitick immune responses and susceptibility to coinfection-induced immune changes observed in the *Mus musculus* mice used in our experimental study may differ from those observed in other mouse species, such as *A. flavicollis* and *A. sylvaticus*. Second, recent comparative immunological analyses show that SPF laboratory mice and those obtained from the wild differ not only in their expression of immune functions but also in specific and generalized immune reactivity. C57BL/6 mice raised under SPF conditions activate natural killer cells less readily than wild *M. musculus* mice (51), which also display a stronger general status of immune cell activation and higher antibody titers and avidities (52). Thus, coinfections with nematodes may differentially affect the success of tick feeding and antitick immunity in wild mice with a highly activated immune system. Third, sex-related differences in the immune reactivity of the host to many

parasite species have been described (47, 53, 54). We restricted our experimental coinfection studies to female mice, but factors such as host sex, breeding condition, nutritional status, and population density may clearly alter immune reactivity to single infections and coinfections with parasites.

Infections with intestinal nematodes affect immune responses to unrelated pathogens, such as bacteria and protozoa (22–28). This results from efficient immune modulation by parasitic worms and the counterregulation of Th1 and associated cytotoxic T cell responses (25, 27), as well as modulated innate effector cell functions during strongly biased Th2 responses (22, 55). Immune control of LD spirochetes depends on the development of Th1 responses in mice and humans (56–59). In the present study, mice coinfected with *H. polygyrus* and *I. ricinus* were only slightly inhibited in their mounting of a Th1 response to *B. afzelii*, as seen by a trend of reduced IFN-γ production by local and systemic T cells in response to the spirochete antigen. Surprisingly, the general capacity for IFN-γ production, as assessed after unspecific CD4$^+$ T cell activation, was significantly elevated even in mice that had been coinfected/infested with nematodes and ticks, further arguing against a counterregulation of Th1 reactivity in the face of two macroparasites coinducing strong Th2 responses. We also assessed whether a concurrent nematode infection increased local and systemic spirochete-specific IL-10 levels; however, local responses were not altered and the *Borrelia*-specific IL-10 production was significantly suppressed in the splenocytes of mice coinfected with nematodes. In line with these data, the presence of an acute worm infection at the peak of systemic Th2 cell reactions did not affect either the susceptibility of mice to *B. afzelii* or spirochetal proliferation or dissemination to different organs. Because two of the three essential components of reservoir competence, susceptibility to repeated tick feeding and susceptibility to the vector-borne pathogen, were not influenced, nor were spirochetal numbers modified by coinfection with *H. polygyrus*, we speculate that the competence of mice as a reservoir for LD spirochetes is not enhanced by the presence of enteric nematodes. Hence, this study indicates that the strong systemic Th2 responses in house mice coinfected with nematodes and ticks fail to affect the success of tick feeding and the control of the causative agent of Lyme disease.

ACKNOWLEDGMENTS

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